

Protein Engineering to Probe the Catalytic Mechanism of Alpha-Class Glutathione *S*-Transferases

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

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May 1997

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ABSTRACT

Glutathione S-Transferases (GSTs) are a large family of isoenzymes that catalyse the addition of glutathione to hydrophobic electrophiles. Over-expression of GSTs in tumours leads to resistance to chemotherapy drugs, thus understanding GST biochemistry is clinically important. However, the catalytic and substrate recognition mechanisms remain poorly understood.

Two rat, alpha-class GSTs, Yc₁ and Yc₂ have 91% homology but have specific activities of 0.01 and 13.2 nmoles/min/mg respectively for the carcinogen aflatoxin-*exo*-8,9-epoxide. The protein structures for each were homology modelled on the coordinates for alpha-class human GSTA1-1. S-aflatoxinyl glutathione modelled into the active sites identifying positions 108 and 208 as important residues. A 'knock-out' double mutant D208MY108L was made in rGST Yc₂ and an 'engineered' E208DH108Y mutant was made for Yc₁.

The mutations reduced rGST Yc₂ activity to <0.01 nmoles/min/mg and increased rGST Yc₁ activity to 0.32 nmoles/min/mg which was further used to protect a human bronchial cell line against aflatoxin B₁. Modelling of S-aflatoxinyl glutathione into huGSTA1-1 suggested the same positions were important in determining its low activity for aflatoxin-*exo*-8,9-epoxide. The double mutant L108YM208D failed to engineer any significant activity for aflatoxin-*exo*-8,9-epoxide into the enzyme.

The C-terminus of huGSTA1-1 was deleted and the kinetics of the truncated enzyme determined in the presence and absence of a synthetic peptide designed to replace the helix sequence. K_{cat} and K_m were modified for the deleted enzyme in the presence of the peptide but k_{cat}/K_m was not, suggesting the helix plays a part in promoting productive substrate binding.

The catalytically important Tyr9 was investigated by NMR. Tyr9 is thought be responsible, in part, for the deprotonation of glutathione during catalysis and as such must have a lower pK_a than tyrosine in solution. Assignment of Tyr9 in the NMR spectrum allowed its titration, confirming that the pK_a of Tyr9 is shifted from 10.0 to 7.72 ± 0.21 .

ACKNOWLEDGEMENTS

I would like to acknowledge Dr G.E. Neal and Mr D.J. Judah for their help and patience in collecting the data for the aflatoxin 8,9-epoxide conjugation activity assays, supplying the monoclonal antibodies and proofreading this thesis. I would also like to acknowledge Dr L-Y Lian and C.S. Allardyce for their work collecting the NMR data presented in this thesis. Grateful thanks also go to Dr. R. Snowden for FACS and cell counting analysis in Chapter 4, Dr A. Pfeiffer and Dr E. Offord at Nestec, Lausanne, Switzerland for the tissue culture cells to work on and Dr K. Lilley and the PNAC lab for automated DNA sequencing and synthesis of the oligonucleotides. Finally I would like to say thank you to Prof G.C.K. Roberts for his supervision and overview of my Ph.D. project.

ABBREVIATIONS

AFB₁	Aflatoxin B ₁
AFBO	Aflatoxin 8,9-epoxide
Ah	Aryl Hydrocarbon receptor
ARE	Anti-oxidant Response Element
BSA	Bovine Serum Albumin
CDNB	1-chloro-(2,4-dinitro)-benzene
CYP450	Cytochrome P450
DNA	Deoxyribonucleic Acid
DNPGSH	2,4-dinitrophenol glutathione
dNTP	2'-deoxyribonucleotide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPN	<i>O</i> -ethyl- <i>O</i> -(4' nitrophenyl)phosphonothioate
EpRE	Electrophile Response Element
FACS	Fluorescent Activated Cell Sorting
FCS	Foetal calf serum
FRET	Flourescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
GRE	Glutocorticoid Response Element
GSH	Glutathione
GST	Glutathione S-Transferase
HMQC	Heteromagnetic Multiple Quantum Coherence
IPTG	Isopropyl-β-D-thiogalactopyranoside
MOAT	Multi-Organic anion Associated Transporter
mRNA	Messenger ribonucleic acid
MRP	Multi-drug Resistance associated Protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NHBE	Normal Human Bronchial Epithelium
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Spectroscopy
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
ROS	Reactive Oxygen Species
SDS PAGE	SDS polyacrylamide gel electrophoresis
TNB	1,3,5 trinitrobenzene
XRE	Xenobiotic Response Element

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Chapter 1

Introduction

1.0 INTRODUCTION.

1.1 Toxic insults come from man-made and natural compounds.

Organisms have to deal with exogenous chemical insults constantly throughout their lifetime. Exposure to toxic compounds can be the result of pollution by the chemical industries, man-made pesticides in the diet or the prescription of drugs. However, the natural environment has proved to be a more important source of biologically active compounds that are produced as organisms compete against each other for space, light and nutrients. In addition to environmental hazards, compounds produced within cells can also prove to be dangerous. Reactive Oxygen Species (ROS), produced as by-products of aerobic respiration and inflammation, can go on to produce harmful compounds by reacting with DNA and lipids (Halliwell and Gutteridge, 1985).

These chemical insults are from structurally diverse and mainly hydrophobic compounds and so are difficult for a single enzyme to deal with because of substrate specificity and water solubility problems. Thus, as the threats from toxic compounds in the environment have changed with time, cells have evolved complex, multi-enzymatic strategies to match. These strategies include such mechanisms as drug efflux pumps (Gottesman and Pastan, 1993), drug sequestration (Sato, *et al*, 1993), repair of drug-target sites (Fox and Roberts, 1987) and the most flexible and important of the strategies, drug metabolism.

1.1.1 Drug metabolism is a multi-component strategy.

The enzymes involved in drug metabolism can be subdivided into three major classes according to their position in the sequence of detoxification reactions. Phase I enzymes, such as the cytochrome P₄₅₀ superfamily, usually act first. The majority of P450 reactions are hydroxylation, N- or O-demethylation to increase the polarity of the hydrophobic substrates. However, these types of enzyme reactions can activate chemicals via their mono-oxygenase activities (Guengerich, 1991) to produce reactive functional groups such as epoxides.

Phase II enzymes can usually deactivate these reactive compounds by reacting with the activating functional group to add a hydrophilic moiety e.g. glutathionyl groups added by Glutathione S-Transferases (Mannervik and Danielson, 1988); glucuronyl groups added by Glucuronosyl Transferases (Bock, 1991) or sulphate groups added by Sulpho-transferases (Falany, 1991). The addition of the hydrophilic

groups means that the compounds become more water soluble and prevents them from partitioning to lipid bilayers where they can effectively evade detoxification.

The resulting conjugates can be further metabolised by phase III enzymes to mercapturic acids (Moss, *et al*, 1985) or exported from the cell by various membrane pumps such as a putative GS-X pump, the multi-organic-anion-transporter (MOAT) and an ATP dependent pump that transfers dinitrophenol S-glutathione and presumably other glutathione conjugates (Hayes and Pulford, 1995). There is also evidence that multi-drug resistance associated protein (MRP) is capable of transporting glutathione conjugates because its activity is inhibited by oxidised glutathione GSSG and glutathione-hydroxynonenol (Jedlitschky, *et al*, 1994, Muller *et al*, 1994)

1.2 Glutathione S-Transferases are Phase II detoxification enzymes.

Glutathione S-Transferases (GSTs) (EC 2.5.1.18) are an integral part of the phase II detoxification mechanism. They are a large family of enzymes and are dimeric proteins of molecular weight approximately 50,000 Da. They are found in nearly all aerobic eukaryotes and prokaryotes and play critical roles in providing protection against electrophiles and products of oxidative stress (Stenborg *et al*, 1992). The family of isoenzymes displays a remarkably broad substrate specificity and members act in concert as 'catalytic scavengers' of reactive and harmful compounds.

While in the majority of cases, GST acts to detoxify compounds there are a few exceptions. A few compounds are unstable when conjugated to glutathione and can be cleaved into further intermediates in a process called thiolysis. This occurs with the insecticide EPN leading the production of *p*-nitrophenol which has to be further metabolised by phenol sulphotransferase or UDP-glucuronosyl transferase. Nitrogen mustards also undergo thiolysis to release a cytotoxic phosphate moiety. Other toxic compounds can exist in a dynamic equilibrium between glutathione-conjugated and unconjugated forms, leading to transport throughout the body, for example methyl isocyanate which was the poison leaked from the Union Carbide plant in Bhopal, India.

In common with many drug-metabolising proteins, gene expression is modulated according to the tissue (Strange *et al*, 1984) and the pattern of gene expression is an important adaptive response, allowing the organism to 'mix and match' the components it needs. However, the adaptive response can lead to problems of drug resistance in neoplastic tissue. Interruption of normal gene control leads to over-expression of GST in these tissues, thus reducing the therapeutic value of some anti-cancer drugs. Association of the export of glutathione-conjugates with MRP makes GST of real pharmacological importance in drug resistance.

1.3 Transcriptional control of Glutathione S-Transferases is important.

This adaptive response of cells to toxic insults is mediated by control of gene expression primarily at the transcription level. Control of GST expression is complicated and is influenced by tissue specificity, sex, species, physiological stress and exposure to toxins. The number of compounds that can induce GST expression is extremely large, although they can be classified into 4 broad categories; (a) polycyclic aromatic hydrocarbons, (b) phenolic antioxidants, ROS and Michael reaction acceptors, (c) phenobarbital and (d) synthetic glucocorticoids. These classes of inducers are likely to have their own separate mechanisms of inducing GST expression in the cells (Hayes and Pulford, 1995).

The classification of inducer compounds has been confirmed by computer analysis of the DNA sequence of 5' non-translated regions of genes. The analyses show that there are response elements present in the transcription promoters of the GSTs analysed. Analysis of GSTA1 and GSTA2 from mice has shown that the promoters are made up from a number of *cis*-acting elements.

1.3.1 *Cis*-acting elements are involved in regulation of transcription

Murine GSTA2 has a xenobiotic response element (XRE) (Rushmore *et al*, 1990) that is sensitive to polycyclic aromatic hydrocarbons and modulated through the Aryl Hydrocarbon (Ah) receptor (Paulson *et al*, 1990, Telakowski-Hopkins *et al*, 1988), a glucocorticoid response element (GRE) (Rushmore *et al*, 1993), a Barbie box sequence thought to be involved in induction of barbiturate responsive protein (Laing *et al*, 1995) and an anti-oxidant response element (ARE) (Rushmore *et al*, 1991).

AREs have high sequence homology to tetradecanoyl phorbol 13-acetate (TPA)-responsive elements (TREs) (Friling *et al*, 1992). TREs, also called AP-1 sites, bind the transcription factor AP-1 which has been identified as a Jun/Fos heterodimer. This prompted speculation that Jun/Fos was involved in the transcriptional regulation of GSTs. However *in vitro* experiments using different members of the Jun (c-Jun, Jun-B, Jun-D) and Fos (c-Fos) family have failed to show AP-1 complex recruitment to this site (Yoshioka *et al*, 1992). However, the AP-1 family is extensive and not all the members have been tested.

Another, related responsive element has also been identified in the 5' non-translated region in murine GSTA1, this is called the electrophile-responsive element EpRE. The element has two non-identical motifs, one of which is an ARE and the other contains the ARE consensus sequence (Friling *et al*, 1992). However, in contrast with a single ARE site, there is evidence to suggest that EpRE can interact

with an AP-1 complex but this interaction is likely to be fairly non-specific as mutagenesis was unable to knock-out AP-1 binding capacity in band-shift assays (Hayes and Pulford, 1995).

While these elements begin to describe how GST expression is modulated in response to chemical stresses, GSTs also show tissue specificity and over-expression in pre-neoplastic tissues. The questions have been addressed by examining transcriptional control of human GSTP1 gene in human mammary carcinoma cell-line MCF7 and a multi-drug resistance derivative VCREMS which shows much increased GSTP1 activity over that present in the parental MCF7. It was found that the Sp-1 transcription factor is important in determining the basal level of expression and is required for optimal activity (Moffat *et al*, 1996), determined by the distance of the element from the transcriptional start site. However, using promoter deletions, Sp-1 was shown not be responsible for the differences in activity between the multi-drug resistance cell-line and the parental cell line.

Further experiments showed that the differences in the cell lines were due to a silencer element present in the parental MCF7 cells (Moffat *et al*, 1996). The repressor bound to the silencer is thought to interact with other transcription factors bound at the promoter, exerting its effect by displacing transcription factors needed for transcription. However, cell specific differences were seen between MCF7 and VCREMS because the composition of the transcription factors at the promoter in each of the cell-lines appeared to determine whether the silencer was able to switch off transcription (Moffat *et al*, 1996)

1.3.2 Inducers of GST activity can be monofunctional or bifunctional

Compounds that induce GST activity can be broadly classified into two groups, monofunctional and bifunctional inducers (Prochaska and Talalay, 1988). Monofunctional inducers can only induce activity in phase II enzymes, whereas bifunctional inducers can induce phase I enzymes as well as phase II enzymes. The distinction between mono- and bi-functional compounds is now blurred because phase I activity induced by the compounds was originally determined by measuring aryl hydrocarbon hydroxylase activity. Therefore, it is possible that compounds could activate phase I enzymes that do not have aryl hydrocarbon hydroxylase activity.

This means that there must be a better definition of mono- and bi-functional inducers. Treatment with bi-functional compounds must stimulate Ah hydroxylase activity as well as phase II activity and so must interact with Ah receptors modulate that gene expression through an XRE dependent mechanism. Therefore it follows that bifunctional inducers induce phase II expression either by acting directly through an

XRE element upstream of a phase II enzyme promoter or by metabolism of the compound by XRE-controlled phase I enzymes whose products can then induce phase II proteins via an XRE-independent mechanism i.e a catalytic cascade (Prochaska and Talalay, 1988). Monofunctional compounds must act independently of XRE control and so they tend to be more structurally diverse than bifunctional compounds. They can either be direct acting or need to be metabolised before acting e.g. ethoxyquin which is metabolised to 6-hydroxy-2,2,4-trimethyl-1,2-dihydroquinoline. Ethoxyquin is involved in the induction of various cytochrome P450s and also rGST Yc₂ (Hayes *et al*, 1991, Hayes *et al*, 1994). Putative ARE sequences have been identified upstream of the promoter of rGSTYc₂ (Pulford and Hayes, 1996). Aflatoxin B₁ metabolism will be dealt with in more detail later.

1.4.1 Glutathione S-Transferases are organised into five main classes

A large number of GSTs have been purified from a wide range of organisms. Most GSTs can be placed into one of seven species independent classes based on immunological, sequence, structure and activity considerations. Six of the classes, alpha, mu pi theta, kappa (Pemble *et al*, 1996) and sigma are cytosolic enzymes and there is a class of membrane-bound proteins called microsomal (Mannervik *et al*, 1985, Meyer *et al*, 1991). However the rules governing the assignment of new GSTs to classes are not absolute. It is generally taken that GSTs that share more than 40% sequence identity are included in the same class and subsequently, any that have less than 30% identity are assigned to separate classes but there are a few cases that lie between these boundaries. For example, the new GST structure from squid does not sit comfortably in any of the established classes and has been placed in a class of its own called sigma (Ji *et al*, 1995).

1.4.2 Evolutionary paths can be predicted from sequence alignments.

Sequence alignments have been carried out between all the GSTs isolated and evolutionary relationships have been postulated. These are summarised in Fig 1.1 below. It shows that alpha, mu and pi are all closely evolutionarily related, while theta and sigma are more distantly related. There appears to be evidence for the evolution of a hydrophobic lock and key mechanism that locates the two subunits properly centred around a phenylalanine residue and helix $\alpha 5$ (Armstrong *et al*, 1996) present in alpha mu and pi. This structure is missing from the sigma protein structure. Mu class then diverged from an alpha/mu/pi progenitor before divergence of the alpha and pi classes because the 3'-noncoding region of mu and theta class genes are highly conserved

as are the corresponding regions of alpha and mu (Pemble and Taylor, 1992). The divergence of alpha from pi produced novel active site interactions (Armstrong, 1997).

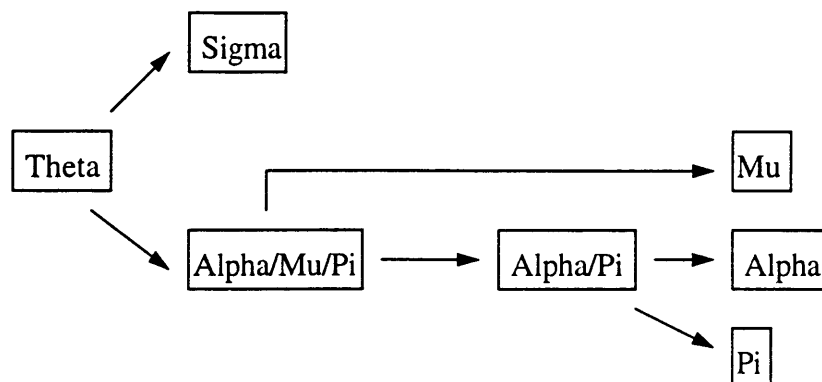


Fig 1.1 Proposed evolutionary relationships among the glutathione transferase classes. Determined by sequence alignments and detail at the dimer interface (Armstrong *et al*, 1996).

1.4.3 3-Dimensional structures show related folds and dimer interactions

If 3-dimensional structures of GST monomers are examined, then the evolutionary relationships suggested are clearly visible. Representative structures for all classes have been solved by X-ray crystallography (Dirr *et al*, 1994, Ji *et al*, 1995, Ji *et al*, 1992, Reinemer *et al*, 1992, Sinning *et al*, 1993, Wilce *et al*, 1995, Cameron *et al*, 1995, Dirr *et al*, 1992). The overall topography of all the transferases are understandably similar and these can be seen in Fig 1.2

All GSTs are dimers of approximately 50kDa and have two active sites per molecule that function independently of each other (Danielson and Mannervik, 1985). Active sites are formed at the dimer interface, corroborating the observation that there is no catalytically active monomeric species. Heterodimers as well as homodimers do exist for GSTs but only monomers of the same class have been seen to interact. This is likely to be because of the symmetrical way that the subunits dimerise, complementary 'locks' and 'keys' are needed and supports the 'lock and key' mechanism for subunit association proposed (Armstrong *et al*, 1996).

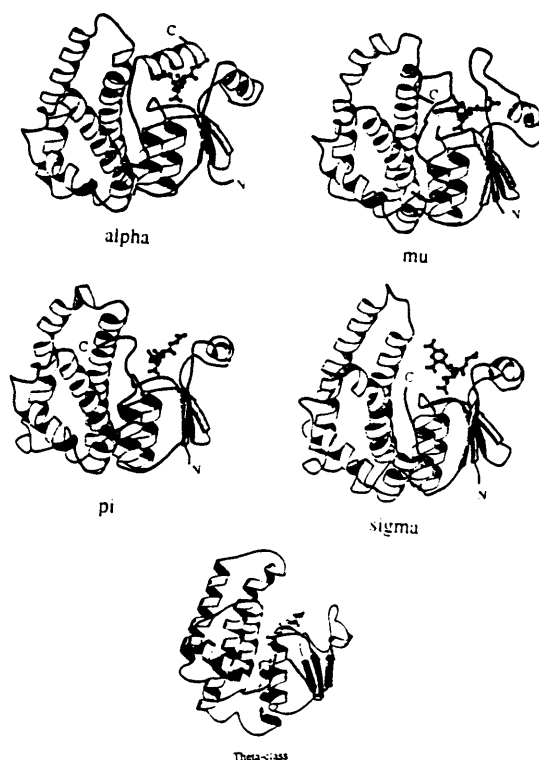


Fig 1.2 Ribbon structures of alpha-class (human GST A1-1 with S-benzylglutathione at 2.6 Å resolution), mu-class (rat M1-1 with (9S, 10S)-9-(S-glutathionyl)-10 hydroxy-9,10 dihydrophenanthrene at 1.8 Å resolution) pi-class (pig P1-1 with glutathione sulfonate at 2.1 Å resolution) and sigma-class (squid enzyme in complex with 1-(S-glutathionyl-2,4-dinitrobenzene) at 2.4 Å resolution. Diagrams from (Vermeulen, et al., 1996)

All GST active sites consist of a N-terminal G-site, where the glutathione binds and a C-terminal H-site where the hydrophobic substrate binds. Although the cytosolic GSTs show overlapping substrate specificities with broad tolerance of both substrate size and electrophilic functional group, each enzyme has its own preferred substrate. GSTs are also implicated in binding and transport of non-substrate compounds and hormones (Litowsky, et al., 1988). Their binding capacities had been identified long before the genes were isolated and were called 'ligandins'. Some isoenzymes show distinctive properties for example, the alpha class enzymes show a non-selenium dependent peroxidase activity with organic hydroperoxides (Prochaska, 1980).

1.5 Alpha-class Glutathione S-Transferases.

Alpha-class GSTs have been cloned from humans, rats and mice and the subunits that have been cloned are shown in Table 1.1.

Organism	New Nomenclature	Old Nomenclature	Organ	Diagnostic substrate	Reference
Rat	rGSTA1	Ya ₁	Liver	Δ^5 andros	(Lai <i>et al</i> , 1984)
Rat	rGSTA2	Ya ₂	Liver	Δ^5 andros	(Pickett <i>et al</i> , 1984)
Rat	rGSTA3	Yc ₁	Liver	CuOOH	(Telakowski-Hopkins <i>et al</i> , 1985)
Rat	rGSTA4	Yk	Liver	4-HNE	(Stenburg <i>et al</i> , 1992)
Rat	rGSTA5	Yc ₂	EQ-Liver	AFBO	(Hayes <i>et al</i> , 1994)
Mouse	mGSTA1	Ya ₁	Liver	-	(Daniel <i>et al</i> , 1987)
Mouse	mGSTA2	Ya ₂	Liver	-	(McLellan <i>et al</i> , 1991)
Mouse	mGSTA3	Yc	Liver	AFBO	(Hayes <i>et al</i> , 1992)
Mouse	mGSTA4	Yk	Lung	4HNE	(Zimnak <i>et al</i> , 1992)
Human	huGSTA1	B ₁	Liver	CDNB	(Board and Webb, 1987)
Human	huGSTA2	B ₂	Liver	-	(Hayes <i>et al</i> , 1989)

Table 1.1. Δ^5 andros Δ^5 androstene-3,17-dione. 4HNE 4-hydroxynonenol. AFBO aflatoxin 8,9-epoxide. CDBN 1-chloro-(2,4)-dinitro-benzene. CuOOH cumene hydroperoxide. EQ- ethoxyquin treated liver. Nomenclature has recently been simplified and is summarised. Other subunits have yet to be unambiguously identified. (Hayes and Pulford, 1995)

In common with mu- and pi-class, alpha-class enzymes form heterodimers as well as homodimers which can be identified by SDS-PAGE, HPLC, isoelectrofocusing and Western blotting. The formation of heterodimers is important because the active-sites of the subunits are independent. Thus, dimerisation leads to the subunits contributing in a dosage-dependent manner to the catalytic activity (Hayes *et al*, 1994) and present a way for organisms to fine-tune their catalytic activities to the environment. The ability of different subunits to dimerise has not been systematically studied but in alpha-class enzymes, it is clear that not all the subunits are able to

hetero-dimerise. For example in rats GSTA1, GSTA2, GSTA3 and GSTA5 are able to hybridise (Hayes *et al*, 1994) but GSTA4 only exists as the homodimer rGSTA4-4 (Alin *et al*, 1989). Solving the crystal structures of these enzymes may show individual differences in the way the proteins can dimerise. To date however, the only experimentally determined structures for alpha-class enzymes belong to huGSTA1-1.

1.5.1 Human Glutathione S-Transferase A1-1.

Alpha-class GSTs are the major GST in human liver, contributing upto 5% of total cell protein. These proteins exist in the liver as homo and heterodimers of huGSTA1-1, huGSTA1-2 and huGSTA2-2 (Stockman *et al*, 1987). Although these subunits have similar catalytic properties, the huGSTA2 subunit has significantly greater selenium independent glutathione peroxidase activity and huGSTA1-1 has a high affinity for Tributyltin acetate which can be used as an inhibitor to distinguish the subunits kinetically (Stockman *et al*, 1987).

The first structure to be published was huGSTA1-1 in complex with S-benzylglutathione (Sinning *et al*, 1993). It showed the overall polypeptide fold closely resembles that of the mu and pi-class proteins. The G-site was similar to previously reported structures with glutathione making a network of specific interactions with polar groups of both subunits of the GST dimer, as would be expected for enzymes that bind glutathione and are evolutionarily related. However, the H-site showed a new and more varied structure when compared with other GST structures, concordant with relaxed substrate specificity.

The monomer is a two-domain structure (fig 1.3a) with connections between the two domains at residues 82 and 192. The first domain which contains the glutathione binding site is an α/β structure with a mixed β -sheet made up of 4 β strand β 1 to β 4 and three α -helices arranged in a $\beta\alpha\beta$ configuration joined to $\beta\beta\alpha$ configuration by helix α 2. The α -helices pack onto the β sheet by hydrophobic interactions. The supposition from sequence alignments that alpha-class enzymes do not have an insertion between β 2 and α 2 which corresponds to the loop over the active site present in mu-class was confirmed. The loop connecting α 2 and β 3 contains Gln54 and Val55 which adopt standard configurations but Pro56 is a *cis* conformation residue. These residues define part of the interactions with the backbone of glutathione with Val55 taking part in H-bonds with the cysteinyl moiety of glutathione.

The second domain is made completely from β -sheets $\beta 4$ to $\beta 9$. The main distinguishing structural feature of the structure was that the C-terminus folds into an extra alpha helix ($\alpha 9$), at least when complexed with S-benzylglutathione. The C-terminal alpha-helix forms part of the H-site and has the appearance of a lid over the active site, replacing the insertion between $\beta 2$ and $\alpha 2$ in non-class enzymes. The dimer interface is made up from hydrophobic and hydrophilic interactions and because of

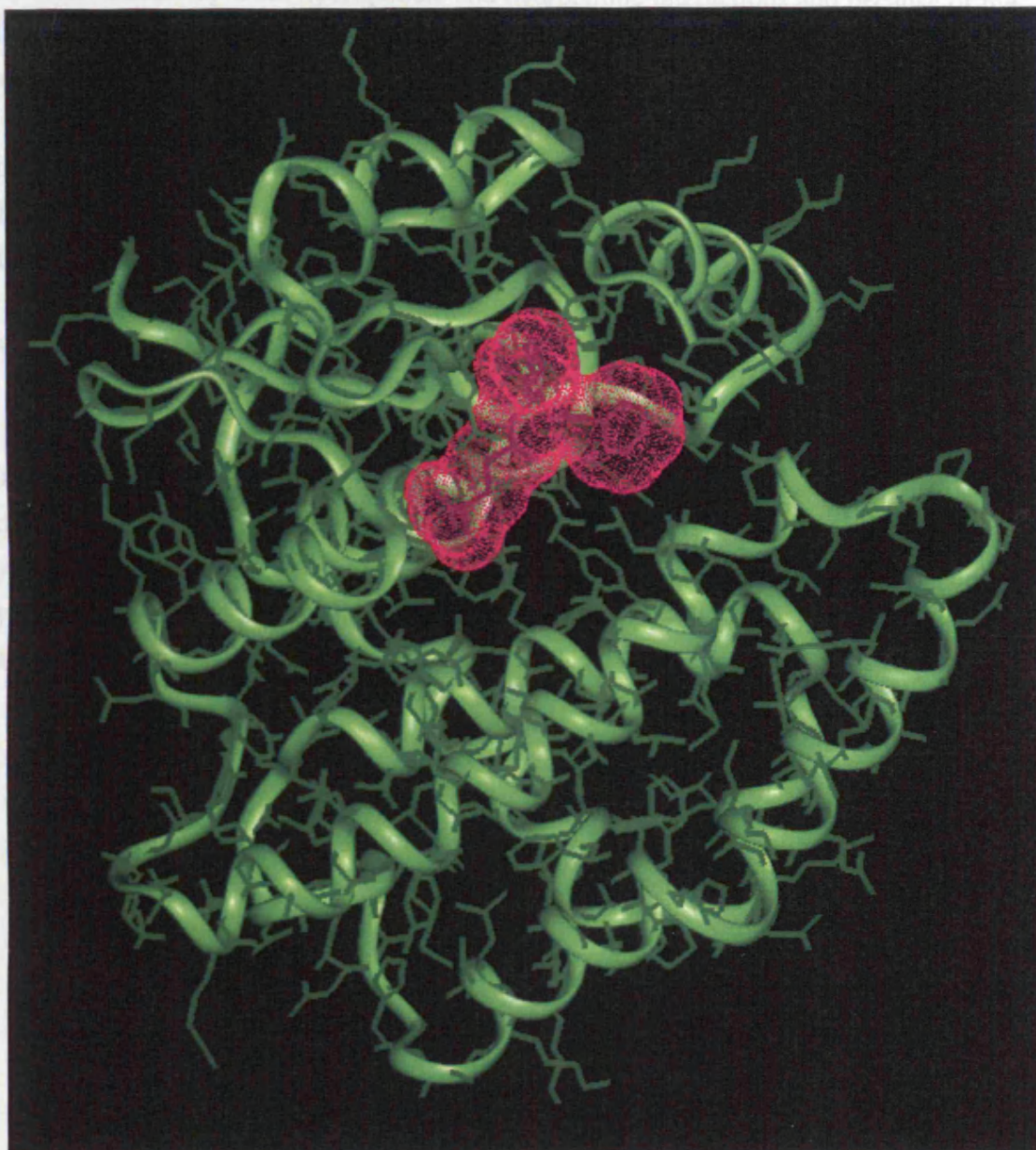


Fig 1.3 O-site interactions between hGSTA1-1 and bound S-benzylglutathione. H-site is not shown. Residues marked * are from the other subunit. (Sinning et al., 1993).

Fig 1.3a Insight representation of the structure of human GSTA1-1 complexed with S-benzylglutathione (Sinning, et al., 1993).

The second domain is made completely from 5 α -helices $\alpha 4$ to $\alpha 9$. The main distinguishing structural feature of the structure was that the C-terminus folds into an extra alpha helix ($\alpha 9$), at least when complexed with S-benzylglutathione. The C-terminal alpha-helix forms part of the H-site and has the appearance of a lid over the active site, replacing the insertion between $\beta 2$ and $\alpha 2$ in mu-class enzymes. The dimer interface is made up from hydrophobic and hydrophilic interactions and because of the domain separation, there is a central cavity that is open to the external solvent and contains around 12 water molecules.

Comparisons of the G-site and H-site with other classes of GSTs can give clues to structure/function relationships when considered with the diverse kinetic characterisations in the literature. The G-site will be considered first followed by the H-site.

1.6 Glutathione binding sites (G-sites) in Glutathione S-Transferases

GSTs from each of the representative classes clearly have a glutathione binding site within the N-terminal portion of the peptide sequence. One feature that is conserved in the crystal structures of alpha-, pi-, and mu-class enzymes appears to be a hydrogen bond between the sulphur of bound glutathione adducts and the phenolic hydroxyl group of a tyrosine side-chain. This interaction can be clearly seen in the G-site of huGSTA1-1.

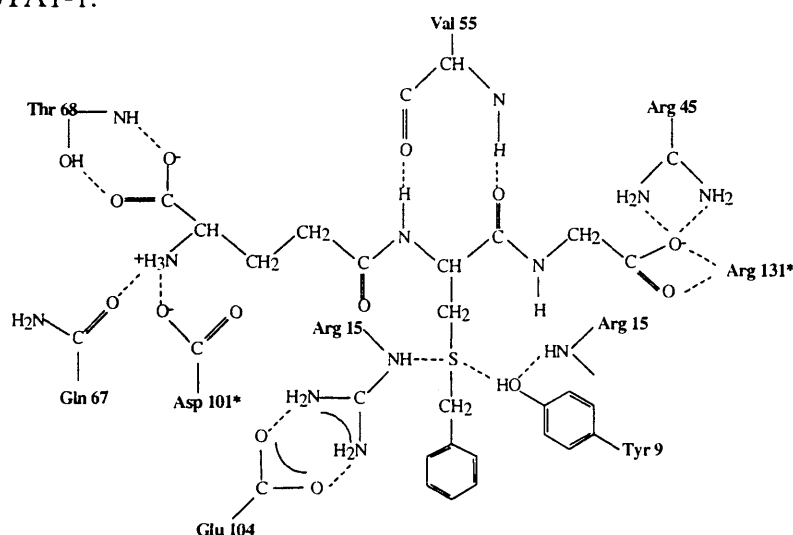


Fig 1.3 G-site interactions between huGSTA1-1 and bound S-benzyl-glutathione via H-bonds and salt-links. Residues marked * are from the other subunit. (Sinning *et al*, 1993)

Crystallographic temperature factors indicate that glutathione backbone is rigidly bound at the G-site. The characteristic network of polar interactions can clearly be seen in the molecular recognition of glutathione in huGSTA1-1. Residues from both subunits are involved and most of the functional groups in the glutathione

molecule are bound by protein with the exception of the glycine and γ -glutamate amide hydrogen. The γ -glutamyl moiety of glutathione serves as the major binding determinant and interacts extensively with a hydrophobic complementary pocket near the subunit interface.

