A THESIS

entitled

EXPLORING THE MOLECULAR MECHANISM OF hA1-1 GLUTATHIONE S-TRANSFERASE, FOCUSING ON THE ROLE OF THE CHARACTERISTIC C-TERMINAL HELIX.

by

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Submitted to the University of Leicester in fulfilment of the requirements for the award of the degree of Doctor of Philosophy.

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October 1996.
ACKNOWLEDGEMENTS.

I would like to thank Roland Wolf for providing the wild-type hA1-1 GST clone and Paul McDonaugh for generating the DNA constructs for the various forms of hA1-1 GST used in these studies. Thanks to Tony Simula for the initial protocol for the production and purification of hA1-1 GST and Ju-Chun Yang for preparing the samples and spectra of the uniformly $^{15}$N labelled protein. For their guidance during the course of my PhD research I would like to thank Lu-Yun Lian and Gordon Roberts, and last, but not least, I would like to thank the Wellcome trust for funding the research.
ABSTRACT
The molecular mechanism of hA1-1 glutathione S-transferase (GST) has been probed using fluorescence and NMR spectroscopy, kinetic analysis and X-ray crystallography. The results show that the mechanism of hA1-1 GST involves hydrophobic substrate activation, and the efficiency of this process is a crucial factor in hydrophobic substrate specificity. Hydrophobic substrate activation can only occur when the glutathione peptidyl moiety has bound, inducing a conformational change in the protein. The main region of the protein that is involved in this conformational change is the characteristic C-terminal helix of hA1-1 GST and the integrity of this region has been shown to be essential for hydrophobic substrate activation. It is thought that the C-terminal helix correctly orientates the hydrophobic substrate in the active site allowing activation to occur. The deletion of the C-terminal helix alters the substrate specificity of the enzyme, with the truncated enzyme having a high activity towards ethacrynic acid, normally a pi-class GST substrate. Thus the characteristic C-terminal helix of alpha class GSTs is a major determinant in substrate specificity, in particular determining the characteristic substrate specificity of hA1-1 GST.
CONTENTS

List of abbreviations v

1. Introduction 1

2. Materials and Methods 17
   2.1. The production and purification of wild-type and specifically mutated hA1-1 GST. 17
      2.1.1. Transformation of E. coli cells. 17
      2.1.2. Test for GST expression. 18
      2.1.3. Production of hA1-1 GST by fermentation. 19
      2.1.4. Purification of hA1-1 GST. 19
      2.1.5. Activity Assay. 21
   2.2. Synthesis and purification of glutathione conjugates. 22
   2.3. Fluorimetric determination of the dissociation constants for ligands binding to GST. 22
   2.4. Spectrophotometric determination of the kinetic constants describing GST catalysis 23
      2.4.1. Determination of the kinetic constants for the conjugation of hydrophobic substrates and glutathione. 24
      2.4.2. Determination of the kinetic constants for small thiol compounds to hydrophobic substrates in the presence of a glutathione analogue. 24
   2.5. NMR spectroscopic studies of hA1-1 GST. 25
      2.5.1. The assignment of ligand spectra. 25
      2.5.2. GST sample preparation. 25
      2.5.3. Protein resonance assignments. 25
      2.5.4. Detection of intermolecular NOEs. 25
   2.6. Crystal studies. 26
      2.6.1. Crystal growth. 26
      2.6.2. Ligand diffusion trials. 26
      2.6.3. Diffraction analysis. 27
   3. Production and purification of hA1-1 GST and glutathione conjugates. 28
      3.1. Introduction. 28
      3.2. Production of wild-type and specifically mutated hA1-1 GST. 28
         3.2.1. Transformation of E. coli strains used in the production of GST by fermentation. 28
      3.3. Purification of hA1-1 GST. 33
      3.4. Production of glutathione conjugates. 37
      3.5. The extinction coefficients of the non-glutathione conjugates used for the determination of the rate of conjugation. 41
   4. Determination ligand dissociation and kinetic constants for wild-type and specifically mutated forms of hA1-1 GST. 42
      4.1. The specificity of substrate binding and catalysis at the G-site. 42
      4.2. The conjugation reaction of small thiol compounds to CDNB in the presence of a structurally similar glutathione analogue. 57
      4.3. Determination of kinetic and dissociation constants for H-site ligands. 62
      4.4. The dissociation constants describing GSDNP binding to hA1-1 GST. 71
      4.5. The dissociation constants of GSDNP to specifically mutated forms of hA1-1 GST. 74
      4.6. Further analysis of the F222Y, Y9F, and truncated mutants. 76
   5. Ligand binding studies using NMR spectroscopy. 80
      5.1. NMR Pulse programs. 80
         5.1.1. 1-D NMR experiments 80
         5.1.2. 2-D Heteronuclear Multiple-Quantum resonance experiments 81
LIST OF ABBREVIATIONS.

GST: Glutathione S-transferase
CDNB: 1-chloro-2,4-dinitrobenzene
DCNB: 1,2-dichloro-4-nitrobenzene
GSDNP: 2,4-dinitrophenylglutathione
GSCNP: 2-chloro-4-nitrophenylglutathione
rpm: revolutions per minute
SA: Specific activity
F_max: the maximum change in fluorescence on addition of ligand to a macromolecule solution
K_d: The dissociation constant for a ligand to a macromolecule.
K_cat: the turnover number (s^{-1})
K_M: The Michaelis-Menten constants (mM) equivalent to the concentration of substrate required to achieve a reaction rate equivalent to half the maximum reaction rate
OD: Optical density
NOE: Nuclear Overhauser Effect
NOESY: Nuclear Overhauser spectroscopy
1. Introduction.

Organisms have developed various defence mechanisms against the spectrum of toxic chemicals, both naturally occurring and environmental pollutants, to which they are continually exposed, including rapid efflux systems, metabolism, sequestration, and the efficient repair of damage. The glutathione S-transferases or GSTs (EC 2.5.1.18) are a family of multi-functional isoenzymes that have both toxin sequestering and metabolising activities. The "Ligandins" were the first GSTs to be isolated for their sequestration properties. They were characterised by their high affinity for metabolites and xenobiotics binding either at the substrate or non-specific binding sites.

Depending on the nature of the ligand, binding can be irreversible, representing a "suicide" reaction usually observed with genotoxic electrophiles such as 3-methylcholanthrene, or reversible as with endogenous lipophilic compounds, such as bile acids, enabling their transport and storage in a more soluble form. Further, reversible binding may buffer the intracellular concentrations of ligands, which is particularly important for effector molecules, such as steroid hormones. Later cytosolic GST activity was shown to be in part a function of the "Ligandins" which are now known as alpha-class GSTs.

The GSTs catalyse the conjugation of the tri-peptide glutathione (the co-substrate) to electrophilic centres on a spectrum of non-polar compounds (the substrates) including products of oxidative stress, environmental pollutants, carcinogens, and anti-cancer drugs. Glutathione conjugation facilitates the detoxification of most substrates, firstly, by blocking an electrophilic centre, directly reducing the reactivity. Secondly, the conjugate is often more soluble than the substrate facilitating further metabolism and preventing partitioning into the lipid membrane. The glutathione moiety also serves as a molecular marker for entry into several metabolic pathways including mercapturic acid biosynthesis and for active efflux from the cell.

Some GST isoenzymes exhibit further properties that are not centred around detoxification. For example, some GSTs have been implicated as being involved in hydrophobic metabolite transport and others may have a structural role. Some have been found to catalyse glutathione dependent reduction, some have a selenium-independent peroxidase function or catalyse or isomerisation reactions and others are involved in the biosynthesis of leukotriene A4 and prostaglandin H2. All catalytic activities exhibited by the GSTs are at least partially dependent on the same mechanism as glutathione conjugation, that is the ability of the enzyme to activate the glutathione thiol, promoting its attack on an electrophilic centre bound in proximity.
Most of the GST hydrophobic substrates are products of cytochrome P450 monoxygenase activity (phase I detoxification reactions), hence the GSTs are categorised as one of the major groups of phase II detoxification enzymes. However, for some substrates glutathione conjugation forms part of the mechanism by which toxic effects are produced. There are three categories of GST assisted toxicity: Thiolysis, reversible conjugation and the production of directly acting toxic conjugates.

Thiolysis occurs when glutathione conjugation leads to cleavage of the substrate and, although one product is conjugated, the unconjugated product is still a chemical threat to the cell. Reversible conjugation occurs with some isothiocyanates and presents a different problem: Conjugation is favoured at high GST concentrations, however after glutathione-facilitated transport to peripheral tissues with low GST concentrations the isothiocyanate can be reformed resulting in cytotoxicity. Finally, in a small number of instances the glutathione conjugate is more toxic than the parent compound, as occurs with some alkyl halides.

The biological importance of the GSTs is illustrated in their ubiquitous and diverse nature: GST isoenzymes have been isolated from bacteria, fungi, yeast, plants and many different animal tissues. Research has mainly focused on the multiple mammalian cytosolic isoenzymes and to avoid confusion an efficient nomenclature system has been proposed. Each isoenzyme is prefixed with letters that correspond to the species of origin, for example human GSTs are prefixed with the letter “h”. Following this is a capital letter abbreviating the gene class that encodes the subunits. Mammalian cytosolic GSTs are encoded by four main species independent gene classes namely, alpha, mu, pi, and theta, abbreviated to A, M, P, and T respectively. The GSTs are dimers of identical or non-identical 25kD subunits from the same gene class. The subunit composition is represented by Arabic numerals, separated by a hyphen. Each naturally occurring subunit that is encoded by a discrete gene is assigned a number sequentially according to the order of its discovery and this number is also assigned to the gene locus. For example, hA1-1 is a human homodimer of the first alpha-class subunits to be characterised, encoded at the gene locus GSTA1. In total ten gene loci encoding cytosolic GSTs have been unambiguously identified: A1, A2 (alpha-class), M1, M2, M3, M4, M5 (mu-class), P1 (pi-class) and T1 and T2 (theta-class). Additionally, two further loci encoding alpha-class GSTs possibly exist. Allelic variants are distinguished by letters after the subunit number, for example hM1a-1a and hM1b-1b are human homodimeric allelic variants encoded by the mu-class gene locus GSTM1.
The control of GST expression is extremely complex and depends on the interplay of numerous factors including species, gender, tissue type and developmental stage. Perhaps one of the most interesting determinants of GST expression is the environment. The in vivo concentrations of some isoenzymes are altered on exposure to chemicals, especially GST substrates, and this is thought to play an important part in resistance to herbicides, pesticides, carcinogens and therapeutic drugs making the GSTs economically and clinically important isoenzymes influencing the effectivity of commercial chemicals. The importance of GSTs in resistance mechanisms is such that the amount and type of GST isoenzymes present can affect the susceptibility of a cell to different toxins.

hA1-1 GST was the first human GST isoenzyme to be isolated, as "Ligandin". It is the major GST found in the human liver, constituting approximately 2% of cytosolic protein,
which is 50% of the total GST concentration\textsuperscript{70}. It is also found in the kidney and several other detoxification tissues\textsuperscript{71,72}. The high abundance of this isoenzyme in these tissues implies it has an important cytoprotection function, particularly against the larger more hydrophobic compounds for which it has a preference. Further, hA1-1 GST has been shown to have a selenium-independent peroxidase function and it has been implicated as being involved in the development of some types of multi-drug resistance\textsuperscript{73}. Solving the structure-function relationship of hA1-1 GST would facilitate the design of specific inhibitors to probe the function of this isoenzyme \textit{in vivo}, giving an insight into the reason behind its high natural abundance. Additionally, some types of multi-drug resistance could be explored and the contribution of GSTs to the total cellular peroxidase activity investigated.

All mammalian cytosolic and nearly all other GST isoenzymes are dimeric, formed by the non-covalent association of two similar subunits that do not spontaneously exchange\textsuperscript{74}. The subunits are held together by stacking forces, hydrogen bonds and hydrophobic interactions. Their association is quite specific and limits the range of subunits that are able to hybridise to members of the same gene class. Further, some subunits from the alpha gene class appear to be unable to form heterodimers, as are some mu-class subunits\textsuperscript{23}. Dimerisation stabilises the tertiary structures of the individual subunits\textsuperscript{75} and is essential for catalytic activity, as the active sites although located predominantly on each subunit involve amino acid residues from both subunits. Dimerisation also shields the hydrophobic surface of the substrate binding site (the H-site)\textsuperscript{19} which is located in a cleft (figure 1.1) between the two subunits, reducing the risk of aggregation.

The overall topology of the GST isoenzymes from all four gene classes is very similar. Each subunit is composed of a single polypeptide chain which folds into two structurally distinct domains separated by an approximately six amino acid linker. Domain I is the smaller domain involving about 40% of the amino acid residues. It folds to form most of the glutathione binding site (the G-site)\textsuperscript{76} and is conserved across the GST gene families and also shares homology with equivalent domains in other glutathione binding proteins. It has an \( \alpha-\beta \) structure; a central four-strand \( \beta \)-sheet (\( \beta 1, \beta 2, \beta 3, \) and \( \beta 4 \) - figures 1.2 and 1.3) of \(-1\-2\-1\) topology, flanked by one helix (\( \alpha 2 \)) on one side and two \( \alpha \)-helices on the other (\( \alpha 1 \) and \( \alpha 3 \)). In alpha-, mu- and pi-class isoenzymes \( \alpha 2 \) is an \( \alpha \)-helix but in theta-class GSTs this is a bent irregular helix. Domain II is \( \alpha \)-helical, the common core being composed of four amphipathic \( \alpha \)-helices (\( \alpha 4-\alpha 7 \)). In alpha-, mu- and pi-class subunits there is a further short two turn helix (\( \alpha 8 \)), however in theta-class isoenzymes there is no secondary structure in this region. Most of the H-site is located in domain II, however the N-terminus of helix 1 forms one side of it. This feature is conserved across the gene-classes as is the involvement of the C-terminus of helix 4, however there are
some inter-gene class variations. Where as the H-sites pi- and theta-class isoenzymes are comparatively open to solvent (figure 1.2), the mu-class isoenzymes have an additional loop between the second β-sheet and second α-helix in domain I, from which two residues, Met34 and Tyr40 (hM1-1 numbering), are postulated to form part of the H-site. As an alternative to this loop, alpha-class isoenzymes have an amphipathic α-helix (helix 9 in figure 1.2 and 1.3) at the C-terminus which covers the ligand binding sites when either glutathione or hydrophobic ligand are bound.

Figure 1.2. Ribbon diagram showing the secondary structure elements that make up the GST subunit determined by X-ray crystallography. The ligands bound at the active site of each isoenzyme are shown in “ball-and-stick” fashion. [Adapted from Wilce et al (1995)77]
Figure 1.3. The structurally based amino-acid sequence alignments of human GST isoenzymes from the four cytosolic gene classes.

[Figure is adapted from Wilce et al (1995) ??]
Key for Figure 1.3.
Amino acid residues implicated as being involved in ligand binding are indicated by the letters H and G depending on whether they are involved in H- or G-site binding, respectively. Boxed residues are conserved within the mammalian cytosolic GSTs. The numbering and secondary structure row (α - alpha helix; β - beta sheet) is based on the hA1-1 GST structure.

α - Alpha-class GST subunit hA178; π - Pi-class hP179; μ - Mu-class hM180; θ - Theta-class hT177.

Each GST isoenzyme has a fairly broad hydrophobic substrate specificity, with regards to both the nature of the functional group which includes carbon, sulphur and nitrogen based centres and the chemical structure of the compound. Essentially the H-site is a large hydrophobic pocket imposing few binding constraints. The salient characteristic for binding is hydrophobicity. However, the structural differences in the H-sites of different isoenzymes appear to influence substrate specificity. The characteristic C-terminal helix of alpha-class isoenzymes could be responsible for the high affinity of these enzyme for more hydrophobic ligands, such as cumene hydroperoxide and Δ²-androstene-3,17-dione possibly by increasing the hydrophobicity of the H-site. Alternatively the C-terminal helix may just shield the H-site from the aqueous environment of the cell allowing the alpha-class enzymes to have a more hydrophobic H-site without increasing the chance of aggregation. It was postulated by Sinning et al. that larger compounds could be partially accommodated at the H-site of alpha-class isoenzymes with some side chains protruding out into the solvent channel. However, the structure of hA1-1 GST in the absence of ligand shows that the C-terminal helix is flexible and becomes slightly distorted when ethacrynic acid or its glutathione conjugate are bound.
Figure 1.4 - The conformation of glutathione and residues involved in its binding in alpha-, mu-, pi- and theta-class GSTs (Adapted from Wilce et al. 1994 and 1995)

Alpha

Mu
(Figure 1.4 continued)
The diversity of the hydrophobic substrate has diverted the focus of mechanistic research towards the activation of the co-substrate, glutathione, which binds at an adjacent site to the hydrophobic substrate named the G-site. The G-site is structurally distinct and does not overlap with the H-site. In contrast to the hydrophobic substrate, the requirement for glutathione is stringent, with only a few structurally similar compounds, such as homoglutathione (γGlu-Cys-Ala), effectively replacing the co-substrate in the enzyme-catalysed conjugation reaction. Although the GSTs do appear to tolerate a range of glycyl-modified glutathione analogues, they are extremely sensitive to modifications affecting the cysteinyl residue and α-carboxy and α-amino groups of the γ-glutamyl residue. It is postulated that the integrity of the cysteinyl residue is essential for the correct orientation of the thiol group in the active site, maximising the efficiency of its activation. The specificity displayed regarding the α-carboxy and α-amino groups of the γ-glutamyl residue is probably because these two functional groups are involved in hydrogen bonds with the protein (Figure 1.4). Nearly all of the carboxyl and amino groups of glutathione hydrogen bond with amino acid residues of the protein, however only pi-class GSTs H-bond with all of these groups. Hence, the stringent specificity that GSTs show for glutathione peptidyl moiety may be because all bonds must be formed for the thiol donor to bind productively. Alpha-class GSTs have been shown to exert the greatest stringency for glutathione although the model of glutathione binding has four hydrogen bonds less than the model for binding of glutathione in pi-class GSTs (figure 1.4).

The literature concerning the structure-function relationship of the GSTs and their catalytic mechanism is extensive, however the exact mechanism is not fully understood. In all four classes of mammalian cytosolic GST a hydroxyl functional group of an amino acid residue near the N-terminus of the protein is positioned in proximity and is postulated to hydrogen bond to the bound glutathione thiol, either directly or through a water molecule, such that the thiol is activated. In mammalian alpha, mu, and pi class isoenzymes an evolutionary conserved tyrosine residue (tyrosine 9 in hA1-1 GST) is responsible for thiol activation and its mutation to phenylalanine severely impairs the catalytic activity of the enzyme. However, in theta-class isoenzymes the equivalent tyrosine does not appear to be positioned in the active site, but as a substitute the hydroxyl group serine 10 (theta-class numbering) is thought to activate the glutathione-thiol group. This is just one example of the differences between GST isoenzymes from different gene classes that have arisen since their genetic divergence, which was probably a result of a theta-gene duplication event. Although isoenzymes within the same gene class share over 70% sequence homology, between gene classes there is less than 30% homology, with theta-class isoenzymes showing most diversity.
The aromatic ring of S-benzyl glutathione is coloured red and the glutathione moiety turquoise, with the thiol in yellow. The position of phenylalanine 10 is highlighted in green in the apoenzyme and orange in the ligand bound form. The C-terminal helix can only be observed in the presence of ligand, possibly due to flexibility, and is shown in green.
Figure 1.6. - Enlarged region of figure 1.5 showing the active site more clearly.
In the presence of an inactive glutathione analogue the rat isoenzyme rM3-3 has been shown to conjugate the thiol donor 2-mercaptoethanol (figure 1.8) to 1-chloro-2,4-dinitrobenzene (CDNB). Hence, it appears that on binding to the glutathione analogue a conformational change occurs enabling the enzyme to activate CDNB. This is thought to be a function of tyrosine 116 which is involved in the activation of phenanthrene 9,10-oxide and the stabilisation of the Meisenheimer complex formed during glutathione conjugation to CDNB (figure 1.8). With 2-mercaptoethanol and an inactive glutathione analogue in place of glutathione the rate of catalysis was significantly reduced, probably because this type of conjugation reaction is independent of thiol activation. In fact this is one of the first experiments that shows the mechanism of GST catalysed conjugation involves enzymatic activation of the hydrophobic substrate and as this is dependent on the presence of the glutathione peptidyl moiety implies that the structural motifs affected by glutathione binding may be involved in the mechanism of hydrophobic substrate activation. However, the only structure of GST to be solved in the absence of ligand is that of hA1-1 GST and the conformational change induced by glutathione analogue binding principally involves the C-terminal helix, which is absent in mu-class isoenzymes. Further, although tyrosine 116 is conserved in pi-class isoenzymes, in
theta-class it is replaced by a tryptophan and in alpha-class a valine residue (V111 in figure 1.3). The mutation of this residue could imply that alpha- and theta-class enzymes do not activate the hydrophobic substrate or another residue in the H-site has this function. Either way, evolutionary divergence has resulted in differences in the molecular mechanism of the isoenzymes from different gene classes. Similar activation by other GST isoenzymes with different hydrophobic substrates has not yet been reported, hence this may be a phenomenon peculiar to mu-class isoenzymes and CDNB. CDNB is used as the standard reaction to monitor GST activity, as most isoenzymes have some affinity for it and conjugation can be easily followed spectrophotometrically (Figure 1.8).  

Some structural and mechanistic studies of hA1-1 GST have been published including 3D structures of this enzyme bound to a hydrophobic ligand (ethacrynic acid) or a glutathione conjugate. Further, the structure of hA1-1 has been published from  

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**Figure 1.8 - The conjugation of glutathione to 1-chloro-2,4-dinitrobenzene.**

![Diagram](image-url)
crystals grown in the absence of additional ligand, however a 2-mercaptoethanol dimer is
in the active site, indicating that this is not a true apoenzyme. Comparing these
structures, the only conformational change that appears to occur on ligand binding
involves phenylalanine 10 and the characteristic alpha-class C-terminal helix (helix 9
figure 1.3): The side chain of phenylalanine 10 undergoes a 90° rotation on ligand
binding and the electron density in the region of the C-terminal helix is "unintelligable" in
the absence of ligand, hence its position has not been defined. It is evident that it cannot
occupy the ligand bound position as it would sterically clash with the side chain of
phenylalanine 10 (figure 1.5). The C-terminal helix also affects the catalytic properties of
the enzyme, as shown by the decreased activity of a mutated enzyme with the terminal 12
amino acid residues deleted and the two new C-terminal residues mutated from aspartic
and glutamic acid to histidine and glycine respectively97. Further, the C-terminal helix
has been shown to be involved in substrate specificity98.

The kinetic analysis of mutated forms of hA1-1 GST has implicated methionine 208 as
being involved in hydrophobic substrate specificity99. This residue is positioned at the
base of the C-terminal helix, in the H-site. When ethacrynic acid binds at the H-site the
side chain of M208 has been shown to be displaced from the H-site such that the C-
terminal helix becomes distorted (figure 1.9). Interestingly ethacrynic acid is a poor
substrate but good competitive inhibitor of hA1-1 GST, hence the location of M208 or
the C-terminal helix may be determining factors in substrate specificity, influencing the
probability of productive binding. If the side chain of M208 is in proximity to hA1-1
GST substrates, it may have a similar function to tyrosine 116 in the mu-class
isoenzyme, stabilising the Meisenheimer complex or even activating the hydrophobic
substrate. The random mutation of M20899 did not reveal how this residue determines
substrate specificity, further no trends were observed between catalytic activity and the
nature of the mutation, hence mutagenesis does not appear to be an ideal technique for
probing the structure-function relationship of this residue.
Figure 1.9. Comparison of the H-site structure with either S-benzylglutathione or the ethacrynic acid-glutathione conjugate bound.

S-benzylglutathione (blue for the glutathione moiety, red for the benzyl moiety and the sulphur atom coloured yellow) is shown to pinpoint the location of the active site. The carbon backbone of the enzyme bound to the ethacrynic acid conjugate is shown in blue and that bound to S-benzylglutathione in green. The side chains of M208 and F10 are shown in ball-and-stick representation, in yellow for the S-benzylglutathione complex and green for the ethacrynic acid complex.
2. METHODS.

2.1. THE PRODUCTION AND PURIFICATION OF WILD-TYPE AND SPECIFICALLY MUTATED hA1-1 GST.

Table 2.1.1. The strains of E. coli used to produce hA1-1 GST.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chromosomal Markers</th>
<th>Amino acid auxotrophic requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1755</td>
<td>ara-137, tonA2, lacY1 or lacZ4, tss-5, supE44, gal-6, λ-, hisG1, rpsL8, 9 or 17, malA1 (λR), xyl-7, mtl-2, ilvA201, metB1, argH1, thi-1</td>
<td>Methionine, Histidine, Arginine.</td>
</tr>
<tr>
<td>PA340</td>
<td>thr-1, fhuA2, leuB6, lacY1, supE44, gal-6, λ-, gdh-1, hisG1, rfbD1, galP63, Δ(gltB-F)500, rpsL19, malT1 (λ), xyl-7, mtl-2, argH1, thi-1</td>
<td>Threonine, Leucine, Histidine, Arginine.</td>
</tr>
</tbody>
</table>

2.1.1. Transformation of E. coli cells.

The wild-type and specifically mutated GST cDNAs (see Acknowledgements on page ii) were sub-cloned into the pGWL11 plasmid as shown in Figure 2.1.1. This plasmid was then used to transform JM109 cells for amplification (see Acknowledgements on page ii). The plasmid was extracted using the Promega magic midiprep package and 10-100ng of plasmid DNA were added to a 100µl aliquot of competent cells in 40% glycerol (prepared according to Sambrook et al.100) defrosted on ice. The cells were then incubated for 30 minutes on ice. DNA uptake was induced by heat shock at 42°C for 90 seconds followed by a 1 minute recovery incubation on ice. An 800µl aliquot of LB (see Appendix 1) was added and the cells incubated for 30 minutes at 37°C. The suspension was centrifuged at 13000 rpm for 3 minutes in a bench top centrifuge and the pellet re-suspended in 200µl of LB before being spread on LB plates (see Appendix 1) containing 100µg/ml ampicillin. The plates were incubated overnight at 37°C and individual colonies were used to inoculate 10ml volumes of 2xYT, which were then incubated for 3 hours at 37°C and 220rpm in an orbital incubator. GST expression was verified (see Section 2.1.2.) and productive cells
were used to inoculate a further 10mls 2xYT broth to an O.D.\textsuperscript{595} of 0.1, read at 595nm on a Phillips PU 8620 UV/VIS/IR spectrophotometer in a 1ml path-length, plastic cuvette against similar broth which had not been inoculated (as for all optical density measurements of broths). The broth was incubated at 37°C until the O.D.\textsuperscript{595} had increased to about 0.5. The cells were chilled on ice for 1 hour and then 4.5mls of 80\% glycerol were added dropwise. 100\mu l aliquots were withdrawn and frozen in pre-chilled Eppendorf tubes on dry ice. The aliquots were stored at -80°C.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2}
\caption{The position of the GST cDNA when sub cloned into the pGWL11 plasmid.}
\end{figure}

2.1.2. Test for GST expression.

Two 5ml volumes of 2xYT broth were inoculated with an overnight culture of transformed \textit{E. coli} cells to an O.D.\textsuperscript{595} of 0.1 and then incubated at 37°C and 220 rpm until the O.D.\textsuperscript{595} had increased to approximately 0.5. GST expression was induced in one of the two volumes by addition of IPTG (Sigma) to a final concentration of 1mM. Both the induced and non-induced samples were incubated for a further 3 hours. The O.D.\textsuperscript{595} of the broth was measured and a volume of the suspension equivalent to 1ml with an O.D.\textsuperscript{595} of 0.5 was centrifuged at 13000 rpm for 5 minutes in a bench top centrifuge. The supernatant was discarded and the pellet was re-suspended in 100\mu l SDS gel loading buffer (see Appendix 1) and incubated in a water bath at 100°C for 5 minutes. The samples were loaded on to a 10\% acrylamide gel along side molecular weight markers of 14.2, 20.1, 24, 29, 36, 45 and 66kD. 100V were applied for 60 minutes in tris-glycine electrophoresis.
buffer (see Appendix 1). The gel was stained using warm comassie blue stain (see Appendix 1) for 20 minutes and destained for between 2 and 4 hours (see Appendix 1). The gels were dried on a SpeedGel SG200 (Savant) gel drier for 75 minutes at 55°C.

2.1.3. Production of hA1-1 GST by fermentation.

The production of GST by fermentation of transformed E. coli was adapted from the guidelines set by Dr Tony Simula (see Acknowledgements on page ii). 100μl of the transformed glycerol stock were used to inoculate 50ml of 2xYT broth in a 500ml conical flask which was incubated overnight at 37°C and 220rpm in an orbital incubator. This culture was used to inoculate two 500ml volumes of warm 2xYT broth in 2l shake-flasks to an O.D. of approximately 0.1. The 2l shake-flasks were incubated for two hours at 37°C and 220rpm after which the O.D.of the culture had increased to approximately 1.0. The production of the recombinant protein was induced by adding IPTG to a final concentration of 1mM, and the protein was harvested after a further 6 hours (see 2.1.4.).

GST selectively labelled with stable isotopes was produced by a similar fermentation except minimal media (see Appendix 1) with specific labelled amino acids (manufactured by GIL) substituted for their unlabelled counterparts (SL medium) was used in place of 2xYT. The yield of selectively labelled protein was optimised by inducing expression when the O.D. of the broth was 2.5 and by reducing the production phase to 4.5 hours.

Deuterated protein was produced by fermentation of transformed E.coli strains NM522 or DL39 in Celltone-D [Martek Biosciences Corporation]. Again, the yield of active protein was maximised by inducing GST production when the medium had an O.D. of 2.5, as little growth occurred after induction. The production period was extended to 10 hours before the protein was harvested as described below.

2.1.4. Purification of hA1-1 GST.

To minimise the denaturation of the recombinant GST, the harvest of the cells and the extraction and purification of the protein were performed in the presence of 1mM dithiothreitol (DTT) at 4°C where temperature control was possible and where it was not apparatus was pre-cooled and samples kept on ice.