1.6.1 Biochemical evidence for the charge state of bound glutathione.

Proposed mechanisms for GST catalysis must be consistent with promoting the nucleophilic addition of glutathione to the substrate bound in the H-site by nucleophilic attack of the glutathione thiol (S_NAr reactions). Alignment of G-site sequences shows that there is an absence of the general acid-base catalytic residues histidine and cysteine that could be expected to carry out such a reaction. An unusual feature of GSTs is the conservation of a hydrogen-bond to bound glutathione adducts, donated by a tyrosine residue but is this residue capable of promoting the nucleophilic attack of glutathione on a substrate?

Ionisation of the sulphydryl group of cysteine gives an intense absorbance between 235nm and 240nm. This absorbance was used to examine enzyme complexes of glutathione (giving E.GSH) and spectrophotometrically transparent γ -L-glutamyl-L-serylglycine (giving E.GOH) by ultra-violet absorption spectroscopy (Graminski *et al*, 1989). The experiments detected a fraction of glutathione molecules in the E.GSH complex that were bound and deprotonated i.e. existed as $E.GS^-$. Graminski *et al* were able to titrate the absorption difference band over the pH range 5-8 to determine a pK_a for bound glutathione. They showed that pK_a for glutathione had shifted from 9.0 in free solution to 6.6 when bound to the enzyme. Thus, it seems likely that GST enzymes are capable of activating the thiol of bound glutathione.

However, there are problems associated with the experiments that Graminski *et al* were unable to resolve. The enzyme complex E.GOH was not perfect because the difference spectra show that E.GOH was not a perfect conformational mimic of E.GSH and raises the question whether the difference band seen was due entirely to deprotonated glutathione over the pH range titrated. This thesis is concerned, in part, with resolving this problem by direct observation of glutathione bound to huGSTA1-1 using NMR and selectively ^{13}C -labelled glutathione.

1.6.2 Is a tyrosine residue involved in the activation of glutathione.

If we take the observation that bound glutathione has a pK_a lower than in free solution, what interactions with the enzyme could be responsible for this shift. The magnitude of the shift suggests that approximately 3.3 kcal/mol of intrinsic binding

energy is used which can be approximated to a hydrogen bond (Graminski *et al*, 1989). From the crystal structures determined, it is clear that tyrosine is capable of forming a hydrogen bond to a bound glutathione thiol group and it was proposed that tyrosine was the acceptor of the proton lost from glutathione.

It is unusual to consider a tyrosine residue for a general base catalyst because it has a pK_a in solution of 10.0 but there is substantial biochemical data to suggest that tyrosine is involved in catalysis. To act as a general base it would have to exist as a phenolate ion, which is unlikely to occur at physiological pH. Difference fluorescence spectroscopy between W21F/Y9F and W21F mutants of ratGSTA1-1 (Atkins *et al*, 1993) shows that the fluorescence contribution of tyrosine 9 could be determined and titrated. Atkins *et al* concluded that the pK_a of tyrosine 9 was shifted from 10.0 to 8.3-8.5.

Unusual activity of an N-terminal tyrosine was first reported in chemical modification experiments with di-ethyl-pyrocabonate (DEPC) (Meyer *et al*, 1993) with ratGSTP1-1. The reaction of one tyrosine with DEPC was anomalous and demonstrated that it had increased electrophilicity, corresponding with a shift in pK_a of around 2.2 units from that of tyrosine in solution which was in agreement with Atkins *et al*. The chemical modification experiments were repeated in the presence of glutathione to determine whether its thiol group interacts with the reactive tyrosine. They found that the presence of glutathione didn't significantly reduce the amount of chemical modification and would argue against a direct role for tyrosine.

However, site-directed mutagenesis of the tyrosine residue in all classes shows that, while the residue may not have a direct role in the activation of the glutathione thiol, it is certainly important for catalysis. Numerous experiments have been carried out to probe the role of tyrosine in the catalytic mechanism by removing the hydroxyl group by mutation to phenylalanine and characterising the resulting activities with model substrates such as CDNB in alpha-class (Stenburg *et al*, 1991, Wang *et al*, 1992), mu-class (Lui *et al*, 1992) and pi-class (Kong *et al*, 1992). All the studies show that removal of the hydroxyl group leads to a decrease in the catalytic activity by approximately two orders of magnitude.

1.6.3 The proposed role for tyrosine in the catalytic mechanism.

When the evidence is considered from spectroscopic and chemical modification experiments, then it is reasonable to propose a mechanism for GSTs as shown in fig 1.4 with a tyrosine activating the glutathione thiol.

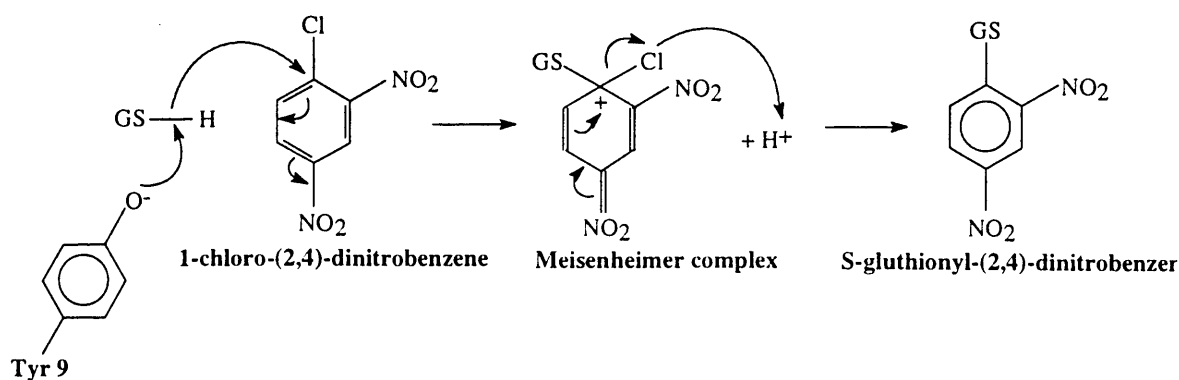


Fig 1.4 Reaction scheme for the addition of glutathione to the general spectrophotometric substrate CDNB. The reaction proceeds through a Meisenheimer complex transition state before decomposing to products.

If the structure of the Meisenheimer complex is examined, then it can be seen that the nature of the functional groups on the benzene ring are important in determining how fast the reaction can proceed. Similarly, the reaction kinetics can be controlled by variation of the leaving group. Using this approach, it was possible to show that the rate limiting step was the formation of the Meisenheimer complex (Chen *et al*, 1988). The existence of the Meisenheimer transition state could be shown by manipulating the functional groups so that there was no appropriate leaving which could then trap the transition state such as 1,3,5-tri-nitro-benzene (TNB).

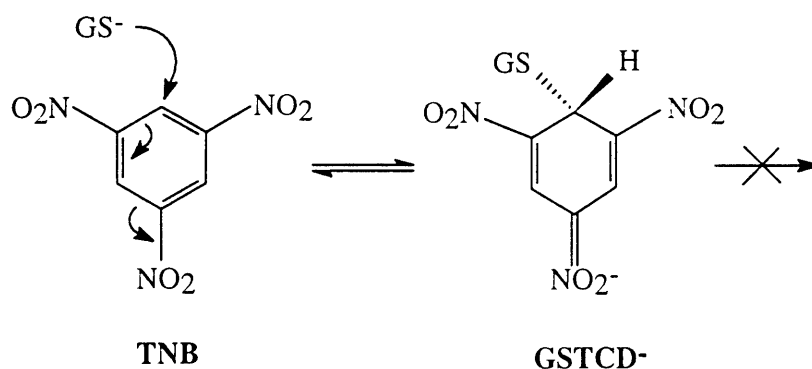


Fig 1.5 Reaction scheme showing the addition of glutathione to TNB to give GSTCD⁻ (1-(S-gluthionyl)-2,4-di-nitro-cyclohexadienate) which cannot decompose to products.

GSTCD⁻ anion can be generated by soaking crystals of huGSTM4-4 with TNB and glutathione (Ji *et al*, 1993) and the deep red colour associated with the GSTCD⁻ complex accumulates in the crystals where it remains long enough for a 1.9 Å resolution structure to be solved of GSTCD⁻ in the active site (Ji *et al*, 1993). The solution kinetics for the formation of GSTCD⁻ has also been characterised (Graminski

et al, 1989) and the two studies show that proposed S_NAr mechanism is valid for GSTs.

1.6.4 The exact role of tyrosine remains obscure.

Despite the validity of the reaction mechanism, there are still unresolved questions and conflicting data about how the enzyme performs it. The spectroscopic titrations from Graminski *et al* show that pK_a for glutathione is 6.5-6.9 and so would advocate a Tyr-OH•••SG arrangement. However, modification (Meyer *et al*, 1993) and difference fluorescence analyses (Atkins *et al*, 1993) show that the tyrosine has significant charged character and so should have a Tyr-O^{δ-}•••^{δ+}H-S^{δ-}G arrangement. These conflicting results can be resolved by adopting an arrangement where the proton sits in an electrostatic 'well' i.e. Tyr-O^{δ-}•••^{δ+}H•••^{δ-}SG (Ji *et al*, 1992, Meyer *et al*, 1993) with exact position determined by the pK_a values of Tyr9 and glutathione. While alpha class enzymes show an unusual pK_a value for the catalytic tyrosine, determination of pK_a value of the catalytic tyrosine in mu class GST has been determined as 10, showing no great change in pK_a from free tyrosine. This suggests that there are differences in the detailed interactions in the active site of each class of enzyme (Xiao *et al*, 1996). However, for alpha-class arrangements of protons are not instructive, the low pK_a value of Tyr9 suggests that a general base mechanism may operate where Tyr9 as a tyrosinate ion and abstracts the proton directly from the glutathione thiol group. However, Tyr9 may form a hydrogen bond with the thiol sulphur which helps to polarise the bond between the sulphur atom and the proton.

1.6.5 Is a conserved arginine involved in stabilising a tyrosinate anion.

The proposed H-bond arrangement attempts to explain the observations made but the existence of a Tyr-O^{δ-} species in alpha class enzymes is uncommon. What components of the enzyme can stabilise the unusual tyrosinate ion inferred? In alpha-class enzymes, Arg15 is a conserved residue that is close enough to take part in H-bonding to the tyrosinate ion and the glutathione thiol. This residue is not seen in the other classes of Glutathione S-Transferase and may explain why the magnitude of remaining catalytic activity for knockout Tyr-Phe mutants is ten-fold higher for alpha-class enzymes than corresponding mutations in the other classes. Arg 15 also interacts with other residues as detailed in fig 1.3.

Extensive mutagenesis has been reported for all arginine residues in huGSTA1-1 While most arginine residues are involved in structural salt-links (Stenburg *et al*,

1991), Arg 15 seems to be important for catalysis Mutation of Arg 15 to Leu (R15L) reduced the catalytic activity 25 fold whereas mutation Arg 15 to Lys (R15K) only reduced the activity 3 fold, suggesting that a positively charged residue at the active site is important. Ultra-violet absorption-difference spectroscopy was used to determine the pK_a of Tyr 9 in the mutants R15L and R15K. The pK_a of Tyr 9 in wild-type protein was determined as 8.2-8.5 (Atkins *et al*, 1993) but in R15L the pK_a was shifted to 8.8 and in R15K to 8.5 (Bjornestedt *et al*, 1995). This shows that the guanidinium group of Arg15 contributes to the electrostatic field around Tyr9 and may stabilise the formation of a tyrosinate ion to some extent. However, even in wild-type protein, the pK_a value is such that there can only be a fraction of Tyr 9 existing as tyrosinate at neutral pH.

1.6.6 A role for water in active site tyrosinate formation.

There are no water molecules seen the alpha-class crystal structure with S-benzyl-glutathione (Sinning *et al*, 1993). Thus, it is likely that during the binding of ligands, water is excluded from the active site. Indeed, in the apo-protein structure (Cameron *et al*, 1995) there is a disordered water molecule that remains within H-bonding distance to the amide backbone of Arg 15.

It is well known that the fluorescence emission of tyrosine is very sensitive to the hydrogen bonding environment and by extension of fluorescence-difference spectroscopy (Atkins *et al*, 1993) it is possible to probe the hydrogen bonding network and the existence of an arrangement like $\text{Tyr-O}^{\delta-} \cdots {}^{\delta+}\text{H} \cdots {}^{\delta-}\text{SG}$ (Ji *et al*, 1992, Meyer *et al*, 1993). To probe the network, excitation-emission correlated spectra were determined for various mutants of rat GSTA1-1. By looking the 2-dimensional plots produced, it was clear that Tyr 9 had two separate excitation-emission components at pH 7.5. One which corresponded to 'normal' tyrosine and the other to strongly hydrogen-bonded tyrosine or tyrosinate (Dietze *et al*, 1996) proving that Tyr 9 is heterogeneous and likely to be in dynamic equilibrium. It is difficult to spectroscopically distinguish between tyrosinate and strongly hydrogen bonded tyrosine but in unliganded protein there are no H-bonding candidates (Cameron *et al*, 1995) within 4Å so it can be inferred that the tyrosinate species exists. Addition of ligands to the apo-protein quenches the tyrosinate component of the spectrum showing that Tyr 9 then becomes indeterminable from other tyrosine residues in the protein.

In the absence of any components that might stabilise the tyrosinate anion directly in unliganded protein, Dietze *et al* speculate that the disordered water molecule seen may prove a likely candidate. They propose that a water molecule is activated by the positive electrostatic field of Arg 15 (Bjornestedt *et al*, 1995) giving it

hydroxide character. The hydroxide ion character water can then lie between the backbone of Arg 15 and the phenol group of Tyr 9.

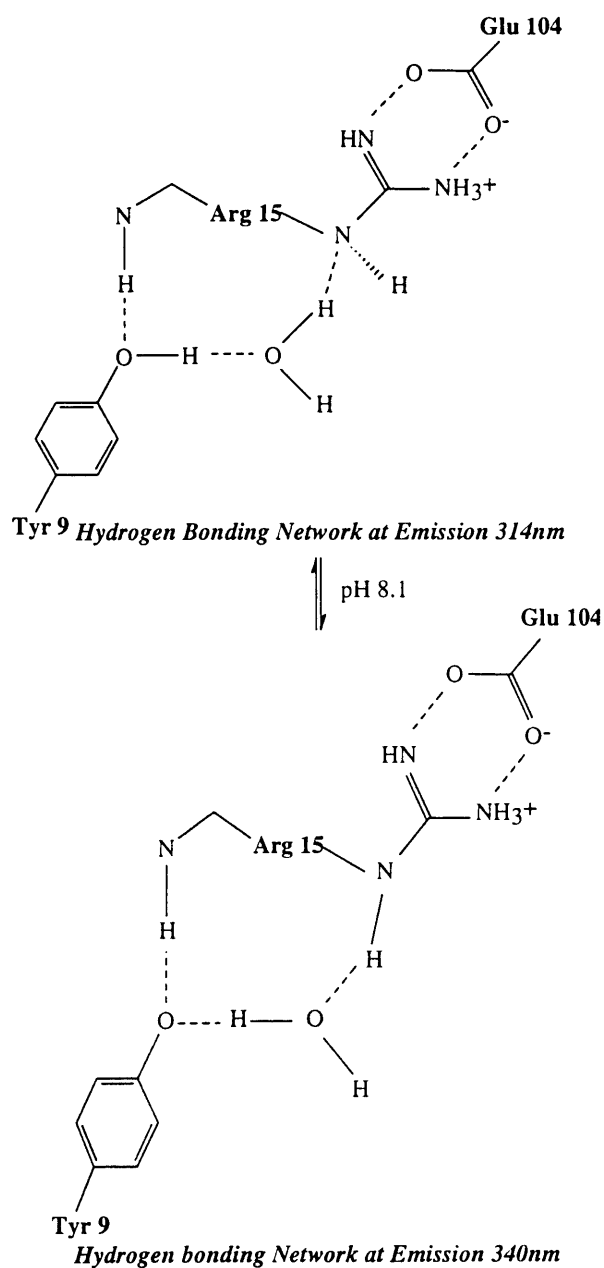


Fig 1.6 Proposed hydrogen-bonding states of Tyr 9 in the absence of ligand. With $pK_a = 8.1$, contributions from both hydrogen bonding states are seen.

1.6.7 Do alpha class enzymes proceed through a general base mechanism?

The hydrogen bonding network described above exists in unliganded protein and must rearrange upon the binding of glutathione or other ligands as the tyrosinate portion of the spectrum is eliminated on binding (Dietze *et al*, 1996). There have been studies of the deuterium isotope effect on k_{cat}/K_m for the reaction of CDNB with glutathione for alpha (Huskey *et al*, 1991) and mu (Parsons and Armstrong, 1996) class enzymes. Both studies show that the effects are small and inverse i.e. $k^H/k^D=0.8-0.9$. This is similar to that seen for the reaction $\text{GS}^-_{(\text{aq})}$ to CDNB and is consistent with desolvation of the thiolate ion and not direct proton transfer between glutathione and tyrosinate, ruling out a general base mechanism. This is further supported by measuring the deuterium effect with tetradeca(3-fluorotyrosyl) mu class enzymes. Here, the addition of a fluorine group on the aromatic ring of every tyrosine residue in the protein guarantees that the residue will be present in the anion O^- form, forcing the enzyme to work via a general base mechanism (Parsons and Armstrong, 1996). k^H/k^D for this modified mu class enzyme is 0.5 and illustrates the what the deuterium isotope solvent effect should be when a general base mechanism operates in alpha and mu class enzymes.

1.6.8 Tyrosine is not essential in all Glutathione S-Transferases.

While a tyrosine 9 is important in alpha- mu- and pi-class enzymes, there are other isoforms where this does not seem to be the case. A Glutathione S-Transferase from *E. coli* was purified and N-terminal sequenced as MKLFYKPGAXSLAS (Nishida *et al*, 1994). Although sequence homologies with mammalian GSTs was low, Tyr 5 was assigned as the catalytic residue. The mutation Y5F was made but this did not result in a significant reduction in the catalytic activity of the enzyme so it seems that in this protein a N-terminal tyrosine is not essential. Mutation of Tyr8 in rat GST1-1 reduces but does not abolish the catalytic activity in the enzyme (Wang *et al*, 1996)

All theta-class enzymes have a conserved N-terminal serine 9, instead of tyrosine, conserving an active site hydroxyl group. Recent crystal structures show that the serine could take part in hydrogen bonding to ligands bound at the active site (Wilce *et al*, 1995). To test the function of serine at the active site, it was mutated to alanine (S9A) in Australian sheep blowfly, *Lucilia cuprina*, theta-class Glutathione S-Transferase. The mutant protein was inactivated and confirmed that serine is an important catalytic residue (Board *et al*, 1995).

1.7.1 The C-terminal helix distinguishes alpha-class GSTs

The distinguishing feature of alpha-class enzymes which sets them apart from the closely related mu and pi classes is the presence of a C-terminal helix. The importance of the helix to catalytic activity in huGSTA1-1 was investigated by deletion (Board and Mannervik, 1991). However as well as deleting the 12 residues that corresponded to the helix, the remaining residues at 209 and 210 were also mutated as shown below.

	210	222
GSTA1-1	PGSPRKPPMDEKSLEEARKIFRF	
GSTdel210	PGSPRKPPMHG-----	

It was shown that those changes diminished the specific activity of the enzyme for CDNB and cumene hydroperoxide. However, the deletion neither abolished GST catalysis nor significantly affected glutathione binding, so suggests that the helix is not directly involved in the catalytic mechanism supporting photoaffinity labelling experiments that the alpha-helix forms a hydrophobic region in the H-site (Hoesch and Boyer, 1989). Inhibition studies with bromosulphophthalein showed that the I_{50} for the truncated protein was unchanged, showing that it was folded correctly despite the extensive deletion

1.7.2 The C-terminus is a dynamic structural motif.

It is unusual for a such a large deletion in a protein's sequence not to have an effect on the folding, however this may be explained in part when crystal structures for huGSTA1-1 complexed with other molecules, such an S-glutathionyl-ethacrynic acid conjugate (GSH-ECA), ethacrynic acid on its own (ECA-alone) and the apo-protein structure (with bound β -mercaptoethanol) are examined (Cameron *et al*, 1995).

When the ECA-GS structure was compared to the enzyme complexed with S-Benzylglutathione (Sinning *et al*, 1993), among the few structural differences that were seen was the position of Met208. The residue had moved slightly out of the H-site in order that the more bulky ECA conjugate could be accommodated and, in turn, caused a degree of disruption in the C-terminal helix. The increased distortion of the helix is reflected in real-space correlation coefficients for the main-chain atoms of residues 208-222. In the S-benzylglutathione structure they are 0.85 but with ECA-GSH they are 0.68, suggesting that with ECA-GSH bound the helix is not in its most 'stable' position (Cameron *et al*, 1995). It was also apparent that the GSH-ECA conjugate was present at the active site in two conformations although models for each of the

1.6.9 Using Nuclear Magnetic Resonance to look at glutathione binding.

This thesis is, in part, concerned with glutathione binding to huGSTA1-1. The activation of glutathione at the active site is important and all proposed mechanisms must include it so it would be important to have direct evidence for thiolate formation. Using edited NMR, it is possible to observe directly the ionisation state of bound glutathione. There is no conformational change associated with ^{13}C -labelled glutathione analogues so the results would be more conclusive than difference spectroscopy (Graminski *et al*, 1989).

It is also possible to look at the ionisation state of Tyr 9 using selectively protonated tyrosine in deuterated protein and site directed mutagenesis and the nature of Tyr 9 is also investigated in this thesis.

1.7 The H-site in Alpha-class Glutathione S-Transferases.

The interactions in the G-site with glutathione are important in GST biochemistry but they are really only half of the story. As previously mentioned, GSTs bind a wide range of substrates and the differences that exist between isoenzymes in terms of the selection of substrates is at least as important as the deprotonation of bound glutathione. Substrate specificity is determined by the geometry of the H-site.

The H-sites in alpha-class GSTs are poorly understood. The only structural data obtained is on huGSTA1-1. The first crystal structure published was in complex with S-benzylglutathione (Sinning *et al*, 1993) which allowed the H-site structure to be clearly defined. The boundaries of the site were; the C-terminus residues of alpha-helix α_4 including Glu104, Leu107, Leu108, Pro110, and Val111; the C-terminal helix, Leu213, Ala216, Phe220 and Phe220, and the loop immediately preceding the helix which includes the residue Met208. While the crystal structure was able to define the site with fine detail, the S-benzyl group of the enzyme-bound inhibitor was too small for any clear interactions with the protein to be seen. It appeared that the H-site had not sufficiently closed around the substrate. This was not surprising because the H-site has to accommodate a wide range of substrates and the site might be expected to have a more relaxed enzyme:substrate interaction when compared to most other enzymes which bind their substrates very selectively.

1.7.1 The C-terminal helix distinguishes alpha-class GSTs

The distinguishing feature of alpha-class enzymes which sets them apart from the closely related mu and pi classes is the presence of a C-terminal helix. The importance of the helix to catalytic activity in huGSTA1-1 was investigated by deletion (Board and Mannervik, 1991). However as well as deleting the 12 residues that corresponded to the helix, the remaining residues at 209 and 210 were also mutated as shown below.

	210	222
GSTA1-1	PGSPRKPPMDEKSLEEARKIFRF	
GSTdel210	PGSPRKPPMHG-----	

It was shown that those changes diminished the specific activity of the enzyme for CDNB and cumene hydroperoxide. However, the deletion neither abolished GST catalysis nor significantly affected glutathione binding, so suggests that the helix is not directly involved in the catalytic mechanism supporting photoaffinity labelling experiments that the alpha-helix forms a hydrophobic region in the H-site (Hoesch and Boyer, 1989). Inhibition studies with bromosulphophthalein showed that the I_{50} for the truncated protein was unchanged, showing that it was folded correctly despite the extensive deletion

1.7.2 The C-terminus is a dynamic structural motif.

It is unusual for a such a large deletion in a protein's sequence not to have an effect on the folding, however this may be explained in part when crystal structures for huGSTA1-1 complexed with other molecules, such as S-glutathionyl-ethacrynic acid conjugate (GSH-ECA), ethacrynic acid on its own (ECA-alone) and the apo-protein structure (with bound β -mercaptoethanol) are examined (Cameron *et al*, 1995).

When the ECA-GS structure was compared to the enzyme complexed with S-Benzylglutathione (Sinning *et al*, 1993), among the few structural differences that were seen was the position of Met208. The residue had moved slightly out of the H-site in order that the more bulky ECA conjugate could be accommodated and, in turn, caused a degree of disruption in the C-terminal helix. The increased distortion of the helix is reflected in real-space correlation coefficients for the main-chain atoms of residues 208-222. In the S-benzylglutathione structure they are 0.85 but with ECA-GSH they are 0.68, suggesting that with ECA-GSH bound the helix is not in its most 'stable' position (Cameron *et al*, 1995). It was also apparent that the GSH-ECA conjugate was present at the active site in two conformations although models for each of the

conformations could not be constructed because the resolution of the data was not good enough.

However, when the structures of ECA-alone and unliganded structures were examined, there were more significant differences in the protein structure. The ECA-alone structure showed clearly that there was only one conformation of ECA bound in the H-site, in contrast to the observations with ECA-GS, and also that ECA was bound nearer to the G-site than would be expected from the structure of the conjugate, partially occupying it. Also, the reactive α,β -ketone group of the compound points away from where glutathione would normally bind, forming a non-productive binding complex in the absence of glutathione (Cameron *et al*, 1995). The C-terminal helix also appeared to be more disordered than the complex with S-benzylglutathione with real-space correlation coefficients of 0.71 and 0.85, respectively. This is unusual as ECA alone should be able to fit in the H-site without causing distortion. Clues to the origin of the disorder may be gained from the apo-protein structure.

When the electron density for the apo-protein was calculated, it was striking to find that there was no density associated with the C-terminal residues 208-222. While there are many examples of 'lids' of active sites that close over the active site while occurs, there was no 'open' conformation of the helix identified. Rather, the helix appeared to be disordered, with some traces of electron density found at low contour levels where the helix was expected to be (Cameron *et al*, 1995).

This may explain why the deletion mutant appeared to be folded correctly (Board and Mannervik, 1991). When unliganded, it seems likely that the protein spends a period of time without any structure at the C-terminus. It would be detrimental to the function of the protein if the disordered C-terminus went on to unfold the rest of the structure. Thus the helix appears to be a dynamic part of the structure which may be important in the binding of substrates, given that it is stabilised by the binding of substrate.

In this thesis, the function of the C-terminal helix is examined by deleting the residues 210-222 while preserving the integrity of the protein by excluding secondary mutations that may otherwise contribute to the activity of the protein. The kinetics of the truncated protein are determined and the effect of adding back the helix as a synthetic peptide is examined to see how the helix might influence substrate binding. Site-directed mutagenesis is also used here to mutate specific phenylalanine residues on the terminal helix in order to assign the resonances of these residues, which can be used to probe its dynamic nature.

1.8 Aflatoxin B₁ metabolism as a model substrate for alpha-class GSTs

1.8.1 The H-site pocket is not easily studied by crystallography

While the C-terminal helix has been relatively easy to study using NMR and crystallography, the residues and interactions of the rest of the residues pocket that make up the hydrophobic H-site, and how that pocket interacts with substrates, has been difficult to study using those techniques. To study these types of interactions, it is necessary to adopt a different strategy.

A successful alternative strategy would ideally involve the identification of related isoenzymes that showed greatly different activities towards a model substrate but that had high sequence homologies. Thus by using sequence alignments, molecular modelling and site-directed mutagenesis it would be possible to determine the amino-acid residues in the H-site that contributed to the differences in activities.

The success of a differential metabolism strategy strongly depends on the choice of a model substrate. As will be discussed, investigation of the species differences of resistance to the mycotoxin aflatoxin B₁ not only created an interesting research topic in mechanistic carcinogenesis but also ultimately led to the use of this system to study the H-sites of alpha-class GSTs. To understand fully how aflatoxin B₁ can be used in this way, it is helpful to understand how the research field developed.

1.8.2 The impact of dietary aflatoxin B₁ on public health

The mycotoxin aflatoxin B₁ is produced by common mould species especially *Aspergillus flavus* and *Aspergillus parasiticus*, and is one of the most potent hepatocarcinogens known (Busby and Wogan, 1984). The moulds that produce aflatoxin B₁ frequently grow on poorly stored grains, cereals and peanuts in warm, humid environments and produce a number of closely related di-furano-coumarin compounds of which aflatoxin B₁ is just one. The moulds and the compounds they produce are a major world-wide public health problem. The effect of human exposure to aflatoxin B₁ in humans has been linked to liver, lung and colon cancer as well as Kwashiorkor and Reye's syndrome (Hall and Wild, 1994, Robens and Richard, 1992).

Aflatoxin B₁ in the diet has been studied epidemiologically in regions that are high risk areas for the development of hepatocarcinoma such as West Africa (Groopman *et al*, 1988), China and Southeast Asia (Ross *et al*, 1992). It has been shown that there is a direct link between the amount of dietary aflatoxin, as measured by urinary markers (Groopman and Kensler, 1993), and risk of hepatocarcinoma. The studies also showed that infection with Hepatitis B virus (HBV) increased the risk of

cancer, the epidemiological group that was most at risk was those that were both infected with HBV and exposed to aflatoxin B₁, suggesting that infection with HBV can increase the toxicity of aflatoxin B₁ (Ross *et al*, 1992). Mice, transgenic for HBV, show increased tumour formation when treated with dietary aflatoxin B₁ and show associated increased levels of Cytochrome P450s that are implicated in the activation of aflatoxin B₁ to a more toxic intermediate (Kirby *et al*, 1994).

Liver cancer is the third most common cause of death from cancer in China (Li *et al*,) and almost half of all estimated liver cancers in the world arise in China (Parkin *et al*, 1984) so the effect that aflatoxin B₁ has on human health should not be underestimated.

1.8.3 Aflatoxin B₁ metabolism.

Aflatoxin B₁ is metabolised by phase I (Forrester *et al*, 1990) and phase II detoxification enzymes (Hayes *et al*, 1991, Gopalan-Kriczky *et al*, 1994). However, as is the case for many other xenobiotic compounds, reactive intermediates of the metabolism, particularly products of phase I enzymes, are more toxicologically important than the parent compound. An overview of the metabolism of aflatoxin B₁ is shown in fig 1.7 below.

The first stage of metabolism of aflatoxin B₁ is NADPH and O₂ dependent and takes place in microsomes, providing the initial evidence that it is Cytochrome P450 dependent (Wolf, 1986). Accordingly, the carcinogenicity of aflatoxin B₁ is linked to the species from which microsomes are prepared and shows that the pattern of CYP450 expression in different species may be involved in the marked differences in sensitivity to aflatoxin. Animals such as the mouse and hamster possess resistance to the toxin while rats, guinea pig and the duck are acutely sensitive (Eaton and Gallagher, 1994)

1.8.4 Microsomal Activation of aflatoxin B₁.

Microsomal activation of aflatoxin B₁ is species dependent (Ramsdell and Eaton, 1990) and relies on CYP450 activity, although levels of activation do not correlate simply with species resistance. For example, rat microsomes are four times more efficient at activating aflatoxin B₁ than humans and, in turn, mouse microsomes are more efficient than rat in activating aflatoxin B₁. However, only the mouse is resistant to the systemic affects of aflatoxin B₁ so while microsomal activation of aflatoxin B₁ is important, there are other factors that contribute to toxicity.

During activation, the terminal furan ring of aflatoxin B₁ is epoxidised to give aflatoxin 8,9-epoxide which is the reactive species that is responsible for DNA damage (Swenson *et al*, 1977). When produced in aqueous solution, aflatoxin 8,9-epoxide has an extremely short half-life of approximately 5 seconds (Ramsdell and Eaton, 1990).

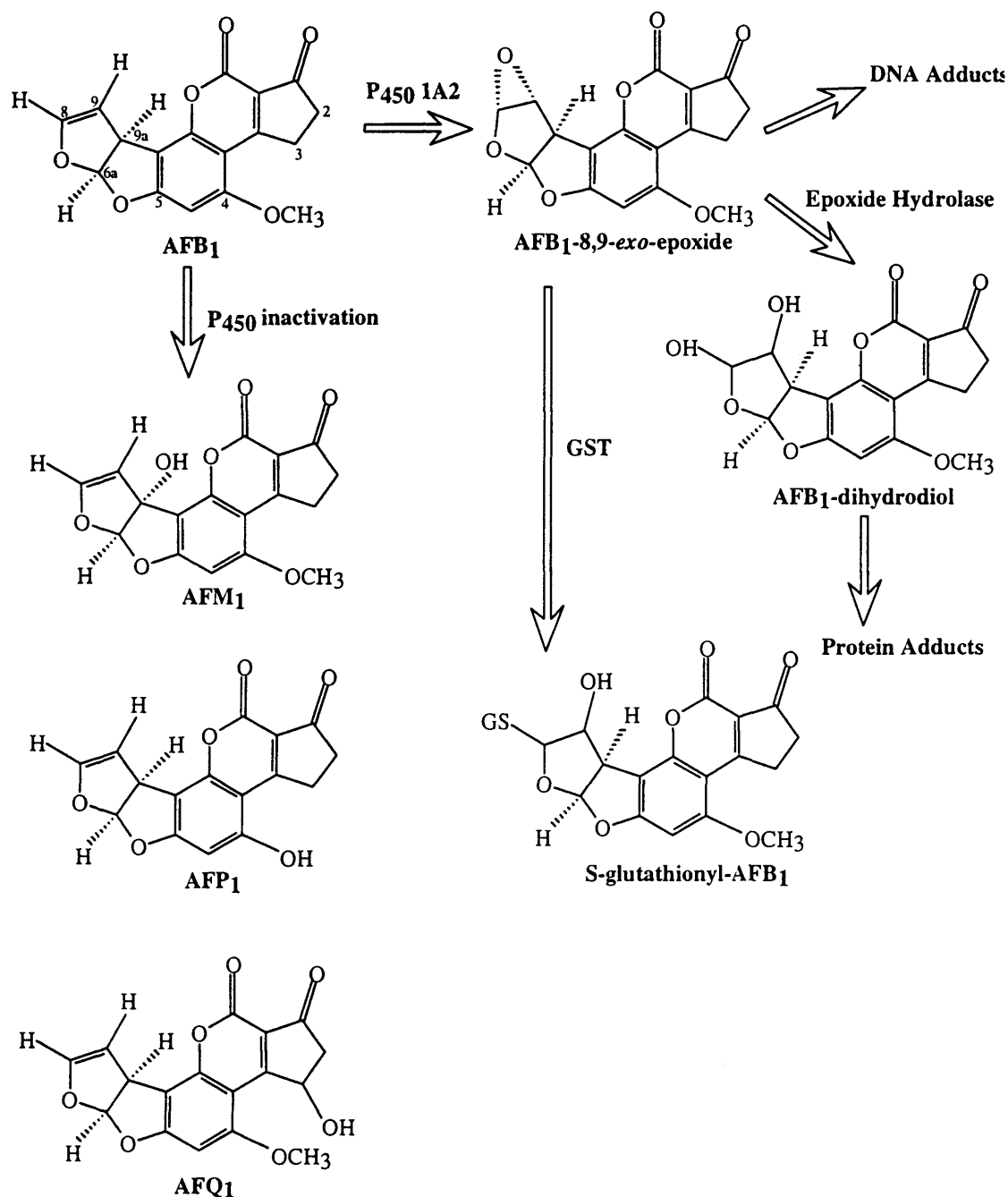


Fig 1.7 The principal metabolic pathways of aflatoxin B₁. The intermediates AFM₁, AFP₁, and AFQ₁ are less toxic than AFB₁. Cytosolic levels of GSTs with aflatoxin 8,9-epoxide conjugating activity are important in determining the level of DNA-AFB₁ adducts formed.

The spontaneous hydrolysis produces AFB₁-8,9-dihydrodiol which may exist in a phenolate resonance form which is capable of forming Schiff bases with primary amine groups in amino acids (Sabbioni *et al*, 1987) and proteins.

The activation of aflatoxin B₁ to aflatoxin 8,9-epoxide by the action of microsomes leads to the formation of two stereoisomers (Raney *et al*, 1992), aflatoxin-*exo*-8,9-epoxide and aflatoxin-*endo*-8,9-epoxide (fig 1.8). There are species differences in the ratio of the epoxides formed. In humans the *exo*-epoxide predominates, while rat and quail microsomes almost exclusively produce *exo*-epoxide. The identities of the epoxides were elucidated by 'trapping' them using glutathione followed by analysis with HPLC and NMR and comparing the spectra to standards made by preparing synthetic epoxides and separating them by crystallisation (Raney *et al*, 1992).

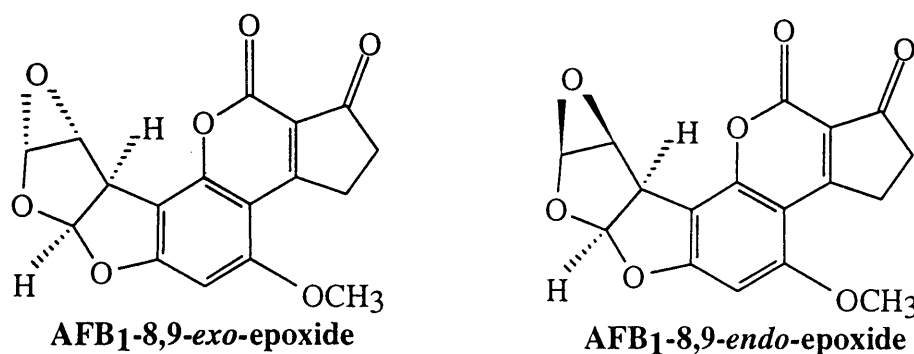


Fig 1.8. Structures of the stereoisomers of aflatoxin 8,9-epoxide showing the relative positions of the oxide group to the plane of the difuranocoumarin rings.

1.8.5 Damage associated with aflatoxin B₁ metabolism

Preliminary data suggests that aflatoxin-*endo*-8,9-epoxide is more resistant to hydrolysis and so could exist in the cell for longer, allowing it to react with cellular targets. These claims were investigated using crystallisation-purified epoxides and the only isomer to bind to DNA was found to be aflatoxin-*exo*-8,9-epoxide (Baertschi *et al*, 1988). Chemical analysis, including NMR, ultra-violet and infrared spectroscopy revealed the nature of the DNA-AFB adduct (Lin *et al*, 1977) to be 8,9-Dihydro-2-(guanylyl-7-yl)-3-hydroxy-aflatoxin B₁. It is now recognised that the binding of aflatoxin-*exo*-8,9-epoxide to the N⁷-guanine group goes through a transition state involving intercalation between the base rings of the DNA helix with a high preference for DNA in a B-conformation (Raney *et al*, 1993).

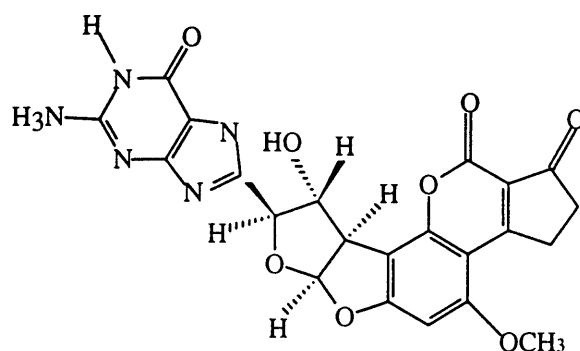
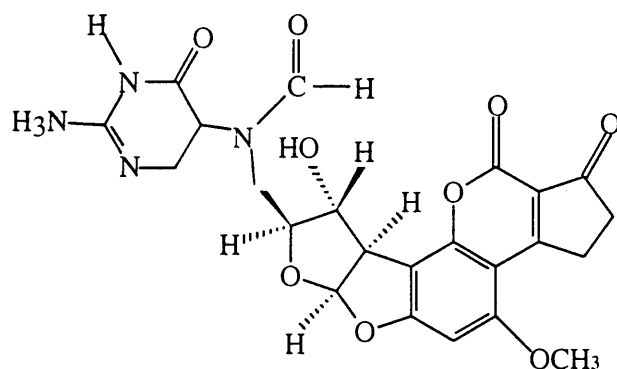
2,3-dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B₁

Fig 1.9. Structure of the main AFB-DNA adduct. The structure was determined by NMR after incubating rat liver DNA with microsomes, NADPH and O₂ to generate the AFB-DNA adducts *in vivo* before releasing the product from polynucleotides by hydrolysis with 0.15N HCl at 100°C (Lin *et al*, 1977)

Although other bases such as adenosine and cytosine, are covalently modified to a limited extent by aflatoxin B₁, the toxicologically important and most abundant adduct is to guanosine, shown in fig 1.9. This may be because it can rapidly undergo rearrangement to a ring-opened form, formamidopyrimidine (FAPY) which is much more stable than N⁷-guanine and can persist in the cell (Lin *et al*, 1977). However, the carcinogenic response appears to correlate with the amount of initial N⁷-guanine adducts in the cell and not to the FAPY adducts. The N⁷-guanine adduct can also spontaneously depurinate to leave an apurinic site in DNA which can become a substrate for error prone DNA repair mechanisms to produce G to T transversions and G to A transitions.

formamidopyrimidine-aflatoxin B₁ (FAPY adduct)

Several genes have been demonstrated to have their function disturbed by AFB-DNA adducts leading to activation of cellular oncogenes. Using appropriate radio-labelled oligonucleotides, it was shown that *ras* oncogenes were activated after

exposure to aflatoxin B₁ (Sinha *et al*, 1988). The nature of the lesion in the genes was a single base pair mutation in the 12th codon of a c-Ki-*ras* oncogene where there was G:C to A:T transition in F344 rats (McMahon *et al*, 1987). N-*ras* alleles have also been found to be activated in liver tumours from rat liver (McMahon *et al*, 1990). However, there is no conclusive evidence for a role for *ras* oncogenes in human aflatoxin-induced hepatocarcinoma (Eaton and Gallagher, 1994).

In addition to the activation of cellular oncogenes, there is evidence to support involvement of the *p53* tumour suppressor gene. Analysis shows a specific G:C to T:A transversion in codon 249 and epidemiological association of the mutation with potential dietary aflatoxin B₁ exposure (Bressac *et al*, 1991, Hsu *et al*, 1991), however the effect of HBV infection is hard to rule out in these studies (Eaton and Gallagher, 1994).

The damage seen to DNA can be repaired by inducible systems. Studies investigating the rate of AFB-DNA adduct clearance shows a clearly biphasic nature. Novel inducible repair systems have been described that appear to be induced when the adduct level rises above 1000 adducts per cell (Kaden *et al*, 1987) which can explain the two different rates of removal of adducts.

1.8.6 Cytochrome P450s and aflatoxin B₁ metabolism

Most of the aflatoxin B₁ consumed in the diet will end up being converted into AFP₁, AFM₁ or AFQ₁ via hydroxylation and *O*-demethylation. None of the species have been shown to bind to DNA at significant levels. Although responsible for the activation to aflatoxin 8,9-epoxide, the CYP450 system must still be considered as an important detoxification process.

In humans, there is evidence for at least five different P450s including 1A2, 2A6, 2B7, 3A3 and 3A4 that are capable of activating aflatoxin B₁ (Forrester *et al*, 1990). As demonstrated by using specific cell lines manipulated by transfection with *vaccinia* virus to express the individual genes (Aoyama *et al*, 1990, Forrester *et al*, 1990). These observations threw into doubt whether CYP 3A4 was the main enzyme involved in bioactivation of aflatoxins (Shimada and Guengerich, 1989). It was later shown that cell lines expressing CYP 1A2 were 3 to 6 fold more effective than those expressing CYP 3A4 (Crespi *et al*, 1991). Further analysis using specific 1A2 and 3A4 inhibitors showed that both enzymes do activate aflatoxin B₁ but that they have different affinities for aflatoxin B₁ (Gallagher *et al*, 1994). CYP 1A2 showed high levels of activation at 16µM aflatoxin B₁ where CYP 3A4 only showed high levels of activation at 128µM suggesting that at concentrations present in the diet, CYP1A2 is the more important enzyme (Eaton *et al*, 1995, Gallagher *et al*, 1994)

1.8.7 Detoxification of aflatoxin-*exo*-8,9-epoxide

As mentioned earlier, the activation of aflatoxin B₁ by microsomes is species dependent and does not necessarily correspond to the systemic effects of the toxin. This is because there are species differences in expression of phase II enzyme that can deal with aflatoxin 8,9-epoxides. The most important of the phase II enzymes involved are alpha-class GSTs and the ability of the expressed GST genes to deal with aflatoxin-*exo*-8,9-epoxide is the major determinant in aflatoxin B₁ toxicity in mammals (Gopalan-Kriczky *et al*, 1994, Hayes *et al*, 1991).

Comparisons of glutathione conjugation to aflatoxin B₁ in mouse (Gopalan-Kriczky *et al*, 1994), rat (Hayes *et al*, 1991) and human cytosols showed that there were quite marked differences between the species tested. The conjugation efficiency of mouse cytosol was 25-fold greater than that of rat liver at 30μM aflatoxin-*exo*-8,9-epoxide. Several human liver cytosols were tested but only very small amounts of AFB-GSH conjugates were detected at the limits of the HPLC method and is at least 30-fold lower than rat liver cytosol (Raney *et al*, 1992). The ability to conjugate aflatoxin-*endo*-8,9-epoxide was also tested. They found that mouse cytosol could only conjugate aflatoxin-*exo*-8,9-epoxide, while rat and human cytosols could conjugate *endo*- and *exo*-epoxides with human liver cytosol conjugating at extremely low rates (Raney *et al*, 1992).

1.8.8 Alpha-class GSTs responsible for AFB-GSH conjugation have been cloned.

In rodents, alpha-class GSTs exist as functional heterodimers which made the attribution of a specific activity to a specific subunit difficult. This problem has been addressed through the molecular cloning of numerous Glutathione S-Transferases from humans, rats and mice. Heterologous expression and purification of homodimeric proteins coupled with extensive characterisation has revealed their individual substrate specificities which can be used to describe the activities of tissue purified heterodimers in a 'dosage dependent' fashion.

This approach allowed the conclusive identification of a GST responsible for the high AFB-GSH conjugation activities (Ramsdell and Eaton, 1990) and cellular resistance to aflatoxin B₁ seen in mice. The gene was cloned from a cDNA library by screening with a mGSTY_a probe and shown to be highly homologous (85%) to a previously cloned and constitutively expressed alpha-class GST in rat called rGSTY_{c1} (Telakowski-Hopkins *et al*, 1985) and thus was called mGST Y_c (Buetler and Eaton, 1992). Northern blotting showed that the gene was constitutively expressed in mouse

liver and heterologous expression in *E. coli* showed that mGST Yc had high specific activity for aflatoxin-*exo*-8,9-epoxide (Buetler and Eaton, 1992). Therefore, resistance to aflatoxin B₁ in mice was ascribed to mGST Yc, but it is interesting that the rat homologue rGST Yc₁ has 1000-fold lower activity towards aflatoxin-*exo*-8,9-epoxide despite the high homology.

1.8.9 Aflatoxin B₁ resistance can be induced in rats.

Feeding Fischer 344 rats with synthetic antioxidants such as ethoxyquin, butylated hydroxyanisole and oltipraz and the barbiturate phenobarbitone has been shown to protect rats against the carcinogenic affects of aflatoxin B₁ (Hayes *et al*, 1991). Treatment with ethoxyquin leads to the induction of heterodimeric forms of GSTs such as GSTY_{a1}Yc₂ and GSTYc₁Yc₂ with the Yc₂ subunit in common. A second, non-GST-dependent detoxification pathway (Judah *et al*, 1993) has been described with an ethoxyquin-inducible aldehyde reductase (AFAR) that converts the protein binding dialdehyde form of AFB-dihydrodiol to the non-binding AFB-dialcohol (Ellis *et al*, 1993 McLellan *et al*, 1994).

Cloning of the cDNA for Yc₂ and subsequent expression in *E. coli* finally showed that this subunit was responsible for ethoxyquin-induced aflatoxin resistance (Hayes *et al*, 1994). The Yc₂ subunit has 150-fold higher activity for aflatoxin 8,9-epoxide than Yc₁ but, in common with mGSTYc, there is very high sequence homology with Yc₁. Protein sequence homology between Yc₁ and Yc₂ is 91% (Hayes *et al*, 1994).

1.8.10 Aflatoxin 8,9-epoxide is a model substrate for H-site interactions

Given the cloning of two subunits, rGST Yc₂ and mGST Yc, that have very high activity for aflatoxin 8,9-epoxide and given that they have very high homology to an almost inactive subunit, Yc₁, there is a natural model system in place for studying substrate specificity. The amino-acids that make up H-site interactions and substrate specificities in alpha-class GSTs, that could not be studied easily by crystallography, can be more easily identified by sequence alignments, molecular modelling and site directed mutagenesis. Aflatoxin B₁ is a good molecule for molecular modelling studies because its conformation is a rigid, planar ring structure and therefore any conformational dynamics of the substrate are likely to be less important than those in the protein.

1.8.11 Important residues for aflatoxin 8,9-epoxide lie between 56-221

In order to identify which residues are important in aflatoxin-*exo*-8,9-epoxide conjugation, chimeric proteins made from mGSTYc and rGST Yc₁ were constructed using convenient restriction sites in the DNA sequences of the two cDNAs (van Ness *et al*, 1994). The results are shown in fig1.10 and show that the residues that confer high aflatoxin-*exo*-8,9-epoxide activity are found between 56 and 221. They are consistent with the region of mGSTYc that would make up the H-site. However, conformation of specific residues is only possible with site directed mutagenesis.

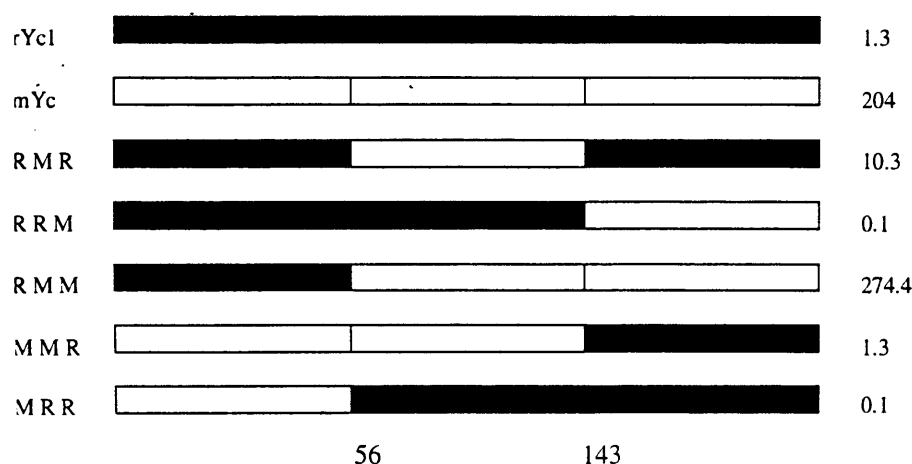


Fig 1.10. Comparison of the catalytic activities of chimeric proteins generated from *StyI* cuts between residues 56-57 and 142-143. The notation denotes which part of the chimeric enzyme came from which species e.g. MRM is mGSTYc 1:56, rGST Yc₁ 57:142 and mGSTYc 143-221

Manipulation of the substrate specificity has been demonstrated using artificial evolution, phage display and panning of random mutated phage (Widersten and Mannervik, 1995). A panel of single point mutations at position 208 has proved to give greatly varied catalytic activities towards a wide range of model substrates (Widersten *et al*, 1994) and shows that the active site is open to manipulation.

In this thesis it is shown that by using molecular graphics and molecular recognition simulations it is possible not only to determine some of the important residues in the binding of the model substrate, aflatoxin 8,9-epoxide, to Glutathione S-Transferases but also to manipulate the enzymatic activity using site-directed mutagenesis. Thus, some of the interactions of substrates with the H-site can be characterised that cannot be seen with X-ray crystallography.

Chapter 2

Engineering Human GSTA1-1 For Aflatoxin B₁ Epoxide Specific Activity

2.0 ENGINEERING HUMAN GSTA1-1 FOR AFLATOXIN B₁ EPOXIDE SPECIFIC ACTIVITY

2.1 Introduction

Identification of important residues in the selection of aflatoxin-*exo*-8,9-epoxide as a substrate in alpha-class GSTs was attempted by engineering activity from mGSTYc into inactive huGSTA1-1. The panel of chimeras of mGSTYc and rGSTYc₁ (van Ness *et al*, 1994) gave a good starting point in the search, as any important residues must lie between 56-221 in mGSTYc. By using site-directed mutagenesis in huGSTA1-1, it was hoped to change the activity of huGSTA1-1 such that it could conjugate aflatoxin-*exo*-8,9-epoxide to glutathione.

Engineering experiments in the H-site of GSTs, huGSTA1-1 in particular, have been successful. Phage display techniques have been used (Widersten and Mannervik, 1995) to select GSTs from random mutagenesis libraries. The random mutagenesis libraries were limited to the residues that line the H-site as defined in the crystal structure (Sinning *et al*, 1993) L107, L108, P110, V111, A216, F220 and F222. The method was able to pull out GSTs with enhanced binding affinities for S-(*p*-carboxyl)-benzylglutathione, however, phage display was unable to select for catalytically competent GSTs and thus would not be applicable for use in aflatoxin B₁ engineering experiments.

Panels of mutants of single residues in the H-site have also been made and characterised against a wide range of potential substrates (Widersten *et al*, 1994). They showed that the character of the residue at position 208 was important in influencing the activity of huGSTA1-1 with aromatic substitution reactions, investigated with 1-chloro-2,4-dinitrobenzene (CDNB). Introduction of side-chains with the ability to donate a proton to a hydrogen bond (histidine, tryptophan and tyrosine) decreased the K_m for the enzyme catalysed reaction with CDNB. However, this did not correlate with an increase in k_{cat} . The results were interpreted as a direct interaction with the negative charge that accumulates on the *para*-nitro group of CDNB in the transition state, particularly in light of results with 4-nitro-benzylchloride showing no great change in K_m . With 4-NBC, the transition state does not interfere with the delocalised electron cloud and so will not affect the charge on the *para*-nitro group.

Thus, it has been demonstrated that activity and binding constants can be manipulated in huGSTA1-1. If important residues can be identified from aflatoxin-

exo-8,9-epoxide active enzymes, there is a good chance that the activity can be engineered into huGSTA1-1.

This chapter describes a strategy to pinpoint important residues rather than using random mutagenesis and screening to create modified enzymes. The strategy involved modelling the reaction product, S-aflatoxynyl-glutathione into the crystal structure (Sinning *et al*, 1993) in place of S-benzyl-glutathione. Identification of substituted residues by sequence alignment and unfavourable interactions between the protein and AFB-GSH allowed candidate residues to be found. These residues were mutated, expressed and purified before being characterised for aflatoxin B₁ metabolism.

2.2 Identification of residues potentially important in aflatoxin-*exo*-8,9-epoxide activity

2.2.1 Sequence alignments of huGSTA1-1 and mGSTYc

The first step in the identification of residues was to perform a peptide sequence alignment between mGSTYc and huGSTA1-1. Completing this step narrowed down the number of candidates to be considered. The sequence alignment is shown in fig 2.1 below

		50
huGSTA1-1	MAEKPKLHYFNARGRMESTRWLLAAAGVEFEKFIKSAEDLDKLRNDGYL	
mGSTYc	--G--V---DG-----PI-----L-TRD--AR--S--S-->	
		100
huGSTA1-1	MFQQVPMVEIDGMKLVQTRAILNYIASKNLYGKDIKERALIDMYIEGIA	
mGSTYc	-----K-----M---I---T--V-->	
		150
huGSTA1-1	DLGEMILLLPVCPPEEKDAKLALIKEKIKNRYFPAPFEKVLKSHGQDYLVG	
mGSTYc	--EI--YY-HM-----E-S--K---QTR----->	
		200
huGSTA1-1	NKLSRADIHVELLYYVEELDSSLISFPLLKALKTRISNLPTVKKFLQP	
mGSTYc	-R-----A-----H-----PGVVDN-----RS-V----->	
		210 220
huGSTA1-1	GSPRKPPMDEKSLEEARKIFRF	
mGSTYc	--Q---FD-A-CV-S-K--->	

Fig 2.1 Sequence alignment of huGSTA1-1 and mGSTYc generated by MacVector. Aligned residues are shown as - and mismatched residues in upper case.

Ten unconserved residues were found to be present around the active site. Most of the other changes were either confined to surface helices or to surface faces of helices which probably arise due to species differences. This analysis was not instructive on its own as any of the unconserved residues around the active site may have been important. The alignment was used to highlight substituted residues in huGSTA1-1 when inspecting the huGSTA1-1 crystal structure (Sinning *et al*, 1993).

2.2.2 Construction of S-aflatoxynyl-glutathione adduct.

To make the reaction product, experimentally determined co-ordinates for bound glutathione were bound to co-ordinates from the crystal structure of aflatoxin B₁. In this way, a very good approximation of the reaction product was produced.

Co-ordinates for the crystal structure of aflatoxin B₁ were down-loaded from the small molecule database at Daresbury, Cheshire, UK and manipulated in InsightII using the BUILDER module (Biosym Technologies). The glutathione moiety was simulated by removing the S-Benzyl-glutathione inhibitor co-ordinates from the crystal structure of huGSTA1-1 (Sinning *et al*, 1993), preserving the correct binding conformation of the γ -glutamyl-cysteinyl-glycine backbone. The benzyl group was deleted to leaving the cysteinyl sulphur atom exposed which was subsequently bound to C₈ of aflatoxin B₁. The aflatoxin B₁ molecule was also hydroxylated at position C₉ to complete the formation of the reaction product. The valencies and potentials were then corrected by hand.

Once the product of the reaction had been built within the computer using BUILDER, the geometry of the molecule was fixed by manual manipulation so that the bond angles around the thiol group were within 0.5° of the geometry of the thiol in the S-Benzylglutathione found crystallised in the protein. The plane of the difuranocoumarin rings of aflatoxin B₁ was also manipulated so that it was in approximately the same orientation as that of the benzyl ring of S-benzyl-glutathione. The stages of construction of the reaction product are shown in fig 2.2. The correct glutathione-aflatoxin conjugate was then ready to be modelled into the active site of GST A1-1.

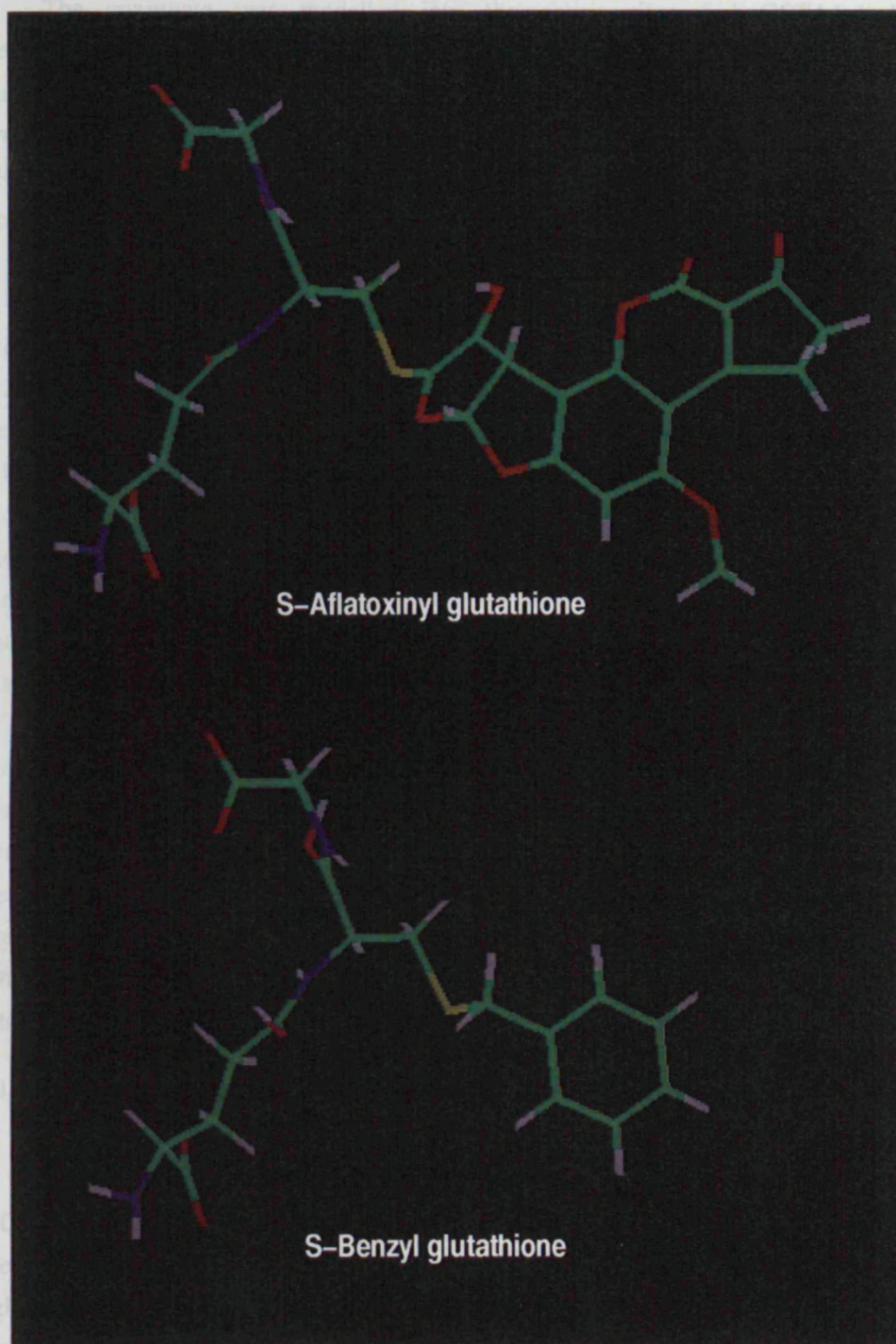


Fig 2.2 Insight illustrations of the stages of construction of S-aflatoxinyll glutathione. The precursor molecules are shown and then the final model ready for modelling.

2.3.3 Docking AFB-GSH co-ordinates into huGSTA1-1 crystal structure

The conjugate was modelled into the active site of huGSTA1-1 by superimposing the glutathione of the conjugate over the glutathione of the S-Benzyl-glutathione originally present in the crystal structure. Once the conjugate was in the correct position, S-Benzyl-glutathione was deleted from the simulation to leave AFB-GSH fairly accurately located in the active site. The BUMP algorithm was then executed to visualise any steric clashes that might have arisen. BUMP highlights atoms that violate van der Waal's radius constraints.

Initially, several residues were identified that clashed sterically with the aflatoxynyl moiety of the simulated reaction product. The most striking clashes were to Met208, in particular, and to Leu213 Ala216 and Ser212 on the C-terminal helix. Several other clashes were seen to conserved residues around the active site including Leu108. The clashes to conserved residues may have arisen for two reasons. They could be a function of the induced fit nature of substrate binding to the enzyme, clearly the active site is going to have a different induced fit conformation when bound to S-Benzylglutathione than it is with the larger, fused ring system of an AFB-GSH conjugate or they arose due to the incorrect position of the conjugate in the active site during modelling which seemed more likely.