The cells were separated from the fermentation broth by centrifugation at 10000rpm for 15 minutes using a JA-10 rotor in the Beckman J2-21 centrifuge. The supernatant was removed and the pellet re-suspended in two pellet volumes of 50mM Tris-HCl, pH 7.2, containing 1mM DTT (Tris buffer). The cytosol was released from the suspended cells using the Power Laboratory press [American Instrument Co., Inc.] and the cell debris was removed by centrifugation at 20000rpm for 1 hour in a Beckman J2-21 centrifuge, using a
JA-20 rotor. The volume of the supernatant (cytosol fraction) was recorded and the GST activity determined (Section 2.1.5.) from which the amount of active enzyme at this stage in the preparation was estimated, allowing the efficiency of the purification procedure to be monitored.

hA1-1 was extracted from the cytosol fraction using a 8.3x1.5cm glutathione-agarose (Sigma) affinity column. A column with these dimensions has the capacity to bind 0.175mmol (8.75g) of GST, about 40 times more than the largest GST yield however the excess binding capacity is necessary to extract nearly all of the GST from the sample and columns with less resin were not as efficient. The resin was prepared following the manufacturer’s guidelines and then equilibrated with at least 250ml of the Tris buffer. The cytosol was applied to the column with a flow rate of approximately 20ml/hour and was washed with Tris buffer until the absorbency of the eluate at 280nm was less than 0.01, when read against the same buffer [Phillips PU 8820 UV/VIS spectrophotometer] in a 1cm path-length, quartz cuvette. The retained hA1-1 GST was eluted with 45mls of 25mM disodium carbonate, pH 10.5. 10ml volumes of the eluate were collected in tubes containing 1ml of 200mM phosphate buffer, pH 6.0, with 1mM DTT. Again, the volume of the eluted fraction was recorded and the GST activity determined. The efficiency of the affinity chromatography purification was calculated from the final yield of GST as a percentage of the amount applied to the column, estimated from the GST activity in the fractions. The efficiency of elution was increased by adding 0.5M sodium chloride and 1mM DTT to the carbonate buffer and binding was enhanced by regenerating the column between runs by washing with 150ml of water, purging with 100mls 0.1M acetate buffer, pH 4.0, containing 1mM DTT and 0.5M sodium chloride, followed by another water wash before re-equilibrating with Tris buffer.

The hA1-1 GST fraction was either dialysed exhaustively against water containing 1mM DTT or 1% 2-mercaptoethanol, de-gassed and sealed against air, or the buffer was changed and the sample concentrated by ultra-filtration, using YM10 diaflo ultra filtration membrane [Amicon Ltd.). Where a more concentrated sample was required Centricon-10 concentrators were used to further reduced the solvent volume. For long term storage (up to three months) the protein was lyophilised and stored at -20°C as GST in solution has a short half-life.

A sample of lyophilised protein was sent for N-terminal sequence analysis and molecular weight determination by mass spectroscopy. Further the absorbency coefficient for the wild-type and truncated (with terminal 10 amino acid residues deleted) forms of hA1-1 GST were determined by quantitative amino acid analysis.

20
2.1.5. Activity assay.

50mM phosphate buffer (pH 6.5) was chilled on ice and used to dilute the concentrated GST or dissolve the lyophilised GST to a concentration of approximately 0.4µM active sites. The concentration was calculated from the absorbency of the GST solution at 280nm, measured against 50mM phosphate buffer using a quartz cuvette and using the absorbency coefficient for GST previously calculated by quantitative amino acid analysis. The activity was determined using the standard GST assay (1mM concentrations of CDNB and glutathione in 50mM phosphate buffer, pH 6.5 at 25°C) as described by Habig et al. on a Hewlett Packard diode array spectrophotometer. The rate of change in absorbency at 340nm with time was corrected for the spontaneous rate (in the absence of enzyme) and used to calculate the rate of GSDNP production in mol s⁻¹ (equation 2.1.5.1) from which the GST activity in the fraction was determined (equation 2.1.5.2).

Let:
- $v_f$ be the GST fraction volume (µl)
- $d$, the dilution factor
- $v_a$, the volume of the GST fraction added to the assay(µl)
- $v$, the assay volume (l)
- $A_p$, the absorbency coefficient of the product, GSDNP (9.6x10⁻⁴ M⁻¹cm⁻¹)
- $\Delta A_{340}$, the rate of change in absorbency at 340nm with time (cm⁻¹s⁻¹)

The rate of GSDNP production in the assay is:

$$\frac{\Delta A_{340} \cdot v}{A_p} \text{ mol s}^{-1}$$  \hspace{1cm} 2.1.5.1.

and the total rate of GSDNP production in the fraction (which is proportional to the amount of GST in the fraction) is:

$$\frac{\Delta A_{340} \cdot v \cdot d \cdot v_f}{A_p \cdot v_a} \text{ mol s}^{-1}$$  \hspace{1cm} 2.1.5.2.

The specific activity (s⁻¹) of A1-1 GST was calculated from the activity of a known concentration of protein by dividing equation 2.1.5.1. by the number of moles of GST in the assay mixture.
2.2. SYNTHESIS AND PURIFICATION OF GLUTATHIONE CONJUGATES.

Glutathione conjugates were prepared non-enzymatically by mixing equimolar amounts of glutathione and the hydrophobic ligand. The ligand was dissolved in the minimum amount of ethanol and then added drop wise to 50mM phosphate buffer pH 6.5 heated to approximately 50°C. An equimolar amount of glutathione dissolved in 5mls of water was added and the mixture incubated for 8 hours at 40°C. The resultant mixture was lyophilised and stored at -20°C. The conjugate was separated from the reactants by reverse phase chromatography. A 250x4.6mm Bio-Rad Hi-Pore reverse phase column was attached to a Gilson HPLC and using a flow rate of 0.5ml/min the column was equilibrated with 20mM ammonium acetate. The filtered sample, dissolved in the same buffer, was loaded and the column washed with this buffer for 10 minutes. The reactants and product were eluted by a gradient of 0-80% of the same buffer in acetonitrile over 25 minutes with a 5 minute wash of 20mM ammonium acetate in 80% acetonitrile. The elution times of the reactants were correlated to the chromatogram of the reaction mixture and the fractions containing the reactants identified accordingly. The fractions containing the product were identified by elimination and the identity confirmed by mass and NMR spectroscopy.

2.3. FLUORIMETRIC DETERMINATION OF THE DISSOCIATION CONSTANTS FOR LIGANDS BINDING TO GST.

Lyophilised GST was dissolved in 50mM phosphate buffer, pH 6.5 and kept on ice. The concentration was determined from the absorbency of the solution at 280nm and an aliquot was diluted to a 2.5ml volume (the sample) in a 1cm path-length fluorescence cuvette. The GST concentration of the sample was usually 0.75μM active sites, but was varied to optimise the fluorescence intensity and obtain a hyperbolic titration curve. A tryptophan reference solution was prepared by diluting tryptophan in 50mM phosphate buffer, pH 6.5 to a concentration which gives the same fluorescence intensity as the sample. 2.5ml of the reference solution were pipetted into a matched cuvette and 2.5-10μl aliquots of 1-100mM ligand solution, depending on the solubility in the phosphate buffer, were added to both cuvettes. The maximum change in volume was 9% on addition of hydrophobic ligands and 4% for other ligands. The aqueous solutions of the hydrophobic ligands used in the titration were prepared by dissolving the ligand in ethanol before dilution in phosphate buffer at 50°C, the final amount of ethanol being less than 1% w/v. The solutions were equilibrated to 25°C before addition to the sample. Using a Perkin Elmer Luminescence Spectrophotometer LS 50B, the wavelengths for excitation and emission were selected to obtain the maximum change in fluorescence due
to ligand binding at 25°C. With all ligands, the greatest degree of fluorescence quenching was achieved with an excitation wavelength of 280nm, recording fluorescence emission at 340nm. The excitation slit width was always 2.5nm to minimise photo decomposition and the emission slit width was varied to maximise the change in fluorescence intensity.

The effect of saturating amounts of glutathione on ligand binding was investigated by the addition of glutathione to a final concentration of 10mM (physiological concentration in the human liver) to both the reference and the sample cuvettes and then titrating the ligand as before. Stoichiometric titrations were also performed, using a 5-10μM GST solution, to make sure that the concentration of ligand binding sites was the same as the concentration of active sites, and therefore the ligand was not binding at additional sites on the protein. The dissociation constant and maximum fractional change in intrinsic protein fluorescence due to ligand binding were determined using equation A2.7 derived in Appendix 2. Each titration was repeated a minimum of four times.

Similarly, the dissociation constants for ANS in the presence and absence of glutathione were determined based on the enhancement of ANS fluorescence at 480nm on excitation at 370nm. ANS was titrated into the GST solution and a phosphate buffer reference solution and the comparative change in fluorescence analysed as a function of ANS concentration according to equation A2.15.

2.4. SPECTROPHOTOMETRIC DETERMINATION OF THE KINETIC CONSTANTS DESCRIBING GST CATALYSIS.

2.4.1. Determination of kinetic constants for the conjugation of hydrophobic substrates to glutathione.

The rate of the reaction catalysed by GST was determined spectrophotometrically, exploiting the change in absorbency of the hydrophobic ligand on conjugation to glutathione (section 2.1.5.). The rate was measured at various concentrations of one substrate with the other constant. The kinetic constants for CDNB, ethacrynic acid (EA) and 3,4-dichloronitrobenzene (DCNB) were determined with a 5mM glutathione concentration. In addition the kinetic constants for CDNB were determined using a 1mM glutathione concentration, as most published studies use this concentration. The kinetic constants for glutathione were determined with 1mM and 1.5mM CDNB. The latter is the upper limit of the solubility of this ligand in phosphate buffer. The maximum concentration of DCNB was also limited by solubility to 1.5mM. Although ethacrynic acid is more soluble than the other two ligands in aqueous solution, the upper limit of the concentration of this ligand for the assays (0.5mM) was limited by its absorption
properties at the assay wavelength (270nm). The assay was repeated a minimum of three
times at each concentration of ligand and all data points were analysed as a hyperbolic
function of substrate concentration using the Enzfitter PC package. The absorbency
coefficients (table 2.4.1.1) for the products of conjugation were taken from the published
literature.

Table 2.4.1.1 Absorbency coefficients for the glutathione conjugates as
described by Habig et al.

<table>
<thead>
<tr>
<th>Hydrophobic ligand</th>
<th>Absorbency coefficient of glutathione conjugate (mM⁻¹cm⁻¹)</th>
<th>Assay wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>9.6</td>
<td>340</td>
</tr>
<tr>
<td>EA</td>
<td>5.0</td>
<td>270</td>
</tr>
<tr>
<td>DCNB</td>
<td>8.5</td>
<td>345</td>
</tr>
</tbody>
</table>

2.4.2. Determination of kinetic constants for small thiol compounds to
hydrophobic substrates in the presence of a glutathione analogue.

Based on the observations of Principato et al., the conjugation of small thiol
compounds to hydrophobic ligands catalysed by GST in the presence of an inactive
glutathione analogue was investigated. All assays were performed in a 1cm path-length
cuvette with 50mM phosphate, pH 6.5 at 25°C. The solutions of hydrophobic ligand
were prepared as described in section 2.3.1. Maximum concentrations of 1.5mM CDNB
or 0.4mM ethacrynic acid were used as hydrophobic ligands, limited by solubility or by
absorbency of the substrate at the standard assay wavelength, respectively. The solutions
were equilibrated at 25°C and the glutathione analogue (S-methylglutathione or γGlu-Ala-
Gly) added to a maximum concentration of 15mM. DTT or 2-mercaptoethanol were used
as the thiol donors at concentrations up to 0.15M. 940μl of the hydrophobic ligand
and glutathione analogue solution were pipetted into 1ml path-length quartz cuvette. The thiol
donor was added, dissolved in a 50μl volume of phosphate buffer and the rate of
spontaneous conjugation monitored for 30-60 seconds before addition of GST in a 10μl
volume. The absorbency was monitored for a further 180-300 seconds and the rate of
conjugation was corrected for the spontaneous rate and the activity (s⁻¹) calculated. The
assay was repeated at least three times for each ligand concentration and all data points
used in the determination of the kinetic constants by analysing the rate of conjugation as a
hyperbolic function of substrate concentration using the Enzfitter PC package.
It was observed that GST catalysed conjugation only occurred in the presence of the small thiol donor, CDNB and the inactive glutathione analogue. The assay mixture was analysed by NMR spectroscopy. The absorbency coefficient for the conjugates were estimated by reacting a known amount of CDNB (2ml of between 0.05 and 0.1μM) in 50mM phosphate buffer pH 6.5 with an excess of the small thiol compound and measuring the absorbency of the resultant mixture against phosphate buffer at 340nm.

2.5. NMR SPECTROSCOPIC STUDIES OF hA1-1 GST

2.5.1. The assignment of the ligand spectra.

The 1-D proton spectrum of 1mM ligand solution, pre-saturated at the water frequency, was assigned by the chemical shifts and coupling constants of the signals, applying homonuclear decoupling where necessary, using a Bruker AX600 NMR spectrometer. The main assignment was carried out at 315K to resolve individual signals and the spectra at other temperatures were assigned by comparison.

2.5.2. GST sample preparation.

0.5mM (dimer) solutions of GST in 50mM phosphate buffer pH 6.5 containing 1mM deuterated DTT were prepared and centrifuged for 3 minutes at 13000rpm in a MSE microcentur bench top centrifuge at 4°C. A 450μl aliquot of the supernatant was transferred into a 5mm Wilmad NMR sample tube which was then flushed with argon and sealed using a NMR tube top and Nesco film. Deuterated phosphate buffer was used when the NMR sensitive nucleus of interest was either $^{13}$C or $^1$H and 10% deuterated buffer used for the observation of $^{15}$N nuclei. In some cases DTT was shown to interfere with the experiment and was omitted from the sample necessitating the use of strictly anaerobic conditions.

2.5.3. Protein resonance assignments.

One dimensional proton, inverse and direct detection $^{13}$C or $^{15}$N spectra (see chapters 5 and 6) were recorded to define the parameters for the 2-D spectra of wild-type hA1-1 GST selectively labelled by amino acids type. Further spectra were recorded for the protein in the presence of a 5-10 fold excess of GSDNP added in a 10μl volume of 50mM phosphate buffer, pH 6.5. In some cases a precipitate was formed on addition of the ligands. The samples were centrifuged and the 1-D proton and 2D spectra were recorded. Using the crystal structures of hA1-1 GST the resonances from the amino acids most affected by addition of ligand were tentatively assigned. Some assignments were then confirmed by comparing the spectra of the wild type protein, selectively labelled by amino acid type, to that of specifically mutated protein similarly labelled.
2.5.4. Detection of intermolecular NOEs.

Computer modelling was used to identify residues in proximity to the bound ligand and selectively labelled protein was produced. Inter- and intramolecular NOEs (see chapters 5 and 6) between protein and ligand protons in proximity were recorded, as described in section 5.1. Some NOEs were assigned to amino acid residues using mutagenesis as described above.

2.6. CRYSTAL STUDIES.

2.6.1. Crystal growth.

Crystals of the hAl-1 apoenzyme were grown using a protocol based on the vapour diffusion method as described by Cowan et al." Wells (of Linbrow tissue culture plates) were filled with 1.5ml of 50mM bistrispropane, pH 6.6-9.6 at 0.2 increments, containing 1% (v/v) 2-mercaptoethanol or 1mM DTT and PEG (polyethylene glycol) 3350 or 2000 (Sigma) from 12.5-27% (w/v) in 2.5% increments. 10μl hanging drops were prepared on 22 x 22mm plastic cover-slips by mixing 5μl of the appropriate well buffer with 5μl of a 30-80 mg/ml solution of GST Al-1 in 10mM Tris-HCl (pH 7.8).

The cover slips were inverted over the wells and sealed with silicone grease. The plates were left at room temperature or in temperature controlled incubators at 20, 21, 22.5 or 25°C and crystals were observed in some wells after 24-72 hours. Crystals of hAl-1 GST bound to various ligands were also prepared using the above protocol but adding an amount of ligand (GSDNP, GSCNP or glutathione) to the enzyme and well buffers equivalent to the concentration of active sites in the hanging drop, or at a 5- or 10-fold excess.

2.6.2. Ligand diffusion trials.

A 1μl volume of ligand dissolved in well buffer was added to hexagonal rod shaped apoenzyme crystals grown at 22.5°C in 20% PEG 2000 pH 7.6 such that the final concentration of ligand was either equivalent to the enzyme concentration in the hanging drop, a five-fold excess or a 10-fold excess. To another crystal, 1μl of well buffer was added as way of establishing a control. The cover slips were replaced over the wells and sealed. The plates were incubated after ligand addition at 22.5°C and the affects observed. Photographs were taken using a Kyowa model RFB-7 microscope fitted with a Praktica BC 1 camera loaded with a Kodak Ektachrome 160T professional colour slide film.
2.6.3. Diffraction analysis.

Crystals grown in the absence of ligand at pH 7.8 with 22.5% PEG 2000 using a 45mg/ml GST solution and glutathione-containing crystals grown at pH 7.8 with 20% PEG 2000 using a 20mg/ml enzyme solution were transferred into the well buffer and drawn into a 0.7mm diameter capillary tube. Excess liquid was removed from around the crystal and a small amount of well buffer placed at both ends of the tube before it was sealed with wax. The crystals were mounted in the X-ray beam with a crystal to detector distance of 100mm. Data were collected (see Acknowledgements page ii) using a Raxis IIc image plate system (Rigaku/Molecular Structure Corp.) mounted on a Rigaku RU200HB rotating anode generator with a copper anode, a nominal focus of 0.3 x 3.0mm and powered at 50KV, 100mA. A graphite monochromator was used to select CuKα (1.5418 Å) radiation. The measurement and reduction of data employed MOSFLM (V5.23; A.G.W. Leslie, LMB Cambridge, UK) and the CCP4 suite (1994).

Cryo-techniques were employed in an attempt to improve the resolution of the structure. Several compounds were tested as cryo-preservationes, with glycerol giving the most satisfactory results. A crystal was transferred to a solution with the same composition as that used for crystallisation except incorporating glycerol to a final concentration of 30% v/v. The crystal was then mounted in a rayon loop and flash-frozen to 100K and mounted in the X-ray beam. Data were collected as described above.
3. PRODUCTION AND PURIFICATION OF hA1-1 GST AND GLUTATHIONE CONJUGATES.

3.1. INTRODUCTION.

The cDNA encoding hA1-1 GST has been cloned and no differences between the native and recombinant protein could be detected. Hence, as the recombinant form was more readily available and easily purified to homogeneity it was used in these studies.

The cDNAs encoding both the wild-type (see Acknowledgements on page ii) and specifically mutated forms of hA1 (see Acknowledgements on page ii) were inserted into the pGWL11 plasmid, flanked by the IPTG inducible promoter, pTacl, and rrnB termination site. The pGWL11 plasmid carries ampicillin resistance genes, allowing the positive selection of transformed cells. The plasmid was used to transform JM109 E. coli cells and amplified, ready for extraction (see Acknowledgements on page ii).

3.2. PRODUCTION OF WILD-TYPE AND SPECIFICALLY MUTATED hA1-1 GST.

3.2.1. Transformation of E. coli strains used to produce GST by fermentation.

The pGWL11 plasmid carrying the wild type or specifically mutated hA1 cDNA was extracted from E. coli IM109 and used to transform several E. coli strains including NM522 enabling the efficient production of unlabelled protein for the determination of kinetic and dissociation constants. Also E. coli strains with auxotrophic requirements for some amino acids were transformed to express GST (see Table 2.1.1.) allowing the production of protein selectively labelled by amino acid type for assignment of NMR resonances. The transformed cells were selected by growth on ampicillin enriched plates and IPTG inducible GST expression was confirmed by gel electrophoresis (Figure 3.2.1.).

Lanes 1 and 6 show the separation of markers with molecular weights of 66, 45, 36, 29, 24 and 20.1kD as indicated on the left hand side of the gel. Lanes 2 and 3 show the E. coli cytosol before and after IPTG induction of wild-type GST expression and lanes 4 and 5 show the cytosol before and after IPTG induction of the expression of the truncated form of hA1-1 GST. In both cases one protein is clearly over expressed on induction with IPTG, migrating between the 24 and 29kD molecular weight markers and as this protein is extracted by glutathione affinity chromatography (lanes 7-9) it can be deduced that the E. coli strains show IPTG inducible expression of GST.

28
Figure 3.2.1.1. Typical gel showing the difference in protein expression in induced and non-induced transformed *E. coli* strains and the purified hA1-1 GST protein against molecular weight markers.

KEY.
Lane:
1 Molecular weight markers
Lanes 2-5 show the migration of the cytosol proteins of *E. coli* transformed to express either wild-type or the truncated hA1-1 GST before and after addition of IPTG as indicated below:
2 Wild-type before induction
4 Truncated hA1-1 GST before induction
6 Markers
8 Purified truncated GST

Table 3.2.1.1. The strains of *E. coli* transformed to express GST.

<table>
<thead>
<tr>
<th>GST type</th>
<th><em>E. Coli</em> Strain.</th>
<th>Selectively labelled protein produced.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type</td>
<td>NM522 DL39</td>
<td>Unlabelled 15N-Phe 15N-Tyr 15N-Leu 15N-Val 13C-Met 15N-Arg</td>
</tr>
<tr>
<td></td>
<td>AB1255 PA340</td>
<td></td>
</tr>
<tr>
<td>Y9F</td>
<td>DL39</td>
<td>15N-Tyr</td>
</tr>
<tr>
<td>L108Y</td>
<td>DL39</td>
<td>15N-Leu</td>
</tr>
<tr>
<td>M208D</td>
<td>PA13</td>
<td>13C-Met</td>
</tr>
<tr>
<td>F220Y</td>
<td>DL39</td>
<td>15N-Phe</td>
</tr>
<tr>
<td>F222Y</td>
<td>DL39</td>
<td>15N-Phe</td>
</tr>
<tr>
<td>ΔCT (terminal 10 amino acid residues deleted)</td>
<td>DL39</td>
<td>15N-Phe 15N-Leu 13C-Arg</td>
</tr>
</tbody>
</table>
3.2.2. Optimisation of the fermentation protocol.

The fermentation protocol was refined for each medium used, to maximise the yield of active protein. The O.D.595 of either NM522 or DL39 E. coli in 2xYT media increased from 1.0 to 4.3±1.5 after a 4 hour production period and to 5.8±0.8 after a further two hours, indicating that growth continued during the production phase. However, using either SL or Celltone-D media very little growth occurred after induction, hence as cell density is directly correlated to the yield of protein (see Table 3.2.2.1.) the yield was improved by inducing protein production when the cell density was between 2 and 3. Thus, the final O.D.595 of the 2xYT broth was greater than either the SL medium (2.5±1.0) or Celltone-D (3.7±0.5). The yield of active protein per litre of minimal medium was lower than that produced per litre of 2xYT however the productivity of the cells in each media was not the same. 26mg of GST were produced per O.D. unit (cm⁻¹) per litre of 2xYT over 6 hours, however using SL medium this was increased to 39mg. This indicates that although maximum cell density achieved in the SL medium appears to be reduced, the productivity of the cells is increased but not to such an extent that it compensates for the reduced growth in this media. The cell density and productivity differences between the two broths appeared independent of the strain of E. coli (either DL39 or NM522) and the same conclusions can be drawn by comparing the yields obtained with a four hour post-induction period. In summary, although the GST expression is higher in the SL medium it cannot support as high a cell density as 2xYT and therefore the yield of active protein from a litre of 2xYT broth is greater.

The yield of selectively labelled protein is further reduced to 78±18mg/l due to the shortened post-induction period adopted for producing labelled protein to minimise mis-incorporation of the label. The longer the production phase, the higher the probability that the E. coli will begin to catabolise the amino acids in the broth using the products for metabolism and re-synthesis of other amino acids. This becomes problematic when the labelled amino acids are degraded and the label is incorporated into another amino acid type, resulting in random labelling (mis-incorporation of the label) within the protein. Mis-incorporation of the label is also reduced by supplying a minimal amount of the labelled amino acid (see Appendix 1), such that there is only sufficient for anabolism and not catabolism. The intensity of the NMR signals can also be reduced by isotopic dilution, where unlabelled amino acids are incorporated into the protein reducing the proportion of labelling. This is minimised by using strains of E. coli that are auxotrophic for the amino acid type being labelled. The combination of the reduced production time and use of minimal medium reduces the yield of active protein to about 52% of that obtained for unlabelled preparations in 2xYT broth.
Site specifically mutated hA-l GST protein was produced in a similar way to the wild-type protein. The DNA constructs were prepared by Paul McDonough for his own studies, however as some mutants were of joint interest, with the mutated residues in proximity to the bound ligand according to the 3D crystal structure of the enzyme59, their availability was exploited for NMR spectroscopic analysis and some kinetic studies. The yield of protein per litre of fermentation broth depended on the mutation made (see Table 3.2.2.1.) but was independent of the E. coli strain used in the fermentation. Particularly low yields of active F220Y and truncated (terminal 10 amino acid residues deleted) proteins were noted, however it was later shown that these proteins have a lower stability compared to the wild-type protein, hence may have become denatured during the course of the production period. Yields of the other mutated forms of hA-l GST expressed in E. coli strain DL39 (Y9F, F222Y, M208D and L108Y) were comparable with the wild-type protein.

A lower growth rate and yield of protein were observed in fermentations using Celltone-D, a commercially available medium for the production of deuterated proteins. 72mg/l of deuterated wild-type GST were produced over a 10 hour production period, extended to compensate for the decreased metabolic rate of the E. coli in this medium, shown by the increased doubling time and reduced productivity.
Table 3.2.2.1. The relationship between the medium, its final O.D.\textsuperscript{595} and the yield of active GST.

<table>
<thead>
<tr>
<th>Broth</th>
<th>GST type</th>
<th>Post-Induction Period (hours)</th>
<th>Final O.D.\textsuperscript{595} of the broth (cm\textsuperscript{-1})</th>
<th>Yield of Active GST (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2xYT</td>
<td>WT</td>
<td>6</td>
<td>5.8±0.8</td>
<td>150±15</td>
</tr>
<tr>
<td>2xYT</td>
<td>WT</td>
<td>4</td>
<td>4.3±1.5</td>
<td>86±13</td>
</tr>
<tr>
<td>SL</td>
<td>WT</td>
<td>6</td>
<td>2.5±1.0</td>
<td>98±11</td>
</tr>
<tr>
<td>SL</td>
<td>WT</td>
<td>4</td>
<td>2.5±1.0</td>
<td>78±18</td>
</tr>
<tr>
<td>Celltone D</td>
<td>WT</td>
<td>10</td>
<td>3.2</td>
<td>72</td>
</tr>
<tr>
<td>2xYT</td>
<td>ΔCT</td>
<td>6</td>
<td>5.2±0.6</td>
<td>93±16</td>
</tr>
<tr>
<td>SL</td>
<td>ΔCT</td>
<td>4</td>
<td>2.8±0.8</td>
<td>52±14</td>
</tr>
<tr>
<td>Celltone D</td>
<td>ΔCT</td>
<td>10</td>
<td>4.1</td>
<td>22</td>
</tr>
<tr>
<td>SL</td>
<td>Y9F</td>
<td>4</td>
<td>2.4</td>
<td>79</td>
</tr>
<tr>
<td>SL</td>
<td>L108Y</td>
<td>4</td>
<td>3.2</td>
<td>88</td>
</tr>
<tr>
<td>SL</td>
<td>M208D</td>
<td>4</td>
<td>3.9±1.2</td>
<td>64±7.0</td>
</tr>
<tr>
<td>SL</td>
<td>F220Y</td>
<td>4</td>
<td>3.2±0.4</td>
<td>42±5.1</td>
</tr>
<tr>
<td>SL</td>
<td>F222Y</td>
<td>4</td>
<td>3.4±0.5</td>
<td>72±9.8</td>
</tr>
</tbody>
</table>
3.3 PURIFICATION OF hA1-1 GST.

The purification of hA1-1 GST was facilitated by the insignificant amounts of any native GST enzyme compared to the amount of hA1-1 over expressed in the E. coli strains used, shown by the inability of the cytosol to catalyse CDNB-glutathione conjugation before the induction of the recombinant GST production. Hence hA1-1 GST was extracted from the cytosol using a single affinity chromatography step (Section 2.1.4.) exploiting the pH dependence of the high affinity of the enzyme for the co-substrate glutathione. The same degree of purification for hA1-1 GST was consistently achieved, as determined from the specific activity of each protein batch (Table 3.3.2.). GST binding to the column was dramatically improved by modifying the protocol (section 2.1.4.) to include a column regeneration step between runs which involves purging the column with low pH buffer. This results in the protonation of the thiol groups of the immobilised glutathione, preventing cross-linking and the covalent binding of cytosol proteins. The low pH buffer will also denature any GST retained in the column, preventing contamination of future preparations which is particularly important when preparing selectively labelled proteins.

The elution of the bound protein was enhanced by adding 0.5M sodium chloride to the elution buffer, resulting in a consistent yield of 90% of the active protein with both wild-type hA1-1 and most mutated derivatives. However, for the truncated and F220Y proteins the protocol was refined by altering the loading buffer to pH to 7.1 and eluting at 10.8 to yield more than 70% of the truncated enzyme and 50% of the F220Y mutant. The cytosol containing F220Y GST was applied repeatedly to the column to extract 80% of the total GST content.

The purity of the protein was assessed by gel electrophoresis and by calculating the specific activity of recombinant protein. Figure 3.2.1.1. shows typical gel produced by the method in section 2.1.2., with the purified wild-type and truncated forms of hA1-1 loaded along with the cytosol from which they were extracted before and after induction, and molecular weight markers. As only one band is shown in the lanes where the purified protein was loaded the sample appears to be homogeneous. Table 3.3.1. shows the specific activity calculated for wild-type hA1-1 GST as 26.9μmol s⁻¹ mg⁻¹ which similar to the published value of 26.3μmol s⁻¹ mg⁻¹. The consistency of the value of the specific activity determined for the wild-type protein indicated that the same degree of purification was achieved repeatedly. Mass spectroscopic analysis of the wild-type and truncated proteins indicates that a single species of the expected molecular weight was present in each sample (Table 3.3.2). Thus for both mutated and wild-type hA1-1 GST a homogeneous protein was obtained.
Table 3.3.1. The specific activity of wild-type GST.

<table>
<thead>
<tr>
<th>pmoles GST in assay</th>
<th>SA (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1</td>
<td>22.0</td>
</tr>
<tr>
<td>6.8</td>
<td>27.9</td>
</tr>
<tr>
<td>5.1</td>
<td>28.4</td>
</tr>
<tr>
<td>4.1</td>
<td>27.3</td>
</tr>
<tr>
<td>2.0</td>
<td>24.3</td>
</tr>
<tr>
<td>1.0</td>
<td>29.7</td>
</tr>
<tr>
<td>0.5</td>
<td>28.7</td>
</tr>
<tr>
<td><strong>Average SA (s⁻¹)</strong></td>
<td><strong>26.9±2.5</strong></td>
</tr>
</tbody>
</table>

Table 3.3.2. Mass spectroscopic analysis of hA1-1 GST.