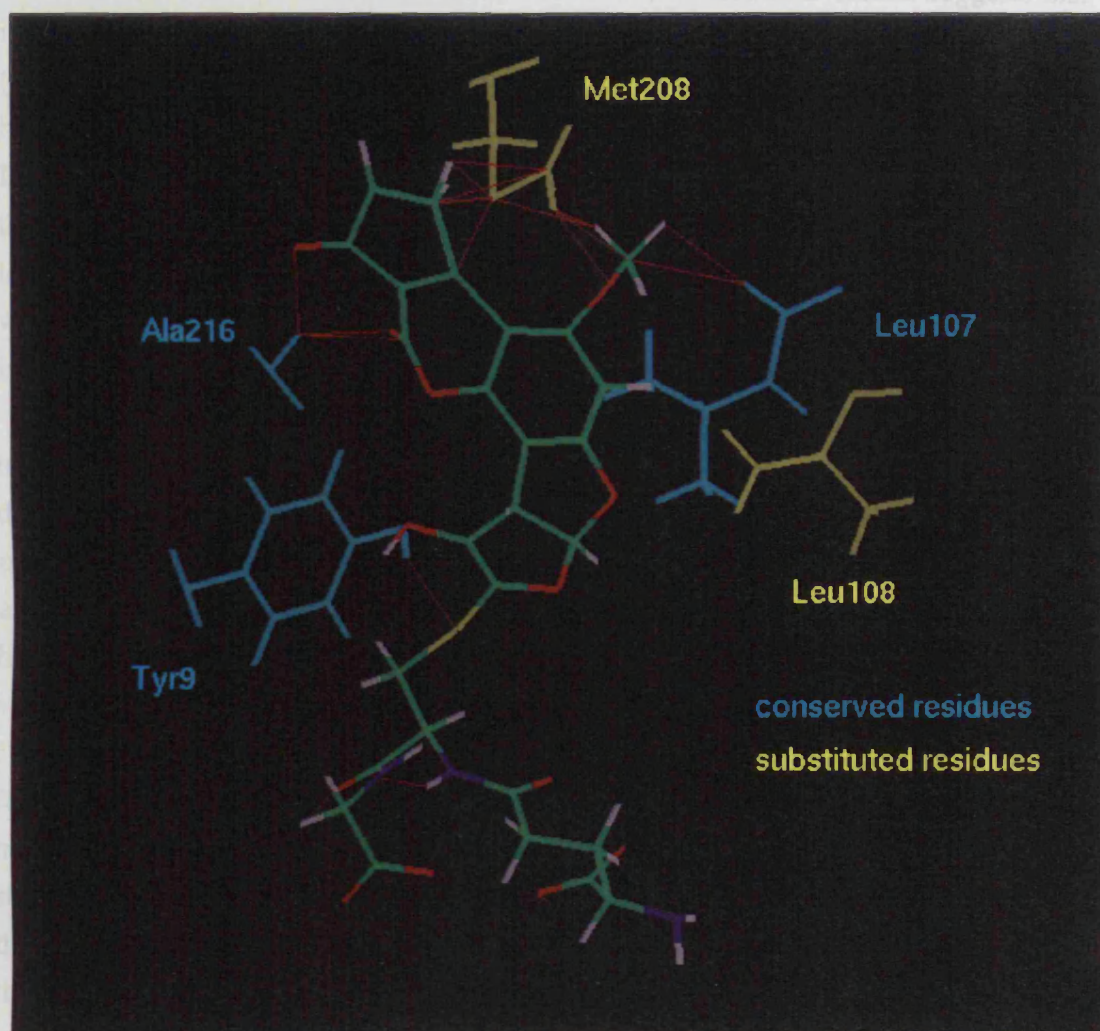
Interestingly, manipulation of the thiol group bond angles of the conjugate while it was modelled within the active site showed that any real deviation of the angles away from those found when crystallised gave massive steric clashes to Phe10. When the angles were returned to normal, these clashes disappeared completely, suggesting that Phe10 may be involved in setting the correct orientation of the sulphur atom of glutathione during catalysis. Indeed with the publication of the apo-protein crystal structure (Cameron *et al*, 1995) showing a different conformation of Phe10 when unliganded then some sort of role must now be considered for this residue.

2.2.4 Analysis of AFB₁-GSH docked model

To further refine the model, the GST polypeptide chain was capped with NH₂ and COOH termini and the forcefield potentials set to pH 6.5. The total model was subsequently subjected to energy minimisation using the DISCOVER module within Insight. DISCOVER manipulated the atoms so that the total energy of the system was reduced, hence removing any energetically unfavourable interactions. Steepest descent conjugate gradients were used with the default AMBER forcefield. Energy minimisation left the model with three major H-site steric clashes after analysis

with GUMP, as shown in fig 2.3. The major clash was still to Met208 and the others were to Leu107 and Ala216. The other clashes were from the glutathione backbone to residues defined as part of the G-site and also from the sulphur atom of the conjugate to Tyr9, showing that the reaction product was likely to be modified into the cox for phase.

Ala216 is a conserved residue for the C-terminal helix. Evidence suggests that



metabolism. From this evidence, it would appear that position 208 is important in aflatoxin B₁ epoxide metabolism.

The modelling experiments were carried out before the publication of the paper (Hayes et al., 1994) reporting the cloning of another GST from rat liver capable of metabolising AFB₁ to a diol (rGST Yc3). Sequence alignments between mGSTYc, rGST Yc2 and huGSTA1-1, shown in fig 2.4, reveal two important residues in the H-site, Asn208 and Tyr107. These positions are substituted in rGST Yc1 and huGSTA1-1 but are conserved in mGSTYc and rGST Yc2. These positions agree with work

Fig 2.3 Illustration of AFB-GSH docked into the crystal structure of huGSTA1-1 active site. Steric clashes are shown in red.

with BUMP, as shown in fig 2.3. The major clash was still to Met208 and the others were to Leu 107 and Ala216. The other clashes were from the glutathione backbone to residues defined as part of the G-site and also from the sulphur atom of the conjugate to Tyr9, showing that the reaction product was likely to be modelled into the correct place.

Ala216 is a conserved residue on the C-terminal helix. Evidence suggests that it would be reasonable to assume that the helix is an interactive part of the active site (Cameron *et al*, 1995, Board *et al*, 1991)(Chapter 5). The evidence suggests that the helix is a dynamic and is disordered in the apo-protein. If the helix is examined using Insight II, it can be clearly seen to be amphipathic. The clash with Ala216 could therefore be due to an induced-fit conformation change; GST is going to close more tightly over a single benzyl-ring substrate than over aflatoxin 8,9-epoxide. Therefore, when a larger product is modelled into the S-benzyl glutathione active site there simply is not sufficient volume available and clashes arise to conserved residues.

Interestingly, there are amino-acid substitutions present in the lid when huGSTA1-1 is compared to mGSTYc. When they are viewed in the 3D structure they do not occur on the substrate binding face of the helix, they seem rather to be 'twisted' out of contact. This may well give a clue as to how the helix acts. Instead of opening and closing over the active site, there may be a more subtle mechanism in which the helix changes its degree of helicity in response to substrates binding at the H-site; this view is supported by the apo-protein structure (Cameron *et al*, 1995).

Met208, however, has much more significance than Ala216. The residue is found in a much more rigid part of the protein structure and the steric clash is much harder to explain in terms of a conformational change. The side chain of the residue sticks through the Aflatoxin ring structure, even after energy minimisation. In GST Yc the residue at position 208 is aspartate and when the mutation M208D is modelled into the GSTA1-1 protein structure, the side chain flips its orientation in the active site and no longer points inwards. This gives much more space in the H-site and nearly all the steric clashes disappear. From this evidence, it would appear that position 208 is important to aflatoxin 8,9-epoxide metabolism.

The modelling experiments were carried out before the publication of the paper (Hayes *et al*, 1994) reporting the cloning of another GST from rat liver capable of metabolising AFB1 8,9 epoxide (rGST Yc2). Sequence alignments between mGSTYc, rGST Yc2 and huGSTA1-1, shown in fig 2.4, reveal two important residues in the H-site; Asp208 and Tyr108. These positions are substituted in rGST Yc1 and huGSTA1-1 but are conserved in mGSTYc and rGST Yc2. These positions agree with work carried out to make chimeric proteins between mGSTYc and rGST Yc1 that showed that residues 53-221 were necessary for AFBO metabolism (van Ness *et al*, 1994).

50

huGSTA1-1 MAEKPKLHYFNARGRMESTRWLLAAAGVEFEEKFIKSAEDLDKLRNDGYL

-PG--V---DG-----PI-----Q-L-TRD--AR-----S.>

mGSTYc --G--V---DG-----PI-----L-TRD--AR--S--S->

RatYc₂ -PG--V---DG-----PI-----N-L-TRD--AR--S--S->

100

huGSTA1-1 MFQQVPMVEIDGMKLVQTRAILNYIASKYNLYGKDIKERALIDMYIEGIA

RatYc₁ -----T-----M-----A--V->

mGSTYc -----K-----M---I---T--V->

RatYc₂ --E-----K-----T-----M-----A--V->

150

huGSTA1-1 DLGEMIL**LL**PVCPPEEKDAKLALIKEKIKNRYFPAFEKVLKSHGQDYLVG

RatYc₁ --D-IV-**HY**-YI--G--E-S--K--D-AR----->

mGSTYc --EI--**YY**-HM----E-S--K--QTR----->

RatYc₂ --EL-V-**YY**-YM--G--E-S--K--D-AR-----Y----->

200

huGSTA1-1 NKLSRADIHLVELLYYVEELDSSLISSFPLLKALKTRISNLPVKKFLQP

RatYc₁ -R-----VY--QV--H-----P-ALAN-----R--V----->

mGSTYc -R-----A-----H-----PGVVDN-----RS-V----->

RatYc₂ -----VS-----H--M-PGIVDN-----R--V----->

220

huGSTA1-1 GSPRKPP**M**DEKSLEEARKIFRF

RatYc₁ --Q---L**E**---CV-S-V--->

mGSTYc --Q---F**D**-A-CV-S-K--->

RatYc₂ --Q---F**D**---CV-S-K--->

Fig 2.4 Sequence alignments of alpha class GSTs produced by MacVector. Residues thought to be important in aflatoxin 8,9-epoxide metabolism are highlighted in bold italics

While the importance of Met208 has been indicated from the molecular modelling studies, Leu108 was identified as a minor interaction before the molecular model was energy minimised. The clash was resolved after minimisation but its potential is indicated in the sequence alignments. It may be that this residue is needed for a more subtle interaction between the protein and the substrate. The model also shows a steric clash to Leu107: slight inaccuracies in the modelling may have led to the identification of this conserved residue, instead of its non-conserved neighbour, Leu108.

Subsequent to the modelling experiments, position 208 was shown to be influential in determining substrate specificity and binding in huGSTA1-1 (Widersten *et al*, 1994, Widersten and Mannervik, 1995) and confirmed that the positions determined in this study by molecular modelling and sequence alignments were likely to be important. Curiously, the mutation M208D was not included in the panel of mutations made by Widersten and co-workers. On the basis of our evidence, it was decided to make huGSTA1-1 more like mGSTYc and rGST Yc₂ by making the mutations M208D, L108Y and the double mutant L108YM208D to see how they may affect activity for aflatoxin-*exo*-8,9-epoxide.

2.3 Construction of dual purpose expression/mutagenesis vector

When starting the project, huGST A1-1 was cloned into the expression vector pEGST (also called pGWL11) from Dr T. Simula (Simula *et al*, 1993) which gave very good expression. In order to carry out oligonucleotide-directed mutagenesis, single-stranded DNA was needed. Unfortunately, the vector lacked the viral origin of replication needed for the production of single stranded DNA. In order to make the mutants in the most efficient way and to avoid excessive subcloning between a mutagenesis and expression vector should any other mutations be required, the f1 origin of replication was amplified from pALTER1 (Promega) using PCR primers that contained the DNA recognition sequence for *SphI*, called F1F and F1R defined in *Materials and Methods*.

The origin was amplified and the fragment was cloned into the unique *SphI* site in pEGST to give the phagemid pEMGST (Expression/Mutagenesis vector). The origin was not cloned directionally and its orientation determines which strand of the phagemid is recovered on production of single stranded DNA. Thus, the orientation of insertion had to be determined before the sense of oligonucleotides needed for mutagenesis could be determined.

The orientation of the origin was determined by isolating single-stranded DNA by infecting *E.coli* JM109, transformed with pEMGST, with the helper 'phage VCSM13. Forward and reverse, internal sequencing primers for huGSTA1-1,

GSTSEQ3 and GSTSEQ4 were annealed to the template and extended with DNA Polymerase I Klenow fragment before being run on a 1% agarose gel. Only one of the primers will be complementary to the single-stranded template and anneal to give a substrate for the polymerase. Only the forward sequencing primer was found to give a double-stranded plasmid band, indicating that this primer was complementary to the template thus demonstrating the orientation of insertion of the f1 origin, fig 2.5

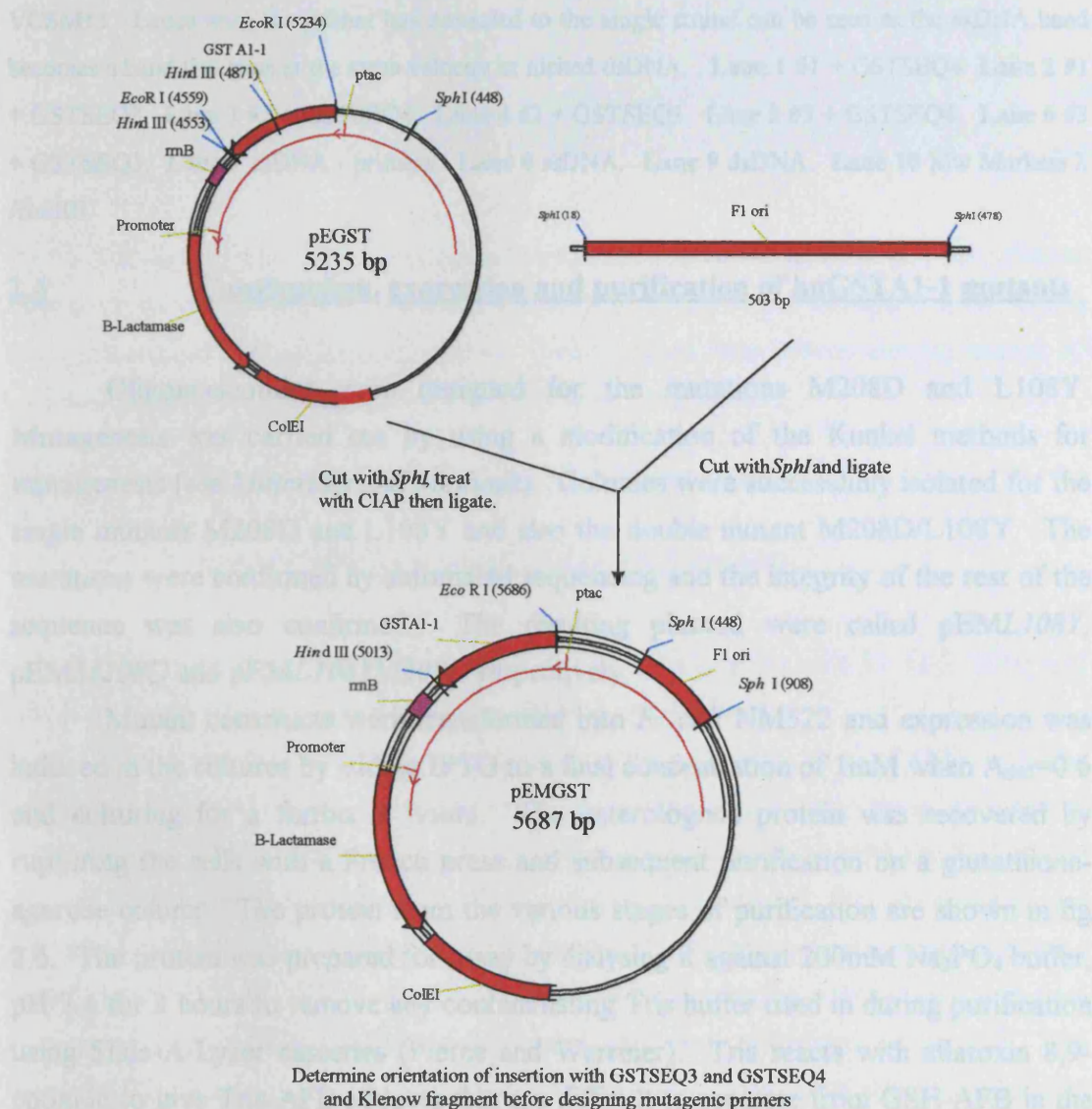


Fig 2.5 Scheme for the construction of pEMGST.

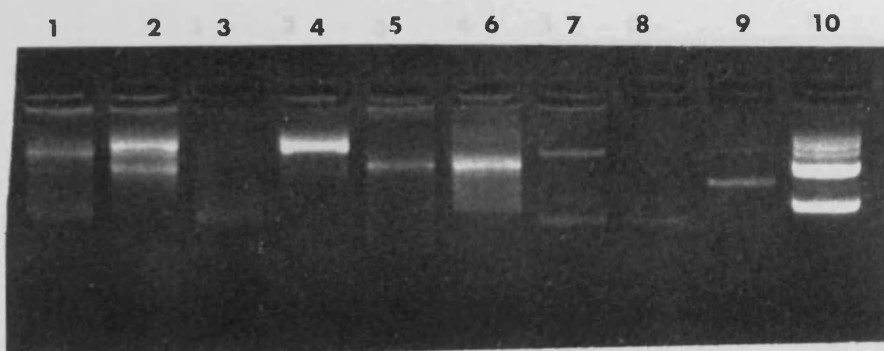


Fig2.5a Gel identifying which phagemid DNA strand is recovered after infection with helper phage VCSM13. Lanes were the primer has annealed to the single strand can be seen as the ssDNA band becomes a band that runs at the same velocity as nicked dsDNA. **Lane 1** #1 + GSTSEQ4 **Lane 2** #1 + GSTSEQ3. **Lane 3** #2 + GSTSEQ4. **Lane 4** #2 + GSTSEQ3. **Lane 5** #3 + GSTSEQ4. **Lane 6** #3 + GSTSEQ3. **Lane 7** ssDNA - primers. **Lane 8** ssDNA. **Lane 9** dsDNA. **Lane 10** Mw Markers λ HindIII

2.4 Construction, expression and purification of huGSTA1-1 mutants

Oligonucleotides were designed for the mutations M208D and L108Y. Mutagenesis was carried out by using a modification of the Kunkel methods for mutagenesis (see *Materials and Methods*). Colonies were successfully isolated for the single mutants M208D and L108Y and also the double mutant M208D/L108Y. The mutations were confirmed by automated sequencing and the integrity of the rest of the sequence was also confirmed. The resulting plasmid were called pEML108Y, pEMM208D and pEML108YM208D respectively.

Mutant constructs were transformed into *E. coli* NM522 and expression was induced in the cultures by adding IPTG to a final concentration of 1mM when $A_{600}=0.6$ and culturing for a further 3 hours. The heterologous protein was recovered by rupturing the cells with a French press and subsequent purification on a glutathione-agarose column. The protein from the various stages of purification are shown in fig 2.6. The protein was prepared for assay by dialysing it against 200mM Na_2PO_4 buffer, pH 7.4 for 3 hours to remove any contaminating Tris buffer used in during purification using Slide-A-Lyzer cassettes (Pierce and Warriner). Tris reacts with aflatoxin 8,9-epoxide to give Tris-AFB adducts that are difficult to separate from GSH-AFB in the HPLC-based assay system, particularly at low levels of product. After dialysis, the proteins were lyophilised in a vacuum drier before resuspending in 200mM Na_2PO_4 buffer, pH 7.4 ready for assay and the concentration of the protein determined by measuring A_{280} .

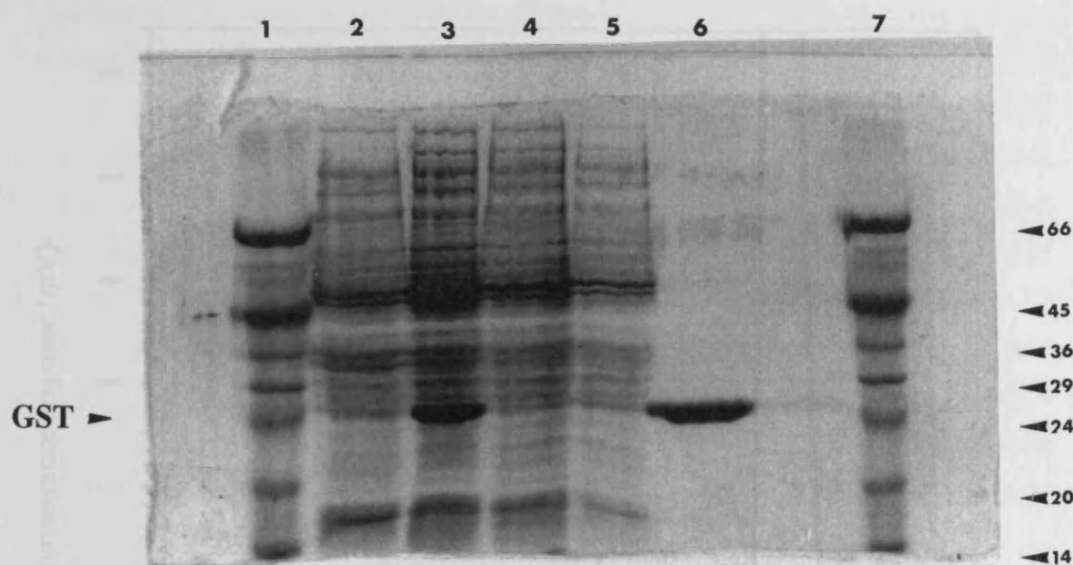


Fig 2.6 Gel showing the stages of purification of huGST from *E. coli* NM522. **Lane 1** Mw Markers. **Lane 2** *E. coli* NM522 - IPTG. **Lane 3** *E. coli* NM522 + IPTG. **Lane 4** Effluent from Column loading showing all GST has bound to column. **Lane 5** Column Wash Effluent showing removal of all non-specifically bound protein. **Lane 6** Elution. **Lane 7** Mw Markers.

2.5 Kinetic analysis of huGSTA1-1 H-site mutants

2.5.1 Specific activity against aflatoxin 8,9-epoxide.

This part of the work was carried out in collaboration with Dr G.E. Neal and Mr. D.J. Judah who carried out the aflatoxin 8,9-epoxide assays.

The assay is a two component assay system (Hayes, et al., 1992) with the aflatoxin 8,9-epoxide being generated *in situ* by microsomes isolated from the livers of Japanese quail. The aflatoxin-*exo*-8,9-epoxide is conjugated by adding the GST to be studied and reduced glutathione. The reaction is incubated for 10 minutes and extracted with acidified methanol. The products of the reaction are separated by HPLC and the GSH-AFB conjugate is identified by measuring column retention time comparing it to an GSH-AFB standard as a control as illustrated in fig 2.7. The amount of conjugated AFBO is determined by the area underneath the corresponding peak calibrated against a column-standard amount of GSH-AFB. With these assays, it is important to keep the protein concentration in the incubations the same because AFBO can react with protein and give lower apparent specific GST activities as a result.

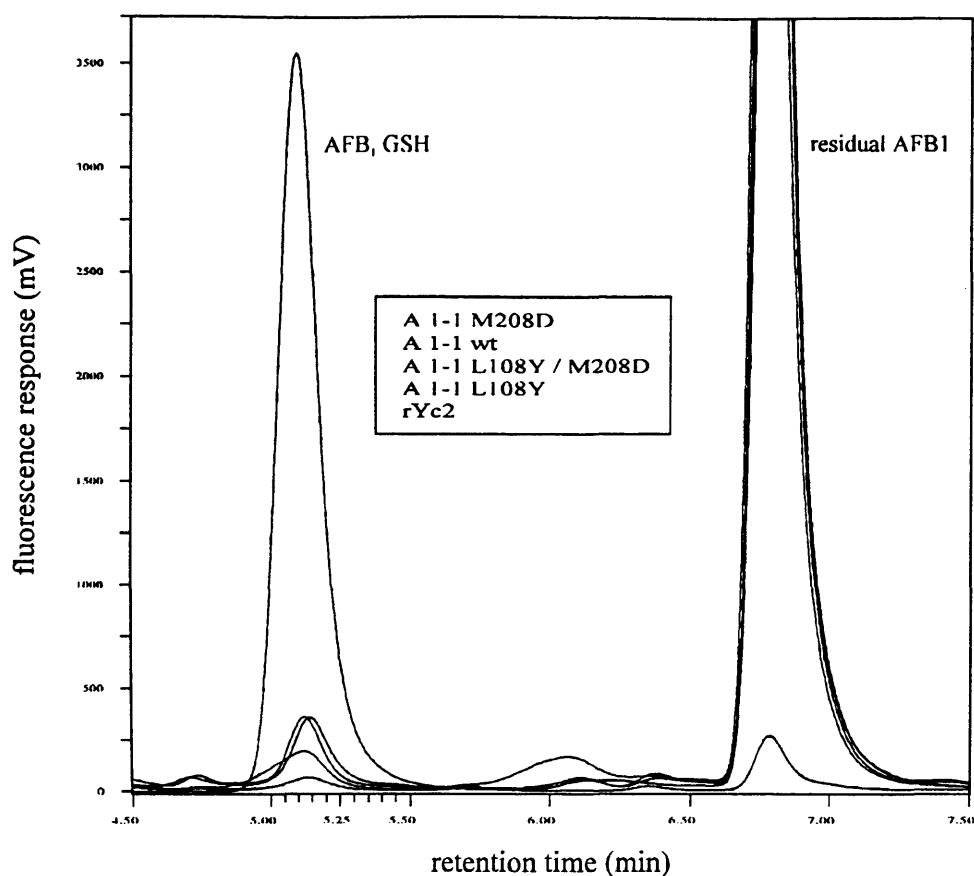


Fig 2.7 Chromatogram generated during kinetic analysis of human GST proteins. The sample for rGST Yc₂ is diluted 5 times so that the peaks in the human samples are not swamped.

The specific activities determined from the chromatographs of the wild-type and mutant proteins are shown in the table 2.1

Protein	Specific Activity. AFB ₁ -GSH nmoles/min/mg
Wild-Type huGSTA1-1	0.0056
L108Y	0.0027
M208D	0.0053
L108YM208D	0.0010
Wild-Type rGST Yc₂	15.220

Table 2.1 Table showing the calculated specific activities for glutathione dependent aflatoxin 8,9-epoxide conjugating activity for human proteins and related mutants. Carried out at 25°C, pH 7.2 and 1mM GSH

The results showed that the wild-type huGSTA1-1 has a very low specific activity for the conjugation of aflatoxin 8,9-epoxide to glutathione of 0.0056 AFB₁-

GSH nmoles/min/mg. This activity is approximately 2700-fold lower than the activity of recombinant rGST Yc₂ which has a specific activity of 15.22 nmoles/min/mg which agrees with the specific activity of 15.03 nmoles/min/mg for aflatoxin 8,9-epoxide previously reported (Hayes *et al*, 1992). Disappointingly, the engineered mutants also have very low activity with aflatoxin 8,9-epoxide, with the double mutant L108YM208D showing the lowest activity. However, this level of activity is likely to be on the limits of detection for the technique which makes accurate comparisons of such low activities difficult. Indeed cytosol from rat liver that has not been treated with ethoxyquin to induce aflatoxin B₁ resistance has an activity of 0.025 nmoles/min/mg which is 4.5- fold higher than wild-type huGSTA1-1. The results however show that there was no significant conjugating activity towards aflatoxin 8,9-epoxide introduced by mutation at positions 108 and 208.

2.5.2 Characterisation of huGSTA1-1 mutants with CDNB.

The proteins were characterised using CDNB as a substrate to confirm that the proteins were folded correctly that despite the lack of activity with aflatoxin 8,9-epoxide, the proteins were still active. The results showed that there was still activity towards CDNB in the preparations (table 2.2).

Protein	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m s ⁻¹ M ⁻¹	Spec. Activity mmoles/min/mg	$\Delta\Delta G$ (kJ/mol)
wt huGSTA1-1	0.59±0.10	32.0±2.9	5.42x10 ⁴	50.66±2.19	0.00
L108Y	1.10±0.14	4.1±0.3	3.73x10 ³	7.84±0.61	+2.23
M208D	0.545±0.14	22.8±7.7	4.18x10 ⁴	4.78±0.50	+0.21
L108YM208D	n.d.	n.d.	-	16.27±0.80	-

Table 2.2 Summary table for the results of the engineering experiments with GSTs. n.d- not determined

The results suggest that the mutations had reduced the levels of activity towards CDNB present in the human proteins. k_{cat} and K_m determinations were possible for all the mutants except L108YM208D. The determinations were carried out in triplicate for L108YM208D but all determinations gave data that could not be fitted to find k_{cat} and K_m . However, the catalytic activity of the double mutant is demonstrated by specific activity determination. Failure to fit the data may reflect the

additive effect of the mutations resulting, a low k_{cat} and high K_m which was incompatible with their determination at the solubility of CDNB at 25°C in aqueous solution.

The levels of activity of the mutants compare quite well with the specific activity of rGST Yc₁ and Yc₂ which are 8.91 ± 0.38 and 4.86 ± 0.21 $\mu\text{moles/min/mg}$ (determined in *Chapter 3*). This suggests that while the mutations had the desired affect of making the human enzyme activity towards CDNB more like the rat enzymes, this was not reflected in activity towards aflatoxin 8,9-epoxide as a substrate. The introduction of negative charge associated with aspartate at position 208 is also consistent with a reduction in the activity with CDNB as predicted by Widersten and co-workers (Widersten *et al*, 1994). The activity in the purified proteins also suggests that the proteins were folded when the aflatoxin 8,9-epoxide and CDNB assays were carried out and the lack of activity in these assays is a result of the mutations not having the intended function.

2.6 Discussion.

Enzymes possibly involved in aflatoxin B₁ resistance in man are poorly characterised. The enzymes responsible remain uncharacterised as experiments with animal models have progressed. While it seems that expression of specific alpha-class GSTs in rodents determines sensitivity, this does not appear to be the case in man. The reason for the disappointing results with the human mutations may be that the human alpha-class enzyme does not represent the correct 'scaffold' around which an aflatoxin 8,9-epoxide metabolising activity can be built.

Evidence exists to suggest that mu-class GSTs are the enzymes that may be important for detoxifying aflatoxin-*exo*-8,9-epoxide in humans (Lui, et al, 1991). Studies of mu-class enzymes from human lymphocyte were carried out using human liver cytosols shown to have significant differences in the amount of mu-class enzymic activity, as measured by the amount of glutathione conjugation to *trans*-stilbene oxide. These cytosols were then used to examine the amount of protection they could give against the formation of AFB-DNA adducts, as measured with [³H]AFB₁. The results showed that the cytosols with high *trans*-stilbene oxide conjugation activity protected the DNA against aflatoxin B₁ adduct to a greater extent than the low activity cytosols.

The genetic locus for mu-class GSTs is polymorphic (Seidegard, et al, 1985). Hepatocellular carcinomas tend to occur in areas where exposure to aflatoxin B₁ is high and also where Hepatitis B infection is endemic, as explained in *Chapter 1*. However, only 20-25% of people infected with Hepatitis B develop hepatocellular carcinoma. Genetic studies of these populations show that there is an increased chance of developing hepatocellular carcinoma if a mutant allele for GSTM1-1 or epoxide

hydrolase is carried (McGlynn, et al, 1995). This further suggests that, for man, alpha-class enzymes do not represent the major detoxification pathway.

Aflatoxin B₁ resistance in human tissue has been demonstrated in normal epithelial cells that have been transfected with P450 1A2 (Mace *et al*, 1994) after treatment with rosemary oil containing carnosol (Andrea Pfeiffer, personal communication) and Western blots for GSTs show that there does not appear to be any significant change in the overall expression levels of GSTs after treatment.

Treatment of rats with ethoxyquin not only induces rGST Yc₂ expression but also an aflatoxin aldehyde reductase (AFAR) (Ellis *et al*, 1993, Judah *et al*, 1993) which appears to be tissue specific in its induction (McLellan *et al*, 1994). The mechanism for the action of AFAR is detailed below (fig 2.8) and shows that the protein affords protection to the cell by detoxifying the dialdehydic phenolate intermediate to form a dialcohol thus interrupting the formation of protein adducts

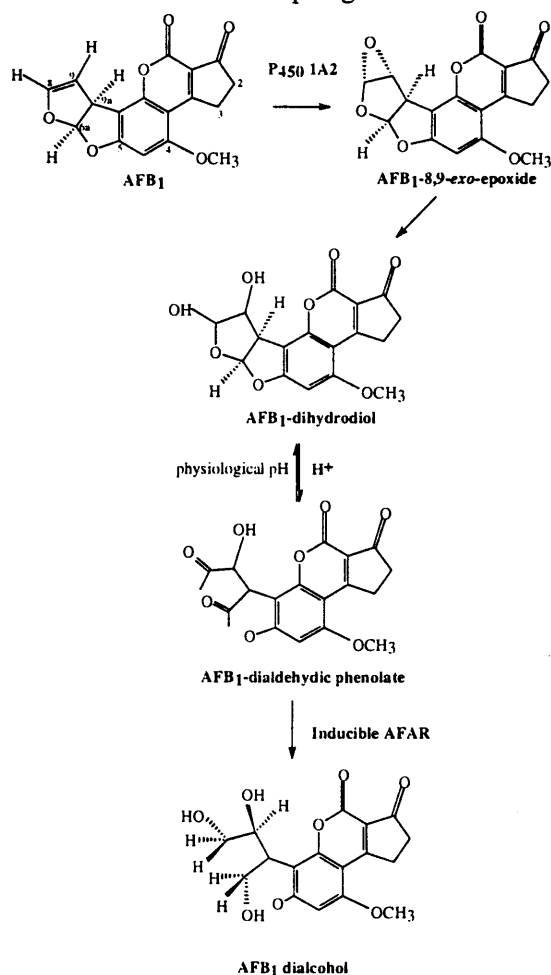


Fig 2.8 Reaction scheme for the action of inducible aflatoxin aldehyde reductase.

While there is no direct evidence for aflatoxin B₁ aldehyde reductase activity in the human bronchial epithelial cells, as epoxide hydrolase as already been suggested to be involved, a human analogue of AFAR should not be ruled out. Experiments would

suggest that this type of activity could be more important in man as the human alpha-class protein seems to be incapable of supporting any great aflatoxin 8,9-epoxide conjugation activity. Of course, from these results alone, it is impossible to determine whether the lack of activity in the mutants was due to the incorrect selection of residues from the modelling experiments. This point is investigated more thoroughly in the next chapter.

Chapter 3

Engineering Rat GSTs for Aflatoxin B₁ Specific Activity

3.0 ENGINEERING RAT GSTs FOR AFLATOXIN B₁ EPOXIDE SPECIFICITY

3.1 Introduction.

Because the results from mutant huGSTA1-1 showed that there was not much activity in the mutants and as such were inconclusive, it was not clear whether docking a glutathionyl-aflatoxin conjugate into the huGSTA1-1 resulted in the correct selection of residues for mutation. It is possible that the numerous other substitutions between inactive huGSTA1-1 and active mGSTYc meant that engineering experiments could only be successful if the entire H-site was transposed (van Ness *et al*, 1994).

The problem could be better addressed by trying to engineer aflatoxin 8,9-epoxide conjugating activity into rGST Yc₁ instead of huGSTA1-1. rGST Yc₁ is more homologous to the highly active mGSTYc, thus presenting a better chance of finding critical residues. Sequence alignments between rGST Yc₁ and mGSTYc showed that candidate residues could readily be identified in rGST Yc₁; the same residues that were picked out as being important from the ligand docking experiments with huGSTA1-1, i.e. positions 108 and 208.

After the completion of the experiments on huGSTA1-1, the cDNA sequence of a new rat GST, rGST Yc₂, was cloned from ethoxyquin treated rat liver, expressed and characterised (Hayes *et al*, 1994). The results showed that the new protein was an alpha-class GST and that it had very high specific activity for aflatoxin 8,9-epoxide. Recombinant, homodimeric rGST Yc₂ was 150-fold more active than rGST Yc₁ towards aflatoxin 8,9-epoxide with specific activities of 30.84 and 0.17 nmol/min/mg respectively (Hayes *et al*, 1994). This was surprising considering that the peptide sequences shared 90% identity (Hayes *et al*, 1994). The cloning of this gene meant that there was an even better model system to study aflatoxin B₁ substrate specificity in GSTs than using mGSTYc and rGST Yc₁. Sequence alignments between rGST Yc₁ and rGST Yc₂ showed, again, that residues at positions 108 and 208 were likely candidates for the dramatic differences in specific activity.

In this chapter I describe work designed to investigate which residues are important in aflatoxin substrate specificity in rat GSTs, using rGST Yc₁ and Yc₂ as model proteins. The three-dimensional structures of both these proteins are calculated by homology modelling and the resulting models used in ligand docking simulations with GSH-AFB conjugates. The quality of the protein-ligand models were tested by computational analysis and also by comparison with experimentally determined intermolecular distances between the protein and the aflatoxin ligand. By careful

docking of a GSH-AFB conjugate into rGST Yc₁ and Yc₂ models, candidate residues were identified and a mutagenesis strategy employed to test whether they were critical in selection of aflatoxin 8,9-epoxide as a substrate. Residues were mutated in rGST Yc₂ to knock out activity and complementary mutations were made in rGST Yc₁ to see whether these residues alone could confer high specific activity towards aflatoxin 8,9-epoxide to an almost inactive protein.

3.2 Homology modelling of rGSTYc₁ and rGSTYc₂

X-ray crystal structures for rGST Yc₁ and are not available, and in order to select residues in a more informed way than by simple inspection of amino-acid sequence alignments alone, suitable models for their 3D structures were needed.

There are many approaches for computational determination of protein structures. These approaches can be divided into broad categories, *ab initio* methods that use thermodynamical properties of atoms and empirical methods (Fasman, 1989), namely homology, or comparative, modelling (Sali and Blundell, 1990, Sali *et al*, 1990). Homology modelling uses experimentally determined structures to predict structures of proteins with similar amino-acid sequences and was chosen in this case because the crystal structure of a closely related GST, huGSTA1-1, had already been solved (Sinning *et al*, 1993).

A homology model has been reported for rGSTM4-4 which was based on the crystal structure of rGSTM3-3 (de Groot *et al*, 1996). The model allowed the identification of structural considerations within the protein that causes differences in the selection of α,β unsaturated ketones as substrates between rGSTM4-4 and rGSTM3-3. The paper demonstrates the general utility for modelling to give an insight into substrate specificity. The model was hand-built by step-wise use of sequence alignment and energy minimisation programs in Quanta version 4.0. For the construction of rGST Yc₁ and Yc₂ models, a purpose-built homology modelling algorithm, MODELLER12 was used.

3.2.1 The MODELLER algorithm

The modeller algorithm (Sali and Blundell, 1993) tries to find the most probable structure of an unknown polypeptide sequence by the satisfaction of a set of spatial restraints derived from one or more known structures. It has been used successfully to generate accurate models of trypsin from two other serine proteases (Sali and Blundell, 1993) and to predict epitope regions in mouse mast cell chymases

(Sali *et al*, 1993). It has proven to be reliable and is fully automated in contrast to methods used to generate other GST models (de Groot *et al*, 1996).

For input, the algorithm needs the sequence to be modelled and at least one homologous protein structure. Modelling is then completed in three steps, alignment of the sequence to known, homologous protein structures, production of the spatial restraints and satisfaction of the restraints by the unknown sequence.

As might be expected, the most important part of modelling is sequence alignment. If only one homologous protein structure is known, as was the case with alpha-class GSTs, then the algorithm aligns the sequences and selects areas of structure based on aligned sequence to contribute to the generation of the restraints used in the production of the final model. These areas of structure identified by homologous sequence are often not contiguous and are called segments. However, if more than one homologous protein structure is identified by the user, the alignment algorithm can also align the 3D structures and use the structural alignment along with the sequence alignment to contribute to the generation of the restraints. All the alignment programs use dynamic programming to allow these multiple alignments to be carried out.

The restraints used in MODELLER do not exclusively come from segments derived from sequence and structure alignment. There is a database within the program that contains structures of 80 proteins organised into 17 families of folds. Statistical analysis of the database gives information about what restraints individual amino-acids have in protein structures such as bond-lengths, dihedral angles etc. The restraints identified from the database are used together with the restraints identified from sequence and structural alignments and combined together with the resolution of the homologous structures to form a 'global' set of restraints that will be used in modelling.

A 'global' set of restraints is more accurately called a molecular probability density function (pdf). This relates to the way that MODELLER expresses the restraints. Expression of restraints as a probability gives more information on the possible values allowed than the mean of the observations alone (Sali and Blundell, 1993) and allows MODELLER more room to select the correct model.

After the formation of the pdf, the unknown sequence is given 3D Cartesian co-ordinates based on the pdf. The most restrained areas will be those segments identified in the sequence alignments while the other parts of the model will be less so. The model is then refined by optimising the Cartesian co-ordinates of the unknown sequence until there is a minimum number of violations of the molecular pdf. The optimisation was a mixture of conjugate gradients for speed and simulated annealing for the large radius of convergence (Sali *et al*, 1990). Further energy minimisation can be carried out automatically in MODELLER to give a refined model.

3.2.2 Homology modelling using MODELLER.

The first stage of modelling is the most important step and the algorithm produced the sequence alignments shown in fig 3.1. to form the segments which went on to contribute to the formation of the molecular pdf.

```

                10         20         30         40         50
human A1-1  MAEKPKLHYFNARGRMESTRWLLAAAGVEFEEKFIKSAEDLDKLRNDGYL
1. Rat Yc1  .PG..V....DG.....PI.....Q.L.TRD..AR.....S.>
2. Rat Yc2  .PG..V....DG.....PI.....N.L.TRD..AR..S..S.>

                60         70         80         90        100
human A1-1  MFQQVPMVEIDGMKLVQTRAILNYIASKYNLYGKDIKERALIDMYIEGIA
1. Rat Yc1  .....T.....M.....A..V.>
2. Rat Yc2  ..E.....K.....T.....M.....A..V.>

                110        120        130        140        150
human A1-1  DLGEMILLLPVCPPEEKDAKLALIKEKIKNRYFPAFKVLKSHGQDYLVG
1. Rat Yc1  ..D.IV.HY.YI..G..E.S..K..D.AR.....>
2. Rat Yc2  ..EL.V.YY.YM..G..E.S..K..D.AR.....Y.....>

                160        170        180        190        200
human A1-1  NKLSRADIHLVELLYVEELDSSLISFPLLKALKTRISNLPVKKFLQP
1. Rat Yc1  .R.....VY..QV..H.....P.ALAN.....R..V.....>
2. Rat Yc2  .....VS.....H...M.PGIVDN.....R..V.....>

                210        220
human A1-1  GSPRKPPMDEKSLEEARKIFRF
1. Rat Yc1  ..Q...LE...CV.S.V...>
2. Rat Yc2  ..Q...FD...CV.S.K...>

```

Fig 3.1 Sequence alignments produced by the MODELLER algorithm (Sali and Blundell, 1993). The alignments for both rGST Yc₁ and Yc₂ are shown and were produced separately. They are shown together here for simplicity.

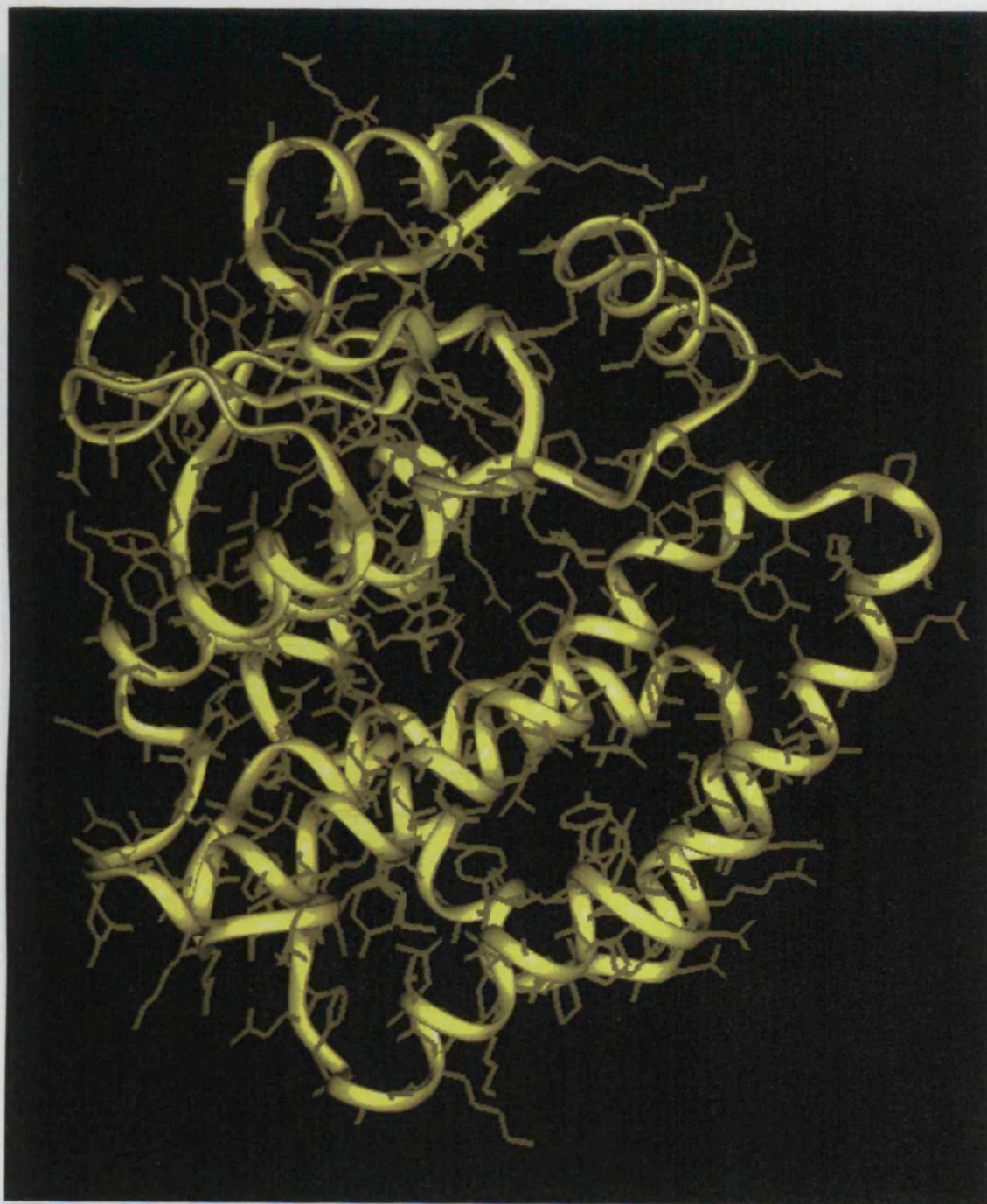
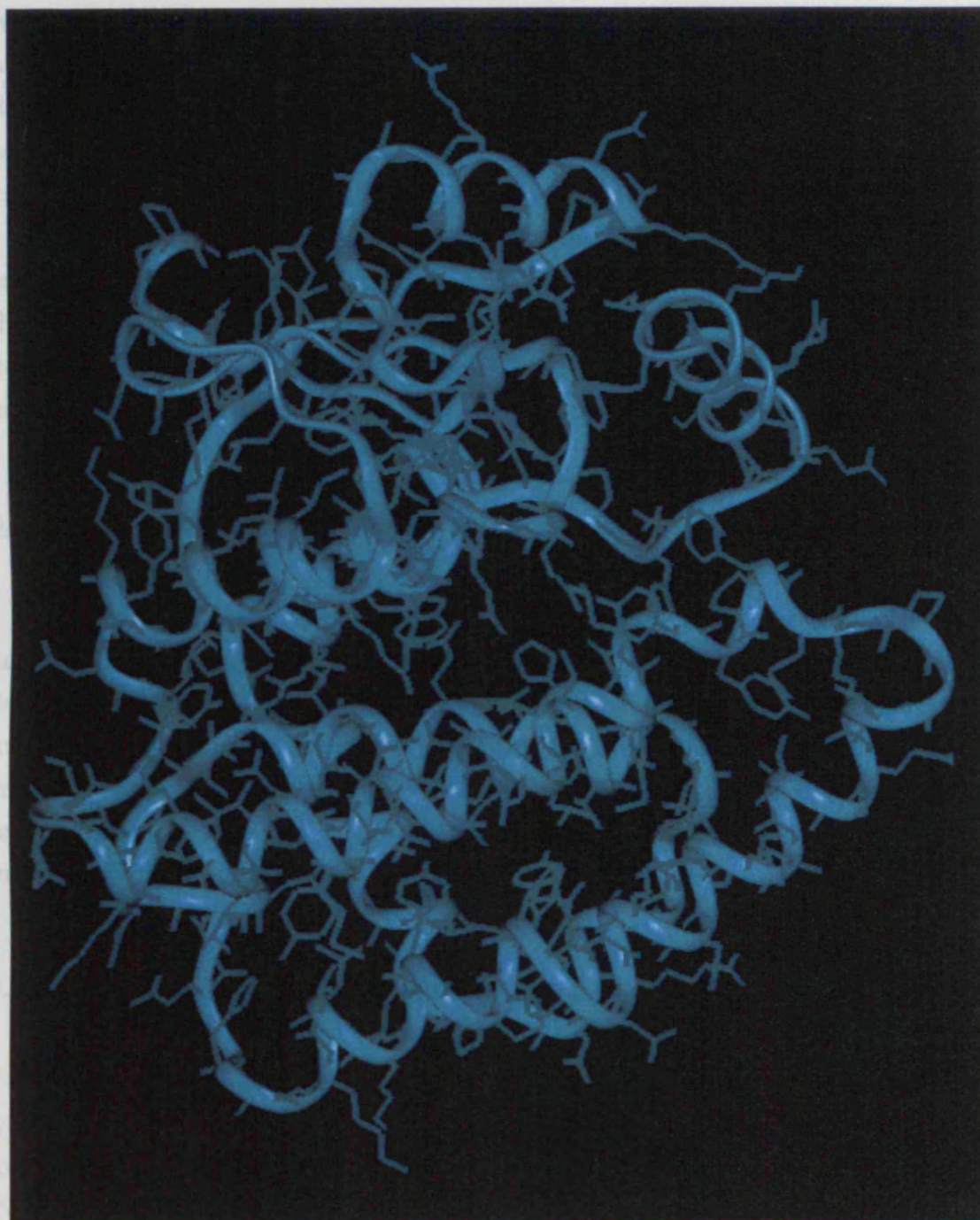


Fig 3.2 Ribbon diagram of modelled rGST Yc₁

As can be seen the previous model was flawed, approximately 30% with no GSTA1-4. The alignment and the conformation showing that there are not likely to be any loops inserted into the structure that MULLER would have to deal with. This demonstrated that homology modelling was likely to give good quality structures



superimposed the structure and ensured that the conjugate was located fairly accurately in the active site of the rat enzyme. In order to resolve any violations in the interactions of the conjugate and the protein, the co-ordinates were run through the energy minimisation algorithm, DISCOVER, to further refine the model.

Steepest descent and conjugate gradients were used in DISCOVER. This type of conjugate gradients are rigorous and effective in finding energy minima but they do not

Fig 3.3 Ribbon diagram of modelled rGST Yc₂

As can be seen the proteins share high homology, approximately 80%, with huGSTA1-1. The alignments are also contiguous showing that there are not likely to be any loops inserted into the structure that MODELLER would have to deal with. This demonstrated that homology modelling was likely to give good quality structures with the high possibility that they would resemble the native protein structure. Note also that MODELLER was only used to predict the monomeric structure. The H-site does not show any dependence on the subunit for its integrity (Danielson and Mannervik, 1985, Sinning *et al*, 1993) and therefore to relieve any complications in the calculation, the structure of the monomeric species was determined.

The co-ordinates predicted for the structures for rGST Yc₁ and Yc₂ are represented as 3D ribbon diagrams produced by InsightII (Biosym) and they are illustrated in fig 3.2 and 3.3. As can be seen, the structures are very similar to that of huGSTA1-1 which was used as a template. However, the results from MODELLER are only the first step in the production of a suitable model.

3.3 Selection of residues for mutagenesis in rGST Yc₁ and Yc₂

This chapter is concerned with identifying residues that are important in accepting aflatoxin 8,9-epoxide as a substrate in rat liver enzymes and this section deals with the second stage of the modelling. It describes how a GSH-AFB conjugate may be accommodated in the active site of the enzyme by molecular modelling and energy minimisation simulations. Analysis and evaluation of models containing the reaction product should give clues as to which residues to mutate and how accurate those predictions are likely to be.

3.3.1 Docking GSH-AFB into homology models of rGST Yc₁ and Yc₂

The computer model of AFB-GSH conjugate that was produced for predicting which mutations to make in huGSTA1-1 in *Chapter 2* was used. The minimised model for huGSTA1-1 containing the conjugate was superimposed on the models for rGST Yc₁ and Yc₂ in turn. The similarity of the peptide backbone structures made the superimposition straightforward and ensured that the conjugate was located fairly accurately in the active site of the rat enzymes. In order to resolve any violations in the interactions of the conjugate and the protein, the co-ordinates were run through the energy minimisation algorithm, DISCOVER, to further refine the model.

Steepest-descent conjugate gradients were used in DISCOVER. This type of conjugate gradients are rigorous and effective in finding energy minima but they do not

have a good radius of convergence. Effective docking of the ligand by superimposition meant that co-ordinates of the ligand were already likely to be inside the effective radius of convergence., making them the method of choice. The AMBER forcefield was used during energy minimisation using the default settings supplied with the DISCOVER package.

Before the algorithm was executed, any invalid atom potentials or valencies identified by DISCOVER, upon initiation of the program, were noted and corrected using the BUILDER module within InsightII. Finally, the docked conjugate was redesignated as part of the protein structure and the whole protein-ligand model was energy minimised.

Upon completion of energy minimisation, the co-ordinates of the conjugate were designated as a discrete molecule and the models produced analysed firstly to check how accurate they were likely to be and secondly to predict what were the major structural determinants likely to affect substrate specificity for aflatoxin-*exo*-8,9-epoxide.

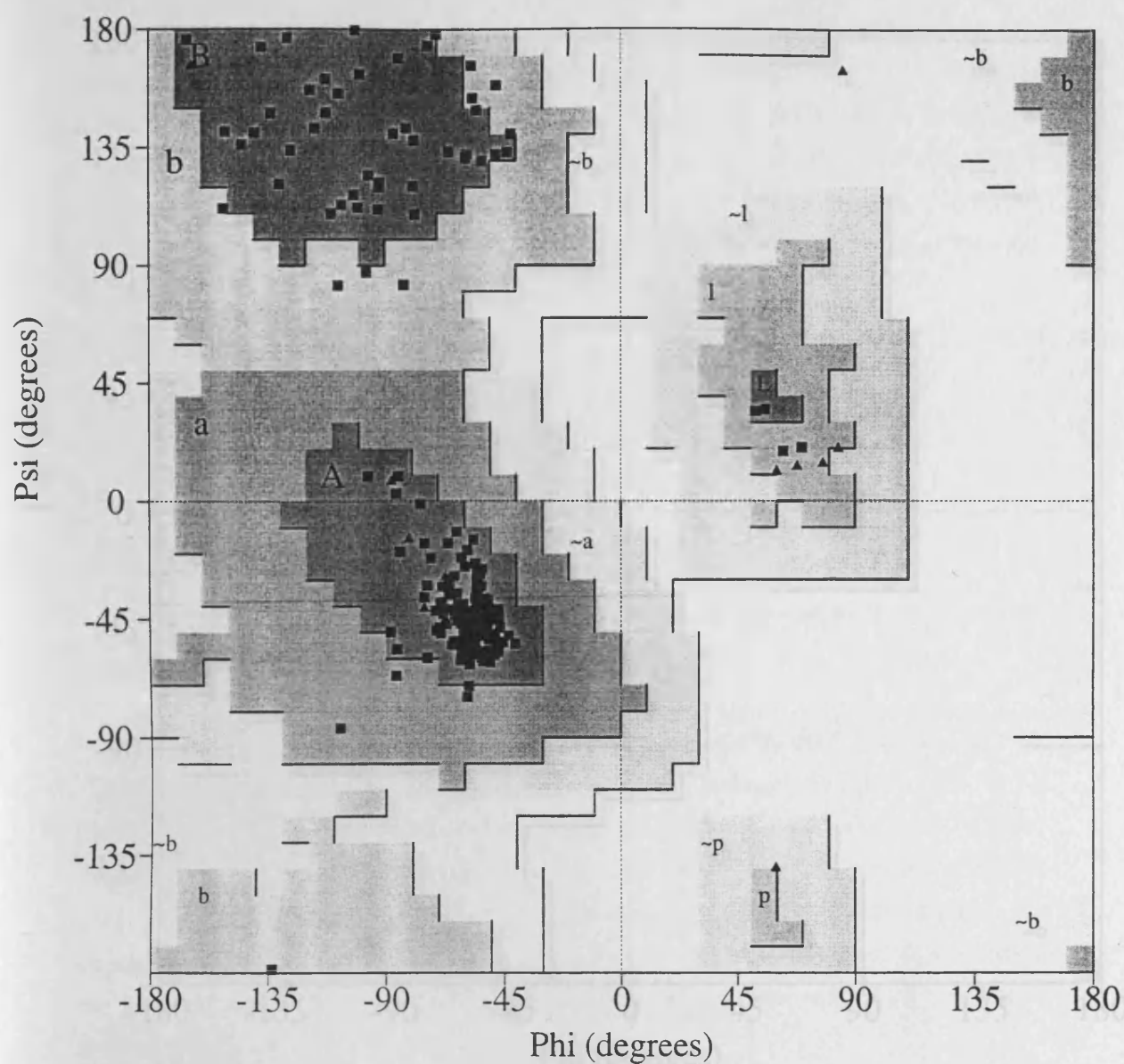
3.3.2 Evaluation of GSH-AFB-rGST molecular models.

There are three main considerations when evaluating molecular models of this type; Is the protein homology model a fair representation of the likely structure in solution ? Is the docked and minimised ligand in the correct place? Given that that the protein model is fine and the ligand is docked in the correct place, what can be said about the structural motifs that govern the acceptance of aflatoxin 8,9-epoxide as a substrate ? With the molecules modelled here, it is necessary to evaluate the accuracy of the predicted structures separately but then compare protein-ligand interactions to identify individual residues as candidates for mutagenesis.

3.3.3 Evaluation of GSH-AFB-rGST Yc₂ molecular model

3.3.3.1 Analysis of Protein Structures

In order to evaluate the protein structure, the co-ordinates of rGST Yc₂ alone were analysed using PROCHECK by Roman Laskowski and Janet Thornton (<http://biotech.embl-ebi.ac.uk:8400>). This program compares the structure against 118 other well defined structures to compare all aspects of the protein's structure such as secondary structure, solvent accessibility, bond-angles and bondlengths. The graphical representations of the analysis of the protein structure are shown in figs 3.4 and 3.5.



Plot statistics

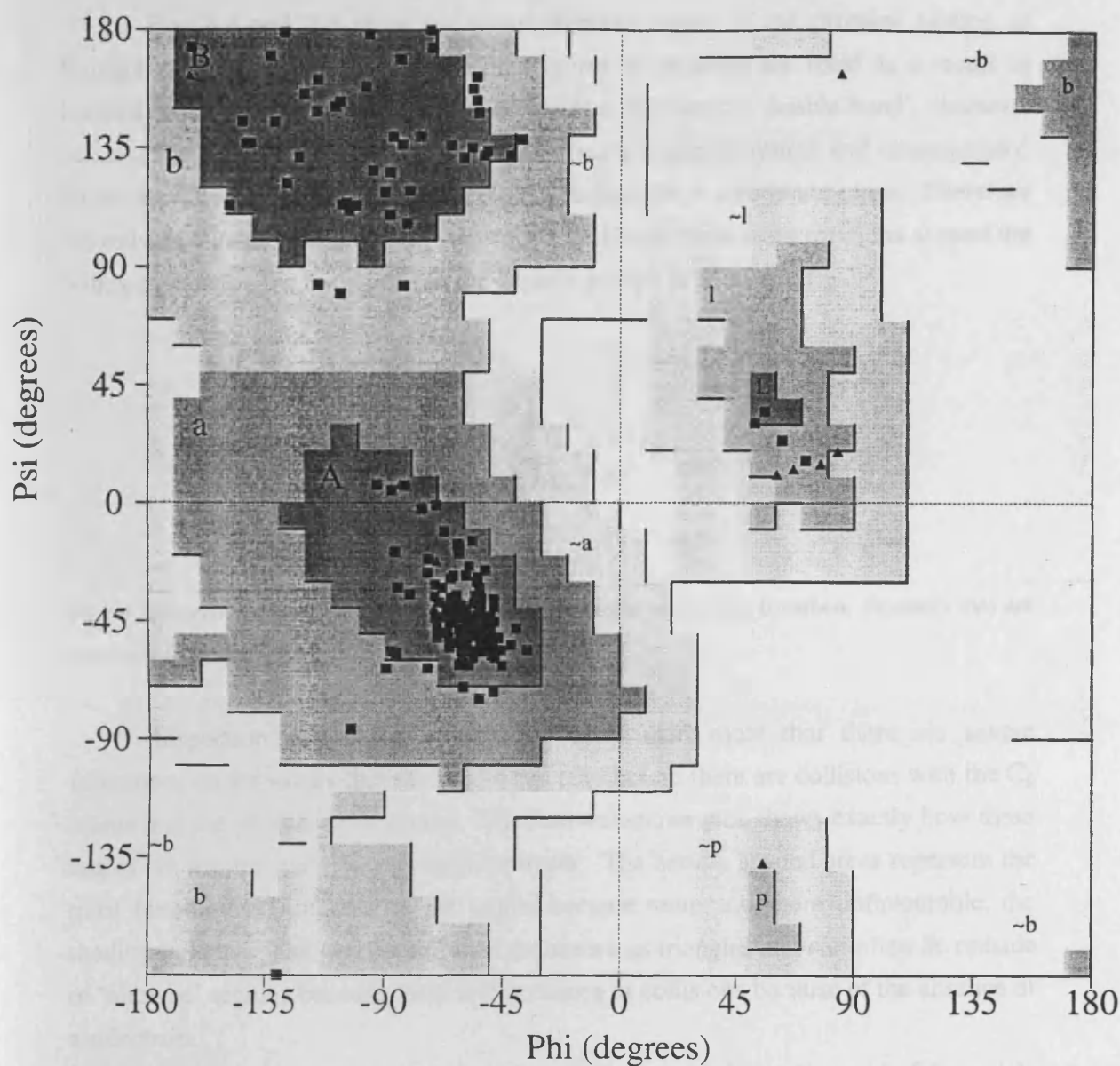
Residues in most favoured regions [A,B,L]	178	92.2%
Residues in additional allowed regions [a,b,l,p]	15	7.8%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%

Number of non-glycine and non-proline residues	193	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	13	
Number of proline residues	13	

Total number of residues	221	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Fig 3.4 Ramachandran plot of rGST Yc₂ structure



Plot statistics

Residues in most favoured regions [A,B,L]	178	91.8%
Residues in additional allowed regions [a,b,l,p]	16	8.2%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%

Number of non-glycine and non-proline residues	194	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	12	
Number of proline residues	13	

Total number of residues	221	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Fig 3.5 Ramachandran plot of rGST Yc₁ structure

Figs 3.4 and 3.5 show backbone dihedral angles of the proteins plotted as Ramachandran Plots. Many geometric features of proteins are fixed as a result of bonded interactions. The peptide linkage has substantial 'double-bond' character because the electrons in the bond delocalise over a π orbital system and consequently, all the atoms together with the connected α -carbons lie in a common plane. Therefore the only adjustable geometries in the peptide backbone come from rotations around the bonds connecting the α -carbons to the peptide groups as shown in fig 3.6

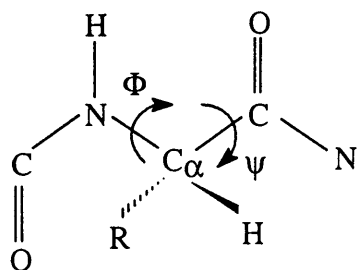


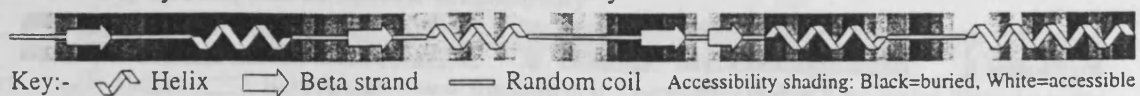
Fig 3.6 Diagram showing the identities of the torsion angles around the α -carbon. Formally they are labelled ϕ (phi) and ψ (psi).

Inspection using solid spheres for atom radii show that there are severe limitations on the values that the angles can take before there are collisions with the C_β atoms and the peptide-bond atoms. The Ramachandran plot shows exactly how these angles are accommodated in protein structure. The heavily shaded areas represent the most favoured regions and as the angles become more and more unfavourable, the shading lightens. The glycine residues are shown as triangles and can often lie outside of 'allowed' regions because there is less chance of collisions because of the absence of a sidechain.

As can be seen from the plot, the distribution of the amino-acids falls mainly within the most favourable regions of the plot. The Ramachandran plot of rGST Yc₂ shows 92.2% lying in the most favoured regions and with rGST Yc₂ and 91.8% for rGST Yc₁. The remaining 8% were found in additionally allowed regions and there were no residues at all in any of the disallowed regions. This shows that as far as the backbones were concerned, there were no unfavourable geometries that would indicate a problem with either of the structures.