<table>
<thead>
<tr>
<th>Run</th>
<th>Wild-type</th>
<th>Truncated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 496.6</td>
<td>24 206.6</td>
</tr>
<tr>
<td>2</td>
<td>25 492.8</td>
<td>24 204.5</td>
</tr>
<tr>
<td>3</td>
<td>25 496.4</td>
<td>24 203.4</td>
</tr>
<tr>
<td>4</td>
<td>25 494.3</td>
<td>24 211.8</td>
</tr>
<tr>
<td>5</td>
<td>25 494.1</td>
<td>24 207.3</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>25 494.8±1.6</strong></td>
<td><strong>24 206.7±3.2</strong></td>
</tr>
</tbody>
</table>

Actual molecular weight as determined from the primary structure.

|               | 25 503.9 | 24 195.2 |
The N-terminal sequence analysis of hA1-1 GST shows that the recombinant protein, produced by fermentation of the transformed E.coli cells and extracted by affinity chromatography had the same N-terminal sequence as the native hA1-1 GST. Further analysis of the recombinant protein has been published by Board and Pierce (1987) showing that the subunits dimerise and the protein retains both GST and glutathione peroxidase activities. The recombinant enzyme also has a similar pi and immunological identity as the native protein. Hence, as the recombinant form of hA1-1 GST appears to be similar to the native enzyme it can be used as a model of the native enzyme to facilitate characterisation.

<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Residue</th>
<th>Amount (pmoles)</th>
<th>Expected residue determined by Sinning et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ala</td>
<td>737.47</td>
<td>Ala</td>
</tr>
<tr>
<td>2</td>
<td>Glu</td>
<td>618.16</td>
<td>Glu</td>
</tr>
<tr>
<td>3</td>
<td>Lys</td>
<td>736.57</td>
<td>Lys</td>
</tr>
<tr>
<td>4</td>
<td>Pro</td>
<td>338.47</td>
<td>Pro</td>
</tr>
<tr>
<td>5</td>
<td>Lys</td>
<td>516.24</td>
<td>Lys</td>
</tr>
<tr>
<td>6</td>
<td>Leu</td>
<td>376.88</td>
<td>Leu</td>
</tr>
<tr>
<td>7</td>
<td>His</td>
<td>273.39</td>
<td>His</td>
</tr>
<tr>
<td>8</td>
<td>Tyr</td>
<td>376.51</td>
<td>Tyr</td>
</tr>
<tr>
<td>9</td>
<td>Phe</td>
<td>293.64</td>
<td>Phe</td>
</tr>
<tr>
<td>10</td>
<td>Asn</td>
<td>270.63</td>
<td>Asn</td>
</tr>
</tbody>
</table>
Table 3.3.5.- The absorbency coefficients determined for the wild type and truncated enzymes by amino acid hydrolysis and quantification are shown along side those estimated for the other mutants by correcting the wild-type coefficient for changes in amino acid residues that contribute to absorbency at 280nm (shown in italics).

<table>
<thead>
<tr>
<th></th>
<th>Number of Tyrosine residues</th>
<th>Absorbency Coefficient for the concentration of subunits (M⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type</td>
<td>10</td>
<td>25 100</td>
</tr>
<tr>
<td>Truncated</td>
<td>10</td>
<td>49 051</td>
</tr>
<tr>
<td>L108Y</td>
<td>10</td>
<td>26 400</td>
</tr>
<tr>
<td>F220Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F222Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M208D</td>
<td>10</td>
<td>25 100</td>
</tr>
<tr>
<td>Y9F</td>
<td>9</td>
<td>23 700</td>
</tr>
</tbody>
</table>

In order to characterise the protein, its concentration must be accurately measured hence the absorbency coefficient for the purified wild-type hA1-1 GST was determined by quantitative amino acid analysis as 0.984mg⁻¹ml⁻¹ (25.087M⁻¹) and for the truncated mutant 2.027mg⁻¹ml⁻¹ (49.051M⁻¹). Thus the deletion of the C-terminal helix increases the absorption properties of the enzyme. Although this was not expected, the quality of the NMR spectra at similar concentrations of the wild-type and truncated enzymes determined using these absorbency coefficients indicate that these values are correct. The absorbency coefficients for other mutants were estimated by correcting the wild-type absorbency coefficient for changes in the phenylalanine or tyrosine content of the protein using the method of Gill and Von Hippel\textsuperscript{103}, and are shown in Table 3.3.5.
3.4. PRODUCTION OF GLUTATHIONE CONJUGATES.

The GST ligands used in these studies include glutathione, hydrophobic substrates (CDNB and DCNB), and the products of their conjugation (GSDNP and GSCNP, respectively). Although the substrates are available commercially the conjugates are not, but were easily synthesised non-enzymatically giving a yield of over 95%. The conjugates were purified to homogeneity using reverse phase HPLC before use (Section 2.2.) and analysed by mass and NMR spectroscopy.

Figure 3.4.1. - Typical elution profile for the purification of GSDNP or GSCNP by HPLC.

On the left hand side of the trace are two axis: The change in O.D. at 340nm which corresponds to the solid black line, and the "% mobile phase" which indicates the amounts of 20mM ammonium acetate (red) and this buffer in 80% acetonitrile (green) being passed though the column.
The reverse phase chromatogram of the reaction mixture used to synthesise the glutathione conjugates has two main absorption peaks at 340nm: One occurring in the gradient and the other when 100% buffer B (20mM ammonium acetate in 80% acetonitrile) was reached. As glutathione does not absorb intensely at 340nm, the two components of the reaction mixture detected are the conjugate and the hydrophobic ligand. Similar HPLC of the latter shows it is eluted with a gradient of 100% buffer B and therefore the conjugate is likely to be the component eluted in the gradient. Both fractions were collected and lyophilised. The identity of the gradient fraction was confirmed as the conjugate by mass and NMR spectroscopy, the results of which are shown below.

**Figure 3.4.2. - The reverse phase chromatogram of CDNB.**

The absorbency of the eluate at 340nm is shown by the black solid lines and the composition of the buffer with time is shown by the red and green lines, representing 20mM ammonium acetate and 20mM ammonium acetate in 80% acetonitrile, respectively.
Figure 3.4.3. - The mass spectrum of non-enzymatically synthesised GSCNP purified by HPLC.

The expected molecular weight of this conjugate is 463g/mol.

Figure 3.4.4. - The mass spectrum of the non-enzymatically synthesised GSDNP conjugate.

The expected molecular weight of GSDNP is 474g/mol. A large amount of thioglycerol is detected in this spectrum, which is a component of the solvent used in the mass spectroscopy.
Figure 3.4.5. - The NMR spectrum of non-enzymatically produced, HPLC purified GSCNP.
The resonance assignments were made as described in Section 5.3.

Figure 3.4.6. - The NMR spectrum of non-enzymatically produced, HPLC purified GSDNP.
The resonance assignments were made as described in Section 5.3.
3.5. THE ABSORBENCY COEFFICIENTS OF THE NON-GLUTATHIONE CONJUGATES USED FOR THE DETERMINATION OF THE RATE OF CONJUGATION.

The absorbency coefficients for the conjugates of CDNB to a range of thiol compounds were determined by reacting a known amount of CDNB with excess thiol and measuring the absorbency of the resultant mixture (section 2.2). In all cases the thiol conjugation had the greatest affect on the absorbency at 340nm, compared to that of CDNB. The absorbency coefficients for the conjugates determined at this wavelength showed a low standard deviation and that determined for GSDNP compared closely to the published value\(^4\). (see Table 3.5.1.). Hence it can be assumed that the absorbency coefficients determined by this method are reasonably accurate.

Table 3.5.1. The estimated absorbency coefficient for conjugates of CDNB and various thiol compounds at 340nm.

<table>
<thead>
<tr>
<th>Thiol compound</th>
<th>Absorbency coefficient (\text{M}^{-1}\text{cm}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>((8.4\pm0.3)\times10^3)</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>((8.2\pm0.2)\times10^3)</td>
</tr>
<tr>
<td>γ-Glu-Cys</td>
<td>((6.2\pm0.6)\times10^3)</td>
</tr>
<tr>
<td>Cys-Gly</td>
<td>((9.7\pm0.2)\times10^3)</td>
</tr>
<tr>
<td>Glutathione (control)</td>
<td>((9.5\pm0.4)\times10^3)</td>
</tr>
</tbody>
</table>
4. DETERMINATION OF LIGAND DISSOCIATION AND KINETIC CONSTANTS FOR WILD-TYPE AND SPECIFICALLY MUTATED FORMS OF hA1-1 GST.

4.1. THE SPECIFICITY OF SUBSTRATE BINDING AND CATALYSIS AT THE G-SITE.

An insight into the substrate specificity at the G-site has been obtained by determining the kinetic and dissociation constants for the co-substrate, glutathione, and fragments of this molecule, namely γ-Glu-Cys and Cys-Gly. Further, the ability of small thiol compounds, such as 2-mercaptoethanol and DTT, to replace glutathione as the thiol donor in the enzyme-catalysed reaction has been investigated. The dissociation constants were determined from the affect of ligand additions on the intrinsic tryptophan fluorescence at 340nm on excitation at 280nm (section 2.3.). hA1-1 GST has a single tryptophan residue, which has been shown from the crystal structure to be in domain I, approximately 17Å from the active site. Ten tyrosine residues are distributed throughout the protein, with tyrosine 9, which is thought to be involved in activating the thiol group of glutathione, positioned in proximity to the active site (see figure 4.1.1). On ligand binding the tryptophan fluorescence was quenched, hence the dissociation constants were determined by analysing this effect, corrected for any quenching induced by non-binding effects, as a quadratic function of the concentration of ligand (see Appendix 2).

The kinetic constants for thiol compounds were determined using spectrophotometric assays similar to those described by Habig et al\textsuperscript{84} (section 2.4.2.) The standard assay for GST isoenzymes, based on the conjugation of CDNB to glutathione, was modified to probe the substrate specificity at the G-site, by replacing glutathione with alternative thiol donors including glutathione fragments, γ-Glu-Cys and Cys-Gly, and thiol compounds with no structural similarities to glutathione (2-mercaptoethanol and DTT). The reaction was monitored spectrophotometrically and the rate of the reaction determined from the increase in absorbency at 340nm using the absorbency coefficients for the respective conjugates listed in table 3.3.1.
Figure 4.1.1. The distribution of the principal amino acid residues that contribute to the intrinsic fluorescence of hA1-1 GST.

The green ribbon represents the \(\alpha\)-carbon backbone of hA1-1, with the tyrosine (yellow) and tryptophan (orange) residues shown in full in ball-and-stick fashion. The position of the active site is shown by the location of the ligand S-benzylglutathione coloured red for the hydrophobic moiety and blue for glutathione and the thiol group is highlighted in yellow.
As the initial rate of the reaction was used to determine the kinetic constants it can be assumed that the reverse reaction, that is the formation of thiol donor and hydrophobic substrate from the conjugate, does not occur and therefore the reaction profile is summarised by equation 4.1.1.a., where $k_1$ is a rate constant describing the binding of the substrate (S) to the enzyme (E) to form the enzyme-substrate complex (ES) and $k_{-1}$ is a rate constant describing the reverse process. The turnover number $k_{cat}$ is collective for all rate constants describing the conversion of the enzyme-substrate complex to free enzyme and product, and also defines the maximal catalytic rate when the substrate is saturating.

\[
[E]_0 + [S] \xrightleftharpoons[k_{-1}][k_1] [ES] \xrightarrow{k_{cat}} [E] + [P] \quad 4.1.1.a.
\]

$[E]$, $[S]$ and $[P]$ symbolise the concentration of free enzyme, substrate and product, respectively, and $[ES]$ is the concentration of the enzyme bound to substrate.

Assuming that steady-state kinetics apply, that is the concentrations of the intermediates in the reaction pathway stay constant while the substrate is converted to product, the rate of formation and breakdown of ES will be the same, and therefore:

\[
k_1[E][S] = (k_{cat} + k_{-1})[ES] \quad 4.1.1.b.
\]

In this situation the Michaelis-Menten constant ($K_M$) can be used to summarise the relationship of the kinetic constants that describe the reaction pathway, as shown in equation 4.1.1.c.

\[
K_M = \frac{k_{-1} + k_{cat}}{k_1} = \frac{[E][S]}{[ES]} \quad 4.1.1.c.
\]

In all of the kinetic assays performed the concentration of enzyme was significantly smaller than the concentration of substrate, thus the concentration of free substrate is approximately equal to the total substrate concentration allowing the rate of enzyme catalysed reaction ($v$) to be described by a hyperbolic function of the substrate concentration commonly known as the Michaelis-Menten equation (4.1.1.d.).

\[
\frac{v}{k_{cat}} \frac{[S]}{[S] + K_M} \quad 4.1.1.d.
\]

The rate of enzyme-catalysed thiol conjugation at a constant concentration of the hydrophobic substrate was analysed as a function of the concentration of the thiol compound using equation 4.1.1.d. and the Enzfitter PC package to determine the kinetic
constants, $K_M$ and $k_{cat}$. Further, using the kinetic constants and the dissociation constant ($K_d$) for the thiol compounds the values of the rate constants $k_1$ and $k_{-1}$ could be estimated by rearrangement of equation 4.1.1.e. and from equation 4.1.1.f.

\[
K_d = \frac{k_{-1}}{k_1} \quad 4.1.1.e.
\]
\[
k_1 = \frac{k_{cat}}{K_M \cdot K_d} \quad 4.1.1.f.
\]

Glutathione was the most efficient thiol donor, with a turnover number of 53 s\(^{-1}\) (table 4.1.1.) at a 1.5mM CDNB concentration. CDNB is not saturating at this concentration, but is close to the upper limit of its solubility in an aqueous solution. At 1mM CDNB the $k_{cat}$ was reduced by 9\% (48s\(^{-1}\);table 4.1.2.), indicating that within these limits the turnover number is still significantly dependent on the concentration of CDNB. The value of $k_{cat}$ if CDNB was saturating was deduced by extrapolation as 74s\(^{-1}\), using the Michaelis-Menten equation and the $K_M$ for CDNB (0.53mM - See section 4.3.3.). The $K_M$ values determined for glutathione to hA1-1 GST are similar at both CDNB concentration (0.32mM).

As the $K_M$ for glutathione is 50 times less than the physiological concentration of glutathione in the human liver, where hA1-1 GST is particularly abundant, the enzyme will be 97\% saturated in this tissue and thus the enzymatic rate of conjugation will be dependent on the binding of the hydrophobic substrate. This is an important feature of catalysis for the function of the GST isoenzymes. Glutathione saturation would imply that conjugation is not limited by the availability of this substrate. Being detoxification enzymes it is most advantageous if the rate of conjugation is limited only by the frequency by which the hydrophobic substrate is encountered, maximising the potential for conjugation and thereby maximising the detoxification potential of the enzymes.
Table 4.1.1. The kinetic constants for glutathione determined at 1.5mM CDNB.

<table>
<thead>
<tr>
<th>Protein Batch</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55±2.6</td>
<td>0.29±0.03</td>
</tr>
<tr>
<td>2</td>
<td>60±1.3</td>
<td>0.31±0.13</td>
</tr>
<tr>
<td>3</td>
<td>47±2.5</td>
<td>0.37±0.09</td>
</tr>
<tr>
<td>4</td>
<td>56±0.9</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>5</td>
<td>46±1.4</td>
<td>0.34±0.07</td>
</tr>
<tr>
<td>Average</td>
<td>53±5.4</td>
<td>0.32±0.03</td>
</tr>
</tbody>
</table>

Figure 4.1.2. - The relationship between GSDNP production (s$^{-1}$) and glutathione concentration at 1mM CDNB.
Table 4.1.2. The kinetic constants determined for glutathione conjugation to 1mM CDNB.

<table>
<thead>
<tr>
<th>Protein Batch</th>
<th>$k_{cat} \text{ (s}^{-1}\text{)}$</th>
<th>$K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50±2.2</td>
<td>0.36±0.07</td>
</tr>
<tr>
<td>2</td>
<td>45±0.6</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>3</td>
<td>47±1.2</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>4</td>
<td>51±1.0</td>
<td>0.31±0.05</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>48±2.4</td>
<td>0.32±0.03</td>
</tr>
</tbody>
</table>

The specificity constant ($k_{cat}/K_M$) for glutathione at 1.5mM CDNB was calculated as $1.65 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ and if CDNB were saturating the specificity constant was estimated as $2.31 \times 10^5 \text{M}^{-1}\text{s}^{-1}$. In both cases the specificity constant is three to four orders of magnitude less than diffusion limited catalysis.

Figure 4.1.3 - Raw data showing the change in fluorescence in the sample and the reference cuvettes with successive additions of glutathione.
Comparing the effect of glutathione addition on the fluorescence of the GST sample and tryptophan reference (figure 4.1.3.) it can be deduced that although there is little effect on the reference fluorescence, indicating that glutathione does not create an inner filter effect, the fluorescence of the protein is quenched. Thus it appears that glutathione binding to hA1-1 GST alters the fluorescence properties of the tryptophan residue, either directly or indirectly, by altering the fluorescence properties of tyrosine residues that are involved in energy transfer to the tryptophan residue (see figure 4.1.1.). Tyrosine 9 is the most likely residue to be involved in the fluorescence quench as it is postulated to hydrogen bond to the thiol group of glutathione and structural changes occur in proximity to this residues when glutathione binds to the protein. Further, this residues is about 16Å from the tryptophan residue which is within the distance range for energy transfer to occur.

**Figure 4.1.4. The stoichiometric titration of glutathione into 1.0μM solution of hA1-1 GST subunits.**

The actual concentration of glutathione binding sites can be deduced as approximately 1μM.

From the stoichiometric titration (figure 4.1.4.) the concentration of glutathione binding sites was deduced as 1.03μM, equal to the concentration of G-sites (1.0μM) and therefore it was concluded within the concentration range used (0.06-18μM) glutathione binds to a single site on each hA1-1 GST subunit. By reducing the concentration of GST to between 0.2-0.3μM active sites a quadratic relationship between the corrected fluorescence intensity and the concentration of glutathione was obtained allowing the dissociation constant for hA1-1 GST binding to glutathione to be determined using
equation A2.7 (Appendix 2), the results of which are shown in table 4.1.3. Each titration was performed on a freshly made GST solution the concentration of which was determined from its absorbency at 280nm and is quoted in table 4.1.3. as the concentration of subunits rather than that of the dimer. The magnitude of this fluorescence change (13% of the initial fluorescence intensity) and the accuracy of the curve fit described by equation A2.7 (figure 4.1.5.) allows the dissociation constant for glutathione to be determined with a good degree of accuracy.

The dissociation constant was determined as 0.25μM (table 4.1.3.) indicating that the enzyme has a high affinity for glutathione. Interestingly the dissociation constant is three orders of magnitude less than $K_M$ (0.32mM), implying that glutathione binding is not the rate limiting step in catalysis. Once formed the enzyme-substrate complex can dissociate into enzyme and product or reform the substrate, depending on the ratio of $k_{cat}$ and $k_i$. In this case $k_i$ is much smaller than $k_{cat}$ and therefore product formation is favoured. This is an important aspect of GST catalysis, as obviously in vivo it is preferable that conjugation is maximised to minimise the toxic effects of the hydrophobic substrate.

Table 4.1.3. - The dissociation constant and maximum change in fluorescence for glutathione binding to hA1-1 GST.

<table>
<thead>
<tr>
<th>[GST] (μM)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta F$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.54</td>
<td>0.20±0.07</td>
<td>15±0.6</td>
</tr>
<tr>
<td>0.62</td>
<td>0.21±0.16</td>
<td>16±0.6</td>
</tr>
<tr>
<td>0.27</td>
<td>0.27±0.04</td>
<td>10±0.5</td>
</tr>
<tr>
<td>0.37</td>
<td>0.25±0.13</td>
<td>12±1.0</td>
</tr>
<tr>
<td>0.54</td>
<td>0.31±0.13</td>
<td>13±1.0</td>
</tr>
<tr>
<td>0.32</td>
<td>0.28±0.13</td>
<td>13±1.0</td>
</tr>
<tr>
<td>0.55</td>
<td>0.24±0.02</td>
<td>12±0.8</td>
</tr>
<tr>
<td>Average</td>
<td>0.25±0.04</td>
<td>13±1.9</td>
</tr>
</tbody>
</table>
The dissociation constant for glutathione to hA1-1 GST is two orders of magnitude less than the published values for glutathione binding to rat mu-class isoenzymes rM3-3 and rM4-4 of 21±4 and 24±4 μM\(^4\) respectively. There are several explanations for these differences: Firstly GST isoenzymes exhibit different stringencies for the thiol donor, hence they may also display different affinities for glutathione binding. Interestingly alpha-class isoenzymes exhibit the most stringent requirement for glutathione compared to isoenzymes from mu and pi gene classes\(^8\). Perhaps the ability of some isoenzymes to accept a wider range of substrates as thiol donors is accompanied by a decreased affinity for the natural co-substrate glutathione. Secondly, the published dissociation constants were deduced by analysing the fluorescence titration curves as hyperbolic functions of glutathione concentration, assuming that the concentration of bound substrate is much less than the free substrate. However, in the early stages of the titration this is not true as the concentration of enzyme in these assays was 26μM active sites and is therefore in excess of the substrate. Hence, the titration curves are best described by a quadratic function (Appendix 2). Nearly all published values of \(K_M\) for glutathione with mu-, pi- and alpha-class enzymes fall in the region of 0.2mM, regardless of the isoenzyme being studied\(^5\). Even with a dissociation constant only one order of magnitude less than the \(K_M\), it can still be deduced that although the difference between the “on” and “off” rate is
not so large, product formation is favoured from the enzyme-substrate complex rather than breakdown to reform the substrates.

hAl-1 GST showed a significantly reduced rate of conjugation with thiol compounds alternative to glutathione. With structurally distinct small thiol groups such as DTT or 2-mercaptoethanol, which could theoretically fit into the G-site, the presence of the enzyme did not appear to increase the rate of spontaneous conjugation, indicating that these compounds do not serve as thiol donors in the two-substrate enzyme catalysed conjugation reaction. Thus the peptidyl moiety of glutathione is important in defining the suitability of the thiol substrate. Further the small thiol compounds did not affect the fluorescence of hAl-1 hence their dissociation constants could not be determined. Similar observations were made on titrating inactivate glutathione analogues, S-methylglutathione and dethioglutathione (γ-L-Glu-L-Ala-Gly) into a GST solution. As the glutathione analogues have been shown to induce similar changes to glutathione in the 1-D proton spectra of hAl-1 GST, it is assumed that they bind to hAl-1 as glutathione does. The hypothesis that S-methylglutathione binds to hAl-1 GST similarly to glutathione is further supported by the kinetic experiments in Section 4.2. where S-methylglutathione is used to kinetically activate the enzyme. Thus, although there is a negligible fluorescence change when inactive glutathione analogues bind to hAl-1 GST, it is likely that they do indeed bind. Similarly, although small thiol compounds do not induce a fluorescence change they may also bind to hAl-1 GST. That the fluorescence quench induced by binding of the inactive glutathione analogue is not comparable to that of glutathione indicates that latter is a function of the accessible thiol group and not the peptidyl moiety.

Figure 4.1.6. - The quadratic curve (equation A2.7) describing the change in fluorescence intensity as a function of Cys-Gly concentration.
Further support for the hypothesis that the thiol group of glutathione is responsible for the change in tryptophan fluorescence is given from the results of the characterisation of glutathione fragment (γ-Glu-Cys and Cys-Gly) binding. Both glutathione fragments induce a fluorescence change of 5%, less than that induced by glutathione but more than that induced by the inactive glutathione analogues. Perhaps the postulated H-bond from the hydroxyl group of tyrosine-9 to the thiol group induces the changes in the fluorescence yield of tryptophan by energy transfer. As the glutathione fragments do not induce the same fluorescence change as glutathione, the complete peptidyl moiety of glutathione appears to be required to attain the maximum change in fluorescence possibly because the peptidyl moiety is required to position the thiol in the active site. Although the fluorescence is quenched by only 5% on binding the glutathione fragments dissociation constants can still be determined from the titration, although with a reduced degree of accuracy compared to those for glutathione.

hAl-1 GST exhibits a high affinity for both glutathione fragments, with the dissociation constants being in the μM range, the same order as that for glutathione itself (table 4.1.6.). The affinity of the enzyme for the glutathione fragments is slightly reduced compared to that of glutathione but the changes in free energy on binding glutathione or the fragments (ΔG) are still similar. From the crystal structure of hAl-1 GST with S-benzylglutathione bound, it is postulated that the γ-Glutamyl moiety of the ligand is involved in at least six hydrogen bonds and the glycy moiety around four (see figure

Figure 4.1.7. The direct affect of Cys-Gly concentration on the fluorescence intensity of the tryptophan reference and hAl-1 GST sample.
1.4). Hence as the difference between the values of $\Delta G$ deduced for glutathione and either of its fragments binding to hA1-1 GST is only equivalent to one hydrogen bond it appears that the glutathione fragments form additional hydrogen bonds when bound at the G-site.

The $K_m$ values for glutathione and both fragments are similar, between 0.32 and 0.37mM, thus there is little difference in the concentration of the thiol compound required to half saturate the enzyme at 1.5mM concentration of CDNB. Using equations 4.1.1, it is possible to deduce the “on” and “off” rates for each substrate and compare these to the turnover number. Both glutathione fragments have a decreased “on” rate, compared to glutathione. Once formed, the fate of the enzyme-substrate complex is defined by the ratio of turnover number and the “off” rate. With all thiol compounds the turnover number is substantially larger than the “off” rate but the magnitude of these differences is reflected in the suitability of the thiol compound for the conjugation reaction: The turnover number for glutathione is three orders of magnitude greater than the “off” rate, for $\gamma$-Glu-Cys the turnover number is two orders of magnitude greater and Cys-Gly there is one order of magnitude difference. Thus although product formation from the enzyme-substrate complex is favoured in all cases, the percentage of enzyme-substrate complex converted to product depends on the thiol compound as follows: glutathione > $\gamma$-Glu-Cys > Cys-Gly. This same order defines the comparative suitability of the three thiol compounds as substrates in the glutathione conjugation reaction. The free energy of activation ($\Delta G^\circ$) can be estimated from the specificity constants and this value is shown in table 4.1.6. for all three thiol donors. The magnitude of $\Delta G^\circ$ is similar for both glutathione fragments, but approximately half that for glutathione. However, the difference of between 3.2 and 4.1kcal/mol only accounts for the formation of one additional hydrogen bond in the mechanism of the reaction involving glutathione compared to that involving the glutathione fragments.

One explanation for the small change in affinity but large change in $k_{cat}$ for the glutathione fragments compared to glutathione, is that a higher proportion of glutathione is bound productively compared to the glutathione fragments. Further, if the fluorescence change induced by G-site binding is indeed representative of thiol activation, then the reduction in fluorescence quenching induced by the binding of the glutathione fragments would imply that a lower percentage of the bound glutathione fragments are activated. Therefore, the integrity of the peptidyl moiety of the glutathione appears to be required for productive binding, optimising the activation of the thiol group.
Table 4.1.6. Summary of the kinetic and dissociation constants for G-site ligands. All $k_{cat}$ values were determined at 1.5mM CDNB and although this is only 74% saturating allows the activity of the enzyme with different thiol compounds to be compared.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G$ (kcal/mol)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$M$^{-1}$)</th>
<th>$\Delta G^*$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
<td>0.25 ±0.04</td>
<td>-9.0</td>
<td>0.32 ±0.03</td>
<td>53 ±5.4</td>
<td>1.7x10$^5$</td>
<td>-7.1</td>
</tr>
<tr>
<td>$\gamma$-Glu-Cys</td>
<td>0.95 ±0.3</td>
<td>-8.2</td>
<td>0.37 ±0.03</td>
<td>0.29 ±0.04</td>
<td>7.8x10$^2$</td>
<td>-3.9</td>
</tr>
<tr>
<td>Cys-Gly</td>
<td>2.0 ±0.6</td>
<td>-7.7</td>
<td>0.34 ±0.04</td>
<td>0.057 ±0.003</td>
<td>1.7x10$^2$</td>
<td>-3.0</td>
</tr>
</tbody>
</table>

More extensive studies on the stringency of G-site substrate specificity have been performed using a range of glutathione analogues systematically modified along the length of the peptide$^{86-88}$. The conclusions of these studies, which used a range of rat GST isoenzymes, were that although substrate specificity is dependent on the isoenzyme studied, a range of modifications of the glycyl moiety can be tolerated, including the substitution of glycine with phenylalanine, but the isoenzymes are sensitive to modification in the cysteinyl and $\gamma$-glutamyl residues. This indicates that the architecture of the G-site is such that there is space around the bound glycyl moiety of glutathione allowing larger residues to be accommodated. Indeed, figure 1.1. shows that the glycyl residue is located at the edge of the G-site, towards the solvent channel and although the side chains of the amino acid residues of the protein are not shown in full, this gives an indication of the different environments that the glycyl and $\gamma$-glutamyl residues of glutathione experience. Figure 4.1.8. shows a different view of the G-site, with the side chains of amino acid residues involved in glutathione binding shown in full. It is apparent that the $\gamma$-glutamyl binding site is more clearly defined by these residues than the glycyl binding site, indicating that the bound $\gamma$-glutamyl moiety can adopt fewer favourable conformations. The only interaction that appears to occur between protein and the glycyl moiety of glutathione is with the $\alpha$-carboxyl group and therefore the nature of the side chain substituted onto this residue appears to have little affect on binding as long as they can be accommodated at the G-site. In contrast the enzyme is extremely sensitive to analogues modified at the cysteinyl residue. It was concluded that the integrity of the cysteinyl moiety is essential for the correct orientation of the thiol group in the active site, and this in turn effects the activity of the enzyme with the thiol compound. Modifications of the $\gamma$-glutamyl moiety indicate that the $\alpha$-carboxyl group and $\alpha$-amino group are
involved in binding to the protein and both of these interactions are observed in the crystal structures of the enzyme (see figure 1.4.). Further, all isoenzymes exhibited a high stereo specific recognition with respect to the γ-glutamyl moiety, with the best overall γ-glutamyl modified substrate not having an equivalent to the α-amino group (for example glutaryl-L-Cys-Gly). Alternative compounds with structural differences in the region of the α-amino group were not good substrates, indicating that the interaction between glutathione and the enzyme in this region is highly specific with slight structural changes leading to steric clashes hindering substrate binding. The structure of the G-site of hA1-1 GST with S-benzylglutathione bound\(^{59}\) is shown in figure 4.1.8. The residues defining the G-site are shown in ball-and-stick fashion coloured pink and yellow, the latter being residues from the second subunit. Clearly there are more residues in proximity to the γ-glutamyl moiety compared to the glycyl moiety of glutathione and therefore there are fewer favourable conformations that the γ-glutamyl moiety can adopt.

Interestingly, the deletion of the α-amino group functional group of the γ-glutamyl moiety was shown to increase the \(K_M\) compared to glutathione of all for isoenzymes tested, whereas the \(K_M\) for Cys-Gly, having the entire γ-glutamyl moiety deleted is similar to that for glutathione. It is possible the bonds between the α-amino group of the γ-glutamyl and GST serve to anchor the peptidyl moiety in the extended conformation characteristic of glutathione bound at the G-site. In the absence of this bond, the extended conformation may not be favoured, and steric clashes could occur between the G-site residues and γ-glutamyl moiety hence the concentration required to half saturate the enzyme is increased. When the glutamyl moiety is deleted these steric interactions would not occur and therefore the \(K_M\) is reduced. Thus the hydrogen bonds that occur along the length of the glutathione peptidyl moiety may be necessary for it to adopt the extended conformation, which in turn may favour productive binding. In the absence of these bonds the extended conformation is less likely to be adopted resulting in steric clashes with the amino acid residues of the protein. The glutathione fragments appear to have a different mode of binding compared to glutathione, which occurs with a similar affinity but results in \(k_{cat}\) being reduced. Therefore, the stringent requirement for glutathione as a substrate is dependent on the thiol group adopting the correct orientation in the active site, which a function of the entire peptidyl moiety.
The α-carbon backbone of the protein is shown in green with the residues postulated to form the G-site shown in “ball and stick” fashion. Those from the same subunit as the ligand is bound are coloured pink and those from the second subunit coloured yellow. The ligand (S-benzylglutathione) is also shown in “ball and stick” fashion, with the glutathione moiety coloured blue, the thiol group yellow and the benzyl moiety red.
4.2. THE CONJUGATION REACTION OF SMALL THIOL COMPOUNDS TO CDNB IN THE PRESENCE OF A STRUCTURALLY SIMILAR GLUTATHIONE ANALOGUE.

DTT and 2-mercaptoethanol do not alone act as substrates for conjugation to CDNB catalysed by GST but in the presence of an inactive glutathione analogue catalysis by a mu-class isoenzyme has been observed. HPLC analysis of the reaction mixture showed that the conjugate was eluted at the same place in the chromatogram as a synthetically produced small thiol donor-hydrophobic ligand conjugate. This observation was applied to hA1-1 GST and expanded to determine the kinetic constants for the reaction. The enzyme catalysed reaction mixture was analysed by NMR spectroscopy and the results compared to a synthetically made conjugate (figure 4.2.1.).

The amount of enzyme in the assay was small compared to the concentration of the substrates, therefore the resonances from the enzyme were masked by the intensity of the ligand signals, simplifying the spectra. Figure 4.2.1.a. and b. show the aromatic region of the proton spectra of enzymatic and synthetic mixtures respectively. The resonances from unconjugated CDNB (4.2.1.c.) can be identified in both mixtures, and by elimination the other three resonances must be generated by the aromatic protons of the conjugate. As these resonances do not have the same frequency as the equivalent resonances of GSDNP (d) it appears that the product is indeed a DTT-CDNB conjugate. Further, the difference spectrum (e) of the 1.8-4.7ppm region, where the DTT (f) and S-methylglutathione (g) resonances are detected, calculated by subtracting the synthetic spectrum (h) from the enzymatic spectrum (i) has the same profile as S-methylglutathione (f), in particular the relative intensities of the resonance signal generated by the S-methyl protons (1.98ppm) are the same indicating that this substrate is not utilised in the reaction and therefore the conjugate formed is indeed that of DTT and CDNB.
Figure 4.2.1. - Proton spectra of the substrates and reaction mixtures of the synthetically and enzymatically made DTT•CDNB conjugate.

Purified DNPGSH.

a) Aromatic region of Synthetic reaction mixture

b) Aromatic region of Enzymatic reaction mixture

c) CDNB.
e) Difference spectrum

f) S-methyl glutathione

h) Synthetic reaction mixture, includes only CDNB and DTT.

i) Enzymatic reaction mixture, including GST, S-methylglutathione, DTT and CDNB.

g) DTT
The kinetic constants for the conjugation of small thiol compounds to CDNB catalysed by hA1-1 GST were calculated for all three ligands and are presented in table 4.2.1. In section 4.3, it is shown that the affinity of the enzyme is affected by the presence of the glutathione peptidyl moiety, either glutathione itself (from the $K_m$ values) or an inactive glutathione analogue (from the dissociation constants) therefore this effect appears to be a function of the peptidyl moiety and is independent of the nature of the thiol group. The $K_m$ values for CDNB in the small thiol•S-methylglutathione•CDNB reaction and the standard reaction are similar, implying that the affinity of the enzyme for CDNB in both reaction types is similar. Thus S-methylglutathione has the same effect on the affinity of the enzyme for CDNB as glutathione does and the reduced turnover number with the small thiol compound is not due to a reduced affinity of the enzyme for CDNB.

1.5mM CDNB was used to represent saturation when determining the kinetic constants for the small thiol compound and glutathione analogue, as for the determination of the kinetic constants of other thiol compounds (section 4.1.). As S-methylglutathione and the small thiol compound together approximate the function of glutathione in the standard reaction it is difficult to draw conclusions by comparing their individual rate constants. Further, as S-methylglutathione is not utilised in the reaction, but is necessary in order for the enzyme•substrate complex to be converted to product, Michaelis-Menten equation do not apply to this situation. However, if the concentration of the substrate required to attain half maximum velocity ($S_{0.5}$) is a measure of affinity, that the $S_{0.5}$ value for either 2-mercaptoethanol or DTT is two orders of magnitude greater than that for S-methylglutathione, indicates that the small thiol compound is unlikely to be competitively inhibiting S-methylglutathione binding. Further the magnitude of $S_{0.5}$ for S-methylglutathione is similar to the $K_m$ for glutathione, which coupled to the effect of the peptidyl moiety of glutathione on the affinity of the enzyme for CDNB indicates that the glutathione analogue binds in a similar way to the co-substrate altering the affinity of the apo-enzyme for CDNB. The kinetic constants determined for the small thiol donor were both similar, implying that the enzyme does not impose restrictions on this substrate.

The turnover numbers for all three “substrates” were between 0.21 and 0.33s$^{-1}$, significantly lower than the turnover number the standard reaction (53-58s$^{-1}$) but still measurable. Thus, by separating the two functions of the glutathione molecule, namely enzyme activation by the peptidyl moiety and conjugation as the thiol donor, the efficiency of catalysis is reduced, most probably because the small thiol compounds are unlikely to be activated by the enzyme as they cannot bind at the G-site simultaneously with the glutathione analogue, the presence of which is essential for catalysis to occur. Thus, this example of catalysis depends solely on the activation of the electrophilic centre of the hydrophobic substrate and is independent of thiol activation and proximity effects.
Table 4.2.1. The kinetic constants determined for the small thiol, CDNB and S-methylglutathione in the conjugation reaction catalysed by hA1-1 GST. (2M= 2-Mercaptoethanol).

<table>
<thead>
<tr>
<th>Varied ligand</th>
<th>Constant ligands and their concentrations (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$S_{0.5}$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2M CDNB</td>
<td>15mM CDNB 15mM SMGSH</td>
<td>0.27±0.12</td>
<td>54±1.9</td>
<td>5.0x10$^{-3}$</td>
</tr>
<tr>
<td>CDNB</td>
<td>15mM SMGSH 125mM DTT</td>
<td>0.33±0.028</td>
<td>0.34±0.015</td>
<td>0.97</td>
</tr>
<tr>
<td>DTT</td>
<td>15mM SMGSH</td>
<td>0.22±0.050</td>
<td>56±0.8</td>
<td>3.9x10$^{-3}$</td>
</tr>
<tr>
<td>CDNB SMGSH</td>
<td>15mM SMGSH 0.368M 2M</td>
<td>0.31±0.0</td>
<td>0.34±0.02</td>
<td>0.91</td>
</tr>
<tr>
<td>SMGSH</td>
<td>1mM CDNB 0.368M 2M</td>
<td>0.21±0.09</td>
<td>0.67±0.18</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Interestingly the rate of conjugation of CDNB to glutathione catalysed by either the Y9F (0.34) or truncated (0.39) forms of hA1-1 are in the same range as the rate of the small thiol-S-methylglutathione reaction catalysed by the wild-type enzyme. It is possible that conjugation catalysed by these two forms of hA1-1 GST also depends on the activation of the substrate rather than the thiol donor. However, the rate of the small thiol conjugation reaction with either mutant appeared to be independent of enzyme concentration, if anything the spontaneous rate showed a slight decrease on addition of enzyme. As these enzymes exhibit a catalytic rate equivalent to 1% of the wild-type rate with glutathione, a further reduction in rate, proportional to that observed for the wild-type enzyme comparing the rate of the small thiol compound-glutathione analogue reaction with the rate of the standard reaction, would imply that the rate of catalysis would be so slight that it would be difficult to detect. However, as a rate of small thiol conjugation to CDNB equivalent to that catalysed by the wild-type enzyme in the presence of S-methylglutathione was not observed, it appears that both of these mutations affect the activation of the electrophilic centre of the hydrophobic substrate. Tyrosine 9 has been implicated as being involved in thiol activation and it could be postulated that this event may result in the activation of the hydrophobic substrate as a secondary event. However, as the small thiol-CDNB reaction takes place in the presence of dethioglutathione, it appears that hydrophobic substrate activation occurs in addition to thiol activation and not because of it. Therefore the lack of activity with the Y9F mutant implies that this mutation directly effects hydrophobic substrate activation.

Catalytic conjugation of small thiol compounds to CDNB in the presence of S-methylglutathione was not observed with ethacrynic acid as a hydrophobic substrate.
However, as turnover number with glutathione is approximately 0.1% (see tables 4.3.3.) of the turnover number with CDNB, and the substitution of a small thiol compound and S-methylglutathione for glutathione reduces the $k_{\text{cat}}$ of the wild-type enzyme with CDNB by around 99% the rate enhancement due to catalysis would be difficult to detect.

4.3. DETERMINATION OF KINETIC AND DISSOCIATION CONSTANTS FOR H-SITE LIGANDS.

Although a broad spectrum of ligands can bind at the H-site of hA1-1 GST not all serve as substrates in the conjugation reaction. CDNB is a well characterised hydrophobic substrate for nearly all GSTs and for this reason it was used as a model substrate in these studies. The kinetic and dissociation constants of ethacrynic acid (EA) and DCNB were also determined. Although ethacrylic acid is a good substrate for some GSTs, particularly pi-class isoenzymes, the activity of hA1-1 GST with this substrate is quite low, hence ethacrylic acid it is often classed as an alpha-class GST inhibitor. In addition the dissociation constants for the fluorescence probe, ANS, were determined both by the effect of ligand binding on intrinsic protein fluorescence and enhancement of ligand fluorescence. The stoichiometry of ANS was determined as 1 binding site per subunit up to a 2-fold excess of ANS, then binding at additional sites occurred. CDNB, DCNB and EA stoichiometry was determined as one binding site per subunit up to 10-fold-excess of ligand. Thus all four ligands could be used to probe H-site binding within the defined concentration range.

Figure 4.3.1. The corrected intrinsic protein fluorescence (2µM active sites) with successive additions of ANS on emitted at 480nm on excitation at 370nm.
The dissociation constants determined for CDNB, binding to the apo-enzyme and in the presence of 20-fold excess of dethio-glutathione (γGlu-Ala-Gly) compared to G-sites, are 2.1 and 0.54μM respectively, showing that the presence of the glutathione peptidyl moiety decreases the dissociation constant for CDNB. In contrast, the presence of either glutathione, S-methylglutathione or dethio-glutathione increases the dissociation constant of the hydrophobic ligand ANS by one order of magnitude. Thus glutathione has a different effect on the affinity of the enzyme for these two hydrophobic compounds. As the affinity for other hydrophobic ligands, such as hemin, increases in the presence of glutathione, the effect of glutathione on ANS binding appears to be the exception rather than the rule. It is possible that ANS may not bind at the same site as the other ligands do, indeed addition of up to a ten-fold excess of CDNB to GST containing a 2-fold excess of ANS did not appear to alter the fluorescence of ANS indicating that the ligands do not bind competitively.

The degree of fluorescence quenching induced by CDNB binding is nearly three times greater in the presence of the glutathione analogue (12% compared with 4.4%). This may imply that in the presence of the peptidyl moiety of glutathione the interaction between CDNB and the enzyme is different compared to that with the apo-enzyme. Either CDNB adopts a different orientation in the H-site of the enzyme•glutathione complex such that fluorescence quenching is increased or the presence of the peptidyl moiety of glutathione enables the enzyme to alter the absorption properties of CDNB, perhaps by catalytically activating it so that conjugation to glutathione is favoured.
Although ANS induces a greater change in fluorescence on binding to GST compared to that induced by CDNB, because it has a strong absorption band at 340nm, and the fluorescence change is increased on binding in the presence of glutathione, the magnitude of this change (132%) compared to that difference in the fluorescence change induced by CDNB in the presence and absence of glutathione (272%) is quite different possibly because the two ligands bind in different ways to the enzyme. DCNB induces a similar fluorescence change in the presence of dethio-glutathione as CDNB, as would be expected as these molecules are so similar, yet the fluorescence change induced by ethacrynic acid, binding under similar conditions is around 58% of that induced by CDNB. This is possibly because of the different absorption properties of the substrates, as CDNB and DCNB have absorption bands in the region of the emission wavelength (340nm) but ethacrynic acid has an absorption maximum at 270nm, closer to the excitation wavelength (280nm).

Table 4.3.1. - The dissociation constants and percent change in fluorescence for hydrophobic ligand binding to the apo-enzyme.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kd (µM)</th>
<th>ΔF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>2.1±0.80</td>
<td>4.4±0.76</td>
</tr>
<tr>
<td>ANS</td>
<td>0.74±0.26</td>
<td>36±12</td>
</tr>
<tr>
<td>ANS*</td>
<td>0.76±0.10</td>
<td>160±21</td>
</tr>
</tbody>
</table>

(*Determined from the change in ligand fluorescence on binding)

Table 4.3.2. The dissociation constants and fluorescence change for hydrophobic ligand binding to the dethioglutathione•GST complex.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kd (µM)</th>
<th>ΔF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>0.54±0.10</td>
<td>12±1.2</td>
</tr>
<tr>
<td>ANS</td>
<td>7.2±3.1</td>
<td>41±8.8</td>
</tr>
<tr>
<td>DCNB</td>
<td>0.77±0.05</td>
<td>11±0.0</td>
</tr>
<tr>
<td>EA</td>
<td>1.6±0.36</td>
<td>7.2±6.6</td>
</tr>
<tr>
<td>ANS*</td>
<td>5.2±1.4</td>
<td>290±59</td>
</tr>
</tbody>
</table>

(*Determined from the change in ligand fluorescence on binding)
Figure 4.3.3. - The change in the corrected fluorescence intensity on successive additions of glutathione to a 0.5µM solution of GST active sites.

Figure 4.3.4. Corrected intrinsic protein fluorescence with successive additions of ANS used to determine the dissociation constant.
The dissociation constants for all four hydrophobic ligands are in the μM range indicating that the enzyme has high affinity for a range of structurally diverse hydrophobic ligands and it is evident with both the hydrophobic substrates and ANS, that glutathione binding at the G-site effects the affinity of the protein for these ligands. Binding of glutathione conjugates at the G-site has been shown by X-ray crystallography to cause structural changes in the C-terminal helix (helix 9) of the protein. It is possible that a similar change occurs on glutathione binding and this is responsible for the change in substrate specificity. The localisation of the helix 9 over the H-site probably increases its hydrophobicity enhancing hydrophobic substrate binding. That the affinity for ANS is reduced in the presence of glutathione may indicate that the glutathione–enzyme complex has a higher affinity for hydrophobic substrates with an electron deficient centre, compared to hydrophobic ligands. If this can be applied on a more general scale, the structural change induced by glutathione binding would reduce the inhibitory effect of hydrophobic molecules, favouring the conjugation function of the GSTs rather than the storage function.

The effect of glutathione binding, increasing the affinity of the enzyme for the hydrophobic substrates is also illustrated in the values of the kinetic constants. At 5mM glutathione the $K_M$ for CDNB was deduced as 0.38mM and the $k_{cat}$ as 58s$^{-1}$. Decreasing the concentration of glutathione to 1mM resulted in a 1.4-fold increase in
Thus it appears in contrast to the effect of the concentration of CDNB (either 1 or 1.5mM) on the $K_M$ for glutathione, the increased concentration of glutathione, from 1mM to 5mM, does affect the $K_M$ of the enzyme for CDNB. That the catalytic rate remains unaffected by the increased affinity of the enzyme for CDNB implies that the kinetic model describing catalysis is over simplified.

As for the kinetic constants describing conjugation with respect to the co-substrate glutathione, the dissociation constant for CDNB (in the presence of dethio-glutathione to more accurately describe the binding of the hydrophobic substrate leading to catalysis) is three orders of magnitude smaller than $K_M$, implying that once the enzyme-substrate complex has formed product formation is favoured over the reformation of the substrates. A similar difference in magnitude is observed on comparing the dissociation constant and Michaelis-Menten constant for DCNB but the difference for ethacrynic acid is only two orders of magnitude, indicating that although the ethacrynic binds to GST with a similar affinity to the other substrates the probability of the enzyme substrate complex forming product is reduced. This implies that the enzyme-substrate complex with ethacrynic acid is less likely to be productive than the complex involving the other two substrates, indicating the hydrophobic substrate can bind non-productively and the probability of this event depends on the nature of the substrates, which in turn effects the rate of the reaction.

**Table 4.3.3. Determination of the kinetic constants for conjugation hydrophobic ligands to glutathione.**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Concentration of glutathione (mM)</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>1.0</td>
<td>58±6.2</td>
<td>0.53±0.08</td>
<td>1.1x10$^5$</td>
</tr>
<tr>
<td>CDNB</td>
<td>5.0</td>
<td>58±8.7</td>
<td>0.38±0.05</td>
<td>1.5x10$^5$</td>
</tr>
<tr>
<td>DCNB</td>
<td>5.0</td>
<td>0.11±0.02</td>
<td>1.3±0.39</td>
<td>8.4x10$^1$</td>
</tr>
<tr>
<td>EA</td>
<td>0.25</td>
<td>0.045±0.006</td>
<td>0.099±0.028</td>
<td>4.5x10$^1$</td>
</tr>
</tbody>
</table>

The kinetic constants for the conjugation of the hydrophobic substrates EA and DCNB were determined at 5mM glutathione. Although the $K_M$ for DCNB was 3.4 times greater than the $K_M$ for CDNB it was still in the mM range and is in part due to a decreased affinity of the enzyme for DCNB, as shown from the comparison of the dissociation
constants for the two ligands: The dissociation constant for DCNB is 1.4 times greater than that of CDNB. The $K_m$ for ethacrynic acid is approximately a quarter of that for CDNB, however as the dissociation constants for ethacrynic acid is greater than that of CDNB this does not indicate that the enzyme has a greater affinity for this substrate. The reduced $K_m$ for ethacrynic acid compared to CDNB reflects the overall efficiency of catalysis with these two substrates: CDNB being by far the better substrate for hA1-1 GST, as shown by the turnover number for CDNB being approximately three orders of magnitude greater than that for ethacrynic acid and hA1-1 GST enhances the spontaneous rate of CDNB conjugation twice as much as that of ethacrynic acid (table 4.3.4). Although the turnover number for DCNB is only 0.2% of that for CDNB, the spontaneous rate of conjugation of DCNB is also reduced as shown in table 4.3.4. This is due to the different functional group at position 2 on the ring. The nitro-group of CDNB can more efficiently stabilise the reaction intermediates due to its electron sharing properties, as opposed to the chloro-group at this position in DCNB. Thus, it appears that the greater turnover number with CDNB partially is due to the intrinsic properties of this substrate.

Figure 4.3.6. Typical data set showing Michaelis-Menten curve fit to describe the effect of changing CDNB concentration on the rate of GSDNP production (s$^{-1}$) at 1mM glutathione concentration.
A good insight into the catalytic ability of an enzyme is obtained by comparing the rate of enzyme catalysed conjugation with the spontaneous rate, as shown for CDNB and DCNB conjugation in figures 4.3.7. and 4.3.8. Due to the different catalytic activities of the enzyme with the two substrates the amount of enzyme used in the assays is defined as that which catalyses conjugation at a rate of 60s\(^{-1}\) at a 1.5mM concentration of the substrate, and this is shown on each figure. Considering first the differences between the spontaneous rates of GSCNP and GSDNP production, with both ligands the rate of conjugation is linear with respect to substrate concentration, however it is apparent that the rate of GSDNP production is twice that of GSCNP production at the same concentration of ligand. These differences are due to the intrinsic properties of the hydrophobic substrate. However, this does not explain the differences in the enzyme catalysed rate of reaction, specifically, it takes 1250 times more enzyme to achieve the same rate of conjugation at 1.5mM DCNB as it does at 1.5mM CDNB. Therefore catalysis is by far more efficient with CDNB. Although the enzymatic rate of ethacrynic acid conjugation is slightly lower than that of DCNB (table 4.3.4.) a direct comparison cannot be made as the concentration of glutathione used in the ethacrynic acid assay was less than that in the DCNB assay in order to reduce the spontaneous rate of ethacrynic acid conjugation.

Table 4.3.4. Comparison of the turnover numbers with the spontaneous rate of conjugation for the hydrophobic substrates with 5mM glutathione.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>Rate of GSDNP production</th>
<th>Percentage increase in rate due to enzyme (% µg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Enzyme catalysed (pmol/s/µg)</td>
<td>Spontaneous (pmol/s)</td>
</tr>
<tr>
<td>CDNB</td>
<td>1.5</td>
<td>1900</td>
<td>30</td>
</tr>
<tr>
<td>DCNB</td>
<td>1.5</td>
<td>0.89</td>
<td>16</td>
</tr>
<tr>
<td>EA</td>
<td>0.5</td>
<td>2.00</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 4.3.7. The rate of conjugation of CDNB.

Figure 4.3.8. The rate of conjugation of DCNB.
Table 4.3.5. The kinetic and dissociation constants determined for H-site substrates in the presence of glutathione.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_d$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>0.54</td>
<td>58</td>
<td>0.38</td>
<td>1.5x10$^5$</td>
</tr>
<tr>
<td>DCNB</td>
<td>0.77</td>
<td>0.11</td>
<td>1.3</td>
<td>8.4x10$^1$</td>
</tr>
<tr>
<td>EA</td>
<td>1.6</td>
<td>0.045</td>
<td>0.099</td>
<td>4.5x10$^1$</td>
</tr>
</tbody>
</table>

4.4. THE DISSOCIATION CONSTANTS DESCRIBING GSDNP BINDING TO hA1-1 GST.

The dissociation constant of the product of CDNB conjugation to glutathione (GSDNP) was determined as 5.6µM with an 89% maximum change in fluorescence. It is interesting to note that such a large fluorescence change was observed compared to the binding of any combination of the substrates. This is probably due to the different absorption properties of the thiol-substituted aromatic ring of GSDNP, which has a strong absorption band at 340nm, the emission wavelength. The ring may also be oriented in a different manner in the active site. Indeed, the crystal structure of a mu-class isoenzyme, solved with GSDNP bound shows that the aromatic ring is located outside of the active site cavity in the solvent channel between the two subunits$^{106}$. Similar positions of the hydrophobic moiety of other glutathione analogues bound to hA1-1 GST (S-benzylglutathione and the ethacrynic acid conjugate) have not been noted but ethacrynic acid is located further in to the H-site pocket compared to this moiety on the glutathione conjugate, thus the hydrophobic substrate is located in a different position compared to this moiety when conjugated to glutathione. That the dissociation constant for GSDNP is larger than for the two substrates is representative of efficient catalysis allowing the substrates to displace the product from the active site.

The difference between the dissociation constants of the substrates and product is further expanded when determined under physiological concentrations of glutathione. In contrast to the effect on CDNB binding, the presence of glutathione decreases the affinity of the enzyme for GSDNP, as shown by the dissociation constant increasing nearly five-fold to 27µM, but this increase was three orders of magnitude smaller than that expected if binding of the product and was strictly competitive deduced from equation 4.4.1.
where \( K'_d \) is the apparent dissociation constant for GSDNP in the presence of a competitive inhibitor of concentration \([I]\), in this instance 10mM, and \( K_i \) is its dissociation constant to the enzyme (0.25\( \mu \)M). Hence \( K'_d \) for GSDNP would be 21mM if binding were purely competitive with glutathione.

![Figure 4.4.1. Corrected change in fluorescence with successive additions of GSDNP.](image)

**Table 4.4.1.** The dissociation constants and maximum change in fluorescence as a percentage of initial fluorescence for GSDNP binding to GST in the presence and absence of glutathione.

<table>
<thead>
<tr>
<th></th>
<th>( K_d ) (( \mu )M)</th>
<th>( \Delta F ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo-enzyme</td>
<td>5.6 ± 0.9</td>
<td>89 ± 13</td>
</tr>
<tr>
<td>plus 10mM glutathione</td>
<td>27 ± 4.3</td>
<td>52 ± 12</td>
</tr>
</tbody>
</table>

It appears that both glutathione and GSDNP can be bound simultaneously by hA1-1 GST, implying that while glutathione is located at the G-site, the aromatic ring of GSDNP can bind at the H-site, with the glutathione moiety possibly being oriented out of
the active site into the solvent channel. The reduced affinity of the enzyme for GSDNP in the presence of glutathione may be because binding to the apo-enzyme occurs at the G-site or that GSDNP occupies both ligand binding sites in the apo-enzyme, thus when the G-site is occupied binding only occurs at the H-site reducing the affinity of the enzyme for the ligand. Alternatively, GSDNP may bind at the H-site in both the presence and absence of glutathione, but the glutathione induced conformational change, that increases the affinity of the enzyme for the hydrophobic substrates, may also decrease the affinity of the enzyme for the product of conjugation. On a wider scale, this would imply that in vivo where glutathione is saturating, the GST function of the enzyme is preferenced above that of sequestering the conjugates. Thus, the potential of the enzyme to detoxify hydrophobic electrophiles is maximised. Unfortunately, the effect of either GSDNP on CDNB binding or vice versa could not be determined as the enzyme could not be saturated with CDNB due to solubility and saturation with GSDNP resulted in complete quenching of intrinsic protein fluorescence.

Figure 4.4.2. Curve fit of GSDNP binding data in the presence of 10mM glutathione (first data set from table 4.4.2).
4.5 THE DISSOCIATION CONSTANTS OF GSDNP TO SPECIFICALLY MUTATED FORMS OF hA1-1 GST.

The dissociation constants for GSDNP to various mutated forms of hA1-1 were determined as for the wild-type. Interestingly all forms of hA1-1 GST showed a similar fluorescence change on binding to GSDNP, implying that the environment of bound GSDNP was not drastically affected by the various mutations. In contrast the dissociation constants, and therefore the affinity of the mutated form of hA1-1 GST, varied according to the nature of the mutation, between 5 and 285%. A summary of the dissociation constants are shown in table 4.5.1.

Table 4.5.1. Summary of GSDNP binding to the wild-type and mutated forms of hA1-1 GST. The Kd and AF values are presented as a fraction of the wild type values (shown in brackets).

<table>
<thead>
<tr>
<th>GST Type</th>
<th>Kd (µM)</th>
<th>ΔF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.6 (1.0)</td>
<td>89 (1.0)</td>
</tr>
<tr>
<td>F222Y</td>
<td>16 (2.9)</td>
<td>74 (0.83)</td>
</tr>
<tr>
<td>Y9F</td>
<td>0.28 (0.05)</td>
<td>81 (0.91)</td>
</tr>
<tr>
<td>L108Y</td>
<td>9.5 (1.7)</td>
<td>82 (0.92)</td>
</tr>
<tr>
<td>M208D</td>
<td>8.2 (1.5)</td>
<td>85 (0.96)</td>
</tr>
<tr>
<td>Truncated</td>
<td>11 (2.0)</td>
<td>73 (0.82)</td>
</tr>
</tbody>
</table>

All mutations affect the dissociation constants of GSDNP to the protein, but to different extents. Surprisingly, the mutation of the terminal residue of the protein, F222, to a tyrosine, effectively introducing a hydroxyl functional group onto this amino acid residue decreases the affinity of the enzyme for GSDNP by the greatest extent. This is surprising because the side chain of phenylalanine 222 in the crystal structure points away from the active site of the protein. However, as the mutation of F222 to tyrosine has introduced a hydroxyl function it is possible that this group may be involved in a hydrogen bond to another amino acid residue, reducing the flexibility of the C-terminal helix which in turn sterically interferes with ligand binding. Further, the complete deletion of the C-terminal helix only increases the dissociation constants two-fold, thus GSDNP binding is more
sensitive to the change in polarity of P222 than the complete deletion of the helix. It was expected that the truncated form of hA1-1 GST would have a reduced affinity for hydrophobic ligands because one of the functions of the C-terminal helix is to enhance ligand binding, possibly by increasing the hydrophobicity of the H-site.

Alterations of amino acid residues in the H-site of hA1-1 GST also reduce the affinity of the enzyme for this ligand: The mutation of L108 to tyrosine and M208 to aspartic acid both have the effect of increasing the dissociation constants 1.7 and 1.5 times, respectively. As both of these mutations are of a similar nature, increasing the polarity of an amino acid residue side chain that is involved in forming the H-site the similar values of the dissociation constants were expected. However, it is interesting to note that these mutations of amino acids within the H-site do not have as great an effect on the binding of GSDNP compared to the mutations affecting the C-terminal helix which was postulated to act only as a lid over the H-site. Therefore it appears that the C-terminal helix is an important determinant in substrate specificity. As GSDNP binding is affected by H-site alterations, it is likely that the aromatic ring of this ligand is located in the H-site, not in the solvent channel as it is in the crystal structure of a mu isoenzyme.

The mutation of tyrosine 9 to phenylalanine is the only mutation shown here that increases the affinity of the enzyme for GSDNP. This is probably because the hydroxyl functional group of tyrosine 9 lies on the boundary between the G- and the H-site and deletion of the hydroxyl group would reduce steric interactions with a ligand binding in this region. As in the absence of glutathione bound GSDNP may occupy both ligand binding sites, its conformation is limited by steric interactions with this hydroxyl functional group. Therefore deletion of this group by the Y9F mutation decreases the restrictions imposed on conjugate binding allowing GSDNP to bind more freely, and thereby increase the affinity the enzyme has for this ligand. This also indicates that for conjugate binding the hydrogen bond between the hydroxyl functional group of tyrosine 9 and the thiol group of the conjugate is not a major driving force in binding. Further, as a similar fluorescence quench is induced by GSDNP binding to both the wild-type and Y9F mutant, this hydrogen bond does not influence tryptophan fluorescence via energy transfer when GSDNP binds, although this may occur when glutathione binds.
4.6. FURTHER ANALYSIS OF THE F222Y, Y9F AND TRUNCATED MUTANTS.