Figs 3.7 and 3.8 show a more detailed analysis of the protein residue-by-residue. The upper panels show a schematic picture of the secondary structure as defined using Kabsch and Sander assignments (Kabsch and Sander, 1983). The shading behind the schemes show the predicted solvent accessibility (Nishikawa and Ooi, 1986), the lighter shading, the more accessible the protein is to solvent. The bottom panel is a chequer-board of G-factors

Secondary structure & estimated accessibility



G-factors

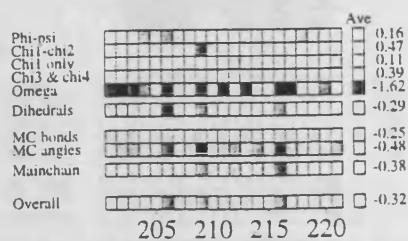
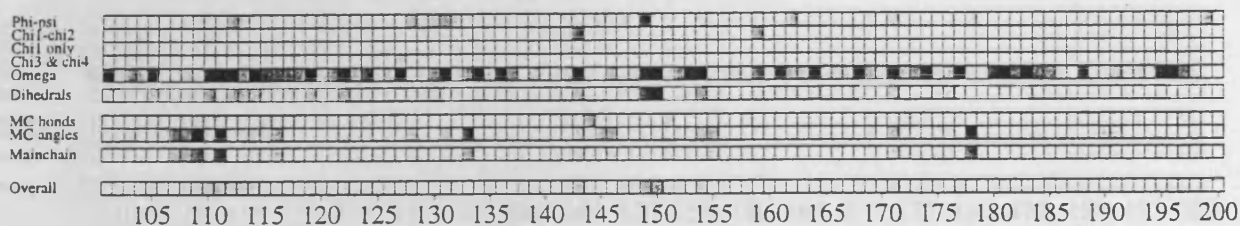
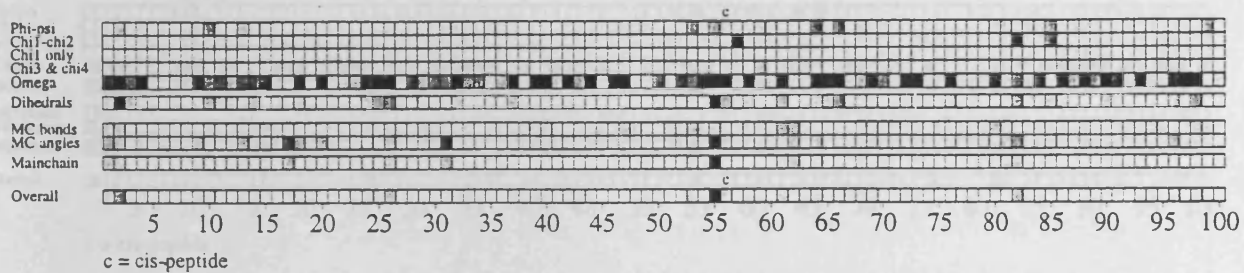
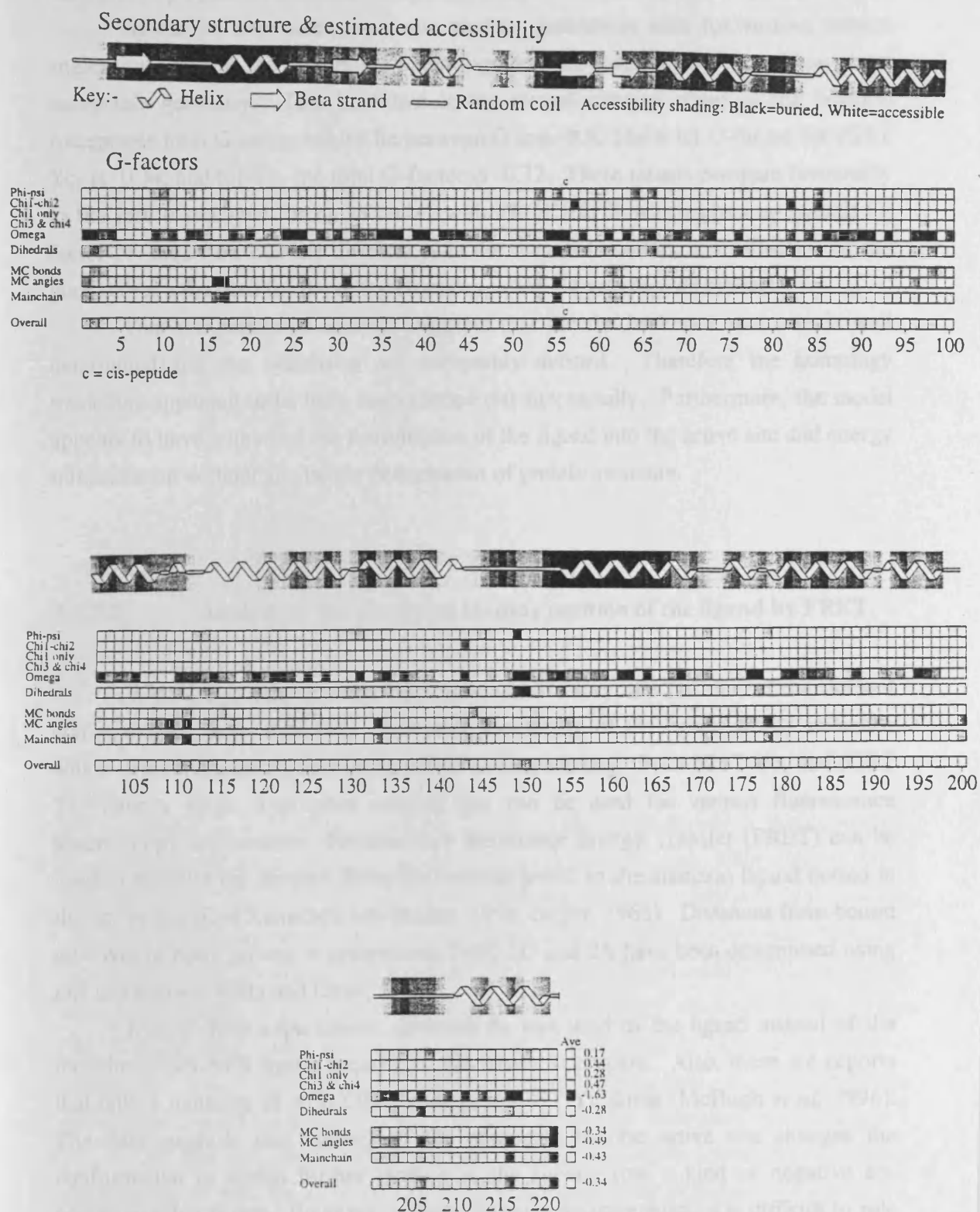


Fig 3.7 Residue by residue analysis of rGST Yc₂

Fig 3.8 Residue by residue analysis of rGST Yc₁

for various protein geometry properties. Each 'G-factor' is a measure of normality for each property based on statistical analysis of the proteins in PROCHECK's database.

As can be seen there are some unusual geometries seen for various torsion angles, but when all the factors are taken together, the vast majority of residues show acceptable geometry. This is shown in the overall chequer-board at the bottom. Acceptable total G-factor values lie between 0 and -0.5. The total G-factor for rGST Yc₂ is -0.34; and for Yc₁ the total G-factor is -0.32. These results compare favourably to the results obtained for huGSTA1-1 with PROCHECK which gives an overall G-factor of -0.03 however the differences in the overall G-factors is a reflection of the poor scores generated for the omega torsions in the Yc polypeptide models.

This computational analysis suggests that the the backbone structure is well determined and the sidechains are acceptably defined. Therefore the homology modelling appeared to be have been carried out successfully. Furthermore, the model appears to have withstood the introduction of the ligand into the active site and energy minimisation without any undue perturbation of protein structure.

3.3.3.2 Analysis of the predicted binding position of the ligand by FRET.

With the protein structure apparently satisfactory, the position of the docked conjugate was now important. Incorrect positioning of the ligand would influence which structural features may be identified from modelling. Both rGST Yc₁ and rGST Yc₂ have a single tryptophan residue that can be used for various fluorescence spectroscopy applications. Fluorescence Resonance Energy Transfer (FRET) can be used to measure the distance from this 'anchor point' to the aflatoxin ligand bound in the active site (Dos Remedios and Moens, 1995, Stryer, 1968). Distances from bound aflatoxin to heme groups in cytochrome P450 1D and 2A have been determined using this technique (Omata and Ueno, 1985).

During these experiments, aflatoxin B₁ was used as the ligand instead of the modelled GSH-AFB ligand because it was easier to prepare. Also, there are reports that only 1 molecule of AFB-GSH binds per mGST Yc dimer (McHugh *et al*, 1996). The data suggests that binding of the conjugate to one active site changes the conformation to inhibit further binding at the second site, a kind of negative co-operativity that is rare. However, without distance measurements, it is difficult to rule out the possibility that the AFB-GSH conjugate is not binding at the subunit interface, in a mode similar to that proposed for the ligandin properties of GSTs. In order to reduce the complexity of the analysis, aflatoxin B₁ alone was used.

In order for fluorescence energy transfer to take place, the absorption spectrum of one of the components must overlap the fluorescence spectrum of the other component. Tryptophan and aflatoxin B₁ are ideally suited in this respect. The graph in fig 3.9 shows portions of experimentally determined the fluorescence emission spectrum of Trp21 and the absorption spectrum of aflatoxin B₁. The overlap in this region means that there is potential for FRET.

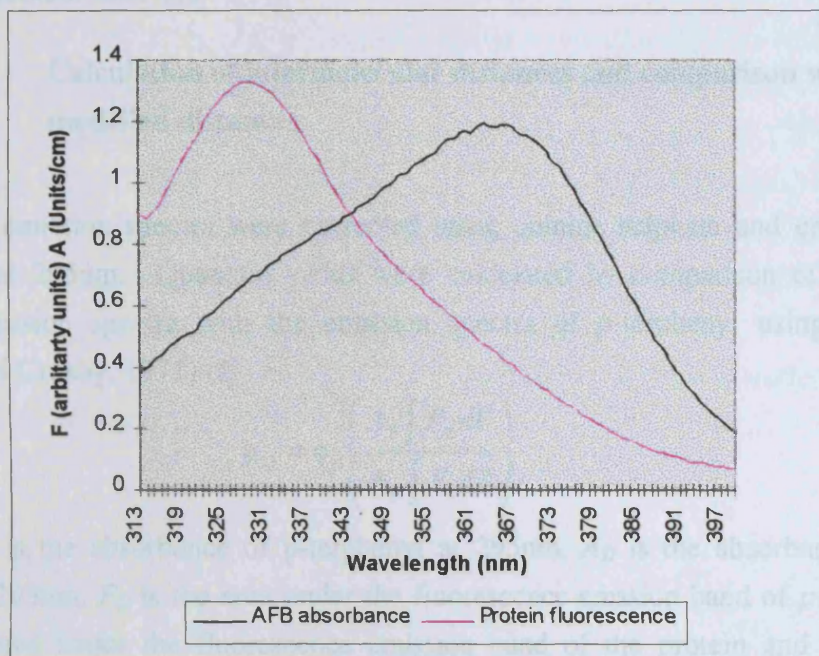


Fig 3.9 Graph showing the spectral shapes of aflatoxin B₁ and rGST Yc₂ and the region of overlap.

FRET analysis does not solely depend on the extent to which absorption and fluorescence emission spectra overlap (J) (Campbell and Dwek, 1984, Selvin, 1995, Stryer, 1968). It also depends on the orientation of the transition dipoles of the acceptor and donor molecules (κ^2) and, critically, the distance between them. (J defined below). The dipole orientation factor can only be accurately calculated from crystal structures, from which distance data can already be extracted, so FRET can only be accurately used to confirm crystal structure distances. However, FRET is most often used when no other source of distance measurement can be used easily. Thus, it is clear that an approximation for κ^2 is needed for distances to be calculated.

K^2 can take on values of 0 to 4. Statistical analysis shows that a random orientation of both the acceptor and donor dipoles gives an orientation factor of $2/3$. This value has been used in previous calculations when measuring distances from aflatoxin B₁ and cytochrome P450s interactions (Omata and Ueno, 1985). Analyses of FRET have also been carried out where spectroscopically determined distances using $\kappa^2=2/3$ were compared with X-ray crystal structures, (Dos Remedios and

1995). Dos Remedios and co-workers show that distances determined using $\kappa^2=2/3$, although not as accurate as crystallographic data, give acceptable estimates of intermolecular distances. Further, they show that differences in distances determined by FRET are very accurate. Therefore, FRET was used to measure the distances of bound aflatoxin B₁ to Trp21 and then compared to the predicted distance in the model to show that the conjugate was likely to be located in the correct place within the homology protein structure.

3.3.3.3 Calculation of intermolecular distances and comparison with modelled distances.

All emission spectra were corrected using quinine sulphate and collected by excitation at 295nm. Quantum yields were calculated by comparison of corrected protein emission spectra with the emission spectra of *p*-terphenyl using equation (Demas and Crosby, 1971) (1)

$$\phi_D = \phi_s \left(\frac{A_s \int F_D dF}{A_D \int F_s dF} \right) \quad (1)$$

Where A_s is the absorbance of *p*-terphenyl at 295nm, A_D is the absorbance of the protein at 295nm, F_D is the area under the fluorescence emission band of *p*-terphenyl, F_s is the area under the fluorescence emission band of the protein and ϕ_D is the quantum yield of *p*-terphenyl, quoted as 0.93. The quantum yield was used to define the Forster distance, R_0 given by the equation (2)

$$R_0 = 979(J\kappa^2\phi_D n^{-4})^{\frac{1}{6}} \quad (2)$$

Where κ^2 is the orientation factor and given the value 2/3, n is the refractive index of water and given as 1.35 and J is the integral of spectral overlap and given by equation (3)

$$J = \frac{\int \epsilon_A(\lambda) F_D(\lambda) \lambda^4 d\lambda}{\int F_D(\lambda) d\lambda} \quad (3)$$

The Forster distance was substituted into equation (4) to calculate intermolecular distances.

$$R = R_0 \left(\frac{1-E}{E} \right)^{\frac{1}{6}} \quad (4)$$

Where E is the efficiency of energy transfer; calculated as described in equation (5)

$$E = \left(\frac{I_{AD}}{I_A} \right) \left(\frac{\epsilon_A}{\epsilon_D} \right) \quad (5)$$

I_{AD} is the area under the corrected fluorescence emission band from the protein:aflatoxin B₁ complex, I_A is the area under corrected fluorescence emission band of aflatoxin B₁ alone, ϵ_A is the extinction coefficient for aflatoxin B₁ at 295nm and ϵ_D is the extinction coefficient of the protein at 295nm.

The equations (1) through (5) are the formal equations and they do not take into account any quench of fluorescence of Trp21 by unbound aflatoxin B₁. Therefore, to take this into account K_d values were determined by fluorescence titration of aflatoxin B₁ and measuring the quench of tryptophan as described in *Materials and Methods*. Determination of K_d meant that at the concentration of aflatoxin B₁ used in the FRET experiments, the fraction of aflatoxin B₁ molecules bound to the protein can be calculated the fraction of absorbance coming from bound aflatoxin B₁ can be known. The error in the determination of K_d values influences the calculation of intermolecular distances more than the error in determining the efficiency of energy transfer thus they are used to generate the errors in distance.

The results determined for each of the parameters defined in equations (1) to (5) are shown in table 3.1.

Protein	K_d (μ M)	J cm^3M^{-1}	Φ $\text{M}^{-1}\text{cm}^{-1}$	R_0 ($\text{\AA}$)	E	R ($\text{\AA}$)	Model Dist ($\text{\AA}$)
Yc ₁	3 ± 1	1.74×10^{-14}	2.09×10^{-3}	24.21	0.69 ± 0.06	20.0 ± 0.3	17.0 ± 2.1
Yc ₂	9 ± 3	1.62×10^{-14}	7.03×10^{-2}	14.38	0.12 ± 0.01	21 ± 1.9	16.9 ± 2.0

Table 3.1 Table to show the distances from Trp21 to aflatoxin B₁ in the active sites of rGST Yc₁ and Yc₂ determined by FRET, compared against the distances predicted by modelling.

When comparing the results from FRET with the model it is difficult to know which atoms to measure the distances from. Therefore in the table, a range of distances are given which are measured from the centre of the imidazole ring of Trp21 to the centre of the nearest and farthest rings of aflatoxin. As can be seen, the distances predicted from the model and the calculated distances are similar. This is an experimental demonstration that the docking of GSH-AFB into the homology models gives co-ordinates that are a good approximation of the actual solution structure. It is also interesting to note that inactive rGST Yc₁ binds aflatoxin B₁ tighter than rGST Yc₂ with $K_d = 3 \pm 1 \mu\text{M}$ and $K_d 9 \pm 3 \mu\text{M}$ respectively. The tighter binding of aflatoxin B₁ to rGST Yc₁ may reflect a different mode of binding that makes rGST Yc₁ inactive towards aflatoxin-*exo*-8,9-epoxide. After validation of both the protein's structures

and the positions of the modelled ligands, the structural features that cause the difference in the specific activities can be investigated.

3.3.3.4 Identification of structural motifs putatively involved in substrate specificity

In order to quickly identify residues that may important in rat GSTs, the display on Insight was set up so that substituted residues in each of the structures could be identified by colour. It was assumed that the main consideration in the differences of specific activity would be steric considerations in the H-site. They may subtly change orientation of the ligand in the active site and have an affect on k_{cat} . Therefore, steric clashes between the protein structure and the docked ligand were identified using BUMP, an algorithm designed to be run within Insight and capable of highlighting atoms that are too close to each other in space. The cut off constraint is generally atoms that are less than 3Å apart.

A number of steric clashes between the ligand and the protein were identified using this method both in the model for rGST Yc₁ and for Yc₂. These violations are shown as red lines in the pictorial representations in figs 3.10 and 3.11.

The molecular modelling results with GSTYc₁ were similar to those seen with human model generated in *Chapter 2*. The most extensive steric clashes were at positions 208 and 111. 208, high-lighted in yellow, was the only steric clash to a sidechain that that was substituted in GSTYc₂ while Y111 clashes may indicate an on-face H-bond and there was some deformation of Y111 as a result of the energy minimisation algorithms. The model also identified L107, just as the modelling identified L107 in huGSTA1-1, however, with this residue conserved between huGSTA1-1, mGSTYc, rGST Yc₁ and rGST Yc₂, this was considered to be a possible modelling inaccuracy. The closest substituted residue to L107 was H108. Also, the model did not show any interactions with the residues that form the C-terminal alpha helix, unlike the results seen with the huGSTA1-1 model

Interestingly, R15 was also predicted to be interacting with the sulphur atom in the conjugate. Difference fluorescence experiments show that mutation of Arg15 to Lysine affects the pK_a of bound glutathione and suggest R15 involvement in the catalytic mechanism (Bjornestedt *et al*, 1995) so this potential interaction is not too surprising,

Other contacts were restricted to the conserved residues that define the glutathione binding pocket, as described in the original crystal structure of the human GST protein (Sinning *et al*, 1993) such as R45, G67 and T68 This suggests that the adduct was

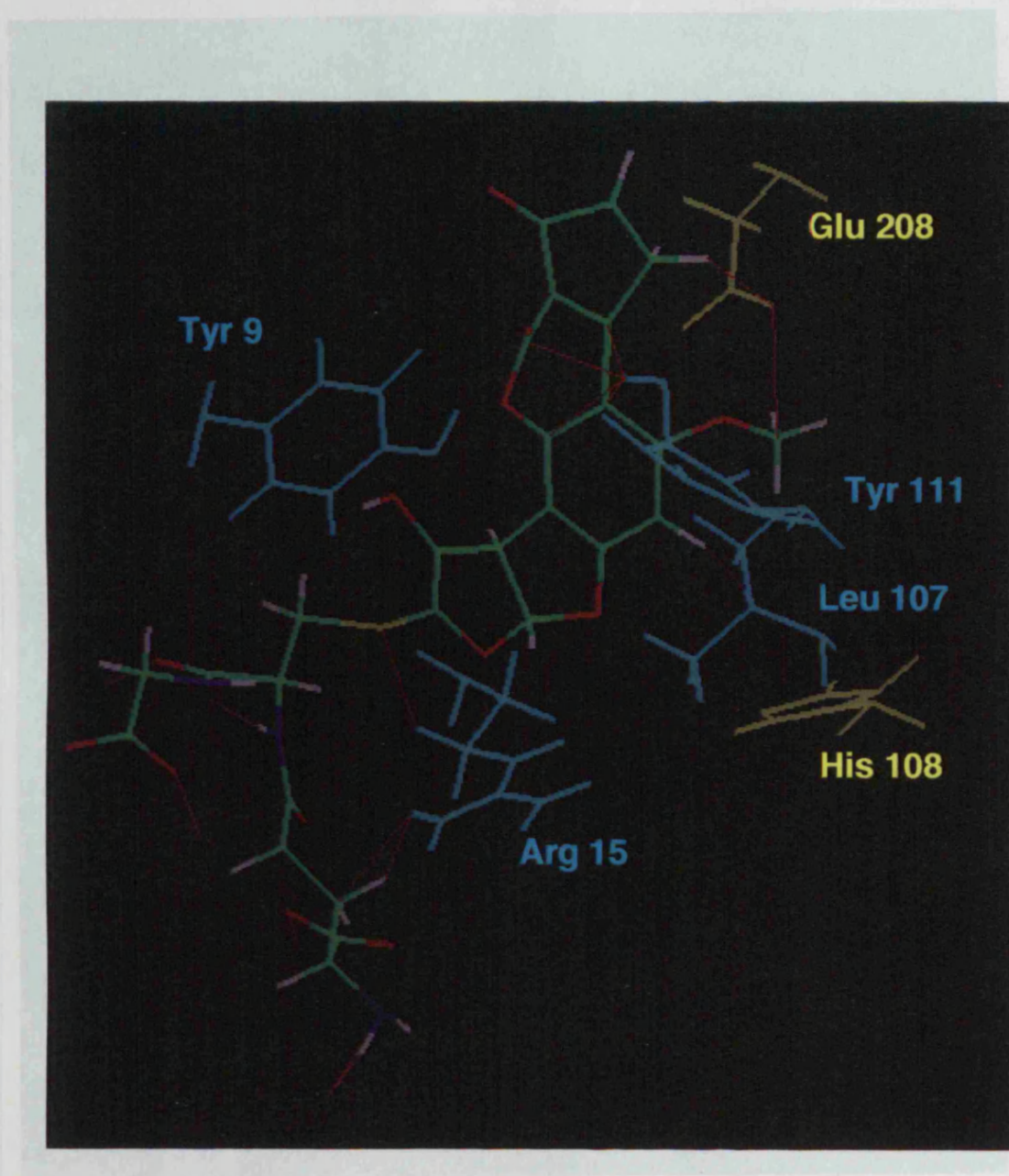
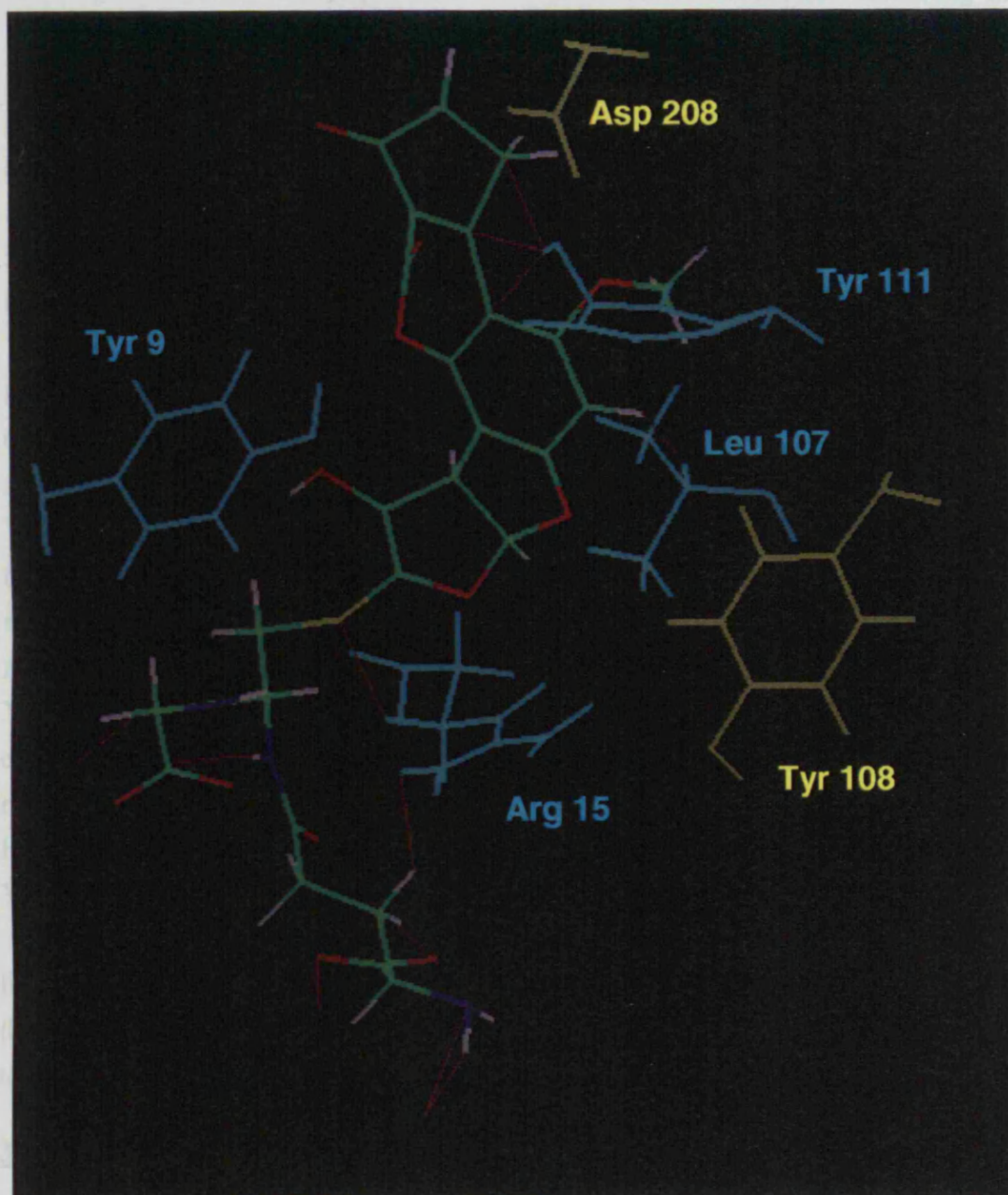


Fig 3.10 GSH-AFB conjugate docked into the modelled active site of rGST Yc₁. Steric clashes, calculated by BUMP are shown in red and substituted residues in yellow.

modelled into the current part of the molecule with the glutathione moiety being anchored to the G-site.

Inspection of the rGST Yc₂ model revealed its close contact with L157, Y111 and the G-site but crucially, position 33 was unoccupied. This modelling thus



In order to test out the ideas about substrate specificity generated from the docking experiments, six sequences encoding the proteins had to be subcloned into expression vectors and mutated. The cDNAs were kindly provided by Prof J. D. Hayes (Biochemistry Department, University of Dundee) as library clones JH24 and JH30 for rGST Yc₁ and Yc₂ respectively. The strategy was to clone them into the

Fig 3.11 GSH-AFB conjugate docked into the modelled active site of rGST Yc₂. Steric clashes, calculated by BUMP are shown in red and substituted residues in yellow.

A pictorial representation of the strategy is shown in Fig 3.12

modelled into the correct part of the protein, with the glutathionyl moiety firmly anchored in the G-site.

Inspection of the GSTYc₂ model revealed the same contacts with L107, Y111 and the G-site but crucially, position 208 was accommodated. This modelling thus supports the hypothesis that the low activity seen with rGST Yc₁ is due in part to steric hindrance of aflatoxin-*exo*-8,9-epoxide binding in the active site. This is further supported by differences between Yc₁ and Yc₂ sequences themselves. In Yc₁ the residue is E208 and in Yc₂ D208, the only difference being the length of the side chain. As the charge at position 208 is conserved, it is unlikely that the residue at the position is involved in the stabilisation of the transition state of aflatoxin-*exo*-8,9-epoxide, unlike the reaction of huGSTA1-1 with CDNB (Widersten *et al*, 1994).

With the modelling experiments identifying of 208 and the contact to L107 as likely contacts, it was decided to see whether AFBO activity could be knocked-out of GST Yc₂ and engineered into Yc₁. The mutations were to be made at positions 208 and 108. 108 was thought to be important because Y108 is conserved between mGST Yc and rGST Yc₂ but is substituted to H108 in rat Yc₁, while L107 is conserved. To test the positions 108 and 208, knock-out mutations were designed to make GSTYc₂ more like GST A1-1, in order to completely inactivate Yc₂. To see whether these residues were responsible alone, experiments were carried out to make Yc₁ more like Yc₂. If these positions were the only important residues, then aflatoxin-*exo*-8,9-epoxide activity should be engineered into Yc₁. Therefore the mutations to be made in rGST Yc₁ were the single mutants H108Y, E208D and the double mutant H108YE208D. Similarly, in rGST Yc₂ Y108L, D208M and the double mutant Y108LD208M.

The modelling results also support the findings from the models made with huGSTA1-1. The selection of the residues at 108 and 208 was justified and failure of the mutations to make any significant change in the specific activity may have been due to the cumulative presence of the number of other substitutions in the H-site.

3.4 Cloning and Mutagenesis of rat GST polypeptides.

In order to test out the ideas about substrate specificity generated from the modelling experiments, the sequences encoding the proteins had to be subcloned into expression vectors and mutated. The cDNAs were kindly provided by Prof J. D. Hayes (Biochemistry Department, University of Dundee) as library clones λJH24 and λJH30 for rGST Yc₁ and Yc₂ respectively. The strategy was to clone them into the expression/mutagenesis vector constructed in *Chapter 2* for huGSTA1-1 using PCR to lift out the cDNAs from the library with the correct restriction sites. A pictorial representation of the strategy is shown in fig 3.12

The genes were amplified by PCR using oligonucleotides YCF, YC1R and YC2R (detailed in *Materials and Methods*) that included restriction enzyme recognition-sequences for *EcoRI* and *XmaI* to allow directional cloning of the amplified genes into the expression vector. The thermocycling program was 94°C for 4 minutes and then (94°C for 1 minute, 65°C for 1 minute and 72°C for 1 minute) for 30 cycles followed by a 4°C soak.

3.4.1 Cloning of rat GST Yc1 and Yc2.

The amplified products were gel isolated using 1% agarose gel electrophoresis and purified using electroelution from the agarose gel-slice. The DNA fragments containing the genes were cut first with *XmaI* and then with *EcoRI* in appropriate concentrations of Pharmacia All-Phor-One Plus buffer, with heat denaturation after each digestion incubation. The vector pEM (expression/mutagenesis vector) was prepared similarly. The genes were ligated in to the vector using T4 DNA Ligase and the supplied buffer overnight at 16°C. *E.coli* JM109 was transformed with the ligation reaction. Plasmid DNA was isolated from returned colonies and recombinant plasmids identified with a diagnostic *NcoI* digest which linearised recombinant DNA. Finally, the entire genes were sequenced using the external sequencing primers PGWLF and PGWLR. The reactions were carried out according to the PRISM Ready Reaction DyeDioxy Terminator Cycle Sequencing Kit and Applied Biosystems Model 373A DNA Sequencer. These results showed that there had been a chance mutation from R82L, fortunately the mutation occurred underneath the sequencing primer RODSEQ and the sequence was corrected by mutagenesis with this primer. The resulting plasmids were called pEMYc₁ and pEMYc₂ and were in a vector suitable for mutagenesis.

3.4.2 Mutagenesis of pEMYc₁ and pEMYc₂

pEMYc₁ and pEMYc₂ were transformed into *E.coli* RZ1032 (F⁺ung⁻ dut⁻). Single stranded DNA was produced by infecting a LB culture at A₆₀₀ of 0.5 with 5x10¹⁰ pfu VCSM13 helper phage and recovered after precipitating the phage particles with Polyethylene Glycol (PEG) partitioning of phage coat protein with phenol and ethanol precipitation of the DNA from the aqueous phase.

To make these mutants, a variation of the method of (Kunkel, 1985) was used as described in *Materials and Methods* (7.3.18, Method 2). The mutations were introduced using the oligonucleotide primers H108Y, E208D, Y108L and D208M and double mutants were made by introducing both of the appropriate primers into the

the same mutagenesis mixture. Colonies returned were screened for discrete mutations using a combination of automated sequencing and manual sequencing using the primers PGWLF and PGWLR. The identification of a mutant by manual sequencing is shown in fig 3.13. The resulting plasmids were designated pEMH108Y, pEME208D pEMH108YE208D; and pEMY108L, pEMD208M and pEMY108LD208M.

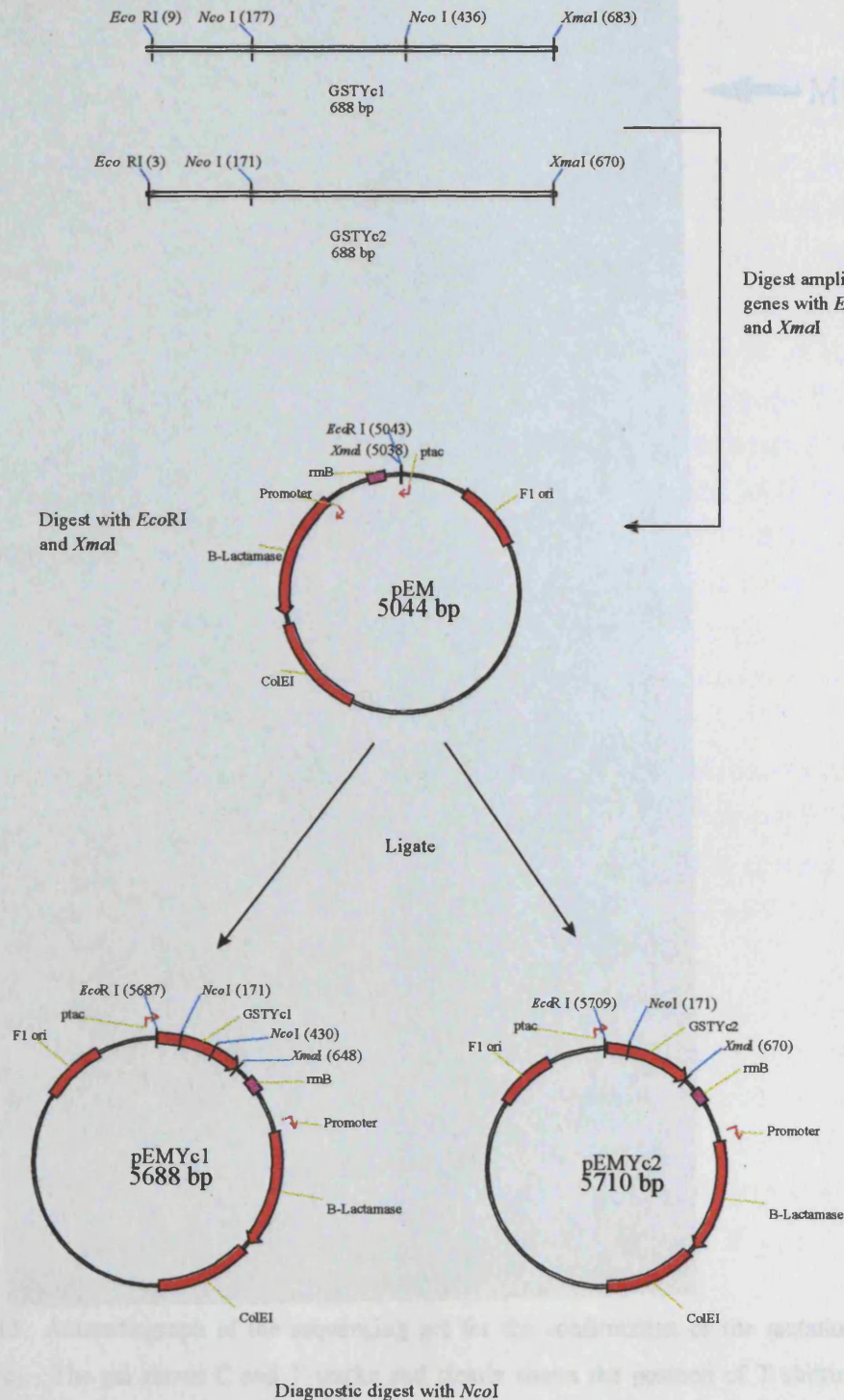


Fig 3.12 Cloning scheme for the construction of mutagenesis/expression vectors for rGSTYc₁ and rGSTYc₂

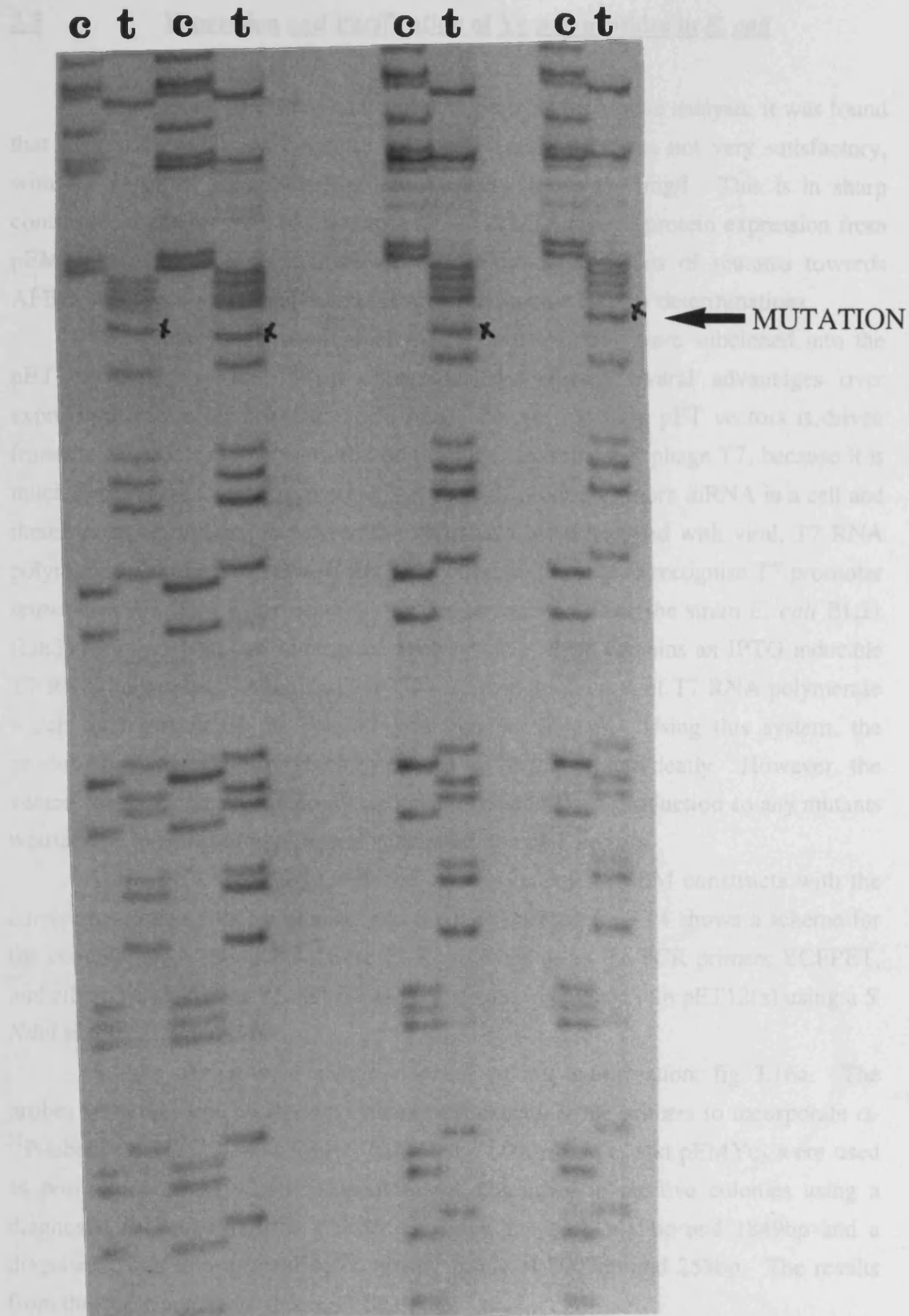


Fig 3.13 Autoradiograph of the sequencing gel for the confirmation of the mutation D208M in rGSTYc₂. The gel shows C and T tracks and clearly shows the position of T shifting showing a successful mutation. The important T is marked with an x. TTTTgaTgaT (aspartate) instead of TTTTaTggaT (methionine).

3.5 Expression and Purification of Yc polypeptides in *E. coli*

During expression trials of the mutant proteins for kinetic analysis, it was found that expression of Yc polypeptides from pEM constructs was not very satisfactory, with the yields of recombinant protein typically less than 1mg/l. This is in sharp contrast to the expression levels seen with huGST. Although protein expression from pEM constructs gave preliminary answers regarding activities of mutants towards AFBO, this was not at all sufficient for accurate specific activity determinations.

To increase the expression of the rat mutants, they were subcloned into the pET expression system. This expression system had several advantages over expression from a *tac* promoter as in pEM. Expression from pET vectors is driven from the extremely strong transcription promoter from bacteriophage T7, because it is much stronger than a *tac* promoter it is capable of producing more mRNA in a cell and therefore more protein. However, the cells have to be supplied with viral, T7 RNA polymerase as indigenous *E. coli* RNA Polymerase is unable to recognise T7 promoter sequences. T7 RNA polymerase is supplied *in trans* by using the strain *E. coli* BL21 (DE3) (Studier, 1986) containing the DE3 lysogen which contains an IPTG inducible T7 RNA Polymerase. Addition of IPTG results in production of T7 RNA polymerase which then transcribes the heterologous gene in *E. coli*. Using this system, the production of recombinant proteins should be increased sufficiently. However, the vector does not contain any origin for single stranded DNA production so any mutants would have to be made in pEM and subcloned into pET vectors.

Again, PCR was used to lift the cDNAs out of the pEM constructs with the correct restriction sites for cloning into the pET vectors, fig 3.14 shows a scheme for the construction. The cDNAs were PCR amplified using the PCR primers YCFPET, and either YC1RPET or YC2RPET as appropriate and cloned into pET12(a) using a 5' *NdeI* site and 3' *BamHI* site.

Positive clones were identified using colony hybridisation, fig 3.16a. The probes were prepared by random priming and extending the primers to incorporate α -³²P-labelled dCTP. *E. coli* JM109 transfected with pEMYc₁ and pEMYc₂ were used as positive controls. Correct insertion was confirmed in positive colonies using a diagnostic *PstI* digestion for pEMYc₂ to give bands of 3419bp and 1849bp and a diagnostic *NcoI* digest for pEMYc₁ giving bands of 5009bp and 259bp. The results from the construction are shown in fig 3.16b.

3.0 Manipulating AFBO activity in Rat Yc Polypeptides

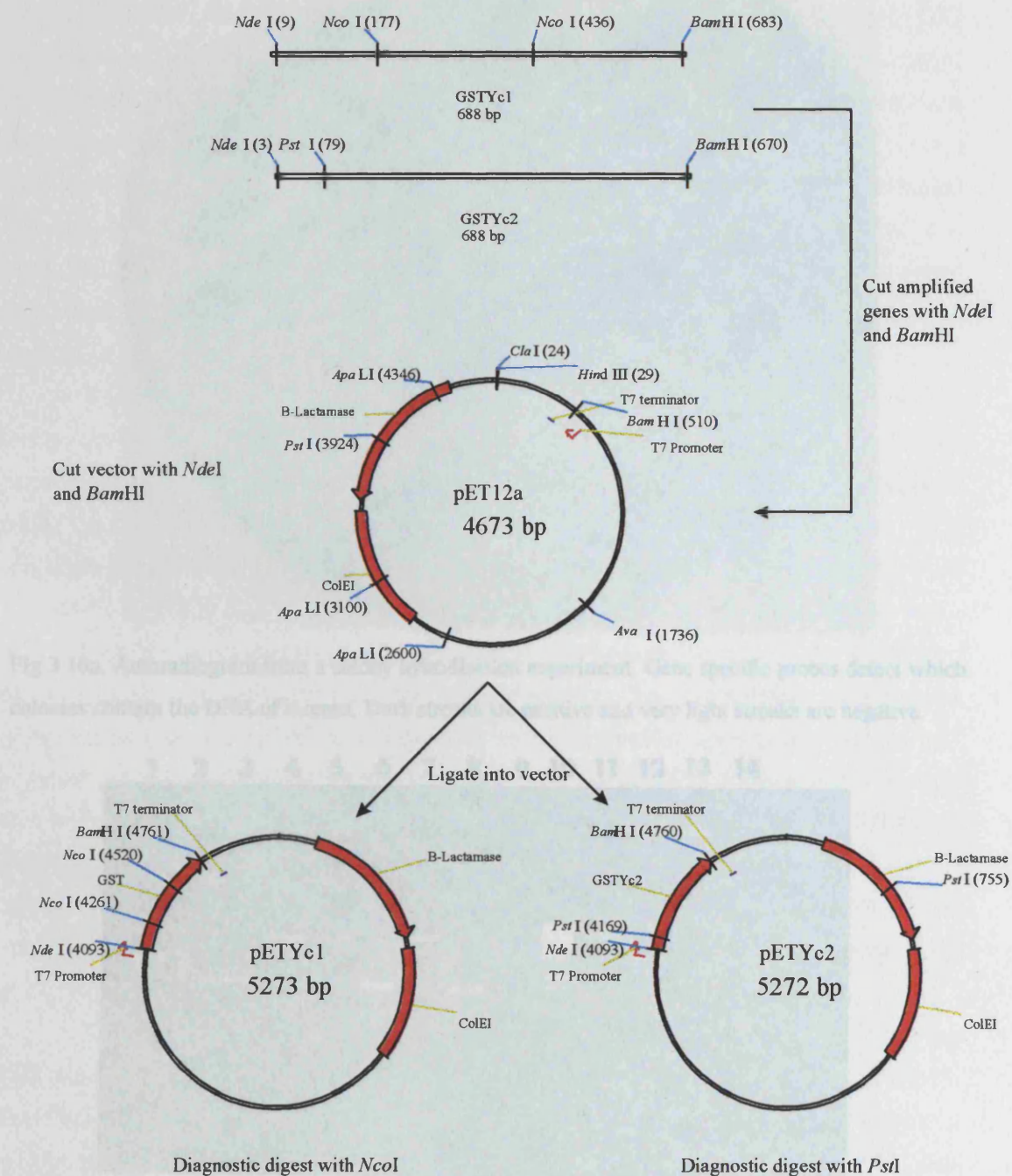


Fig 3.15 Construction scheme for pET Yc₁ and pET Yc₂.

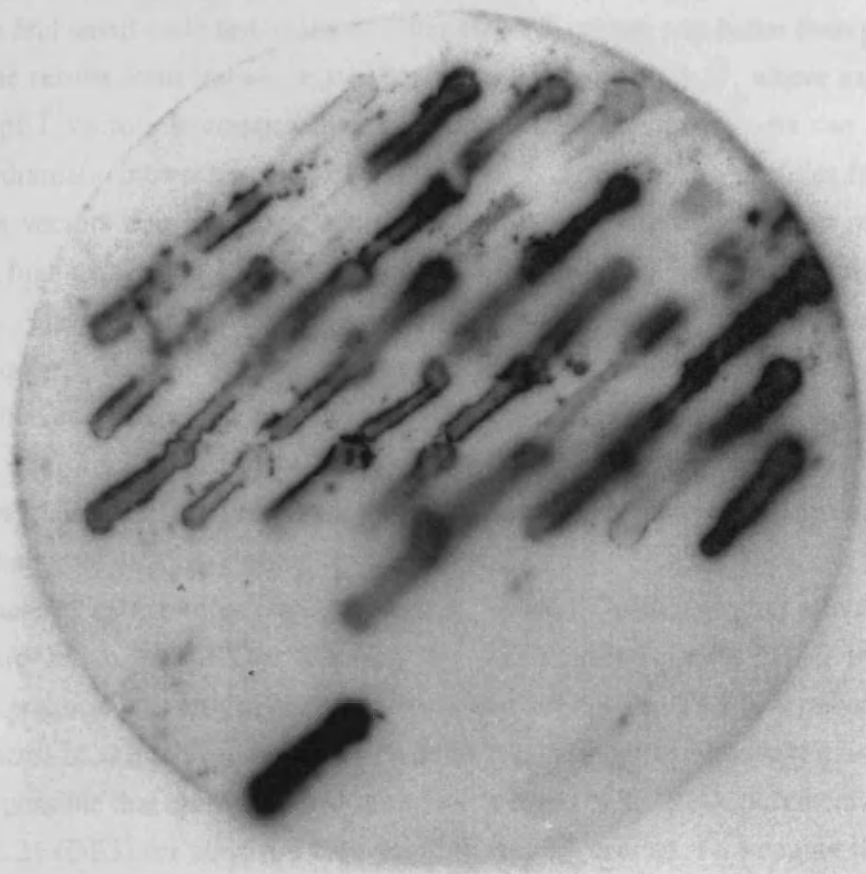


Fig 3.16a. Autoradiogram from a colony hybridisation experiment. Gene specific probes detect which colonies contain the DNA of interest. Dark streaks are positive and very light streaks are negative.

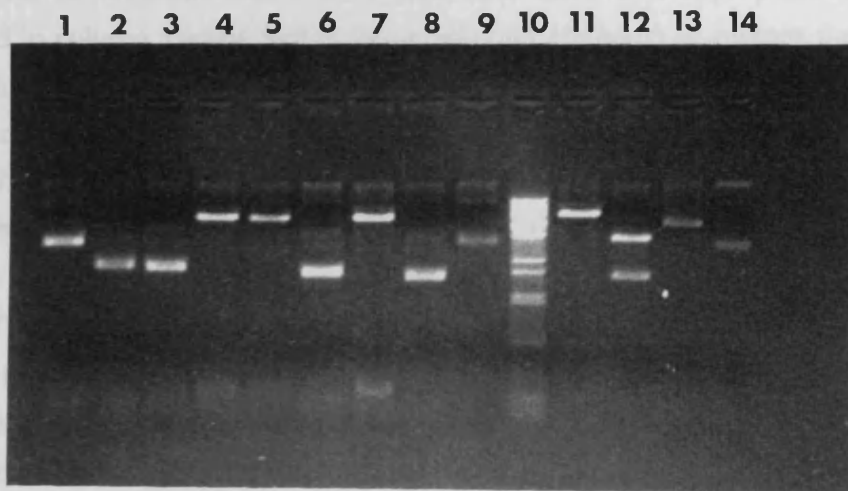


Fig 3.16b. Gel showing the successful construction of pET-GST expression vectors. Lane 1 Undigested plasmid pET12a DNA. Lanes 2-8 are pETYc₁ *Nco*I digests Lanes 4, 5 and 7 show correct insertion of Yc₁ cDNA, liberating fragments of 5009bp and 259bp. Lane 9 Undigested plasmid pET12a DNA. Lane 10 λ *Hind*III Markers. Lanes 11-13 are pETYc₂ *Pst*I digests with Lane 12 showing correct insertion of Yc₂ cDNA; liberating fragments of 3419bp and 1849bp. Lane 14 Undigested plasmid pET12a DNA.

Finally, the constructs were used to transform *E.coli* BL21(DE3) and the expression tested in a 5ml small scale test to see whether the pET system was better than pEM.

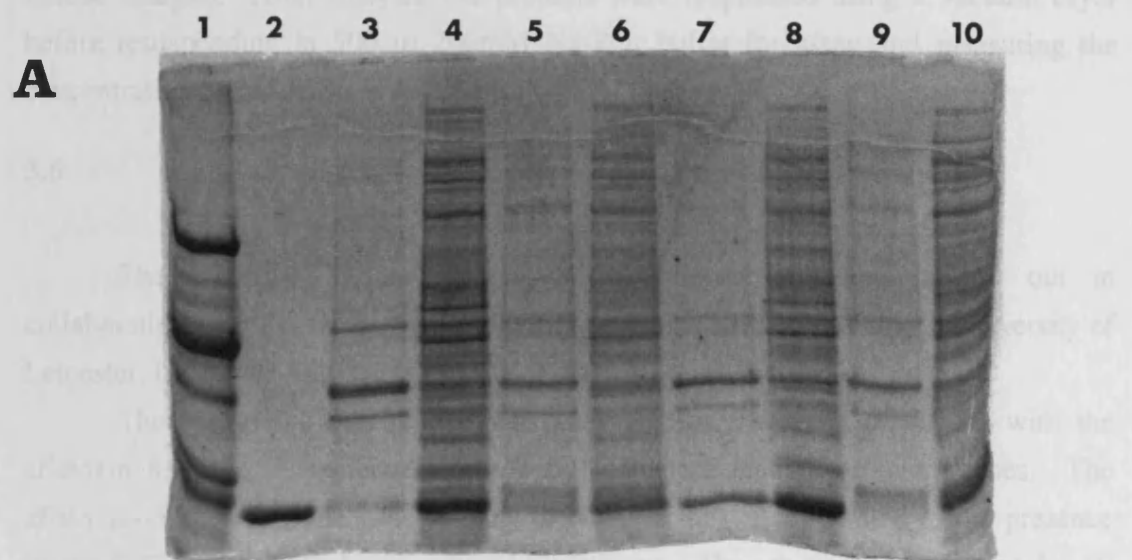
The results from the expression tests are shown in fig 3.17, where expression from the pET vectors is compared to that from the pEM vectors. As can be seen, there is a dramatic increase in the amount of expression of Yc polypeptides from pET expression vectors due to the increased strength of the viral transcription promoter. However, high expression levels such as these are often associated with inclusion body formation. The cellular folding pathways become saturated with heterologous protein and subsequently the protein is precipitated and stored in electron-dense bodies called inclusion bodies. Recombinant protein in this form is difficult to purify because it is insoluble. In order to test whether the protein was expressed in a soluble form, expression trials were performed to optimise the culture conditions to maximise the amount of soluble protein returned for purification.

Bacterial cell cultures were grown at 30°C and 37°C to see what effect varying temperature had on rGSTYc₂ production. The requirement for IPTG to induce protein expression was also tested. As mentioned previously, T7 RNA polymerase is under control of the *lac* promoter which gives 'leaky' control of transcription. It is therefore possible that there are already sufficient levels of T7 RNA polymerase within *E. coli* BL21 (DE3) for adequate expression from pET vectors. By keeping the levels of T7 RNA polymerase restricted in the cells, it may be possible to stop the saturation of the folding pathways and keep the protein soluble. To test whether recombinant protein was forming inclusion bodies, cells were taken after appropriate culture and sonicated in column loading buffer (*Materials and Methods*) to rupture the cell walls and release soluble protein. The sonicate was subsequently centrifuged at 13,000rpm in a microcentrifuge to pellet cellular and insoluble debris and thus separated soluble protein from inclusion body. The protein samples were then boiled in SDS-PAGE loading buffer and separated on a 10% SDS-PAGE gel. The results are shown in fig 3.17.

The results clearly show that there is some inclusion body formation using the pET vectors but that there is a predominance of soluble protein. The gel also shows that 'leaky' transcription from the *tac* promoter is sufficient to drive adequate protein expression. Therefore, it was decided to express the protein at 37°C but without IPTG induction thus reducing the chances of bacteriophage infection during production of the protein.

3.5.1 Purification of rat Yc polypeptides from *E. coli*

All the rat Yc proteins were purified using glutathione-agarose affinity matrix columns as described in *Materials and Methods* and the purity confirmed with SDS-PAGE analysis showing only a single band for each protein preparation.



Gel A. 1 Mw Markers. 2 Yc₁ standard. 3 Insoluble fraction 30°C - IPTG. 4 Soluble fraction 30°C - IPTG. 5 Insoluble fraction 30°C + IPTG. 6 Soluble fraction 30°C + IPTG. 7 Insoluble fraction 37°C -IPTG. 8 Soluble fraction 37°C -IPTG. 9 Insoluble fraction 37°C +IPTG. 10 Soluble fraction 37°C + IPTG.

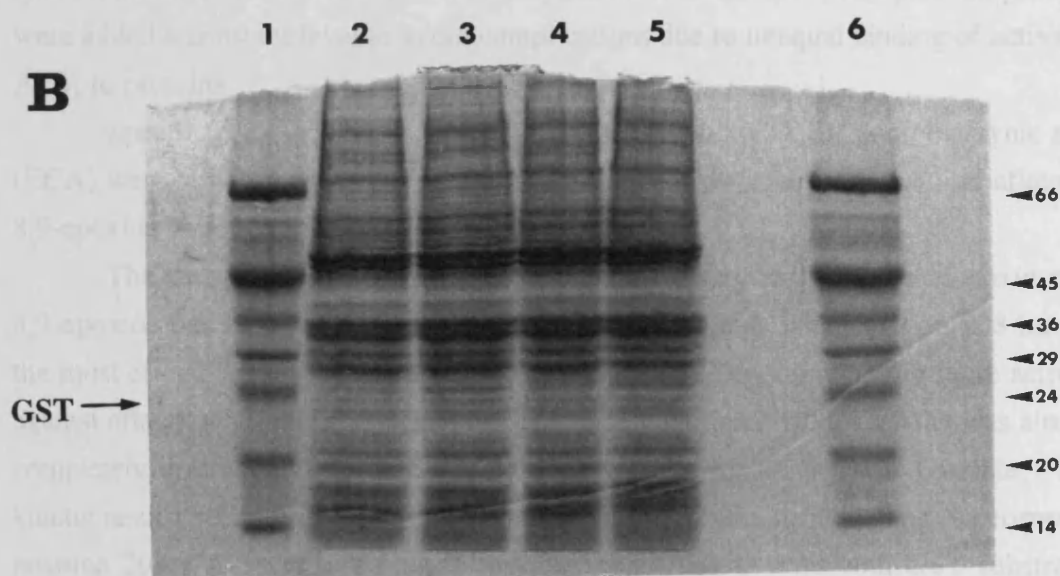


Fig 3.17 Gels showing comparisons of pET expression trials (Gel A) and expression levels from pEM (Gel B) expression vectors. 1 Mw Markers. 2 pEMYc₁ - IPTG. 3 pEMYc₁ + IPTG. 4 pEMYc₂ - IPTG. 5 pEMYc₂ + IPTG. 6 Mw Markers.

Subsequently, the protein solutions were dialysed against 200mM Na₂PO₄ buffer, pH 7.2 using Slide-A-Lyzer cassettes (Pierce and Warriner UK) to remove any traces of Tris buffer which was used in buffers during purification. The dialysis step was necessary because Tris forms conjugates with aflatoxin-*exo*-8,9-epoxide to give compounds that are very difficult to distinguish from GSH-AFB conjugates during kinetic analysis. After dialysis, the proteins were lyophilised using a vacuum dryer before resuspending in 500 µl 200mM Na₂PO₄ buffer for assay and measuring the concentration by absorbance at 280nm.

3.6 Assay results from purified rGST Yc₁ and Yc₂ mutants.

The aflatoxin 8,9-epoxide assays in this section were carried out in collaboration with Dr. G. E. Neal and Mr D. J. Judah, MRC Toxicology, University of Leicester, Lancaster Rd, Leicester.

The assay is a two component assay system (Hayes *et al*, 1992) with the aflatoxin 8,9-epoxide generated *in situ* by Japanese quail liver microsomes. The aflatoxin-*exo*-8,9-epoxide generated by the microsomes is conjugated in the presence of the GST to be studied and reduced glutathione. The reaction is incubated for 10 minutes and extracted with acidified methanol. The products of the reaction are separated by HPLC and the GSH-AFB conjugate is identified from its retention time (compared to a GSH-AFB standard). The amount of conjugated AFBO is determined by the area underneath the corresponding peak calibrated against a column-standard amount of GSH-AFB. With these assays, at the low levels of activity present, proteins were added a constant level to avoid complications due to unequal binding of activated AFB₁ to proteins.

Specific activities for 1-chloro 2,4 dinitrobenzene (CDNB) and ethacrynic acid (ECA) were also determined to see whether any changes in activity towards aflatoxin 8,9-epoxide were specific.

The data in Table 3.2 clearly shows that the ability to conjugate aflatoxin-*exo*-8,9-epoxide has been knocked out of Yc₂ by the mutations with position 208 having the most effect. 108 was less important, with mutated Yc₂ still retaining more activity against aflatoxin 8,9-epoxide than Yc₁. The double mutant Y108L D208M was almost completely inactive and so no accurate quantification of activity was possible. The kinetic results seem to agree with the findings of the molecular modelling experiments, position 208 was likely to be more important than 108. Assays with GST substrates such as CDNB and ECA show that specific activity for these substrates is also changed but, crucially, that the loss in activity for aflatoxin 8,9-epoxide is selective thereby proving that the mutant proteins are still active and that loss of aflatoxin 8,9-epoxide activity is specific to the mutations and due to general effects through misfolding. The

differences in activity towards CDNB and ECA are difficult to interpret and such unexplained differences in specific activity for H-site mutants has been previously reported for position 208 in huGSTA1-1 (Widersten *et al*, 1994).

Protein	Specific Activity CDNB (μ mmoles/min/mg)	Specific Activity ECA (μ moles/min/mg)	Specific Activity AFBO (nmoles/min/mg)
Yc ₁ wild type	8.91 $\pm$ 0.38	0.65 $\pm$ 0.07	0.01
Yc ₁ H108Y	4.30 $\pm$ 0.51	1.56 $\pm$ 0.08	0.01
Yc ₁ E208D	7.76 $\pm$ 0.17	1.23 $\pm$ 0.58	0.01
Yc ₁ E208D H108Y	4.07 $\pm$ 0.10	0.39 $\pm$ 0.04	0.30
Yc ₂ wild type	4.86 $\pm$ 0.21	0.26 $\pm$ 0.14	13.20
Yc ₂ Y108L	8.82 $\pm$ 0.15	0.49 $\pm$ 0.40	0.32
Yc ₂ D208M	0.14 $\pm$ 0.01	0.09 $\pm$ 0.04	<0.01
Yc ₂ Y108L D208M	0.56 $\pm$ 0.10	0.17 $\pm$ 0.08	<0.01

Table 3.2 Table showing the specific activities determined for rGST Yc polypeptide and mutants. 200mM NaPO₄ pH 6.5, 2mM glutathione, 1mM CDNB 200 μ M ECA 25°C.

The data for the engineering mutations in Yc₁ also confirms the importance of positions 108 and 208 but only when combined in a double mutant protein. The activity in the engineered Yc₁ H108Y E208D showed a 30-fold increase from 0.01 to 0.30 nmoles/min/mg. However, this falls short of the 13.2 nmoles/min/mg target of Yc₂ wild-type protein. A 30-fold increase in activity means that in energetic terms, the mutations are approximately halfway to engineering full activity in rGST Yc₁, an extra 44-fold increase of activity would be needed to make Yc₁ work as well as Yc₂ with aflatoxin 8,9-epoxide. Mutation of these positions points to the fact that there may be some steric considerations when aflatoxin 8,9-epoxide binds that could affect activity. However, it is unclear whether full activity can only be engineered by complete mutation of substituted residues around the H-site of Yc₁ (van Ness *et al*, 1994) or whether there are other subtle differences in the mechanisms of Yc₁ and Yc₂ that are causing differences seen in activity.

3.7 Analysis of aflatoxin B₁ binding by FRET spectroscopy

To investigate what the differences may be in the mutant proteins that influences the specific activity toward aflatoxin 8,9-epoxide, distances from Trp21 to aflatoxin B₁ bound in the H-site were measured by FRET as had previously been carried out to validate the molecular models. The mutants with the greatest change in activity were investigated which meant that the double mutants H108YE208D and Y108LD208M were compared to wild-type rGSTYc₁, rGSTYc₂ and also to huGSTA1-1 as the mutations in rGSTYc₂ were designed to make it more like huGSTA1-1 as regards its specific activity to aflatoxin-*exo*-8,9-epoxide. Table 3.3 shows the values determined for the parameters needed to satisfy equations (1) to (5). Again corrections were made for the efficiency of transfer by determining the K_d values.

Protein	K_d (μ M)	J cm^3M^{-1}	Φ $\text{M}^{-1}\text{cm}^{-1}$	R_0 ($\text{\AA}$)	E	R ($\text{\AA}$)
A1-1	2.0 ± 0.5	1.66×10^{-14}	2.80×10^{-3}	14.22	0.14	19.3 ± 0.2
Yc ₁	3.0 ± 1.0	1.74×10^{-14}	2.09×10^{-3}	24.21	0.69	20.0 ± 0.3
H108YE208D	6.1 ± 1.9	1.68×10^{-14}	6.30×10^{-3}	16.29	0.31	18.6 ± 0.7
Y108LD208M	6.7 ± 1.5	1.74×10^{-14}	1.20×10^{-3}	12.36	0.07	19.2 ± 0.3
Yc ₂	9.0 ± 3.0	1.62×10^{-14}	7.03×10^{-2}	14.38	0.12	21.1 ± 1.9

Table 3.3 Results from FRET analysis of aflatoxin B₁ binding in the H-site and distances to Trp21. K_d values were determined independently by fluorescent titration.

3.8 Discussion

Examination of the K_d values determined table 3.4 suggests there is an inverse correlation between the strength of binding of aflatoxin B₁ and specific activity with aflatoxin 8,9-epoxide (table 3.4). The more inactive the protein, the lower the K_d value. This may reflect different modes of binding, where an inactive protein binds aflatoxin B₁ in a tighter and spatially distinct way.

However, there is no clear trend when the distances from Trp21 to aflatoxin B₁ are compared. Indeed, some care must be taken when comparing distances determined by FRET. On binding, there is often a blue shift of the fluorescence emission of tryptophan because of the exclusion of water from the H-site. This may well affect the value of spectral overlap, J, and give errors when converting transfer efficiencies to

distances. Also, it is assumed that κ^2 will be 2/3 for all the proteins, however this may not be strictly true when comparing different proteins although κ^2

Protein	CDNB (mmoles/min/mg)	EA (umoles/min/mg)	AFBO (nmoles/min/mg)	K_d (μ M)	R ($\text{\AA}$)
huGSTA1-1	50.66 $\pm$ 2.19	-	<0.01	2.0 $\pm$ 0.5	19.3 $\pm$ 0.2
Yc ₁	8.91 $\pm$ 0.38	0.65 $\pm$ 0.07	0.01	3.0 $\pm$ 1.0	20.0 $\pm$ 0.3
H108YE208D	4.07 $\pm$ 0.10	0.39 $\pm$ 0.04	0.30	6.1 $\pm$ 1.9	18.6 $\pm$ 0.7
Y108LD208M	0.56 $\pm$ 0.10	0.17 $\pm$ 0.08	<0.01	6.7 $\pm$ 1.5	19.2 $\pm$ 0.3
Yc ₂	4.86 $\pm$ 0.21	0.26 $\pm$ 0.14	13.20	9.0 $\pm$ 3.0	21.1 $\pm$ 1.9

Table 3.4 Summary table for specific activity determinations, K_d and distance measurements between Trp21 and aflatoxin B₁

values are not expected to change upon mutation (Dos Remedios and Moens, 1995) and the distances measured for wild-type protein and its respective double mutant should be comparable. The errors in determination of the distances also make interpretation of trends in the data difficult. If Trp21 was closer to the H-site then small differences in the distance could be more easily resolved.