Table 4.6. The kinetic constants for mutated proteins are displayed and these values as ratios to the wild-type values are shown in brackets. \( \Delta \Delta G^* \) was calculated using equation 4.5.1. below.

\[
\Delta G^* = -RT \ln \left( \frac{k_{cat}}{K_M} \right)_{\text{mutant}} \div \left( \frac{k_{cat}}{K_M} \right)_{\text{wild-type}}
\]

<table>
<thead>
<tr>
<th>Mutated protein type</th>
<th>Varied ligand</th>
<th>Constant ligand</th>
<th>( K_M ) (mM)</th>
<th>( k_{cat} ) (s(^{-1}))</th>
<th>kcat/KM (s(^{-1})M(^{-1}))</th>
<th>( \Delta \Delta G^* ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CDNB 5mM GSH</td>
<td>0.38 ±0.05 (1.0)</td>
<td>58 ±8.7 (1.0)</td>
<td>1.5x10^5 (1.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT GSH 1.5mM CDNB</td>
<td>0.32 ±0.09 (1.0)</td>
<td>53 ±5.4 (1.0)</td>
<td>1.7x10^5 (1.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT EA 5mM GSH</td>
<td>0.099 ±0.028 (1.0)</td>
<td>0.045 ±0.006 (1.0)</td>
<td>4.5x10^2 (1.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y9F CDNB 5mM GSH</td>
<td>0.59 ±0.12 (1.1)</td>
<td>0.34 ±0.03 (5.9x10^-3)</td>
<td>5.8x10^2 (3.9x10^-3)</td>
<td>-3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y9F GSH 1.5mM CDNB</td>
<td>0.33 ±0.033 (1.0)</td>
<td>0.22 ±0.01 (4.2x10^-3)</td>
<td>6.7x10^2 (3.9x10^-3)</td>
<td>-3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F222Y CDNB 5mM GSH</td>
<td>0.84 ±0.13 (1.5)</td>
<td>55 ±4.4 (0.95)</td>
<td>6.5x10^4 (0.43)</td>
<td>-0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F222Y GSH 1.5mM CDNB</td>
<td>0.16 ±0.01 (0.50)</td>
<td>37 ±0.45 (0.70)</td>
<td>2.3x10^5 (1.4)</td>
<td>-0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACT CDNB 5mM GSH</td>
<td>10 ±0.72 (18)</td>
<td>0.39 ±0.10 (6.7x10^-3)</td>
<td>3.9x10^1 (2.6x10^-4)</td>
<td>-4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACT GSH 1.5mM CDNB</td>
<td>0.37 ±0.04 (1.2)</td>
<td>0.27 ±0.01 (5.1x10^-3)</td>
<td>7.3x10^2 (4.3x10^-3)</td>
<td>-3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACT EA 5mM GSH</td>
<td>0.14 ±0.06 (0.44)</td>
<td>2.0 ±0.27 (44)</td>
<td>1.4x10^4 (31)</td>
<td>+2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The turnover number deduced for either CDNB or glutathione as substrates in the conjugation reaction catalysed by Y9F was less than 1% of the wild-type value. This is partially due to the reduced efficiency of catalysis as the mutated enzyme has a higher affinity for the product compared to the wild-type enzyme thus the product is less easily displaced from the active site by the substrates. If the $K_M$ is a measure of affinity, as the value of this constant for both substrates is very similar to the wild-type enzyme it appears that the Y9F mutation does not dramatically affect the affinity of the enzyme for its substrates. The decreased value of the turnover number, compared to the wild-type enzyme, is consistent with the postulated role of tyrosine-9 in reducing the $pK_a$ of the glutathione thiol. The Y9F mutated hAl-1 GST has been further characterised by Stenberg et al. The activity of the mutated protein was also found to be significantly less than that of the wild-type protein and, although the published study was carried out at different substrate concentrations and 30°C, the specificity constant for CDNB was 5% and for glutathione 2% of the wild-type values under similar conditions, thus the comparative activity of the Y9F and wild-type enzymes are similar to the results presented in table 4.6.1. Similarly, the mutation of equivalent tyrosine residues in mu and pi class isoenzymes reduces the rate of enzyme catalysed conjugation. Therefore the involvement of tyrosine 9 and equivalent residues in the catalytic mechanism of the alpha, mu, and pi class isoenzymes appears to be conserved. Although these residues are involved in catalysis, the integrity of the hydroxyl function is not essential for catalysis to occur, as all tyrosine to phenylalanine mutants still show some catalytic activity. The magnitude of the value of $\Delta \Delta G^*$ for both substrates in the Y9F catalysed conjugation (-3.3 kcal/mol) could account for an additional hydrogen bond being involved in the activation of the substrates in the wild-type mechanism, which is in accordance with the role of tyrosine 9 in hydrogen bonding to the thiol group of glutathione. However, it must be emphasised that tyrosine 9 has also been implicated as being involved in the activation of the hydrophobic substrate, which, although is dependent on the peptidyl moiety of glutathione being bound at the G-site, is independent of the thiol group of glutathione (see section 4.2. and Chapter 8).

The F222Y mutation has the least effect on catalysis, with the $\Delta \Delta G^*$ values being -0.49 kcal/mol for CDNB and -0.18 kcal/mol for glutathione, indicating that the activity of the enzyme and its affinity for the substrates is similar to that of the wild-type. The $K_M$ for CDNB is increased in comparison to the wild-type $K_M$, by 1.5 times, but the turnover number remains the same. The it appears that the mutated enzyme has a decreased affinity for CDNB as the $K_M$ is increased and the $k_{cat}$ for glutathione is less than that for CDNB, indicating that 1.5mM CDNB is no longer saturating. The $K_M$ for glutathione is however half that for the wild-type enzyme, indicating that the enzyme is
saturated with this substrate at a lower substrate concentration compared to the wild-type enzyme. However these changes are very subtle and rather than implying the F222 is involved in catalysis may indicate that the mutation causes slight changes in the region of the active site, reducing the catalytic efficiency.

In contrast the deletion of the C-terminal helix has increased the $K_M$ for both glutathione and CDNB by 1.2 and 18 times respectively. This is due to the altered affinity of the enzyme for these ligands, as shown by the dissociation constants (tables 4.6.2.). The dissociation constants for glutathione to the truncated form of hA1-1 GST is 8.5 times greater compared to the wild-type dissociation constants and for CDNB 18 times larger. The reduced affinity for CDNB was expected as the deletion of the C-terminal helix was postulated to reduce the hydrophobicity of the H-site. As the terminal residues of the helix (220-222) are close to the G-site, the reduced affinity for glutathione indicates that these residues may influence ligand binding. The turnover number of this substrate is dramatically reduced implying that glutathione may not be binding productively to the truncated enzyme. The helix may be involved in promoting the substrates to bind in the active site such that catalytic activation is maximised. The flexibility of the helix has been shown to alter on ligand binding (from crystal studies)\(^*\) such that it becomes localised over the active site of the enzyme. For this change to occur the side chain of phenylalanine 10 must rotate so that the side chain of phenylalanine 220 can be accommodated. With these changes occurring in proximity to tyrosine 9, which is involved in the activation of glutathione, it is possible that in the absence of the helix the environment of bound glutathione is not the same as that in the wild-type enzyme therefore either due to the conformation of the residues in the active site or the orientation of the glutathione thiol, catalytic activation is reduced. It has been shown that the truncated mutant does not catalytically activate CDNB (section 4.2), hence the C-terminal helix appears to be involved in this aspect of catalysis, either directly activating the electrophilic centre or altering its position in the H-site so that the potential for activation is maximised (for further discussion see chapter 8).

**Table 4.6.2. The dissociation constants for CDNB and glutathione to the truncated enzyme.**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_d$ (μM)</th>
<th>$\Delta F$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
<td>1.7 ±0.8</td>
<td>21 ±3.7</td>
</tr>
<tr>
<td>CDNB</td>
<td>10 ±1.8</td>
<td>25 ±3.8</td>
</tr>
</tbody>
</table>
Interestingly, the catalytic activity of the truncated enzyme with ethacrynic acid is substantially increased compared to that of the wild-type enzyme with $K_M$ being about half and the turnover being 44 time that of the wild-type values. Hence it appears that the substrate specificity of the enzyme has been substantially altered by deleting the C-terminal helix. The high activity with ethacrynic acid is more characteristic of the pi-class GSTs, compared to alpha-class isoenzymes. Interestingly the pi-class isoenzymes have a more open active site and lack the C-terminal helix of the alpha-class GSTs. That both the $K_M$ and turnover number with ethacrynic acid are affected implies that the effect of the deletion of the C-terminal helix on the substrate specificity of the enzyme is more than a function of affinity changes. It has been established that the wild-type enzyme activates CDNB as well as glutathione in the conjugation reaction however this activation cannot be detected with ethacrynic acid as a substrate. This could imply that the deletion of the C-terminal helix alters the ability of the enzyme to activate its substrates, enabling the activation of ethacrynic acid but not CDNB.
5. LIGAND BINDING STUDIES USING NMR SPECTROSCOPY.

5.1. NMR pulse programs.

Quantisation rules restrict the number of orientations in space that an atomic nucleus, with an angular momentum quantum number I and an associated nuclear magnetic moment, can adopt to \((2I+1)\). Spin-\(\frac{1}{2}\) nuclei are of particular interest as they can only adopt two states in an applied magnetic field. Normally the energies of these states are degenerate, however when a magnetic field is applied small energy gaps are created between these levels. Consequently, at equilibrium slightly less nuclei will be in the highest energy state compared to the lowest, the latter being aligned with the applied magnetic field, hence there is a net magnetisation in this direction (termed \(M_z\)). Further, each nucleus contributes a rotating magnetic moment in the \(xy\) plane, however these are random hence there is no net magnetisation in this plane. If a pulse of electromagnetic radiation is applied at the Larmour frequency, which is the frequency of radiation required to promote transitions between the high and low energy states, the Boltzmann population difference between the two states will be altered. In the extreme case the population of the low and high states is inverted. The transitions also bring the rotating magnetic moment of similar nuclei into phase such that they precess together at their Larmour frequency, that is they have phase coherence resulting in magnetisation in the \(xy\) plane rotating at the Larmour frequency described by the vector \(\mathbf{M}\), which is a sum of the magnetisation in the \(xy\) plane \((M_{xy})\) and \(M_z\). In most NMR experiments a \(90^\circ\) pulse is applied which results in the magnitude of \(M_z\) becoming zero and therefore \(\mathbf{M}\) is equivalent to \(M_{xy}\). After this pulse the nuclei will relax back to their original state emitting energy which can be recorded with time to produce a free induction decay (FID) spectrum. Applying a Fourier transform to this data results in a plot of the frequency of the recorded emission against its intensity and the line width of these signals depends on the time taken for \(M_{xy}\) to relax back to the equilibrium value, which is directly correlated to the molecular motion of the molecule.

Several different NMR pulse programs were used to record the resonances of the NMR sensitive nuclei in the sample. These include 1-D experiments that were used to assign the ligand resonances, monitor changes in the protein spectra with successive additions of ligand and define the parameters required to record 2-D spectra, as described below.

5.1.1. 1-D NMR experiments.

Three types of 1-D experiment were applied to GST: 1-D proton, 1-D direct detection \(x\)-nucleus and 1-D indirect \(x\)-nucleus. As, even with deuterated buffers, the amount of water is such that resonances from these protons swamp the proton spectra, these were
recorded with water pre-saturation. The Larmor frequency of the water protons was
determined by recording a 1-D proton spectrum with no saturation. Pre-saturation was
then incorporated into the pulse program at this frequency prior to the 90° pulse. Due to
the pre-saturation at the Larmor frequency of the water protons, their energies will not
display the Boltzmann distribution and therefore when the 90° pulse is applied to the
sample the water protons will not exhibit phase coherence and no resonance signal will be
detected. The 90° pulse does, however, result in the other protons in the sample
exhibiting phase coherence and when the pulse is stopped the nuclei relax back to their
original states and generate a FID which excludes the resonances of water protons. A
similar experiment can be applied to other NMR sensitive nuclei (x-nuclei) such as
carbon-13 or nitrogen-15, recording their resonances directly however presaturation of
the water protons is not necessary when detecting these signals. The receptivity of these
nuclei is much less that of protons, hence the signal to noise ratio in these experiments is
reduced.

As an alternative to the direct detection of resonance of the x-nucleus changes in their
Larmor frequency can be monitored by observing the frequency of the attached protons
using an “indirect” experiment. The pulse program used in this experiment is similar to
that used for the 1-D direct detection for the x-nucleus, except an additional pulse is
incorporated that transfers the phase coherence from the x-nucleus to attached protons
before it is detected. Thus, only the resonances of protons attached to the x-nucleus are
detected and the changes in the Larmor frequency of these resonances can be correlated
to similar changes in the x-nucleus. However, the sensitivity of detection by the inverse
method is significantly greater than that of the direct detection method, as the former relies
on the receptivity of the protons and not the x-nucleus.

5.1.2. 2-D Heteronuclear Multiple-Quantum Resonance Experiments
(HMQC).

The 1-D experiments can be collected in 2-dimensions, reducing signal overlap and
therefore increasing the ease of interpretation of the spectra. FIDs are collected as
described for the 1-D experiment, but with incremental values of times for coherence
transfer (\(t_1\)). Thus a matrix of data is collected, each row of which is one FID. Applying
a Fourier transform to each row gives a series of spectra with differing times of coherence
transfer. Applying a second Fourier transform down the columns of this data results in a
two dimensional plot with the Larmor frequency of the nucleus on one axis and the
frequency of the second axis will be a function of the acquisition time (\(t_2\)). Figure
5.1.2.1. shows the pulse sequence for the standard 2D (HMQC) NMR experiment. The
upper line shows the proton pulses and below it are the x-nucleus pulses. The spin of
each proton is transferred into the xy plane by the 90° pulse along the x-plane (90x).
After a time $\Delta$ to allow the different spins separate, a 90° pulse is applied to the x-nucleus, such that they also rotate in the xy plane allowing coherence transfer for a time (t1). Half way through this time a 180° proton pulse is applied that essentially reverses the order of the proton spins resulting in the spins becoming re-focused at time t1. Then a 90° pulse is applied to the x-nuclei which transfers the coherence back to the protons for detection. During detection broadband heteronuclear decoupling is applied.

**Figure 5.1.2.1. The HMQC pulse sequence**

5.1.3. The NOESY experiment.

The NOESY (Nuclear Overhauser Effect Spectroscopy) can be used to identify nuclei that are in proximity in space but not linked through bonds. Figure 5.1.3.1. shows a standard NOE pulse program. The proton spins are transferred into the xy plane by the first 90° pulse and precess for time t1. The proton spins will separate out during this time according to their Larmour frequency. The second 90° pulse triggers the coherence transfer between nuclei in proximity for time t_m (the mixing time) before the third pulse (the detection pulse) is applied. The recorded cross-peaks that are a result of transferred phase coherence will have a frequency that is a function of both the frequency of the precession of the spins in t1 from which the phase coherence originated and the frequency of the spins to which the phase coherence was transferred during t_m. Similarly, cross peaks are recorded due to chemical exchange, that is when a nucleus is transferred from one site to another during the course of the experiment, and therefore the cross peaks of the NOE experiment are a result of both NOEs and chemical exchange. This type of cross-peak can be differentiated using a ROESY experiment (Rotating-frame Overhauser Spectroscopy). The rotating frame in which the data is collected (as oppose to the laboratory frame of NOESY experiments) results in the NOE cross peaks having the opposite phase to the proton resonance signals, thus they appear as negative signals. In contrast the sign of the exchange cross peaks is not affected and therefore these signals have the same sign as the proton resonance signals.
Coherence transfer between nuclei in proximity in space, generating NOE signals, only occurs when the nuclei are in slow exchange, on the NMR time scale, which in turn is defined by the internal motion of the molecule. When considering ligand binding, the exchange rate can be defined by the differences between the chemical shifts of the free and bound protons. If this is large then exchange will be slow, the free and bound ligand resonances are individually resolved and coherence transfer can occur between the protein nuclei and the ligand protons. If the exchange rate is fast, the detected resonances of the ligand will be a weighted average of the free and bound resonances.

Figure 5.1.3.1. The NOE pulse sequence

5.2. LIGAND PROTON RESONANCE ASSIGNMENTS

The glutathione conjugates of DCNB and CDNB were prepared and purified as described in section 2.2. Resonance assignments of protons of the glutathione moiety were made with GSCNP as a model ligand based on chemical shifts, coupling constants and the effects of homonuclear decoupling. In order to resolve the GSCNP proton resonances at 4.72ppm from the resonance of the water protons, which fall in the same region of the spectrum at 298K, the temperature was increased to 315K resulting in a 0.1ppm upfield shift of the water proton resonance. Figure 5.2.1. compares the proton spectra at this temperature with no homonuclear decoupling applied (d, e, and f) with a similar spectrum, except applying homonuclear decoupling at 2.07ppm (a, b, and c). Irradiating the sample at this frequency caused the triplet at 2.46ppm to become a singlet (c and f). This indicates that the protons generating the resonance at 2.46ppm are only coupled two equivalent protons resonating at 2.07ppm. Thus, it can be deduced that the resonance at 2.07ppm is generated by two equivalent protons coupled to two groups of protons: one resonating at 3.64-3.81ppm (b and e) and the other, at 2.46ppm, has no protons on adjacent atoms. By correlating this information with the structure of GSCNP, the resonance at 2.07ppm can be assigned to the protons attached to the β-carbon of the
glutamyl residue. Hence the resonance of the \( \gamma \)-protons can be assigned to that at 2.46ppm and the \( \alpha \)-proton to 3.64-3.81ppm.

Continuing in a similar fashion, spectra in figure 5.2.2. show the effect of homonuclear decoupling at 3.45ppm on the resonance at 4.72ppm: the triplet forms a singlet. This indicates that the protons resonating 4.72ppm are coupled to two similar protons that have a resonance frequency of 3.45ppm. Correlating this to the structure of GSCNP the resonances at 4.72 and 3.38 ppm can be assigned to the protons attached to the \( \alpha \)- and \( \beta \)-carbon atoms of the cysteiny l moiety, respectively.

The spectra in figure 5.2.3. show that by irradiating the sample at 4.72ppm, the resonance assigned to the protons attached to the \( \alpha \)-carbon of the cysteine residue, the resonances in the region of 3.45ppm resolve into two signals (3.47 and 3.42ppm). This indicates that the protons attached to the cysteiny l \( \beta \)-carbon are not equivalent, each experiencing a slightly different chemical environment and therefore resonating at a slightly different frequency. This is a common phenomenon exhibited by equivalent protons attached to carbon atoms adjacent to chiral centres, thus it is common to protons attached to the \( \beta \)-carbon atoms of amino acids. This effect is more pronounced in the spectra of S-iodobenzylglutathione and S-methylglutathione (figure 5.2.5.).

Figure 5.2.1. Spectra of GSCNP with (a, b and c) and without (d, e and f) homonuclear decoupling at 2.07ppm.
Figure 5.2.2. Spectra a and c show the proton resonances of GSCNP with and without homonuclear decoupling at 3.45ppm, respectively. Resonances effected by the decoupling are enlarged in parts b and d.

Figure 5.2.3. Spectra showing the effect of homonuclear decoupling applied to the sample at 4.72ppm on the proton resonances at 3.38ppm. The spectra c and d are not decoupled whereas a and b have homonuclear decoupling at 4.72ppm.
The protons attached to the amide and carboxyl groups will be in exchange with solvent at pH 6.5, which in these experiments was deuterated, thus the resonances of these protons will not be detected. Therefore the only protons within the glutathione moiety remaining to be assigned are those attached to glycyl residue. From the expected chemical shift and by elimination, the resonances of these protons can be deduced to be in the region of 3.64-3.81ppm, overlapping with those from the protons attached to the α-carbon of the γ-glutamyl moiety.

The resonances from the aromatic protons of GSCNP were assigned on the basis of coupling constants and chemical shifts. GSCNP has three protons on the aromatic ring at positions 3, 5 and 6. As there are no protons adjacent to position 3 this resonance will not be coupled, appearing as a single line. Thus, the resonance of proton 3 can be assigned to the signal at 8.35ppm, the most down field signal in the spectrum. Resonances from the protons at positions 5 and 6 will be coupled to each other and, as expected the other two resonances in the aromatic region of the proton spectrum of GSCNP are doublets. That at 8.18ppm was assigned to the proton at position 5 and 7.66ppm to position 6 on the basis of their expected chemical shifts (figure 5.2.4). The chemical shift of aromatic protons depends on the nature of the other groups substituted on to the ring as the greater the electron density around the proton the smaller the chemical shift will be. Nitro-groups withdraw electrons from the ortho and para carbons and increasing the electron density at the meta positions, thus the electron density of proton 5 will be reduced and the electron density of proton 6 increased by the nitro group at position 4. The thiol group at position 1 will reduce the electron density around proton 6, but not to such an extent that the chemical shift is increased above that of proton 5 because of the electron donating properties of nitro group 4. Therefore it is expected that proton 6 will have a greater electron density than proton 5 generating the most upfield resonance. The aromatic resonances of the other two conjugates were similarly assigned and these assignments are shown in figure 5.2.5.b and 5.2.5.c.
Table 5.2.1. Ligand resonance frequencies and their assignment.

<table>
<thead>
<tr>
<th>Resonance (ppm)</th>
<th>Frequency</th>
<th>Assignment</th>
<th>Number of protons in each environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.07</td>
<td>β-Glu</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2.46</td>
<td>γ-Glu</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3.42-3.48</td>
<td>β-Cys</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3.64-3.81</td>
<td>α-Glu and α-Gly</td>
<td>1 and 2</td>
<td></td>
</tr>
<tr>
<td>4.72</td>
<td>α-Cys</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7.63</td>
<td>Aromatic 6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>8.18</td>
<td>Aromatic 5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>8.33</td>
<td>Aromatic 3</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.2.4. Enlarged region of the proton spectrum of GSCNP at 298K showing the aromatic proton resonances.

The resonances of the protons in S-iodobenzylglutathione, GSDNP, glutathione and S-methylglutathione were assigned by comparing the spectra of these ligands with that of GSCNP. The resonances of the α-carbon protons show some differences in the different ligands, with those attached to the glycy1 and cysteinyl residues being more affected by the nature of the S-linked moiety compared to the proton attached to the α-carbon of the glutamyl residue, the latter group having a resonance frequency of between 3.68-3.78ppm in all ligands. The resonance frequency of the protons attached to the α-carbon of cysteine of GSCNP and GSDNP are around 4.7ppm however these protons in glutathione and S-methylglutathione resonate at 4.5ppm and in S-iodobenzylglutathione at 4.31ppm. The protons attached to the α-carbon atoms of the glycy1 residue in the S-substituted glutathione analogues superimposed with those of the glutamyl residue,
whereas in glutathione itself the α-glycyl protons exhibit a down field shift compared to the equivalent resonances in the other ligands, resolving the resonances of these and the α-glutamyl protons. In most spectra the β- and γ-glutamyl resonances were most upfield except in that of S-methylglutathione were the protons of the S-linked methyl group had a smaller resonance frequency. In the spectrum of GSDNP some residual acetonitrile from the purification protocol can be detected resonating at 1.88ppm. The resonances of the protons attached to the cysteinyi β-carbon show the most deviation between the ligands as expected due to the direct linkage between this carbon and the thiol group. It was shown by homonuclear decoupling that the chemical shifts of each of the two protons attached to the cysteinyi β-carbon of GSCNP are slightly different, hence they are experiencing slightly different chemical environments. The resonances from the β-protons in glutathione overlap with one another, such that the peak height of this signal is greater than that in the spectra of the other ligands. The spectra of S-methylglutathione and S-iodobenzylglutathione show that the resonance frequencies of the β-cysteinyi protons are significantly different allowing them to be individually resolved.
Figure 5.2.5. Spectra of the ligands used in these studies at 298K assigned by chemical shifts, coupling constants and by comparison to the spectrum of GSCNP.
Table 5.2.2. Summary of resonance assignment of the ligands at 298K.

<table>
<thead>
<tr>
<th>Resonance assignment</th>
<th>Glutathione</th>
<th>S-methylglutathione</th>
<th>GSDNP</th>
<th>GSCNP</th>
<th>S-iodobenzylglutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-methyl</td>
<td>-</td>
<td>2.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-Glu</td>
<td>2.18</td>
<td>2.16</td>
<td>2.15</td>
<td>2.07</td>
<td>2.13</td>
</tr>
<tr>
<td>γ-Glu</td>
<td>2.51</td>
<td>2.51</td>
<td>2.52</td>
<td>2.46</td>
<td>2.49</td>
</tr>
<tr>
<td>β-Cys</td>
<td>3.91</td>
<td>2.84 and 3.02</td>
<td>3.41-3.52</td>
<td>3.42-3.48</td>
<td>2.81 and 3.00</td>
</tr>
<tr>
<td>α-Glu and α-Gly</td>
<td>3.78</td>
<td>3.72-3.77</td>
<td>3.73-3.85</td>
<td>3.64-3.81</td>
<td>3.69-3.82</td>
</tr>
<tr>
<td>α-Cys</td>
<td>4.52</td>
<td>4.59</td>
<td>4.81</td>
<td>4.72</td>
<td>4.37-4.42</td>
</tr>
<tr>
<td>Aromatic6</td>
<td>-</td>
<td>-</td>
<td>7.90</td>
<td>7.63</td>
<td>7.17</td>
</tr>
<tr>
<td>Aromatic5</td>
<td>-</td>
<td>-</td>
<td>8.48</td>
<td>8.18</td>
<td>7.39</td>
</tr>
<tr>
<td>Aromatic3</td>
<td>-</td>
<td>-</td>
<td>9.05</td>
<td>8.33</td>
<td>7.82</td>
</tr>
<tr>
<td>Aromatic4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.72</td>
</tr>
</tbody>
</table>

In addition to CDNB and DCNB, ethacrynic acid was used to investigate H-site binding. Ethacrynic acid is a larger hydrophobic ligand compared to the substituted single ring aromatic compounds CDNB and DCNB. The crystal structure with ethacrynic acid bound at the H-site has been solved and shows that the side chain of methionine 208 has been pushed out of the H-site resulting the C-terminal helix being distorted compared to when S-benzylglutathione is bound. Further, ethacrynic acid binds deeper in the H-site compared to the hydrophobic moiety of the glutathione conjugate hence it was postulated that by comparing NOEs between residues at the bottom of the H-site to CDNB and ethacrynic acid it could be determined if all hydrophobic ligands bind in this way. The proton spectrum of ethacrynic acid was assigned on the basis of chemical shifts and coupling constants and is shown with the structure of ethacrynic acid in figure 5.2.6.
Figure 5.2.6. The assigned proton spectrum of 0.5mM ethacrynic acid in 50mM phosphate buffer pH 6.5, at 298K.
5.3. LIGAND TITRATIONS

The 1-D proton spectra of hA1-1 GST were recorded with successive additions of ligand. Due to the number of proton resonances in the protein and the line broadening these spectra are complex allowing only a few resonances to be resolved individually. The proton spectrum of apo-enzyme GST shows the majority of the resonances are clustered between 0.2-4.2ppm with some aromatic signals between 6.5 and 7.5ppm, thus ligands were selected with resonance signals in different regions of the spectrum to the apo-enzyme so that some of the resonances of the two species could be distinguished allowing the titration to be more effectively monitored. As the majority of the glutathione resonances fall between 2 and 4ppm, glutathione conjugates were used in the titration, enabling the ligand additions to be monitored by observing the aromatic proton resonances. The Figures 5.3.1.- 5.3.3. show the proton spectra of hA1-1 GST with successive additions of S-iodobenzylglutathione, GSCNP and GSDNP.

Addition of S-iodobenzylglutathione to a concentration equivalent to 0.41 of the concentration of hA1-1 GST active sites results in subtle changes in the protein proton resonances. A resonance can be resolved at -0.13ppm which does not appear at this frequency in the apo-enzyme. Other protein proton resonances are affected throughout the spectrum but are not as clearly resolved as this example. Further additions of ligand result in progressive changes in the protein proton resonances however the complexity of the spectra is such that it is difficult to resolve individual resonances. However, the resonances of the ligand aromatic protons can be detected clearly over the protein proton resonances between 0.41-0.66 equivalents of S-iodobenzylglutathione to binding sites. As more ligand is added the aromatic resonances progressively move down field towards the free resonance frequencies. Thus it can be concluded that S-iodobenzylglutathione binding to GST is in fast exchange. The chemical shift of the ligand proton resonances, observed over the protein proton resonances, represents a weighted average of the chemical shift in the free and bound states. Therefore, as the chemical shift of the resonances is proportional to the amount of ligand in each state, it can be analysed as a quadratic function of the concentration of ligand to determine the dissociation constant. However, the protein concentration used in these titrations is 150 times larger than the dissociation constant determined by fluorescence titrations for GSDNP, another glutathione conjugate, hence the NMR titration is representative of stoichiometric binding. Indeed, the value of the dissociation constant was estimated by this method to be 1.06±0.04mM which is the close to the concentration of the dimer in the titration and is not a good indication of the true dissociation constant. The estimated resonance frequencies of the bound protons are shown in italics in table 5.3.1. Apart from the large concentration of protein used in these titrations there are several other inaccuracies
associated with this method for determining dissociation constants: Slight changes in the resonance frequency of the protons (0.05 ppm) are correlated to large changes in the concentration of the ligand and the ligand resonances at low concentrations are difficult to determine over the protein proton resonances, in the case of S-iodobenzylglutathione these resonances only become apparent between 0.44 and 0.61 equivalents of S-iodobenzylglutathione to active sites, hence the number of data points that can be used to describe the binding is restricted.

Table 5.3.1. The chemical shift of the aromatic protons of S-iodobenzylglutathione with increasing concentration of this ligand in 0.75 mM active site solution of GST. [The chemical shift of the bound protons was determined as described above and is shown in italics.]

<table>
<thead>
<tr>
<th>Ratio of S-iodobenzylglutathione to active sites</th>
<th>Chemical Shift (ppm) of aromatic protons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1.0:0.0 (free)</td>
<td>7.818</td>
</tr>
<tr>
<td>2.30:1.0</td>
<td>7.777</td>
</tr>
<tr>
<td>2.05:1.0</td>
<td>7.774</td>
</tr>
<tr>
<td>1.69:1.0</td>
<td>7.771</td>
</tr>
<tr>
<td>1.44:1.0</td>
<td>7.768</td>
</tr>
<tr>
<td>1.17:1.0</td>
<td>7.765</td>
</tr>
<tr>
<td>0.92:1.0</td>
<td>7.756</td>
</tr>
<tr>
<td>0.66:1.0</td>
<td>7.742</td>
</tr>
<tr>
<td>0.41:1.0</td>
<td>7.727</td>
</tr>
<tr>
<td>(bound)</td>
<td>7.72</td>
</tr>
</tbody>
</table>
Figure 5.3.1. Proton spectra of hA1-1 GST (0.75mM active sites) and increasing concentrations of S-iodobenzyl glutathione. Part a shows all proton resonances and parts b and c enlarged regions of interest.
Figure 5.3.1. (continued).
Figure 5.3.2. Proton spectra describing the titration of GSCNP into 0.75mM active site solution of hA1-1 GST. Part a shows all proton resonances and parts b and c enlarged regions of interest.
Figure 5.3.2. (continued).
Table 5.3.2. The chemical shift of the aromatic protons of GSCNP with increasing concentration of this ligand. The bound chemical shift of the protons determined as described above are shown in italics.

<table>
<thead>
<tr>
<th>Ratio of GSCNP to active sites</th>
<th>Chemical Shift (ppm) of aromatic protons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1.0:0.0 (free)</td>
<td>8.363</td>
</tr>
<tr>
<td>7.73:1.0</td>
<td>8.287</td>
</tr>
<tr>
<td>6.07:1.0</td>
<td>8.282</td>
</tr>
<tr>
<td>4.33:1.0</td>
<td>8.270</td>
</tr>
<tr>
<td>3.40:1.0</td>
<td>8.266</td>
</tr>
<tr>
<td>2.52:1.0</td>
<td>8.262</td>
</tr>
<tr>
<td>1.11:1.0</td>
<td>8.254</td>
</tr>
<tr>
<td>(bound)</td>
<td>8.23</td>
</tr>
</tbody>
</table>

The titration of GSCNP into Ha1-1 GST solution is shown in figure 5.3.2. The aromatic signals of the free ligand are not clearly distinguishable until the ligand is in 2.5 times excess of H-sites; the aromatic region of the spectra is enlarged and shown as figure 5.3.2.b. The upfield methyl region of the spectra is also enlarged as individual protein proton resonances within this region can be observed, however there are no obvious changes in the resonances by which the binding of GSCNP can be characterised. The free ligand signals are not observed until the ligand is in excess of the H-sites, probably due to exchange broadening, therefore it appears that binding is in intermediate exchange, hence the dissociation constants for GSCNP to Ha1-1 GST cannot be determined by NMR spectroscopy.

The aromatic proton resonances of free GSDNP can be detected at a similar concentration as the equivalent signals were detected in the GSCNP titration (figure 5.3.3.b.), that is when the conjugate is approximately in 2.5 times excess of the number of H-sites. Further, these resonances are in the same position as the free resonance signals indicating that GSDNP is in intermediate-slow exchange. Hence the dissociation constant for
GSDNP cannot be determined from the changes in the ligand resonance frequency. Addition of GSDNP to hA1-1 GST alters some protein resonances. Of particular use to NMR studies is the upfield methyl proton resonance shift that occurs during the course of the titration (see figure 5.2.3.c.). It is difficult to say which apo-enzyme resonance undergoes this shift, possibly that at 0.05ppm, however as this is on the edge of the cluster of protein proton resonances it is unclear if this resonance indeed moves upfield or the observed resonance shift originates as one of the superimposed resonances with a greater frequency than 0.2ppm. However, this upfield methyl shift can be characterised in the spectra of hA1-1 bound to GSDNP. It appears the resonance broadens such that it is indistinguishable from the baseline up to a 1:1 ratio of GSDNP and H-sites. However, as the GSDNP concentration increases further the resonance signal at -0.45 ppm becomes increasingly more evident. If GSDNP binding is in slow exchange the aromatic resonances will only be detected at two frequencies corresponding to the bound and free states of the ligand. This is particularly useful when trying to identify NOE cross peaks between the protein and the ligand as the ligand resonances can be clearly identified and the chemical shift is not dependent on the concentration of the ligand. GSDNP was the only conjugate tested that exhibits slow exchange hence it was used in the subsequent ligand binding studies with its precursors glutathione and CDNB.
Figure 5.3.3. Proton spectra of hA1-1 GST showing the change in resonances on addition of GSDNP. Part a shows all protein proton resonances and parts b and c show the regions where the free ligand signals are observed and the changes in the upfield region of the spectra, respectively.
Figure 5.3.3. (continued).
A similar change in the upfield region of the proton spectra of hA1-1 GST was observed on addition of the hydrophobic ligand ethacrynic acid. Although ethacrynic acid is a substrate for glutathione conjugation catalysed by some GST isoenzymes, particularly pi-class, the activity of hA1-1 GST with ethacrynic acid is so low that it is often classed as an inhibitor of this enzyme. Ethacrynic acid has been shown by X-ray crystallography to bind to the H-site of hA1-1, thus the inhibition is competitive with respect to the hydrophobic substrate. As ethacrynic acid is a larger ligand than either DCNB or CDNB, it occupies more of the H-site and therefore using this ligand it may be possible to detect additional intermolecular NOEs. The binding of ethacrynic acid was characterised by observing the changes in the proton spectra of hA1-1 GST with increasing concentrations of this ligand (figure 5.3.5.). In addition to the upfield methyl shift (figure 5.3.5.c.), the only clear difference between the spectra was the detection of the free ligand signals when ethacrynic acid was in two-fold excess of H-sites. The free signals of ethacrynic acid can be detected when the ligand concentration is in 1.6-2-fold excess of that of the active sites appearing at the same resonance frequency as those in the absence of protein, indicating that ethacrynic acid is in slow exchange. As some of the free ethacrynic acid signals (Figure 5.2.6. resonances c and d) appear in a region of the protein spectrum where there are few protein resonances indicates that ethacrynic acid could be a useful ligand for NMR studies.

5.3.5. Ethacrynic acid was titrated into a 1mM H-site solution of GST resulting in a similar upfield methyl shift to that observed with GSDNP (5.3.5.c.). The free ligand signals were observed above a 2-fold excess of ligand (5.3.5.b.).
5.4 \textit{1}H 2-D LIGAND BINDING STUDIES.

Due to the complexity of the 1-D spectra, 2-dimensional spectra were generated by recording a series of FIDs, with incremental times for coherence transfer and performing two Fourier transforms on the data. Two-dimensional NOE experiments were recorded on 1mM hA1-1 GST samples containing a 5-fold excess of GSDNP. This ligand was used as it is more soluble in aqueous solvent, compared to its hydrophobic substrate precursor (CDNB), but it retains the aromatic protons generating resonances in a region of the spectrum where there are comparatively few protein proton resonances. An NOE spectrum of hA1-1 GST in the presence of a 5-fold excess of GSDNP is shown in figure 5.4.1. Cross peaks are shown between the two resonance frequencies of a proton experiencing chemical exchange and protons exhibiting both inter- and intramolecular NOE. This makes the upfield region of the spectra (-1.5ppm) complex due to the vast number of protein proton intramolecular NOEs. However the aromatic regions of the spectrum is less crowded and cross peaks can be individually resolved. The aromatic proton resonances of free GSDNP, assigned from the 1-D proton titration in section 5.2.5. are labelled on the diagonal in figure 5.4.1., which includes proton resonances from the protein and ligand. Clear cross peaks can be detected between all three free GSDNP resonances. The free resonance at 9.05ppm shows an exchange cross peak with a resonance at 8.12ppm, indicating that the bound proton at position 3 on the aromatic ring of GSDNP resonates at 8.12ppm. The free resonances from proton 5 and 6 (at 8.42 and 7.81ppm respectively) share an intramolecular NOE, shown by the cross-peak between these two resonances. Three further cross peaks are detected between the resonance at 8.42ppm, with resonances at 7.08, 6.93, and 4.98ppm. These cross-peaks could either be exchange peaks, between the free and bound proton resonance frequencies or intermolecular NOEs with the protein. Rotating-frame Overhauser spectroscopy (ROESY) can be used to distinguish between exchange and NOE cross peaks as the rotating frame in which the data is collected (as oppose to the laboratory frame of NOESY experiments) results in the NOE cross peaks having the opposite phase to the proton resonance signals, whereas chemical exchange peaks have the same phase. The ROESY experiment of hA1-1 GST with GSDNP bound is shown in figure 5.4.2. with the positive signals (including the proton resonances and the exchange cross peaks) shown in black and the negative cross-peaks (corresponding to NOE cross-peaks) in red. The resonances between 7.08 and 6.93 are clearly exchange peaks, possibly implying that the bound state of GSDNP has two conformations in which the proton attached to carbon 5 resonates at a slightly different frequency. Two conformational states have also been detected for the ethacrynic acid moiety of the glutathione conjugate using X-ray crystallography\textsuperscript{82}, thus it appears that this may be common to the hydrophobic moiety of
glutathione conjugates. Cross peaks between the free resonance of the proton attached to carbon 6 and resonance at 7.23, 7.08, and 6.88ppm are detected (figure 5.4.1.). From the ROESY experiment it can be deduced that the most downfield signal (7.23ppm) is a NOE however the other two resonance are exchange peaks, again implying that the bound form of GSDNP has two conformers, resulting in the proton at position 6 generating two resonances. Interestingly, the frequency of the bound resonances of protons at position 5 and 6 are similar whereas the free resonance signals show over 0.5ppm difference.

Figure 5.4.1. The NOE spectrum of hA1-1 GST in the presence of a five-fold excess of GSDNP.
Figure 5.4.2. The ROESY experiment with hA1-1 GST in the presence of a 5-fold excess of GSDN\(^2\) showing the positive signals (including proton resonances and exchange peaks) in black and negative signals (NOEs) in red.
The bound signals of CDNB were similarly assigned using 2-D NMR techniques, however due to the lower solubility of this ligand in aqueous solvent (≤1.5mM) in order to achieve a suitable ratio of ligand binding sites to ligand a much reduced concentration of protein was used (0.2mM active sites). In turn the spectra generated were of poor quality. To improve the resolution deuterated protein was used, hence the number of intramolecular protein proton NOEs was minimised allowing the cross peaks involving CDNB resonances to be identified. Better spectra were recorded in the presence of S-methylglutathione (figure 5.4.3), perhaps due to the higher affinity of the glutathione-enzyme complex for CDNB and perhaps due to the increased stability of the glutathione bound protein. Of particular interest are the cross peaks observed in the presence of S-methylglutathione between the free resonance of the CDNB proton at position 6 (7.85ppm) and a resonance at 7.20ppm, indicating that perhaps the latter resonance is generated by this proton in bound CDNB. Although exchange cross peaks between the free resonance of the CDNB proton at position 5 are not detected the bound resonances can be assigned by the intramolecular NOE between this and the proton at position 6, indicating the bound proton 5 resonates at 7.05ppm.

Due to the production method of the media used to make deuterated protein, positions 3 and 5 of tyrosine amino acids residues are protonated. Thus in the spectra of the deuterated protein strong resonances can be detected in the region of 6-7ppm generated by these tyrosine protons. A cross peak can be detected between one of these tyrosine protons resonating at 6.49ppm and a resonance at 7.20ppm. That this cross-peak could represent an intermolecular NOE between the CDNB proton at position 6 and a proton attached to a tyrosine, possibly tyrosine 9, at position 3 or 5.

Figure 5.4.4. shows the cross-peaks generated by deuterated hA1-1 GST bound to CDNB. The only exchange cross-peaks detected are between the free and bound states of the CDNB proton attached to carbon 6. These spectra are too poor a quality to deduce any firm conclusions about the mode of CDNB binding in the presence and absence of S-methylglutathione.
Figure 5.4.3. The NOE experiment performed on deuterated hA1-1 GST in the presence of an equimolar concentration of CDNB to active sites and a two-fold excess of S-methylglutathione.
Figure 5.4.4. The NOE spectrum of hA1-1 GST in the presence of 0.7 equivalents of CDNB to active sites.
Comparing the 1-D proton spectra of wild-type, Y9F and the truncated hA1-1 GST (figure 5.4.5.), the resonance at 6.50 does not appear to be generated by Y9 or affected by the deletion of the C-terminal helix as differences between the mutated and wild-type hA1-1 spectra are in the region of 6.65 and 6.8ppm. Further, the resonance at 6.5ppm does not appear to be affected by the titration of CDNB into the GST solution (figure 5.4.6.).

**Figure 5.4.5.** Spectra showing the proton resonances of wild-type (a), Y9F (b), and the truncated hA1-1 GST (c) in the absence of ligand.

The titration of CDNB into the wild-type deuterated protein is shown in figure 5.4.6. Free CDNB resonances can be detected when the ligand is above a concentration equivalent to one third of the concentration of binding sites. Further these signals have the same resonance frequency as the free ligand signals, indicating that they are not a result of fast exchange, which was expected because of the comparatively large difference in the resonance frequencies of the free and bound protons of CDNB. The resonances of proton 5 are in the same region of the spectra as an impurity spike at 8.46ppm, thought to be a contaminant of the deuterium oxide used as a solvent, however the CDNB resonance can be seen as a shoulder on the downfield side of this signal. The other two CDNB proton resonances can be clearly resolved. Some changes in the residual protein protons can be detected on the addition of CDNB, in particular the tyrosine resonances show gradual alterations as the ligand concentration increases: The resonance at 6.36ppm shifts slightly downfield closer to the resonance at 6.39ppm and the resonance at 6.77ppm becomes less intense as the CDNB concentration increases, probably because this peak is
composed of at least two resonance signals, one of which is affected by CDNB binding shifting downfield such that it appears on the side of the resonance at 6.77ppm at a 1.61 fold excess of CDNB. The resonance signals from CDNB protons 5 and 6 in the bound state are expected at 7.20 and 7.05ppm respectively, however these signals are not clearly detected over the baseline of the spectra. This is probably due to the increased line width of the resonances in the bound state compared to the free state, as bound CDNB adopts the molecular motion of the enzyme. Further the 2-D spectra indicate that CDNB may be bound in two conformational states thus, the concentration of bound CDNB protons in each state is less than the protein concentration (0.2mM) implying that the signals from bound CDNB will be weak.
Figure 5.4.6. Proton spectra of deuterated wild-type hA1-1 showing changes in the region of 6-10ppm on addition of CDNB.
Addition of CDNB to the deuterated truncated protein yields similar results in that the free signals of CDNB are detected above a 0.3 ratio of CDNB to active sites and the bound resonances are not detectable (Figure 5.4.7.). The tyrosine resonances in the deuterated protein differ from those of the wild-type enzyme, indicating that although the deletion does not include any tyrosine residues some of the environments are altered. It could be expected that the deletion would affect the resonance of tyrosine 9, which is in the active site and therefore in proximity to the terminal residues of the helix. However, it is difficult to assign the resonances of Y9F by comparing the spectrum of this mutant with the wild-type enzyme (figure 5.4.5.) as there is a significant amount of overlap of the resonance signals. It does appear, however, that the most upfield resonance at 6.37ppm is not detected in the Y9F spectrum and the intensity of this resonance is also dramatically reduced in the spectrum of the truncated enzyme, perhaps if this is a proton resonance of tyrosine 9, because the more open environment of the active site in the truncated enzyme results in an increased rate of exchange of these protons with the solvent, which in this case was deuterated resulting in the proportion of protonation being decreased. Alternatively the reduced intensity could be a result of this residue exhibiting an increase degree of conformational exchange in the absence of the C-terminal helix. Due to the proximity of the C-terminal residues of the helix, particularly F220, it could be postulated that the C-terminal helix is either directly or indirectly involved in stabilising tyrosine 9. It is interesting to note that the most upfield tyrosine resonance in the wild-type spectrum is also affected by CDNB binding, adding further support for its tentative assignment to tyrosine 9, as this residue is in close proximity to bound CDNB. Addition of S-methylglutathione to either the wild-type or truncated form of hA1-1 GST in the presence of CDNB (at a ratio of 0.7 to active sites) does not appear to affect the free CDNB signals nor make the bound signals visible (figures 5.4.8. and 5.4.9.). When S-methylglutathione binds to the wild-type enzyme-CDNB complex the most upfield tyrosine resonance (6.36ppm) shifts to 6.39ppm. S-methylglutathione does not further effect on the resonances at 6.77ppm but results in a shoulder forming on the upfield side of the resonance at 6.39ppm. This implies that this signal comprises of more than one resonance superimposed, one of which is affected by S-methylglutathione resulting in an upfield shift. Similar effects are not observed in the truncated protein, however the mutation has altered the resonance frequencies of some tyrosine resonances hence the effect of S-methylglutathione binding on the resonances in the region of 6.62-6.71 are probably equivalent to those observed in the wild-type spectra.
Figure 5.4.7. Proton spectra recorded after incremental additions of CDNB to the truncated enzyme.
Figure 5.4.8. Proton spectra recorded after incremental additions of S-methyl glutathione to the wild-type enzyme containing 0.7 equivalents of CDNB.

Figure 5.4.9. Proton spectra recorded after incremental additions of S-methyl glutathione to the truncated enzyme containing 0.7 equivalents of CDNB.
6. THE USE OF SELECTIVE LABELLING TO STUDY LIGAND BINDING.

6.1. INTRODUCTION.

NMR studies are normally limited to small proteins and peptides, and the GST sub-unit with 222 amino acid residues represents the upper size limit of proteins amenable to NMR studies. That GST is a dimer introduces two further difficulties: Firstly the dimer may be asymmetric resulting in equivalent nuclei from each sub-unit having a different resonance frequency. Secondly, the total molecular weight of the molecule, that is 51kD, determines the mobility of the enzyme in solution, which in turn affects the line widths of the NMR signals: The larger the molecule, the slower its molecular motion and therefore the broader the resonance signals become. Broad resonance signals have a reduced peak height as the area under the resonance signal is proportional to the number of nuclei that resonate at that frequency. Broad signals also exhibit a greater degree of overlap with adjacent signals, hindering the resolution of individual resonances. Thus the use of conventional techniques for resonance assignments, involving the transfer of phase coherence between adjacent NMR-sensitive nuclei, cannot be applied with much success to hA1-1 GST. Therefore in order to assign the resonances of hA1-1 GST an alternative technique was employed, using site-directed mutagenesis to identify resonances of individual amino acid residues and selective labelling to facilitate the observation of individual resonances.

The spectra of uniformly $^{15}$N-labelled hA1-1 GST in the presence (red) and absence (black) of GSDNP are shown in figure 6.1.1. The regions of the spectra containing the resonance of the amide nitrogen nuclei are shown in 6.1.1.a. Theoretically there are 209 backbone amide resonances, one from each amino acid residue, excluding prolines, in the GST sub-unit, and 24 resonances from the amide nitrogen nuclei in the side chains of asparagine and glutamine residues. These side chain amide resonances would be localised in the upper region of the spectrum, approximately between 100-115ppm in the nitrogen dimension, and are easily recognised as commonly the equivalent protons attached to the amide nitrogens have different resonance frequencies. Thus two resonance signals are generated, both with the same nitrogen resonance frequency but differing in the proton resonance frequency. However, due to the line width and number of resonances, few can be resolved individually, hence it is difficult to deduce the exact number of resonances. The line width of these resonances was reduced in similar spectra by using deuterated $^{15}$N labelled protein, as shown in figure 6.1.2. The line widths of the resonances from the deuterated protein (red) are markedly decreased compared to the protonated sample (black) allowing nearly all of the resonances to be resolved.
individually. However the number of resonances still makes alterations in the spectrum due to ligand binding or mutagenesis difficult to differentiate, but it can be estimated that approximately 14% of the backbone amide resonances are affected by ligand binding.

Figure 6.1.1.a. The $^1$H-$^{15}$N HMQC spectrum of uniformly $^{15}$N-labelled hA1-1, showing the amide resonances.

Figure 6.1.1.b. The $^1$H-$^{15}$N HMQC spectra of arginine side chain resonances in the presence and absence of GSDNP.

Protein samples and spectra were prepared by J.-C. Yang (see Acknowledgements).
The arginine guanidino group side chain resonances are shown in Figure 6.1.1.b. Approximately ten resonance signals are shown, which indicates that not all of the twelve arginine $^{15}$N resonances are resolved. Interestingly, at least three of these resonances are shown to change frequency or peak height on ligand binding and all resonances show a reduced line width in the presence of ligand.

The amide cross-peaks shown in the uniformly labelled spectra have a range of peak heights. There are several possible explanations for this. For example, some resonances could have a greater height due to more than one amide nitrogen nucleus resonating at the same frequency and as the spectra are so complex it is difficult to say whether each resonance signal integrates for a single nucleus. Alternatively the range of peak heights may be a result of chemical or conformational exchange. The protons attached to the amide nitrogen atoms of surface residues are more likely to exhibit chemical exchange with the solvent compared to residues that are buried within the protein molecule, increasing the line width and therefore reducing the peak height of the recorded resonance. Conformational change can also effect the line width of the resonances,
depending on the exchange rate of the signals. When regions of the protein are in slow conformational exchange the amide nitrogen resonances of flexible regions of the protein are more likely to be weak due to the increased number of chemical environments experienced by the nucleus. If the nucleus is located on a portion of the protein that is in fast conformational exchange the detected resonances will be an average of the resonances in all conformations, but can still be detected. Conformational exchange can either effect entire structural motifs of the protein or single nuclei, both being a result of the internal molecular motion of the protein defining the correlation time of the nucleus.

Changes in resonance frequency arise from to changes in the chemical environment, either due to alterations in the electron density of the NMR-sensitive nuclei, due to the proximity of other nuclei or chemical bonding or conformational changes. From the crystal structure of hA1-1 GST bound to S-benzylglutathione 10% of the residues of hA1-1 GST that are in proximity to the bound ligand can be identified. These include, in the G-site, Y9, R15, R45, V55, Q67, T68, D101 and R131, and in the H-site F10, A12, R13, Q14, E104, L107, L108, P110, V111, M208, L213, R217, and F220. All of the G-site residues are postulated to hydrogen bond to glutathione: Hydrogen bonds involving R45, R131, Y9, D101 and Q67 are through the side chains of these residues, the other three residues (V55, T68 and R15) hydrogen bond through the backbone amide or carboxyl groups. Thus, it would be expected that the resonances of the constituent nuclei of these residues would be effected by ligand binding. Resonances can also be indirectly effected by ligand binding, due to a conformational change rather than the proximity of the ligand.

Comparing the 3-dimensional crystal structures of hA1-1 GST in the presence and absence of a glutathione conjugate (S-benzylglutathione) two regions of structural change can be identified: The side-chain of phenylalanine-10 undergoes a 90° rotation and the C-terminal helix (helix-9) becomes less flexible, docking over the H-site. Therefore the resonances of these residues are likely to be effected by ligand binding.

In order to investigate further the affects of ligand binding on the protein resonances the spectra need to be simplified so that specific resonance changes can be observed. Using auxotrophic E.coli strains DL39 and AB1255 transformed to express hA1-1 GST a range of selectively labelled proteins were produced. The E. coli were grown in minimal media supplemented with specific amino acids containing NMR sensitive stable isotopes. Thus, these amino acids were incorporated into the GST such that it was selectively labelled by amino acid type. This allowed the NMR spectrum to be edited to contain only resonances from one type of amino acid residue allowing these to be studied independently. Primarily, this allows resonances to be assigned by amino acid type thus the affects of ligand binding on each type of amino acid residue can be assessed. For
example, on addition of ligand only one tyrosine amide resonance is affected (see figure 6.2.1.) and by correlating the NMR spectral changes to the 3-dimensional structure of the protein tentative assignments can be made. Tyrosine-9 is most probably the resonance that is affected by ligand binding as this residue is in close proximity to the bound ligand and this tentative assignment can be confirmed by mutagenesis (section 6.2.).

6.2. $^{15}$N-TYROSINE LABELLED hA1-1 GST.

The 2D spectra of hA1-1 GST selectively labelled with $^{15}$N-tyrosine were recorded for the apo-enzyme and the enzyme in the presence of a 5-fold excess of GSDNP. Figure 6.2.1. shows the two spectra overlaid, with the spectrum of the apo-enzyme in black and that of the GSDNP complex in red. Ten resonances can be detected in both spectra, generated by the amide nitrogens from the ten tyrosine residues in hA1-1 GST. However, one signals has a different resonance shift in the presence of ligand, labelled a in both spectra, becoming superimposed with resonance b when GSDNP is bound. As tyrosine 9 is the only tyrosine residue postulated to be in proximity to the bound ligand, this resonance was tentatively assigned to the amide nitrogen of tyrosine 9. The assignment was confirmed by comparing the spectra of wild-type and Y9F hA1-1 GST selectively labelled with $^{15}$N tyrosine (figure 6.2.2.). One resonance is absent in the spectra of Y9F in both the presence and absence of ligand and therefore this can be assigned by elimination to tyrosine 9, and is labelled a in the wild-type spectra.

Figure 6.2.1. - 2D spectra of hA1-1 GST selectively labelled with $^{15}$N tyrosine in the presence (red) and absence (black) of DNPGSH.
6.3. 15N-ISOLEUCINE LABELLED hA1-1 GST.

The spectra of hA1-1 GST selectively labelled with 15N-isoleucine in figure 6.3.1. show that the resonances of the amide nuclei of isoleucine residues are similar in the presence of glutathione or GSDNP. Comparing the changes induced by GSDNP binding (figure 6.3.2.) two resonances (d and j) have an increased intensity in the presence of ligand. Although not all resonances in the presence and absence of ligand are exactly superimposed, ten resonances show changes of less than 0.1 ppm in each dimension. Of the other five resonances c shows a 0.15 ppm shift in the proton dimension, and the resonance frequencies of j, o, n and m are affected to a greater extent. These resonances are replaced by resonances p, q and r in the spectra recorded in the presence of ligand, hence not all are individually resolved when ligand is bound. It is possible that the fifteenth resonance is not detected in the presence of ligand due to its weak intensity. However, this is uncharacteristic of the changes that occur on ligand binding, generally leading to increased resonance intensities. In instances such as this 1D slices of the 2D spectra can often be more informative, identifying resonances that have become superimposed.
Figure 6.3.1. - The spectra of hA1-1 selectively labelled with $^{15}$N-isoleucine in the absence of ligand (black), with a 5-fold excess of glutathione (purple) and after the *in situ* formation of GSDNP (red).

Figure 6.3.2. - The spectra of hA1-1 selectively labelled with $^{15}$N-isoleucine in the presence (red) and absence (black) of GSDNP.
Figure 6.3.3. shows 6 1D slices with the resonances labelled accordingly with the 2D spectra. Spectra A shows the changes that occur in the 2-D spectra at 117.3ppm on addition of ligand. The resonances m and k are both visible in this slice of the apo-enzyme spectrum, however on addition of ligand one, apparently m is no longer visible. In contrast, an additional resonance becomes evident on addition of ligand in spectra B (117.1ppm). Although the peak heights of the signals show considerable variation, it is clear that on addition of glutathione apo-enzyme resonance j undergoes a 0.1ppm downfield shift in the proton dimension and becomes superimposed with an additional resonance that is not detected in this 1D slice of the apo-enzyme. On addition of GSDNP these resonance become resolved and are labelled p and r. Spectra C show the resonance h and o, detected in the 124.5ppm 1D slice. It is evident that although each apo-enzyme resonance is generated by a single nucleus the intensities of the signals are quite different. On addition of ligand resonance o is no longer detected in this or other 1D slices, however it is unlikely that it has become weaker and therefore indistinguishable with the baseline as generally ligand binding improves the quality of the spectra. This resonance could have become superimposed with a more intense resonance, such as resonance f in spectra E, the height of which is so much greater than that of o it would be difficult to detect this resonance in this situation. Spectra f show a small shoulder has appeared on resonance i when GSDNP is bound, which could account for the disappearance of resonance o. Unfortunately the varying peak heights make tracking down resonances effected by ligand binding difficult, even when using 1-D slices.
Figure 6.3.3. 1-D slices of the spectra in figure 6.3.2.

A)  

GSDNP-GST

B)  

Glutathione-GST

C)  

GST

D)  

GSDNP-GST

Glutathione-GST

GST

124
6.4. $^{15}$N-VALINE LABELLED hA1-1 GST.

Figure 6.4.1. The standard 2D (a) and water flip-back (b) spectra of $^{15}$N-Valine labelled hA1-1 GST.

A range of peak heights was also observed in the 2D spectra of hA1-1 GST selectively labelled with $^{15}$N-Valine. However, exactly ten resonances corresponding to the ten valine residues in hA1-1 GST can be resolved indicating that signal integrates for one equivalent valine amide nitrogen from both sub-units. Thus the peak height is affected by the line width of the resonance, which in turn is an effect of the correlation time of the nucleus. Figure 6.4.1. shows the results of using different pulse programs on the same sample to generate the 2-D spectra. Comparing the contours of the $^{15}$N-valine...
resonances in the apo-enzyme recorded using the standard 2D HMQC experiment (section 6.1) with those recorded using the water flip back experiment there are some differences in the peak heights of the resonances detected in each spectrum. The water flip back experiment incorporates an additional pulse to return the water protons to the z-axis before acquisition so that amide nitrogen resonances attached to protons in exchange with the water protons can be detected similarly to those that are not in exchange with the solvent. Hence, if resonances are weak in the standard 2D experiment due to proton exchange with the solvent, then in the water flip back spectrum these resonances will have a similar peak height as the other resonances in the protein. Although the intensities of some signals are more equivalent using the water-flip back experiment, for example the more intense resonances in the standard experiment (b, c, h-g, and j) are recorded with a similar intensity to resonances f and e using the water-flip back pulse sequence. However there is still a range of peak heights implying that proton exchange is not entirely responsible for this effect, but there is a degree of conformational exchange in the molecule. This indicates that there are fluctuations in the internal motions of the protein that are not uniform through out.

The 2D spectra of hAl-1 GST selectively labelled with 15N-Valine were recorded for the apo-enzyme and the enzyme in the presence of a 5-fold excess of GSDNP. Figure 6.4.2. shows the two spectra overlaid, with the spectrum of the apo-enzyme in black and that of the GSDNP complex in red. Ten resonances are detected in both spectra, generated by the amide nitrogens from the ten valine residues in hAl-1 GST. Eight of the resonances are not significantly affected by ligand binding, the other two show slight resonances shifts: The apo-enzyme resonance b moves 0.1ppm downfield in the proton dimension and d shifts 0.1ppm upfield in the proton dimension and 1.5ppm in the nitrogen dimension. Thus two of the amide resonances affected by ligand binding in the uniformly labelled spectra (figure 6.1.1.a.) can be assigned to valine residues. From the crystal structure two valine residues are shown to be in proximity to the ligand: one at the G-site (V55) and the other at the H-site (V111). Thus it is probable that the resonances affected by ligand binding are generated by the amide nitrogen nuclei in these two residues.

What is notable about these and other spectra comparing the protein resonances in the presence and absence of ligand, is that the line width of the resonances in the presence of ligand are significantly less making the signals more clearly detectable above the noise. Thus ligand binding appears to reduce the conformational flexibility of the protein. This conformational change not only effects the NMR spectra but also the stability of the enzyme: The apo-enzyme denatures rapidly and changes in the apo-enzyme proton spectra can be observed after 72 hours storage at 4°C in the NMR tube. In the presence
of ligand, however, GST is more stable and can be stored at 4°C for several weeks before any changes in the proton spectra can be detected.

Figure 6.4.2. The 2D-spectra $^{15}$N-valine labelled hA1-1 GST in the absence (black) and presence (red) of GSDNP.

6.5 $^{15}$N-ARGININE LABELLED hA1-1 GST.

The spectra of $^{15}$N-arginine labelled hA1-1 GST are shown in figure 6.5.1. In addition to the backbone amide resonances, arginine residues have three further nitrogen atoms in the side-chain. However as some protons will be in rapid exchange with water, the only side-chain resonance to be detected in these spectra are those of the Ne nucleus. Only the backbone amide resonances are displayed in figure 6.5.1.a. This is possible as the resonance frequencies of the $^{15}$N atoms of the side chain and backbone differ markedly, thus the distribution of the resonances in the F1 dimension (y-axis) allows the backbone amide resonances to be selected independently of side chain resonances. In the apo-enzyme (black) only eleven of the twelve amide arginine resonances are resolved, but on addition of ligand all twelve resonances become visible. The heights of the resonances in the spectra also show significant differences and addition of ligand causes changes in peak height as well as frequency shifts. In the apo-enzyme five $^{15}$N amide resonances (c, g, i, j, and k) of arginine residues are strong and four are very weak (a, b, d, and h). On addition of ligand two of the weak resonances (d and h) become significantly more intense and a resonance that was not detectable in the apo-enzyme becomes visible; however due to the resonance shifts that occur on ligand binding it is difficult to identify positively the origin of this resonance further than saying it is either resonance o, m, n or
Seven resonances remain at approximately the same frequency (a, b, c, d, e, g, and i) whereas the apo-enzyme resonance h undergoes a 1ppm shift in the F1 dimension and a 0.1ppm shift in F2. In the presence of ligand, the resonances equivalent to f, j and k are difficult to positively identify, however it is likely that resonance m corresponds to resonance j. The spectrum recorded in the presence of GSDNP shows two resonances flanking apo-enzyme resonance k (n and l) either of which could be the equivalent. Apo-enzyme resonance f has undergone a substantial shift on binding to ligand and an additional resonance is detected, thus the ligand-bound resonance corresponding to f could either be one of the resonances flanking k (n or l) or resonance o. Of the twelve arginine amide resonances five are affected more than the other seven on addition of GSDNP. Thus five more amide resonances affected by ligand binding can be assigned to arginine residues. Correlating the changes in the NMR spectra on addition of ligand with the 3-dimensional structure of hA1-1 GST, three arginine residues are thought to be involved in hydrogen bonding at the G-site (R15, R45 and R131) and two are in proximity to the hydrophobic ligand, possibly forming part of the H-site (R13 and R217). Hence the five arginine residues affected by ligand binding can be tentatively assigned to R13, R15, R45, R131 and R217.

Figure 6.5.1. The amide region of the 2D spectra of 15N-arginine labelled hA1-1 GST in the absence (black) and presence (red) of GSDNP.
Figure 6.5.2. The positions of the arginine residues in hA1-1 GST relative to the bound ligand S-benzylglutathione.

The carbon backbone of the protein is shown in green with the arginine residues shown in full in “ball and stick” fashion. All arginine residues from subunit A are shown, coloured yellow, and those from subunit B in proximity to the bound ligand are shown in pink. The ligand (S-benzylglutathione) is also shown in “ball and stick” fashion with the glutathione moiety coloured blue, the thiol group yellow and the benzyl moiety coloured red.

Interestingly, not all $^{15}$N-arginine resonances were detected in the apo-enzyme yet twelve signals were apparent in the presence of ligand. In the absence of glutathione-analogue or hydrophobic ligand the position of C-terminal helix (helix 9), characteristic of the alpha-class GSTs, cannot be accurately defined as the electron density in this region is weak and incomplete (see Chapter 7), particularly in the region of R217. Thus it is possible that the undetected resonance in the apo-enzyme spectrum may correspond to R217. If this tentative assignment is correct it would appear that the conformational flexibility observed in the crystal structure of the apo-enzyme is a real effect, also influencing NMR spectra. Further, as the resonance of R221 can be detected, by elimination, it would appear that this tentative assignment implies that the flexibility of the helix is not uniform along its length.
Standard 2D spectra were recorded for the truncated mutant of hA1-1 GST selectively labelled with $^{15}$N-arginine and are shown in figure 6.5.3. Resonances b, c, d, e, g, h, and i, are in approximately the same place in both the wild-type and truncated spectra, recorded in the presence of GSDNP. Wild-type resonances a, l, m, n, and o are replaced by resonances 1 and 2 in the truncated spectrum. It is likely that resonance a which is extremely weak in the wild-type spectrum is not detected in the truncated spectra due to the reduced concentration of protein in the latter samples as the truncated protein is less soluble in phosphate buffer. However resonances l, m, n, and o are strong and therefore that these for resonances are replaced by truncated resonances 1 and 2 implies that two of these wild-type resonances are from arginine 217 and 221, deleted by the truncation, and the other two are in proximity to this region hence are affected by the deletion, resonating at positions 1 and 2 in the truncated spectrum. The same resonances that are effected by ligand binding are also effected by the deletion of the C-terminal helix, showing that they are in proximity to the active site and the C-terminal helix. It is also interesting to note that some resonances in the truncated spectrum, particularly l and h have positions that are more similar to the apo-enzyme that the ligand bound wild-type enzyme. This could imply that in the apoenzyme the C-terminal helix is not in proximity to these residues but on ligand binding effects their environment (for further discussion see chapter 8).

**Figure 6.5.3. The spectra of wild-type (black) and truncated (red) hA1-1 GST in the presence of GSDNP.**
6.6 $^{15}$N-PHENYLALANINE LABELLED hA1-1 GST.

The spectra of $^{15}$N-phenylalanine labelled hA1-1 also showed fewer resonance signals in the apo-enzyme than in the presence of ligand. In the absence of ligand only nine of the ten phenylalanine amide resonance signals can be detected and among those there is dramatic variability in peak height. In particular the resonance at (7.7,111)ppm (resonance $a$ in figure 6.6.1.) is considerably weaker than the other eight resonances. On addition of ligand this resonance increases in strength and shows a frequency shift. In total four resonances undergo shifts on ligand binding: resonance $a$ and resonances $e, f,$ and $i$. Resonance $f$ shows an approximately 1ppm increase in frequency in both dimensions but the other resonance shifts are more dramatic: the apo-enzyme resonances $e$ and $a$ are replaced by resonances $l$ and $m$ such that it is difficult to tell which apo-enzyme resonance has moved to which position on ligand binding by frequency alone. The intensity of resonance $l$ is much less that of $m$, indicating that it could correspond to the weaker apo-enzyme resonance $a$, and that resonance $m$ corresponds to apo-enzyme resonance $e$, however this correlation is not definite. The most dramatic effects of ligand binding are that resonance $i$ in the apo-enzyme shifts to position $j$ without showing any significant change in intensity and the tenth resonance, undetected in the absence of ligand appears, labelled $k$. However, according to the structural predictions, only three phenylalanine residues were postulated to be affected by ligand binding: F10, F222 and F220. F10 is adjacent to Y9, the resonance frequency of which has been shown to be affected by ligand binding (section 6.2.). On binding to ligand the side chain of F10 has been shown to undergo a 90° rotation, allowing the C-terminal helix to dock over the H-site. If the side chain of F10 did not rotate it would sterically clash with the side chain of F220, one of the residues on the C-terminal helix (Chapter 7). The C-terminal helix has two phenylalanine residues: F220 and F222, the latter being the terminal residue of the protein, thus three phenylalanine resonances were postulated to be affected by ligand binding. That two other phenylalanine amide resonances are affected indicates that the predictions derived from the residues shown to be in proximity to the bound ligand in the crystal model are not entirely accurate. These resonance changes may be a result of a conformational change that occurs on ligand binding, as oppose to the ligand being in proximity to these residues. This conformational change may be the same effect that is observed with glutathione binding, altering the affinity of ligands that bind at the H-site and enabling the enzyme to activate the hydrophobic substrate. As only a few resonances are altered it is likely such a conformational change would be localised at the active site (for further discussion see Chapter 8).
Figure 6.6.1. The 2D spectra of hA1-1 GST selectively labelled with 15-N phenylalanine in the absence (black) and presence (red) of GSDNP.

Figure 6.6.2. F222Y hA1-1 GST selectively labelled with 15N-phenylalanine in the absence (red) and presence (black) of GSDNP.

Figure 6.6.3. Spectra of 15N-phenylalanine labelled truncated hA1-1 GST in the presence (red) and absence (black) of GSDNP.
Several techniques can be employed to confirm the tentative assignments of the phenylalanine residues, however due to the size of the protein and therefore the line width of the resonance signals not all can be applied to GST. Selective labelling by amino acid type can be used to assign backbone amide resonances of some amino acids by double labelling the sample with both a $^{15}$N amide labelled and a $^{13}$C a-carbonyl labelled amino acid, such that the two NMR-sensitive isotopes are adjacent. Thus the two nuclei are coupled, resulting in resonance splitting.

This method was applied to GST in an attempt to assign the amide resonance of F220. hAl-1 GST double labelled with $^{15}$N-phenylalanine and $^{13}$C a-carbon isoleucine was prepared and NMR spectra recorded in an attempt to identify the amide resonance of F220 by the coupling effect of the adjoining $^{13}$C at the a-carbonyl position of isoleucine 219. However, resonance at (6.4,122) labelled k in figure 6.6.1., tentatively assigned as that of F220 was not detected above the noise level in the double labelled experiment. This could be a result of the splitting, halving the intensity of an already weak signal, thus reducing its peak height and preventing its detection. No splitting was observed in the other nine resonances implying that these phenylalanine amide nitrogens were not bonded to isoleucine a-carbonyl atoms. However, due to the line breadth of the other signals it is possible that a resonance may be split into two signals which overlap as the coupling constant of the $^{13}$C a-carbonyl atoms is very small and the absence of the resonance k is due to some other effect. Although no significant difference in the peak heights of the other signals in the single and double labelled experiments were observed, due to the range of peak heights observed in GST, the assignment of resonance k to F220 must be viewed with some caution. Hence, double labelling experiments were not used further.

An alternative approach to resonance assignments was using mutagenesis. By comparing the spectra of wild-type and point mutated hAl-1 GST selectively labelled by amino acid type, the resonance generated by the wild-type residue that has been point mutated can be identified by elimination. Two mutants of hAl-1 GST, each with one of the C-terminal phenylalanine residues mutated to a tyrosine residue, selectively labelled with $^{15}$N-phenylalanine were prepared and spectra recorded. The spectra of F222Y hAl-1 GST selectively labelled with $^{15}$N-phenylalanine in the presence and absence of GSDNP are shown in figure 6.6.2. Comparing these spectra with those of the wild-type (figure 6.6.1.) it is interesting to note that there are five differences, mostly affecting the resonances altered by ligand binding in the wild-type protein. This provides support to the theory that the amide nitrogens generating the resonances that are affected by ligand binding are in proximity to F222, and are therefore located in the active site. The most significant differences are that the wild-type resonances labelled i and j in figure 6.6.1.
are not detected in the F222Y mutated spectra hence these resonances must be generated by the amide nitrogen of F222 in the absence and presence of ligand, respectively. The assignment of the resonance of F222 indicates that the chemical environment of this residue is dramatically affected by ligand binding, as would be expected by the helix docking over the H-site in the presence of ligand. The other four resonances that differ in the spectra of F222Y compared to the wild-type show only subtle changes. Resonance f is affected by ligand binding in the wild-type spectra, showing 0.15ppm shift in the proton dimension and a 1ppm shift in the nitrogen dimension, but in the spectra of the F222Y protein the positions of resonance f are approximately the same. Resonance d in the wild-type protein spectra does not show any significant change, but in the spectra of F222Y shows a 0.1ppm shift in the proton dimension on ligand binding. However, both of these differences between the wild-type and F222Y spectra are very slight. A more dramatic difference is shown in the resonance frequency of resonance m, which in the wild-type protein spectrum in the presence of GSDNP has a frequency of (8.6,113.3) and the F222Y spectrum resonates at (7.73,114.2). Interestingly, resonance k, tentatively assigned to F220 is much more intense in the F222Y spectrum compared to the wild-type spectrum with GSDNP, however this resonance can still not be detected in the apo-enzyme. This implies that the environment of the nuclei generating resonance k is further stabilised by the F222Y mutation, perhaps the hydroxyl functional group H-bonds to another residue stabilising the C-terminal helix in the ligand-bound form. This would also explain in the decreased affinity of this enzyme for the product analogue GSDNP (section 4.4.)

The assignment of F220 was not as clear cut as that of F222. The F220Y mutated form of hA1-1 was difficult to purify, as binding to the affinity column was more pH dependent than the wild-type enzyme. However, by reducing the pH of the loading buffer to 7.1 and increasing the pH of the eluting buffer to 10.8 over 80% of the protein was extracted from the cytosol, giving a yield of around 42.1mg/l. The protein was less soluble in 50mM phosphate buffer pH 6.5, attaining a maximum concentration of around 0.3mM active sites (determined using an absorbency coefficient of 26400M⁻¹active sites - table 3.3.5.). This resulted in a poor signal to noise ratio, necessitating the use of many scans in each experiment, and consequently long run times. Unfortunately, even in the presence of DTT and under anaerobic conditions, the enzyme was significantly denatured after 6 hours; the activity was less than half of the initial activity and the proton NMR spectrum showed broader resonances and additional resonances uncharacteristic of hA1-1 GST. Thus the assignment of F220 could not be obtained from this mutant.

The truncated form of hA1-1 GST (with the terminal ten amino acid residues deleted) was also less soluble than the wild-type protein, however it was more stable than the F220Y
mutant allowing experiments to run to completion before signs of protein denaturation were detected. Using the truncated form of hA1-1 GST selectively labelled with $^{15}$N-phenylalanine, spectra in the presence (red) and absence (black) of GSDNP were recorded and are shown in figure 6.6.3. Six resonances in the apo-enzyme spectrum are in almost identical positions in both the wild-type and truncated spectra, labelled b-d and f-h in figures 6.6.1. and 6.6.3. Further apoenzyme wild-type resonance e and truncated resonance 2 are in comparable positions. The wild-type apo-enzyme resonances a and i, the latter having been assigned to the amide resonance of F222 are not detected in the truncated protein, however another resonance is detected, labelled 1 in figure 6.6.3. Hence, only eight resonances are detected in the truncated apo-enzyme, which implies that all of the $^{15}$N-phenylalanine resonances in the truncated mutant are detected. In contrast only nine of the ten resonances in the wild-type apoenzyme are detected implying that the resonance of F220 is that which is not detected in the wild-type apoenzyme. On addition of ligand to the truncated protein, wild-type resonance j, assigned to F222 is not detected, as expected. Eight resonances are detected six of which have equivalent resonance frequencies to the wild-type spectrum in the presence of ligand (b-d and f-h).

However, as in the apo-enzyme there are some differences: The wild-type resonances 1, k, j and m are replaced by truncated resonances 1 and 3. It appears that wild-type apo-enzyme resonance a has moved to position 1 in the truncated enzyme and, whereas in the wild-type enzyme this resonance is affected by ligand binding moving to position 1 or m, in the truncated enzyme there is no significant change. Further, it is possible that wild-type apo-enzyme resonance e has shifted slightly to adopt position 2 in the truncated apo-enzyme and is still affected by ligand binding, moving to position 3 on addition of GSDNP. Thus it appears that the ninth and tenth wild-type resonances detected in the presence of ligand, missing in the truncated enzyme are j, assigned to F222, and k and as with the double labelling experiment, the results indicate that the amide nitrogen of F220 is undetected in the absence of ligand but resonates at position k in the presence of GSDNP.

Interestingly, there seems to be a core of $^{15}$N-phenylalanine resonances that show relatively few changes either on addition of ligand or in either of the two mutant spectra (b-d, g and h), indicating that the effects of ligand binding do not affect the whole protein. The other five resonances show varying degrees of change. Resonance f is slightly affected by the addition of ligand in both the wild-type and truncated protein, but not in F222Y. In all apo-enzyme spectra the position of this resonance is similar, the differences being in the spectra recorded in the presence of ligand: The wild-type resonance is shifted down field relative to the apo-enzyme resonance, in F222Y it is unaffected and in the truncated mutant it shifts up field. That the apo-enzyme resonances are similar, implies that the environment of this phenylalanine reside is not affected by the
mutations, and therefore it appears that this phenylalanine residue is not in proximity to the C-terminal helix. However, from the changes that occur on ligand binding, it is evident that this residue is either in proximity to the ligand, which is accommodated in a different way in each form of hA1-1 GST, or conformational changes occur in the protein which effect the environment of this residue.

6.7. 15N-ASPARTIC ACID LABELLED hA1-1 GST.

Unfortunately the spectra of hA1-1 GST labelled with 15N aspartate showed more resonances than expected, indicating that some cross labelling had occurred. There are twelve aspartate residues and the apo-enzyme shows sixteen resonances: twelve intense signals and four weaker resonances. It is possible that these four weaker resonances correspond to asparagine or possibly glutamate residues and the reduced intensity is due to isotopic dilution as the media had been supplemented with unlabelled asparagine and glutamate, the other asparagine or glutamate resonances being so weak that they are not detected. However, due to the range of resonance intensities observed in other spectra, this is a dangerous assumption. The protein was made in the presence of a large excess of unlabelled asparagine and using E.coli DL39, that are auxotrophic for aspartate and asparagine, perhaps a greater level of correct labelling would be achieved by reducing the amount of labelled aspartate, increasing the amount of unlabelled glutamate, shortening the production period, and using E.coli strains auxotrophic for aspartic acid, asparagine,
glutamine and glutamic acid. The heights of the resonance signals do not appear to be affected by ligand binding, however 6 resonances show frequency shifts on ligand (GSDNP) binding, only one of which is a weak signal (labelled a in figure 6.7.1.). The other apo-enzyme resonances that show shifts are b-f, the latter resonance being affected the least. As only one aspartic acid is implicated as being involved in ligand binding it is difficult to assign these resonances, however, as with the spectra of $^{15}$N-phenylalanine labelled GST, it appears that more resonances are affected by ligand binding those from residues in proximity to the bound ligand.

6.8. $^{15}$N-LEUCINE LABELLED hA1-1 GST.

Figure 6.8.1. The 2D-spectra of hA1-1 GST selectively labelled with $^{15}$N leucine in the presence (red) and absence (black) of GSDNP.

Figure 6.8.2. The 2D-spectra of L108Y (red) and wild-type (black) hA1-1 GST in the absence of ligand.
The apo-enzyme spectrum of hA1-1 GST selectively labelled with $^{15}$N leucine shows thirty resonance signals of varying peak heights. The height of some resonance signals is not much greater than the noise level, however they are in similar positions in both the presence and absence of GSDNP. The addition of ligand affects most resonances, altering the height or resonance frequency. Only ten resonances are not significantly affected, numbered 1-10 in figure 6.8.1. A further 8 resonance (number 11-18) show shifts of less than 0.1 ppm in the proton dimension and resonance 19 shows a slight shift in the nitrogen dimension. The apo-enzyme resonances 20-26 are replaced by resonances 31-33 in the presence of ligand, there possibly being two resonances at position 31. Resonances 27-30 show shifts greater than 0.1 ppm but can still be correlated to a resonance in the spectra recorded in the presence of ligand. Not all resonances are individually resolved in the spectrum in the presence of GSDNP spectrum, probably because of signal overlap. Due to the number of leucine resonances it is difficult to follow spectral changes that occur on ligand binding and therefore difficult to tentatively assign the resonances. Certainly more resonances are affected by ligand binding than are thought to be in proximity to the bound ligand from the crystal structure.

Using L108Y hA1-1 GST spectra of the protein selectively labelled with $^{15}$N-Leucine were recorded in the presence and absence of GSDNP. The apo-enzyme spectrum showed no significant differences with the wild-type spectrum (figure 6.4) hence it appears that the resonance of L108Y is either weak and not detected in the wild-type
spectrum or superimposed with another resonance. In the presence of GSDNP the resonance from the amide nitrogen of L108Y can still not be resolved as although most of the wild-type resonances are closely matched by resonances from the mutated protein, there are some intensity changes, in particular the L108Y resonance at (6.66,111.9) is much more intense than the wild-type resonance, and some frequency shifts.

6.9 THE $^{13}$C RESONANCES OF THE SIDE-CHAIN CARBONS IN METHIONINE, LEUCINE AND PHENYLALANINE RESIDUES.

The differences in the amide resonances of hA1-1 in the presence and absence of ligand can be used to deduce some of the structural changes that occur in the protein on ligand binding and implicate the amino acid residues that interact with the ligand on a molecular basis. However, in most cases it is the side-chain functional groups that interact with the ligand and not the alpha-carbon backbone. Using the 3-D crystal structure of the enzyme in the presence and absence of ligand residues thought to be in proximity to the bound ligand were identified. Of these methionine 208, leucine 108 and three phenylalanine residue (10, 220 and 222) were thought to be of most use in characterising ligand binding at the H-site.

The side chain of methionine 208 protrudes into the H-site pocket and is thought to interact with the bound hydrophobic substrate influencing substrate specificity. Further, this residue has been shown to be displaced from the H-site when larger ligands, such as ethacrynic acid, are bound indicating that its assignment could be used to characterise ligand binding. The 2D spectra of $^{13}$C-methyl methionine labelled hA1-1 GST in the absence and presence of GSDNP are shown in figure 6.9.1. Seven resonances are detected, corresponding to the methyl carbons of the seven methionine residues in hA1-1 GST. On addition of ligand the frequency of two resonances alters significantly: one labelled c in figure 6.9.1. shows a 0.25ppm shift downfield in the proton dimension and the other labelled g shows 0.8ppm shift upfield in the carbon dimension. Thus either resonance could be tentatively assigned to M208. In accordance with the effects of ligand binding on the backbone amide resonances of hA1-1 GST, the majority of the side chain resonance also do not appear to be affected by ligand binding.

Similar spectra of $^{13}$C-methyl methionine labelled a M208D mutated hA1-1 GST (figure 6.9.2.) were recorded in the presence and absence of GSDNP to assign this resonance. The spectra of M208D in the presence and absence of ligand show six individual signals. However the apo-enzyme spectra of the wild-type and M208D proteins differ in that the wild-type resonance g is not in the same position in the M208D spectrum and the intensity of resonance 2 in the M208D spectrum is increased in comparison to the other resonances. Thus it is difficult to assign the resonance of the methyl methionine carbon
of M208 from the apo-enzyme spectra alone. However on addition of ligand M208D resonance 3 moves towards the position of resonance g in the wild-type spectra, indicating the two resonances are probably equivalent. Further a frequency shift corresponding to that of resonance c in the wild-type spectra is not observed indicating that the carbon of the methyl-methionine at residue 208 resonates at (2.05,15.7), position e in the wild-type spectra. Interestingly the spectra of the wild-type and M208D mutated proteins are more similar in the presence of ligand than in the absence. This is probably because the side chain of M208 points into the active site, thus a change in polarity, introduced by its mutation to an aspartate residue could affect the resonances of other residues in proximity to the H-site. However, when the H-site is occupied the main effect would be on the resonances of the ligand and not the protein, thus the other $^{13}$C-methyl methionine resonances in the spectra of M208D are more similar to the wild-type resonances in the presence of ligand.

**Figure 6.9.1.** The 2D spectra of $^{13}$C-methyl methionine labelled wild-type hA1-1 recorded in the presence (red) and absence (black) of GSDNP.
Figure 6.9.2. The 2D spectra of $^{13}$C-methyl methionine labelled M208D GST recorded in the presence (red) and absence (black) of GSDNP.

The assignment of the resonances of the methyl carbon of M208 was of particular interest because NOE experiments detected a NOE between the methyl carbon of methionine 208 and a proton resonating at 9.05 ppm, which is the same resonance frequency as that of the proton attached to carbon 3 of bound GSDNP (see section 5.1.). Thus, these two nuclei are in close proximity showing unambiguously that the benzyl group of GSDNP is located in the H-site when bound to hA1-1 GST. Figure 6.9.3.a. shows the results of the HMQC-NOE experiment with the proton resonance frequency of the x-axis and the carbon resonance frequency on the y-axis. Additionally, a 1-D version of the spectrum in figure 6.9.1. in the presence of GSDNP is displayed on the y-axis, labelled b. Starting with the 1-D spectrum, 7 resonance signals are observed that correspond to the methyl carbons of the 7 methionine residues in hA1-1 GST and these resonances are labelled to correspond to those in figure 6.9.1., where resonance c was assigned to M208. Various cross peaks are shown in part a of figure 6.9.3. which include NOEs detected between the $^{13}$C nuclei and protons that are in proximity to them. These include both the protons of the protein and also the ligand. Of particular interest is the cross peak labelled with * in figure 6.9.3.a. The resonance frequency of this cross-peak (9.05, 15.11) indicates that it is between the $^{13}$C methyl nucleus of M208 and a proton with the same resonance as proton 3 of free GSDNP. This ligand resonance is quite distinct as it falls in a region of the spectrum where there are few protein proton resonances, therefore the cross-peak labelled * in figure 6.9.3.a. appears to be a transferred intermolecular NOE between the
protein and ligand. The proton resonance frequency corresponds to free ligand resonance rather than the bound resonance (expected at 8.1 ppm) because the NOE is transferred from the bound to the free state. That a similar NOE cannot be detected between M208 and the bound proton is probably due to the bound ligand resonances adopting the protein line widths and therefore the peak heights are such that they are indistinguishable from the base line (for further discussion see chapter 8).

Figure 6.9.3. The NOE spectrum of $^{13}$C-methyl methionine in the presence of GSDNP.

The spectra of $^{13}$C-side-chain phenylalanine labelled hA1-1 GST in the absence of ligand (black) and in the presence of either GSDNP (red) and ethacrynic acid (green) were recorded and are shown in figure 6.9.4 and 6.9.5. Resonance shifts do occur on binding to either ligand, some of which are similar and others clearly different, however analysis of these spectra is difficult due to the degree of signal overlap.
Figure 6.9.4. Spectra of hA1-1 GST selectively labelled with $^{13}$C-phenylalanine in the presence (red) and absence (black) of GSDNP.

Figure 6.9.5. Spectra of hA1-1 GST selectively labelled with $^{13}$C-phenylalanine in the presence (green) and absence (black) of ethacrynic acid.
Similarly the resonances of $^{13}\text{C}$-side chain leucine labelled GST showed a significant degree of overlap, thus although changes occurred on addition of ligand their exact nature could not be defined. Hence although selective labelling edits the spectra compared to uniform labelling, it does not also follow that all resonances will be resolved. The amide resonances show a much wider distribution, allowing in most cases all the resonances of each amino acid type to be individually resolved, although in some cases the complexity is such that changes in the resonances that occur on ligand binding cannot be clearly identified. In contrast the $^{13}\text{C}$-resonances of the side chain nuclei in leucine or phenylalanine resides do not show a considerable frequency distribution and are clustered in the same region of the spectrum, hence not all of the signals can be resolved. There are a similar number of phenylalanine residues (10) as methionine residues (7) however, by labelling the amino acid at just one position in the side chain, such as the methionine example in figure 6.9.1., the spectra are much less crowded allowing the changes that occur on ligand binding to be followed with accuracy. In turn, the simplicity of the methionine spectra allowed the assignment of the resonance of M208 by mutagenesis.

Figure 6.7. Spectra of $^{13}\text{C}$-leucine labelled hA1-1 GST in the presence (red) and absence (black) of GSDNP.
7. CRYSTAL STUDIES.

7.1 INTRODUCTION

The interactions of proteins with their ligands can be studied at atomic detail using X-ray crystallography. This technique exploits the ability of a beam of X-rays, with a wavelength of approximately 1.5 Å, to be scattered by atoms. Crystals are used as the regular packing of the molecules amplifies the patterns produced by the scattering, which is based on the diffraction and recombination of X-rays on interaction with the atoms. The effect is similar to that when other forms of electromagnetic radiation, such as light, interact with objects, hence by refocussing the diffracted rays an image of the molecule can be obtained. However there is no equivalent to the lens for focusing X-rays, hence the patterns are recorded using a digital image plate as a series of spots. The intensities of the spots are proportional to the square of the amplitude of a wave of electron density, which can be derived by applying the Fourier transform to the intensity data. In addition to amplitude, the wave also has phase, which is calculated from the changes in the recorded X-ray diffraction pattern on soaking the crystals with heavy atom markers - a procedure known as isomorphous replacement. Alternatively, if a similar structure has been solved, the phase problem can be solved by comparing the model of the unknown structure with the solved structure - a technique known as molecular replacement. Once the amplitude and the phase of the electron waves are known, the electron density map can be plotted and the primary sequence of the protein used to model a structure.

Many structures of GST isoenzymes in complex with various ligands have been published, including four structures of hA1-1 GST: bound to S-benzylglutathione, ethacrynic acid or its glutathione conjugate and in the absence of ligand, with electron density thought to correspond to a 2-mercaptoethanol dimer lodged in the active site. The latter structure is of particular interest as the C-terminal helix was described as having "uninterpretable" electron density hence its position was not defined. The protocol used to prepare the crystals was not published, however it is likely that a similar method was used as described in this report section 2.6.1. necessitating the use of a reducing agent such as 2-mercaptoethanol or DTT to prevent the protein from precipitating out of the hanging drop when the well buffer is added.
7.2. CRYSTAL GROWTH IN THE ABSENCE OF LIGAND.

Crystals of the hAl-1 GST were successfully obtained without addition of ligand and the crystal growth conditions have been defined. In general crystallisation of the ultrafiltration-concentrated enzyme occurs at a lower pH than that of the lyophilised enzyme. For the lyophilised enzyme, good quality crystals are formed between pH 8.4 and 9.4. In the case of the enzyme concentrated by ultrafiltration, good quality crystals are formed between pH 7.0 and 8.0. A PEG concentration of 20% produced the best crystals, however this seemed independent of the type of PEG, either 2000 or 3350. Growth was strongly dependent on the temperature, with reasonable quality crystals only appearing in plates stored at 22.5°C. Slight alterations in the temperature (±0.2°C) resulted in little or no growth. The best crystals were produced in hanging drops with a concentration of 35mg/ml of enzyme. Crystallisation occurred very rapidly, with reasonable sized crystals apparent after 24 hours but the crystals began to crack and degrade after 72 hours hence they were harvested after 48 hours.

Figure 7.2.1. shows some hAl-1 GST crystals grown in the absence of ligand at pH 7.6 in 20% PEG 2000 over a 24 hour period. These and similar crystals were used for X-ray diffraction analysis and ligand diffusion studies as described below. These crystals are hexagonal rods, however other forms of crystal have been produced. As conditions move away from those optimal for production of the hexagonal rods, hexagonal crystals are formed or numerous fine needles too small for X-ray studies. In some instances a precipitate was noted in the hanging drop which was presumed to be the enzyme, as all other constituents have a high solubility.

Figure 7.2.1. hAl-1 GST crystals grown in the absence of ligand at pH 7.6 in 22.5% PEG 2000 over a 24 hour period.
7.3. LIGAND DIFFUSION STUDIES.

The diffusion of different ligands resulted in a different affect on the apo-enzyme crystals. Addition of glutathione resulted in rapid cracking of the crystals which remained in the same form and were still birefringent (figure 7.3.1.). The higher the concentration of glutathione the more apparent the affect became. This implies that the crystal packing adopted by the enzyme in the absence of ligand is not suitable for the glutathione bound enzyme. On addition of ligands GSDNP and CDNB the coloration transferred from the hanging drop into the crystal over a period of 15 minutes, so that the hanging drop became colourless again. Apart from the colour change, the affect of GSDNP on the crystals was much like that of glutathione, in that the crystals showed a mosaic pattern of cracks. Producing GSDNP in situ, by adding CDNB and glutathione simultaneously again resulted in the coloration being transferred from the hanging drop into the crystal so that the hanging drop became colourless, and a mosaic of cracks appeared in the crystal, similar to that observed with either glutathione or the purified conjugate. However, quite a different effect was observed on the addition of CDNB alone. The hexagonal rod apo-enzyme crystal began to crack and partially dissolve. After 24 hours crystals had formed that were extremely thin in one dimension (figure 7.3.2.) but were birefringent. As the results of GSDNP and glutathione diffusion were similar, but different from the diffusion of CDNB into apo-enzyme crystals, it is likely that the conformation adopted by the GST•GSH and GST•GSDNP complexes are more similar to each other than that of the GST•CDNB complex.

Figure 7.3.1. The results of diffusion of a 5-fold excess of glutathione into crystals of hA1-1 GST grown in the absence of ligand.
Figure 7.3.2. The results of diffusion of a 5-fold excess of CDNB into crystals of hA1-1 GST grown in the absence of ligand.

Figure 7.3.3. The results of diffusion of a 5-fold excess of GSDNP into crystals of hA1-1 GST grown in the absence of ligand.

As the apo-enzyme crystals crack on ligand binding, diffusion is not a suitable method to obtain crystals of hA1-1 GST in complex with ligand for X-ray diffraction analysis and for this purpose co-crystallisation yields crystals of a superior quality, as it ensures that the crystal packing is not restricted by that formed in the absence of ligand.
7.4 CO-CRYSTALLISATION.

Crystals were produced in the presence of GSDNP, glutathione or CDNB. In the presence of glutathione or GSDNP crystallisation occurred more rapidly compared to the free protein hence in order to produce reasonable size crystals the concentration of protein was reduced to 15mg/ml in the hanging drop. The crystals containing 10mM glutathione were colourless (figure 7.4.1.) however those containing a 5-fold excess of GSDNP were yellow (figure 7.4.2.) due to the coloration of the ligand. Interestingly all of the coloration was localised in the crystal and not in the hanging drop. In the presence of CDNB (7.4.3.) the crystals were flat, birefingent needles, mainly forming around the outer edges of the hanging drop. The reduced volume of these crystals, compared to the apo-enzyme crystals, indicates that they would not be suitable for diffraction analysis. Crystals containing ligand were more stable than those produced in its absence, particularly those with the glutathione peptidyl moiety, and did not show signs of degradation for up to one week. Thus it appears that the changes in the stability of the protein noted in NMR studies also apply to the crystallised enzyme.

Figure 7.4.1. Crystals of the enzyme-glutathione complex.
Co-crystallisation studies show that it is considerably easier to produce crystals of adequate quality for X-ray diffraction if a glutathione peptidyl moiety was bound compared to the crystals of the enzyme in the absence of ligand.
7.5 DIFFRACTION ANALYSIS.

Crystals grown in the absence of ligand (figure 7.2.1.) were analysed using X-ray techniques by P. Moody (see Acknowledgements). Initial studies at room temperature allowed the space group and cell dimensions to be determined. Data from a 2.0° oscillation exposure was subjected to the auto-indexing routine of Kabsch (1993) that gave a cell consistent with the C2 space group \(a=101.0\) \(b=95.4\) \(c=105.5\) \(\alpha=\gamma=90^\circ\) \(\beta=92.4^\circ\). Thus, although the rod shaped crystals had a hexagonal cross section the space group was monoclinic, like the crystals used to solve the published structure of hA1-1 GST in the absence of ligand\(^\text{2}\). Collection of a full dataset to 2.5 Å allowed the cell to be refined to \(a=100.91\) \(b=95.17\) \(c=105.14\) \(\alpha=\gamma=90^\circ\) \(\beta=92.455^\circ\). These values were within 1% of the published values\(^\text{9}\) \(a=100.8\) \(b=95.4\) \(c=105.2\) \(\alpha=\gamma=90^\circ\) \(\beta=92.5^\circ\). After 45 frames of 1.5° the quality of the diffraction had visibly deteriorated, however 88% of data had been collected with an average 1.5-fold redundancy and an \(R_{\text{merge}}\) of 5.4% allowing the structure to be solved by molecular replacement with reasonable accuracy.

There were no differences between this and the published structure of hA1-1 GST in the absence of ligand\(^\text{9}\). The major differences between these and the structures solved in the presence of ligand that the side chain of phenylalanine 10 undergoes a 90° rotation of the ring, as shown in figure 7.5.1. The "apo-enzyme" position of F10 is shown in blue and the position of this side chain in the presence of S-benzylglutathione is shown in green. The rotation of the side chain of F10 allows the C-terminal helix to dock over the H-site. If this rotation did not occur the side chain of F220 would sterically clash with that of F10. Thus although the position of the C-terminal helix cannot be defined absolutely in the absence of ligand, it is evident that it cannot adopt exactly the same position as it does in the presence of ligand because of this interaction.
Figure 7.5.1. The positions of F10 and F220 in the absence (blue) and presence (green) of S-benzylglutathione (hydrophobic moiety shown in red, glutathione moiety in blue with thiol coloured yellow).

Figure 7.5.2. Positive electron density (blue) that is detected in the active site of the enzyme.
The electron density maps calculated from the diffraction patterns of the crystals in the absence of ligand were calculated for amino acid residues 5 to 210, omitting the first five residues and the C-terminal helix. Figures 7.5.2. and 7.5.3. show positive electron density that cannot be accounted for by these residues. In figure 7.5.2. the active site is shown. The H-site is on the left-hand side, defined by the positions of L107, M208 and V111, labelled accordingly in the figure. In this hydrophobic pocket positive electron density is observed due to the C-terminal helix (shown in blue). Similarly in the G-site (on the right hand side of the figure defined by the positions of T68, and Y9) positive electron density can be detected. The only component of the hanging drop that could be responsible for positive electron density in the ligand binding sites is 2-mercaptoethanol, introduced to prevent the protein denaturing when the precipitant is added. It is possible that by using strictly anaerobic techniques the requirement for 2-mercaptoethanol could be eliminated allowing the production of true apo-enzyme crystals, however as yet this has not been attempted.

Positive electron density was also detected in the region where the C-terminal helix is located in the presence of ligand, as shown in figure 7.5.3. This indicates that although the C-terminal helix was omitted from the model there is electron density that describes its position in the ligand-bound form. This electron density is considerably weaker than that describing residues 5-210, however residues 211-215 appear to be reasonably well defined, adopting the same position as in the presence of ligand. Weaker electron density
can be detected for the residues further along the helix, as shown in figure 7.5.4. The pink contours correspond to those shown in figure 7.5.3. and the blue and green contours correspond to progressively weaker electron density. Hence electron density can be detected in the region of the C-terminal helix, however it is considerably weaker than that describing the rest of the protein thus the position of the C-terminal helix in the absence of ligand cannot be defined with the same degree of accuracy as the rest of the structure. However, it does appear that residues 211-215 have similar positions in the apoenzyme as in the presence of ligand.

**Figure 7.5.4.** An increased number of contour levels can be used to describe the electron density of the C-terminal helix, pink representing 2.5Å, blue 2.8Å and green 3.0Å.
Figure 7.5.5. The electron density in the absence of ligand describes the position of the C-terminal helix in the ligand bound hA1-1 GST.

The green contours represent positive electron density that can be correlated to atoms in the model of the protein. Blue contours describe positive electron density that does not correlate to atoms in the model and red contours show where electron density is expected from the model, but has not been detected.

If the entire model of the protein (from residue 5 to 222) is fitted to the electron density, three types of contour can be used to describe the model, as shown in Figure 7.5.5. The blue contours show positive electron density that cannot be accounted for by residues 5-222, which is thought to be a 2-mercaptoethanol dimer in the active site. The green contours show regions of electron density that are accurately described by the model based on the structure of the protein bound to S-benzylglutathione, and as before this includes the main bulk of the protein, up to residue 215. Residues 216 and 217 are described by red contours, indicating that there is no electron density in this region to describe these residues, however 218 is well defined. In contrast there is no electron density in the region of 219, but the rest of the helix, with the exception of the side chain of F220, is reasonably well defined. Hence it appears that the flexibility of the C-terminal helix is not uniform along its length, with regions of increased flexibility around residues 216, 217, 219 and 220. Similar observations were made from the NMR spectra of hA1-1 selectively labelled by amino acid type (section 5.4.) where the amide resonance of F222 could be detected in the absence of ligand but that of F220 could not. However the areas of flexibility defined by crystallography are not exactly the same as those defined by NMR as all isoleucine amide resonances could be detected in the absence of ligand, indicating that the resonance of I219 is also detected. This could be a result of the
NMR studies centring on the backbone amide resonances, hence the side chain resonances of I219 may not be detected in the apo-enzyme due to the flexibility detected by X-ray studies. Eleven of the twelve amide resonances of arginine residues were detected in the apo-enzyme. In the light of the crystal studies the undetected resonance is likely to be that of R217, which has poorly defined electron density.

In an attempt to improve the resolution of the C-terminal helix cryo-techniques were employed. However, apart from slightly reducing the cell dimensions there was little difference in the resolution of the data at either 298 or 100K. It was thought that this was in part due to the crystals used to collect the data at 100K being of smaller volume hence the two data sets are not directly comparable. Data has also been collected for the GST-glutathione complex at room temperature, indicating that these crystals diffract to around 2.4Å, however time did not permit a detailed study of this data.
8. CONCLUSIONS.

GST is approaching the upper size limit of proteins amenable to NMR studies, hence the primary aim of these studies was to determine which NMR experiments could be applied to GST with success. Uniformly labelled spectra were too complex for detailed interpretation (section 6.1.), however using selective labelling by amino acid type the number of resonances in the spectra could be edited such that there was little or no signal overlap, facilitating interpretation. Selective labelling of aspartate residues was unsuccessful, resulting in some cross labelling, which could probably be avoided by using strain of *E. coli* auxotrophic for glutamate as well as aspartate (section 6.7.). However spectra of GST selectively labelled with six other types of amino acid were recorded successfully (sections 6.2-6.6. and 6.8-6.9). Deuteration was shown to dramatically reduce the line width of the resonances (section 6.1.) however this is a costly procedure hence has limited applicability in practise. Using deuterated protein the ligand proton resonances can be observed more clearly (section 5.4.). However the bound signals were still difficult to detect in 1D spectra. 2D NOE experiments allowed the assignment of the bound aromatic resonances of GSDNP however the assignment of the equivalent resonances of CDNB was not so straightforward as the spectra were of considerably poorer quality. This experiment has the potential to be refined to enable the detection of the bound hydrophobic substrate resonances as with the GSDNP example. If this could be achieved NMR would have a crucial role in probing the structure-function relationship of hA1-1 GST: The bound hydrophobic substrate resonances could be observed in the presence and absence of a glutathione analogue providing a “spot-test” for hydrophobic substrate activation where the kinetic assays fail, due to the low activity of the enzyme with some substrates and the poor solubility of the substrates in aqueous solvent. Already NMR has been applied to hA1-1 GST with some success, providing more evidence for a glutathione induced conformational change and enabling some conclusions to be drawn about the mode of substrate binding.

Several lines of evidence suggest that on binding glutathione induces a conformational change in hA1-1 GST which alters the catalytic properties of the enzyme. The NMR spectra of selectively labelled GST show that on binding to GSDNP approximately 14% of amide resonances are effected, most of which have been assigned by amino acid residue type: About twelve amide resonances effected by ligand binding are from leucine residues, one from a tyrosine residue, five from isoleucine residues, two valine, five arginine, and five phenylalanine. Hence ligand binding effects only a small portion of the total resonances mainly due to the proximity of the bound ligand to the amino acid residues, altering the environment and therefore the resonance frequency. In some instances the same number of resonances of one type of amino acid residue are effected
by ligand binding as are in proximity to the bound ligand, for example only one tyrosine residue is effected by ligand binding and tyrosine 9 is the only residue of this type in proximity to the bound ligand, allowing tentative assignments to be made. However not all spectra show such clear results. Of the ten phenylalanine residues five are affected by ligand binding, yet only three are in proximity to the active site. That more resonances are effected by ligand binding than are in proximity to the ligand itself implies that a conformational change may be induced in the protein. As the majority of resonances are unaffected by ligand binding, the conformational change appears to involve only a small portion of the protein probably the region of the active site. This conformational change could explain the differences in the properties of the apo- and glutathione-bound enzyme. Glutathione binding increases the efficiency of CDNB conjugation in three ways: The affinity for the hydrophobic substrate is increased (section 4.3.), the affinity for the product of conjugation is decreased (section 4.4.) and the enzyme is able to catalytically activate the hydrophobic substrate (section 4.2.). Thus the substrate can more effectively displace the conjugate from the active site, resulting in a more efficient mechanism of catalysis. The hepatic glutathione concentration is such that in vivo the enzyme will be saturated with this substrate and therefore the potential for glutathione conjugation to the hydrophobic substrate, and therefore detoxification is maximised.

The glutathione induced conformational change has been shown to be a function of the peptidyl moiety of glutathione (section 4.1. and 4.2.), hence one of the reasons for the highly stringent requirement for glutathione (section 4.1.) could be that the entire peptidyl moiety is required for this conformational change to occur. It is also postulated that the peptidyl moiety of glutathione is required to correctly orientate the thiol group in the active site so that the thiol group is activated. Indeed the free energy of binding for glutathione and the glutathione fragments shows that the fragments cannot bind in the same way in the active site as the equivalent region of glutathione. However both glutathione and the fragments are bound with similar affinity, the dissociation constant being in the μM range. Thus the reduced turnover number with the glutathione fragments appears to be because of the reduced efficiency of the catalytic mechanism. This implies that the glutathione fragments are binding non-productively, either because they are unable to induce the catalytically activating conformational change or because the thiol group is incorrectly positioned at the active site. Interestingly, the turnover number for both glutathione fragments were similar, compared to that of glutathione, whereas structurally the γGlu-Cys fragment is much more similar to glutathione than it is to the Cys-Gly. Thus it appears that the integrity of the entire peptidyl moiety is required for maximum catalytic efficiency. Further evidence to support this is shown by the magnitude of the fluorescence change induced by G-site binding. This appears to be a function of the thiol group rather than the peptidyl moiety however the maximum
fluorescence change is only achieved by glutathione itself. Thus it is possible that the fluorescence change is also a function of thiol activation, the potential for which is maximised when the peptidyl moiety is intact. Comparing all of the kinetic constants, it appears that the rate of catalysis with respect to the G-site substrate is not so much limited by substrate binding but by some other event, possibly thiol activation.

The crystal structure of hA1-1 GST in the absence of ligand, but with some electron density in the active site, postulated to be a 2-mercaptoethanol dimer, has been solved and on comparison to the ligand-bound structure\textsuperscript{73,79} shows some structural differences: The side chain of F10 rotates about 90° so that F220 can adopt the ligand bound conformation. Without this rotation the two residues would sterically clash. In the absence of ligand the electron density of the C-terminal helix is weak, particularly in the region of residues 216-222, hence the positions of these residues of the protein are ambiguous. It does appear that K218, R221 and F222 are in similar positions in both the apo- and ligand bound structures, however the electron density of A216, R217, I219 and the side chain of F220 is not clear. The weaker electron density probably indicates that these regions of the protein are subject to conformational change and it appears that this is not uniform along the length of the helix. NMR results also show that there are varying degrees of internal motion along the length of the helix, but there are some differences compared to the crystal results, for example the $\text{^{15}N}$ resonance of I219 is detected but there is no electron density describing this residue in the apo-enzyme crystal structure. The difference between the NMR and crystal results could be due to the 2-mercaptoethanol in the active site of the crystallised enzyme or crystallisation itself is effecting the structure, such that there are differences in the structure of this and the true apo-enzyme in solution used in NMR studies. Alternatively structures may be similar but the mobility of the helix may be such that the NMR signals of I219 are detected although the electron density is not. This in turn implies that the helix is oscillating between a few conformational states with similar energy levels such that they all have a similar occupancy and therefore the electron density is weakened. Assuming that the rate of exchange between these states is right the resonance would be detected as the weighted average of the resonances in all states, which correlates to the NMR data in that one resonance for each isoleucine residue is detected.

Electron density of the aromatic ring of F220 is not complete in the apo-enzyme however as the side chain of F10 has rotated such that if F220 was in the ligand bound position the two aromatic rings would sterically interact, hence it is likely that there are perturbations in the structure of this region compared to when the ligand is bound. Interestingly, the position of F10 is much better defined than that of F220. Thus it appears that the changes in the position of F10 alters both the position and motion of F220, and due to the
latter effect the exact orientation of this residue cannot be defined. The NMR resonance of the amide of F220 cannot be detected in the apo-enzyme, however there is electron density for this region in the crystal structure, again showing the differences in the two techniques. In this instance it appears that in the crystal structure the region of F220 is either stabilised, possibly due to the electron density in the active site, or although there is still mobility one conformation is favoured above the others allowing electron density to be detected whereas resonances become broad and indistinguishable with the base line. Independently of the differences in the two techniques the same overall conclusions regarding the flexibility of the C-terminal helix in the apo-enzyme can be drawn: The C-terminal helix is more flexible in the absence of ligand however this flexibility is not uniform along its length. In particular the terminal residues (from 216-222) are more flexible, possibly due to the steric interaction between F220 and F10 in the apo-enzyme position causing disruption of the helix. That the two models, one based on NMR and the other crystallography, are not identical may imply that the molecule in the active site of the crystallised enzyme has changed the properties of the C-terminal helix, hence it would be advantageous to solve the structure in the absence of this component.

With the C-terminal helix deleted the NMR spectra are considerably different from the wild-type spectra. Spectra were recorded in the presence and absence of GSDNP for the truncated protein labelled with either $^{15}$N-arginine (section 6.5.) or phenylalanine (section 6.6.). In both instances the spectra of the truncated and wild-type enzymes were more similar in the presence of ligand than in the absence, however in the case of the $^{15}$N-arginine spectra two resonances in the truncated spectrum with GSDNP bound were more similar to the wild-type apoenzyme than the GSDNP complex. If these are resonances from residues that are not involved in ligand binding but are close to the active site, these results support the hypothesis that the C-terminal helix is flexible in the apo-enzyme, the resonances having the same chemical shifts as if the helix were absent. Alternatively these resonances may be from residues involved in ligand binding (R15 and R131 hydrogen bond to bound glutathione), and their similarities to the apo-enzyme resonances may imply that ligand binding is effected by this mutation. Thus the reduced turnover number of the truncated enzyme may not be so much a function of the C-terminal helix being directly involved in catalysis, but may imply that this region of the protein is involved in the correct positioning of the substrates in the active site so that the potential for their activation is maximised. By assigning more resonances of NMR sensitive nuclei in proximity to the bound ligand, these two hypotheses could be differentiated, however the truncation does effect other resonance signals hence this would not be a straightforward exercise. Alternatively, the structure of the truncated mutant with glutathione bound could be solved by X-ray crystallography and, by
comparison with a similar structure of the wild-type enzyme, differences in the mode of glutathione binding could be established.

The importance of the C-terminal helix in the mechanism of hA1-1 GST is shown by the results in sections 4.2, 4.5 and 4.6. Deletion of the C-terminal helix reduces the affinity of the enzyme for CDNB, but not to such an extent to explain the dramatically reduced rate of conjugation, being just 1% of the wild-type rate. This could be because the truncated enzyme does not activate the hydrophobic substrate as shown in section 4.2. What is interesting is that the truncated mutant has a dramatically increased rate of catalysis with ethacrynic acid as a hydrophobic substrate compared to the wild-type enzyme. Hence the C-terminal helix determines the substrate specificity of alpha-class GSTs by altering the affinity of the enzyme for the hydrophobic substrates and enabling the enzyme to activate the hydrophobic substrate. The molecular mechanism of this activation is unclear, but it also involves tyrosine 9 and is independent of thiol activation (section 4.2). Thus it appears that tyrosine 9 is involved in the activation of both substrates, and accordingly the turnover number of the Y9F mutant is approximately 1% of that of the wild-type protein.

Hydrophobic substrate specificity is partially dependent on the ability of the enzyme to activate the substrate. hA1-1 GST has been shown to have a high affinity for a range of hydrophobic ligands however not all are efficiently conjugated to glutathione. In particular, CDNB is by far a better substrate for conjugation compared to DCNB and EA, as the rate of spontaneous conjugation is enhanced considerably more by the presence of enzyme than with the other substrates. NMR has also been used to investigate hydrophobic substrate binding. This has been characterised by the detection of a transferred NOE between the aromatic protons of GSDNP and the methyl group of M208. This is particularly interesting as it has been shown that to accommodate larger substrates, such as ethacrynic acid, M208 is pushed out of the active site resulting in the C-terminal helix becoming distorted. As the C-terminal helix is the principle region of the enzyme involved in the glutathione-induced conformational change that allows the hydrophobic substrates to be activated, the disruption of the helix may result in the enzyme being unable to activate ethacrynic acid, thereby effecting substrate specificity. The ^15N amide resonances of Y9, P220 and P222 have also been assigned and all are effected by ligand binding, adding further support to their roles in the catalytic mechanism of GST, including the enzyme-activating conformational change. However, the application of NMR to GST is limited by the size of the protein, reducing sensitivity, and therefore the assignments were based on the comparison of similarly labelled wild-type and specifically mutated forms of GST. This is laborious and does not always yield the desired results and therefore it is impractical to consider assigning the majority of the
protein resonances in this way. As the use of assignments is to identity which residues are in proximity and possibly bonding to the ligand it is much more practical to consider only assigning those residues in the active site and using crystallography to view overall changes in the protein.

As an alternative to observing the protein resonances, the ligand resonances can be characterised by deuterating the protein. This reduces the intensities of the proton resonances of the protein hence the bound ligand resonances can be detected more easily. The bound resonances of the aromatic protons of GSDNP have been assigned and show that the binding to GST does not effect the resonances of all protons equivalently. In all cases the bound resonances showed an up field shift, indicating that the protons are shielded from the applied magnetic field to a greater extent in the bound form, as would be expected as the electron density of the H-site is greater than that in the aqueous solvent. The protons at position 4 and 6 show a 1ppm shift whereas that at 5 shifts by 1.5ppm. This is probably an effect of the structure of the H-site, with each proton being in proximity to different residues with different shielding effects. Binding of CDNB has shown that the resonances are similarly effected, however due to the reduced solubility of this ligand changes in the aromatic resonances due to the binding of S-methylglutathione, resulting in hydrophobic substrate activation could not be detected. The rewards of being able to define an NMR system that would be able to detect hydrophobic substrate activation, and thereby determine this aspect of hydrophobic substrate specificity, are immense, especially since alternative methods of characterising substrate specificity are hindered by the low activity of the enzyme with some substrates and inaccuracies associated with the insolubility of the ligand. Hence if sensitivity of the NMR experiments can be optimised to detect the bound CDNB resonances and this method could be applied to other substrates, the technique could revolutionise studies of GST enzymes.
APPENDIX 1.

SI. Broth.

12.5 g anhydrous di-sodium hydrogen phosphate
7.5g anhydrous potassium di-hydrogen phosphate
4.0g succinic acid
0.5g ammonium chloride
500mg alanine
550mg glycine
100mg histidine (hydrochloride)
650mg glutamate (free acid)
420mg lysine
250mg methionine
400mg arginine
200mg asparagine

230mg isoleucine and leucine (55mg if labelled)
170mg tyrosine (35mg if labelled)
230mg valine (100mg if labelled)
400mg aspartic acid (380mg if labelled)

The mixture was made up to 900ml with water and the pH adjusted to 7.2 with 5M sodium hydroxide. The volume was then adjusted to 950ml. This was then autoclaved at 20 atmospheres for 15 minutes for sterilisation.

A second solution, to be sterilised by filtration through 0.2μ arcodiscs (Gelman Sciences) and then added to the first solution, was prepared as follows:

2g serine
230mg threonine
50mg tryptophan
400mg glutamine
4.5g glucose
20mg thiamine hydrochloride
19mg calcium chloride
2.5mg manganese sulphate
100mg ampicillin (sodium salt)
130mg phenylalanine (100mg if labelled)

2xYT formulae

16g/l Bacteriotryptone
10g/l Yeast Extract
5g/l Na Cl autoclaved for 15 minutes at 20 atmospheres and then cooled. 0.1mg/l of ampicillin (Sigma) filtered through 0.2μ arcodiscs was added just before inoculation.
LB (per litre)
10g Bacteriostrogate
10g Sodium Chloride
5g Yeast Extract

LB plates
LB containing 12.5% agar (w/v)

Tris Glycine electrophoresis buffer.
15mM TRIS pH 8.3
250mM GLycine
0.1% SDS

Comassie blue stain
40%ethanol
10%acetic acid
2.5g Comassie blue stain

Destain
30% methanol
10% Acetic acid.

Buffer A formulae
20mM Tris-base
2mM dithiothreitol (DTT)
0.25M NaCl
pH 7.4

SDS gel loading buffer
50mM Tris pH6.8
100mM DTT
2% SDS electrophoresis grade
0.1% bromphenol blue
10% glycerol
APPENDIX 2.

The observed fluorescence intensity of a sample \( F_{\text{obs}} \) is equal to the true fluorescence \( F \) multiplied by a factor that accounts for the inner filter effect and lamp drift \( \alpha \). Similarly, the observed fluorescence in a reference cuvette containing tryptophan to the same optical density at the excitation wavelength as the sample cuvette \( F_{\text{ref}} \), is equal to the true fluorescence \( F_f \) multiplied by the same factor \( \alpha \). Hence the ratio of the observed fluorescence in the sample and reference cuvettes is the equivalent to the ratio of the true fluorescence values.

\[
\frac{F_{\text{obs}}}{F_{\text{ref}}} = \frac{F}{F_f} \tag{a2.1}
\]

For a system where ligand binding results in fluorescence quenching, the true fluorescence intensity in the sample can be divided into a fluorescence contribution from the free protein and a contribution from the bound protein:

\[
F = F_b \left( \frac{ES}{E_T} \right) + F_t \left( \frac{E_T - ES}{E_T} \right) \tag{a2.2}
\]

Where \( ES \) is the concentration of complex at equilibrium, \( E_T \) is the total concentration of ligand binding sites on the protein, \( F_t \) is the fluorescence intensity if all sites are free and \( F_b \) is the fluorescence if all sites are occupied. Thus the maximum change in true fluorescence \( F_m \) is given by subtracting \( F_b \) from \( F_t \) and therefore:

\[
F = F_t - F_m \left( \frac{ES}{E_T} \right) \tag{a2.3}
\]

Dividing this equation by \( F_t \) and substituting equation a2.1 into equation a2.3 gives:

\[
\frac{F_{\text{obs}}}{F_{\text{ref}}} = \frac{F_t}{F_t} \left( \frac{F_m}{F_t} \right) \frac{ES}{E_T} \tag{a2.4}
\]

Consider a system containing the following components, described by:

\[
S + E \leftrightarrow ES \tag{a2.5}
\]

where \( S \) is a ligand that on binding to protein \( E \) causes intrinsic protein fluorescence quenching and \( ES \) is the complex formed.

The binding constant \( (K_d) \) for \( S \) to \( E \) will be given by:

165
\[ K_d = \frac{([E_r]-[E_S])([E_r]-[E_S])}{[E_S]} \quad a2.6 \]

The value of \([E_S]\) can be solved quadratically and substituting this into equation a2.4 gives:

\[
\frac{F_{obs}}{F_{ref}} = \frac{1}{E_r} \left( \frac{K_d + [E_r] + [S] \pm \sqrt{[(K_d + [E_r] + [S])^2 - 4[E_r][S]]}}{2} \right) \quad a2.7
\]

Thus, by plotting the initial ratio of the observed fluorescence in the sample and the reference solution \((F_s/F_r)\) subtracted from the ratio of observed fluorescence in the sample and reference solutions \((F_{obs}/F_{ref})\) against ligand concentration fluorescence the dissociation constant for the protein-ligand complex and the maximum change in true fluorescence as a ratio of true fluorescence if there is no binding can be deduced.

For a system where the fluorescent species is the ligand, which undergoes enhancement on binding to the protein, the true fluorescence of the sample \((F)\) is the sum of the fluorescence intensity contributions of the free and bound ligand and can be expressed mathematically as:

\[ F = F_b + F_f + F_m \quad a2.8 \]

Where the reference cuvette contains only free ligand, and therefore the true fluorescence in this cuvette \((F_r)\) is equal to:

\[ F_r = F_f[S] \quad a2.9 \]

As \(F_b\) is related to \(F_f\) by a quenching constant \((k)\), the term \(k \cdot F_f\) can be substituted in equation a2.9 place of \(F_b\) and on dividing this by equation a2.21 and rearranging we have:

\[ \frac{F}{F_r} = \frac{[E_S] \cdot (k - 1)}{[S]} + 1 \quad a2.10 \]

The maximum change in true fluorescence due to ligand binding \((F_m)\) is equal to the difference between the true fluorescence when \([E_S]=0\) and \([E_S]=0\). On rearrangement this gives:

\[ k - 1 = \frac{F_m}{F_f \cdot [E_f]} \quad a2.11 \]
Therefore:

\[
\frac{F}{F_r} = \frac{F_m}{F_r} \cdot \frac{[ES]}{[S]} + 1 = \frac{F_{os}}{For} \tag{a2.12}
\]

Multiplying by \(F_{or}\) (the observed fluorescence in the sample) gives:

\[
F_{os} = \frac{F_m \cdot For}{F_r \cdot [E_r]} \cdot \frac{[ES]}{[S]} + \frac{For}{For} \tag{a2.13}
\]

As:

\[
F_{or} = \alpha F_r = \alpha F_r [S] \tag{a2.14}
\]

Substituting equation a2.13 into a2.14 gives, on rearrangement:

\[
F_{os} \cdot For = \alpha F_m \frac{[ES]}{[E_r]} \tag{a2.15}
\]

As for the fluorescence quench [ES] can be solved using a quadratic function allowing the dissociation constants and maximum change in fluorescence to be deduced.
REFERENCES.