Looking at the distances determined for rGST Yc₂ and its double mutant, Y108LD208M, the distances are 21.1 $\pm$ 1.9 $\text{\AA}$ and 19.2 $\pm$ 0.3 $\text{\AA}$ respectively. They show that is likely that the nearer aflatoxin B₁ binds to Trp21, the more inactive the protein is towards aflatoxin-*exo*-8,9-epoxide, with Y108LD208M being almost inactive with aflatoxin-*exo*-8,9-epoxide but with the magnitude of the error in the determination of the intermolecular distance with rGST Yc₂ it is difficult to say this definitely. The mutations were designed to make rGST Yc₂ more like huGSTA1-1 and if the distance from Trp21 to rGST Yc₂ in the human enzyme is considered, it appears that Y108LD208M now binds aflatoxin B₁ in a very similar position with huGSTA1-1 binding aflatoxin B₁ 19.3 $\pm$ 0.2 $\text{\AA}$ from Trp21 and Y108LD208M binding aflatoxin B₁ 19.2 $\pm$ 0.3 $\text{\AA}$ from Trp21.

However, when the distances are examined for rGST Yc₁ and its double mutant H108YE208D, the trend does not appear to hold. H108YE208D binds aflatoxin B₁ 18.6 $\pm$ 0.7 $\text{\AA}$ away from Trp21 and is 30-fold more active than wt rGST Yc₁ which binds aflatoxin B₁ 20.0 $\pm$ 0.3 $\text{\AA}$ away. This could reflect a binding position that gives a higher specific activity but also hints that substitution of H-site residues alone may not lead to complete engineering of activity into rGST Yc₁.

However, if all the distance measurements are taken together they show that there are not any large changes in the position of binding of aflatoxin B₁ in the H-sites of the enzymes. This suggests that if there are steric considerations in binding of

aflatoxin B₁ that influence specific activity then they are subtle and it is not clear whether the basis of the selectivity in rGST Yc₁ and Yc₂ lies solely in the H-site.

What is clear is that there are some important residues that together with Y108 and D208 that affect activity with aflatoxin-*exo*-8,9-epoxide. These residues must lie between 53 and 221 as identified with mGST Yc/rGST Yc₁ chimeras (van Ness *et al*, 1994). A likely candidate for an additional residue is L104.

In huGSTA1-1 and rGST Yc₁, both inactive with aflatoxin-*exo*-8,9-epoxide, the residue at position 104 is glutamate. The crystal structure of huGSTA1-1 shows a hydrogen bond between G104 and R15 and it must be assumed that there will be a similar H-bond in rGST Yc₁. However, in mGST Yc and rGST Yc₂, the residues at position 104 are leucine and isoleucine respectively which cannot H-bond with R15.

R15 is shown in the molecular models interacting with the sulphur atom in the conjugate and has been shown to be important in catalysis (Bjornestedt *et al*, 1995), lowering the pK_a of bound glutathione. Thus it could be argued that the constraining of R15 in huGSTA1-1 and rGST Yc₁ could be detrimental in catalysing the reaction of glutathione with aflatoxin-*exo*-8,9-epoxide.

There have been reports that huGSTA1-1 may be involved in the metabolism of aflatoxin B₁ (Simula *et al*, 1993). In this work, *Salmonella typhimurium* tester strains were transfected with various GSTs and incubated with a number of toxins including aflatoxin B₁. Phenobarbitone-treated microsomal protein was plated along with aflatoxin B₁ and the cells to activate aflatoxin B₁ to aflatoxin 8,9-epoxide. The results show that the cells were protected 10-fold against aflatoxin B₁ by expressing huGSTA1-1, however they were unable to show that the protection seen was glutathione dependent (Simula *et al*, 1993). Considering that the K_d for aflatoxin B₁ binding to huGSTA1-1 was determined at 2.0±0.5 µM, it suggests that the protection seen in these experiments was due to huGSTA1-1 binding any free aflatoxin 8,9-epoxide rather than conjugating it to glutathione.

A number of *Salmonella* tester strains have been constructed (Shimada *et al*, 1996, Simula *et al*, 1993) and show sensitivity to aflatoxin B₁ toxicity. These strains could be the key to identifying exactly which residues are important in selecting aflatoxin 8,9-epoxide as a substrate. An elegant tester strain was constructed in *Salmonella typhimurium* NM5004 (Shimada *et al*, 1996). The strain is transformed with a plasmid containing mGSTYc and also with a reporter construct containing an *umu* gene transcription promoter upstream of *lacZ*. *Umu* gene expression occurs in response to cytotoxicity and toxic insult, therefore in the fusion construct, any increase in toxicity is accompanied by an increase in β-galactosidase activity. Treatment of the parent strain (i.e. the one without GST) showed a very high response to aflatoxin B₁.

GSTs have also been shown to be open to forced evolution techniques (Gulick and Fahl, 1995, Widersten *et al*, 1995). Both labs used random mutagenesis of the

residues of the H-site of GST combined with either a screening system in *E. coli* looking for cells that were able to withstand the toxic insult (Gulick and Fahl, 1995) or a phage display system that looked for novel binding activities (Widersten and Mannervik, 1995). Enzymes were created that were capable of metabolising mechlorethamine (Gulick and Fahl, 1995) and showed novel binding properties for para-substituted benzyl compounds (Widersten and Mannervik, 1995). However, with aflatoxin B₁, phage display techniques would not be suitable because inactive enzymes already bind aflatoxin B₁ tightly. It could be possible to create a more elegant forced evolution strategy than previously reported for GST.

Sexual PCR could be used to create libraries of mutant GSTs (Crameri *et al*, 1996, Smith *et al*, 1994). The approach is an *in vitro* technique for the recombination of homologous genes. The genes are fragmented into random pieces and subsequently PCR assembled into full length genes. Inevitable mismatching occurs as the gene fragments are annealing creating changes in the DNA sequence, further errors are introduced by error-prone *Taq* polymerase. The result is that mutations and cross-overs are fixed into the DNA sequences, just as in 'real' evolution. The technique lends itself immediately to the problem of what residues are important in aflatoxin B₁ metabolism. There is a high-activity gene, rGST Yc₂ and two inactive genes, rGST Yc₁ and huGSTA1-1. Sexual PCR could be used to 'infuse' the right components for aflatoxin 8,9-epoxide metabolism into the inactive genes, thereby identifying which residues are important. The pool of fragments generated from either huGSTA1-1 or rGST Yc₁ could be seeded with fragments from rGST Yc₂ before reassembly and recovery of full gene products by PCR. Cross-overs would be extremely likely because of the high homology between the genes. The resulting full length products could be cloned into expression vectors to create an expression library. All that would be needed is an appropriate screen for aflatoxin 8,9-epoxide metabolism.

A *Salmonella* tester strain similar to the one created by Shimada and co-workers could be constructed. Instead of an *umu*"*lacZ* fusion it would be possible to construct an *umu*"*gfp* reporter construct to link the effect of a toxic insult with expression of Green Fluorescent protein from *Aequoria victoria* (Chalfie *et al*, 1994, Kain *et al*, 1995, Ormo *et al*, 1996, Plautz *et al*, 1996, Prasher *et al*, 1992). GFP is a versatile reporter protein that fluoresces without any exogenous substrate addition and can be expressed in bacterial cells. Although not suitable for Fluorescence Activated Cell Sorting (FACS) in its wild-type form, mutagenesis has been used to manipulate the excitation and emission spectra such that it is now optimised for FACS (Cormack *et al*, 1996). Linkage to this reporter gene would mean that varying degrees of *umu* induction could be isolated and quantified. All that would be required would be to transform the strain with the sexual PCR expression library and use FACS or a hand-held ultra-violet light source to identify cells that did not show induction of *umu*-

dependent expression of GFP. By repeating the cycle of sexual PCR, screening and increasing the aflatoxin B₁ concentration, novel constructs could be isolated that would have a good chance of being good metabolisers of aflatoxin 8,9-epoxide.

Chapter 4

Protection of Human Bronchial Epithelium Cells by Engineered GSTs

4.0 PROTECTION OF HUMAN BRONCHIAL EPITHELIAL CELLS BY ENGINEERED GSTs

4.1 Introduction

After considering the results of the manipulation of aflatoxin metabolism in rat GSTs, it was decided that the mutants showing the greatest change in specific activity, H108YE208D and Y108LD208M, should be used to see whether the new activities could be used to protect living cells against the cytotoxic effects of aflatoxin. The *in vitro* assays are necessarily artificial and the activities measured may not reflect what the engineered proteins may do in a cellular compartment. In order to address this, the expression of mutants in tissue culture systems was pursued to assess whether perhaps the mutant proteins performed better inside the cell than the *in vitro* assays suggested.

This is not a novel approach; expression of a particular GST in cell culture to see how it may affect the cells' resistance to toxins has been widely attempted with varying degrees of success. The first published evidence that transiently transfected cells could be protected from toxic insults came from experiments with rGST2-2 (Manohoran *et al*, 1987). The cDNA was cloned downstream of an SV40 promoter and expressed in COS cells. It was shown that there was a 20- to 30-fold enrichment of the cells expressing GST after treatment with benz[a]pyrene(±)-*anti*-diol epoxide (Manohoran *et al*, 1987) and 1.3- to 2.9-fold resistance to chlorambucil and melphalan, (Pulchalski and Fahl, 1990). Other GSTs were also used such as rGSTM1-1 and rGSTP1-1 against a wide variety of compounds and they gave between 1.3- and 1.5-fold resistance as seen previously with rGST2-2 (Pulchalski and Fahl, 1990). Cells expressing combinations of GSTs such as rGST2-2 and rGSTM1-1 are 25% to 75% more resistant to chlorambucil than those cells that express either GST alone (Manohoran *et al*, 1991).

HGSTP1-1 cDNA has also been used to confer 3.0- fold resistance to adiamycin and 7.9-fold resistance to the toxic affects of ethacrynic acid (Nakagawa *et al*, 1990). The involvement of GSTs in the metabolism of chemotherapy agents has focused attention on how the ability of transfected GSTs to metabolise anti-cancer drugs. Stable expression of rGSTA3-3 in MatB cells has lead to resistance of 6- to 12-fold for melphalan, 10- to 16-fold for mechloroethamine and 7- to 30-fold resistance for chlorambucil (Schechter *et al*, 1993).

There is clear evidence that if the correct GSTs are used in the right cell lines, then resistance to certain compounds can be demonstrated. It also appears that resistance is influenced by the level of expression of the heterologous GST. Stably expressing the proteins in the cell lines appears to be more effective (Schechter *et al*,

1993, Manohoran *et al*, 1987, Manohoran *et al*, 1991) than transient expression (Pulchalski and Fahl, 1990).

4.1.1 Choice of expression vector and cell line.

In the type of experiment planned it is important to chose the right cell line. The cells must be able to take up aflatoxin B₁ and activate it to the genotoxic aflatoxin 8,9-epoxide. These criteria were fulfilled by BCMV1A2 cells (Mace *et al*, 1994). The cell line was constructed by immortalising Normal Human Bronchial Epithelium (NHBE) with SV40 T-antigen to form the cell line BEAS-2B, retaining many of the normal phenotypic characteristics (Mace *et al*, 1994). Once the cells were immortalised, they were stably transfected with the vector pCMV1A2 which contained the human cytochrome P450 1A2 cDNA. Cells containing plasmid, integrated into the genome, could be isolated using a Geneticin G418 resistance marker encoded by the plasmid and the resultant cell line was called BCMV1A2. BCMV1A2 gave active Cytochrome P450 1A2 protein and were 400-fold more sensitive to the cytotoxic affects of aflatoxin B₁ than the parent BEAS-2B cell lines (Mace *et al*, 1994) agreeing with other observations that CYP1A2 is the major enzyme in the activation of aflatoxin B₁ (Crespi *et al*, 1991, Eaton *et al*, 1995, Gallagher *et al*, 1994). DNA-aflatoxin adduct formation, measured with ³H-labelled aflatoxin, was also increased in the cell lines. So these cells were very suitable for testing how mutant GSTs could protect cells from aflatoxin toxicity. Their increased sensitivity to aflatoxin B₁ also meant that exposure to aflatoxin during the experiments could be kept as low as possible.

The choice of expression vector was more difficult. BCMV1A2 cells were already G418 resistant, so any mammalian expression vectors using this selection marker could not be used. The vector chosen was pSVZeo from Invitrogen. This vector drives transcription of heterologous cDNA using a SV40 Early Promoter and encodes a resistance protein for Zeocin (Calmels *et al*, 1991, Drocourt *et al*, 1990, Gatignol *et al*, 1988). It can be co-transfected with plasmids conferring G418 resistance and both vectors can be selected simultaneously by using Zeocin and G418. Hygromycin B resistance-based vectors were not considered because they are often an unreliable in exerting selection pressure in mammalian cells.

Zeocin was isolated from *Streptomyces verticillus* as a copper chelating glycopeptide and belongs to a family of structurally related bleomycin/phleomycin-type antibiotics from *Streptomyces*. Zeocin is more toxic than G418 and hygromycin B on mammalian cells with a selection range of 50 to 500 µg/ml Zeocin as compared with 500 to 1000µg/ml G418. Chelation of Cu²⁺ gives Zeocin a blue colour. The copper-chelated form is inactive but when the antibiotic enters the cell, the copper cation is

reduced from Cu^{2+} to Cu^+ and removed by sulphydryl groups. Removal of copper results in activation of Zeocin which then binds to DNA, cleaving it and causing cell death. The exact mechanism of action is not known, although it is thought to be the same as bleomycin and phleomycin due to its structural similarity to these drugs and its inhibition by the *Sh ble* resistance protein (Calmels *et al*, 1991, Drocourt *et al*, 1990, Gatignol *et al*, 1988).

The vector drives transcription of cloned cDNA from the SV40 early promoter and its associated enhancer elements giving strong constitutive expression but in a copy number independent, position dependent manner. The construction of the vector also means that there is great flexibility, it can either be used as a transient transfection vector or a stable transfection vector, depending on what is most appropriate. The configuration of the plasmid is shown in Fig 4.1.

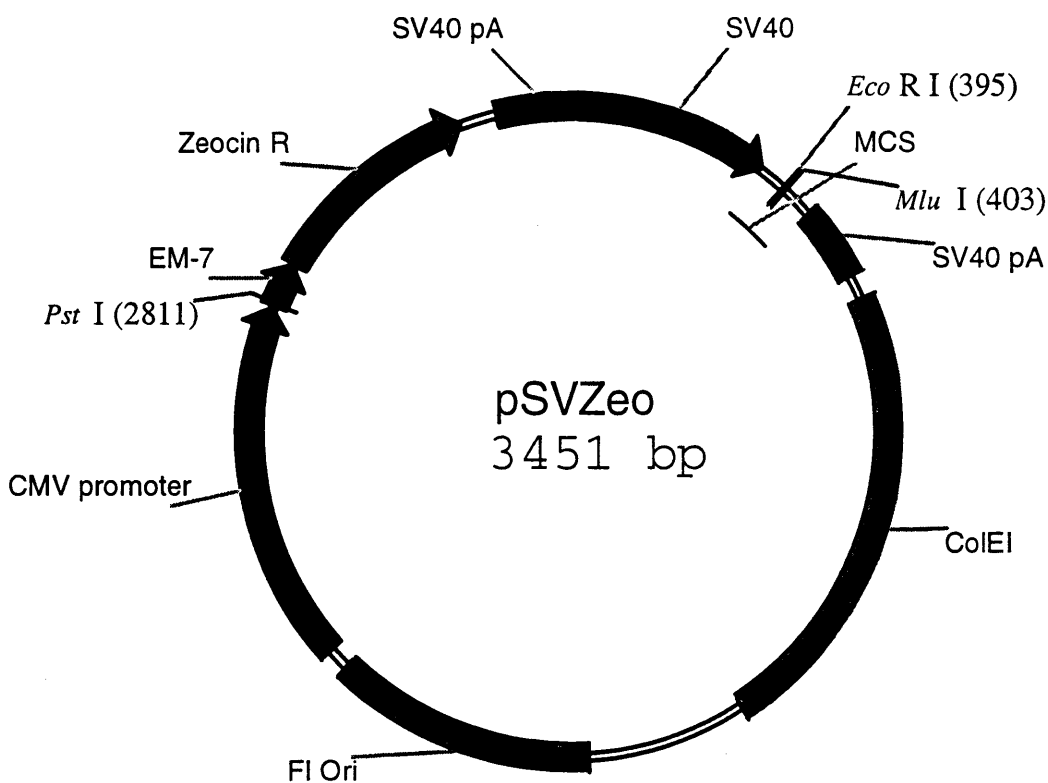


Fig 4.1. Diagram showing the configuration of the mammalian expression vector pSVZeo (Invitrogen).

4.2 Construction of stable cell lines with pSVZeo.

Construction of stable cell lines was the preferred way of co-expressing GSTs with P450 1A2 because, once the expression construct has been integrated into the genome, a homogeneous population cells can be generated by clonal expansion in which heterologous protein levels can be determined by quantitative Western blot. Moreover, the cell lines can be used repeatedly because the constructs can be maintained by continuing to grow the cells under selection with zeocin and G418.

In order to make cell lines stably transfected with pSVZeo, the expression vectors must be properly constructed and the tolerance of the native cell lines to Zeocin must also be determined. The concentration of antibiotic used during the selection after the introduction of the DNA is critical; if the concentration is too high then the resistance mechanisms on the plasmid will be overcome and if the concentration is too low then there will be the chance that cells not containing the construct of interest will survive the selection.

4.2.1 Construction of mammalian expression vectors for rGST.

In order to obtain optimum heterologous protein expression, the cDNA must be cloned downstream of the transcription start site of the SV40 early promoter and upstream of a polyadenylation site, maximising the chances that the cDNA will be transcribed and the mRNA will be stable inside the cell. However, efficient initiation of translation must also accompany transcription if adequate levels of enzymatically active protein are to be produced. Efficient translation initiation has been shown to rely partly on the sequence of six bases around the initiation codon (Kozak, 1987). Analysis of the non-coding sequence of highly expressed mRNAs revealed there is a conserved sequence of T(or C)GGTUCC (Kozak, 1987) around the methionine initiator codon, marked in bold. Codon usage at the 5' end of the transcript is also a consideration. Sub-optimal codon usage can lead to the formation of secondary structure, sequestering the translation initiation and inhibiting translation efficiency (Kozak, 1990). All these factors were taken into account when designing the cloning strategy for constructing the mammalian expression vectors.

PCR was used to lift out the parental wild-type and engineered double mutant cDNA sequences from the appropriate pET bacterial expression vectors. PCR allowed the correct restriction sites for directional cloning and a Kozak sequence to be introduced into the amplified genes by correct design of the primers. *Pfu* polymerase was used in all amplifications to minimise the chance of introducing extra mutations into the cDNAs.

4.0 Protection of Cultured Cells by Transfection with GST

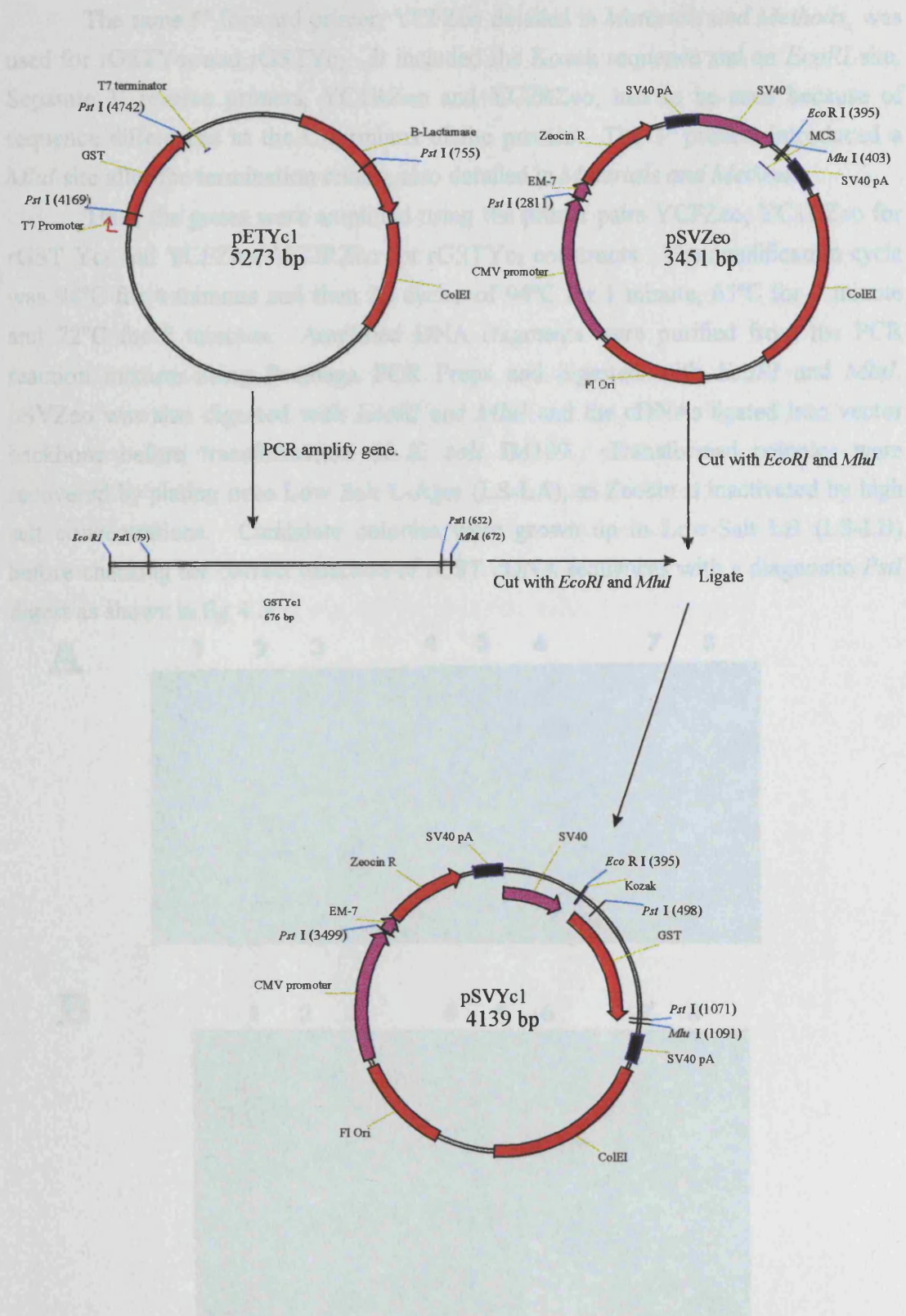


Fig 4.2. Diagram showing the strategy for constructing mammalian expression vectors containing rGST Yc₁. rGSTYc₂ constructs do not have a *Pst*I site at 498bp and only give 2 bands on digestion.

The same 5' forward primer, YCFZeo detailed in *Materials and Methods*, was used for rGSTYc₁ and rGSTYc₂. It included the Kozak sequence and an *EcoRI* site. Separate 3' reverse primers, YC1RZeo and YC2RZeo, had to be used because of sequence differences at the C-terminus of the protein. The 3' primers introduced a *MluI* site after the termination codon, also detailed in *Materials and Methods*.

Thus, the genes were amplified using the primer pairs YCFZeo; YC1RZeo for rGST Yc₁ and YCFZeo; YC2RZeo for rGSTYc₂ constructs. The amplification cycle was 94°C for 4 minutes and then 30 cycles of 94°C for 1 minute, 65°C for 1 minute and 72°C for 2 minutes. Amplified DNA fragments were purified from the PCR reaction mixture using Promega PCR Preps and digested with *EcoRI* and *MluI*. pSVZeo was also digested with *EcoRI* and *MluI* and the cDNAs ligated into vector backbone before transformation of *E. coli* JM109. Transformed colonies were recovered by plating onto Low Salt L-Agar (LS-LA), as Zeocin is inactivated by high salt concentrations. Candidate colonies were grown up in Low-Salt LB (LS-LB) before checking for correct insertion of rGST cDNA sequences with a diagnostic *PstI* digest as shown in fig 4.3.

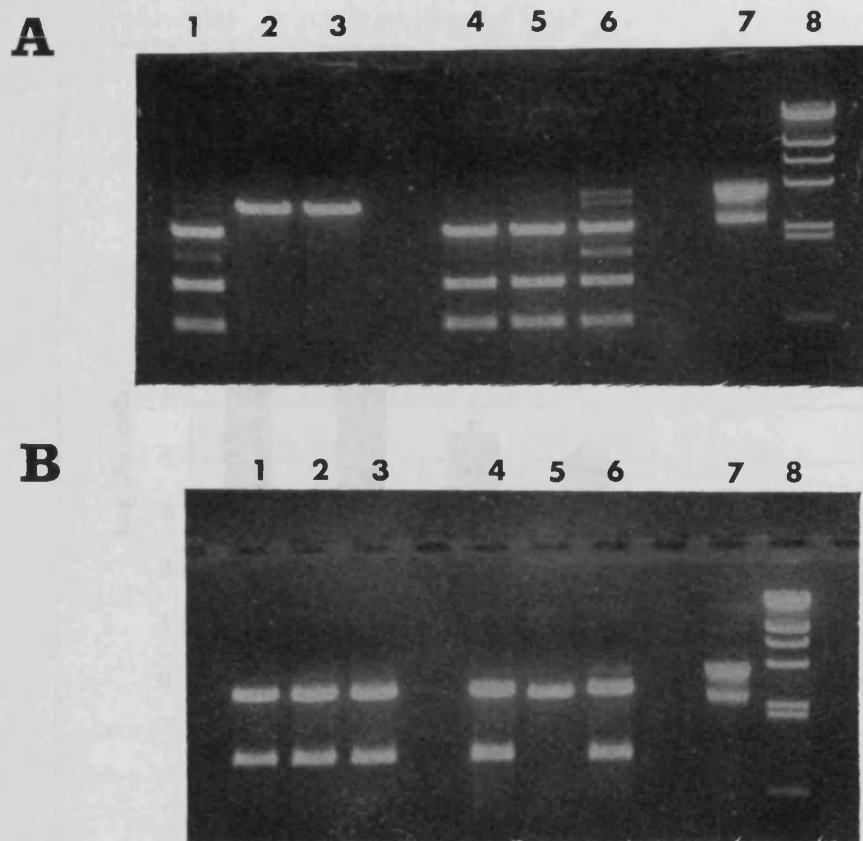


Fig 4.3 Construction of pSVGST mammalian expression vectors. **A.** 1-3 pSVYc₁ candidates' *PstI* digests. 4-6 pSVY108LD208M candidates' *PstI* digests. 7 uncut pSVZeo. 8 λ HindIII Mw markers. **B.** 1-3 pSVYc₂ candidate *PstI* digests. 4-6 pSVH108YE208D candidates' *PstI* digests. 7 uncut pSVZeo. 8 λ HindIII Mw markers.

4.2.2 Intrinsic Zeocin resistance in BCMV1A2 cells.

In order to exert the correct amount of selection pressure when constructing stably transfected cell lines, the lowest concentration of zeocin that will kill all the cells in 7 days must be determined. To accomplish this, 1×10^5 cells are plated into 2" diameter tissue-culture plates and LHC-9 growth medium containing various concentrations of zeocin are added. After 7 days, the cells were examined and the viable cells identified using trypan blue staining and counted using a haemocytometer. Invitrogen advise that the likely optimal zeocin concentration is usually between 50 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$.

However, using this range of concentrations with BCMV1A2 cells led to all the cells being killed after 7 days, showing that they were much more sensitive to the effects of zeocin than the cell lines tested by the company. By subsequent reductions of the concentration of zeocin used, concentrations of zeocin were found that were tolerated by the cells, with the cells tolerating 3.75 $\mu\text{g/ml}$ zeocin but not 5.00 $\mu\text{g/ml}$ zeocin as shown in fig 4.4. It was on the basis of these experiments that the a concentration of zeocin of 5.00 $\mu\text{g/ml}$ zeocin was chosen to isolate stable transfectants.

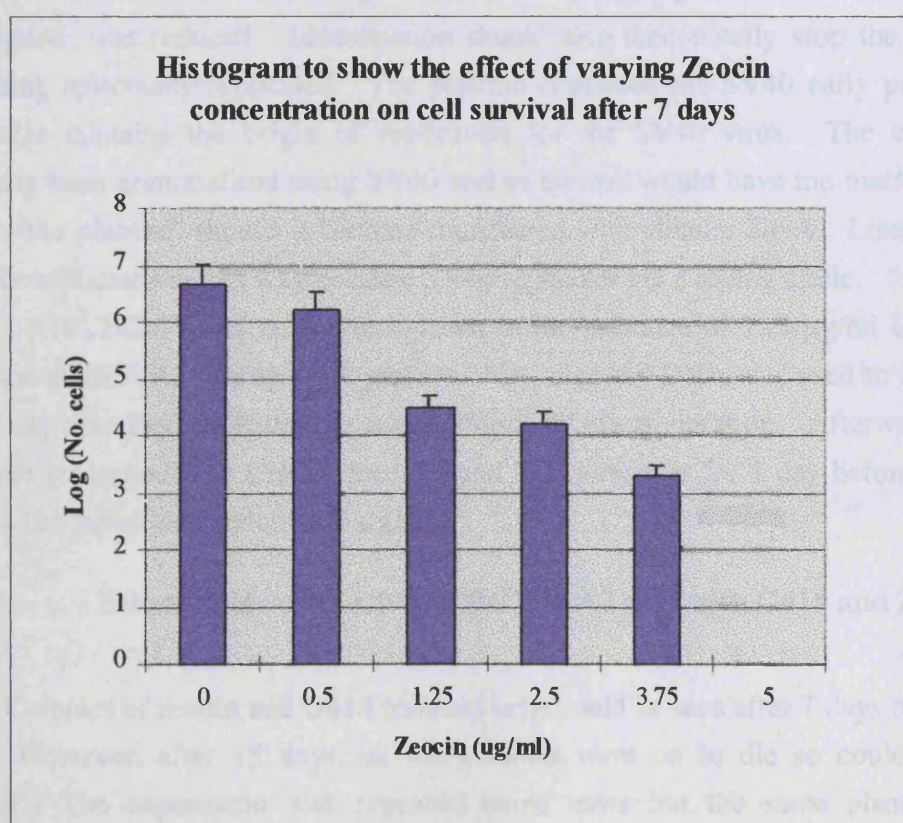


Fig 4.4. The results of the zeocin tolerance testing. BCMV1A2 cells survived zeocin at 3.75 $\mu\text{g/ml}$ but not at 5.00 $\mu\text{g/ml}$.

4.2.3 Transfection of BCMV1A2 cells with pSVZeo GST constructs.

After determination of the concentration of zeocin to be used the introduction of the plasmid DNA to the cells could be tackled. Large amounts of plasmid DNA are used during transfection and large scale plasmid preparations from 250ml LS-LB cultures of *E.coli* JM109 transformed with the plasmids were needed. The best results were obtained using Wizard Maxi-prep kits (Promega). The DNA was run on a 1% agarose gel to check that there was no RNA contamination and the concentration of the DNA determined by measuring the absorbance at 260nm if no RNA contamination was seen.

25µg of plasmid DNA were digested for 24 hours with *NotI* which was a unique restriction site outside of the coding regions, polyadenylation signal sites and the promoter/enhancers of both the zeocin resistance marker and the GST gene. Complete linearisation was checked for by running aliquots of the digest on a 1% agarose gel. The constructs were linearised because free DNA ends are more recombinogenic than circular DNA. Thus, the chance of integration was increased and the risk of recombination occurring in the GST coding region, which will interrupt transcription, was reduced. Linearisation should also theoretically stop the plasmid from being episomally replicated. The plasmid contained the SV40 early promoter, which also contains the origin of replication for the SV40 virus. The cells had previously been immortalised using SV40 and so the cell would have the machinery to replicate the plasmid, should it become transfected with circular DNA. Linear DNA cannot be replicated in this way because SV40 replicates via a rolling circle.

1 x 10⁷ BCMV1A2 cells were grown in the presence of 125 µg/ml G418, to ensure the pCMV1A2 plasmid was present. *NotI* digested DNA was used to transfect the cells as described in *Materials and Methods* by electroporation. Afterwards, the cells were resuspended in LHC-9 medium and left to recover for 1 day before adding G418 at 125 µg/ml and zeocin at 5 µg/ml.

4.2.4 Selection of co-transfected BCMV1A2 cells with G418 and Zeocin.

Colonies of zeocin and G418 resistant cells could be seen after 7 days on all the plates. However, after 15 days, all the colonies went on to die so could not be analysed. The experiment was repeated twice more but the same phenomenon occurred each time. It was clear that electroporation was working because, based on the tolerance experiments, all the untransfected cells should be dead 7 days post-selection but some colonies were surviving between 10 and 14 days after the antibiotics had been added, indeed the control where no pSV construct had been added all went on to die after 10 days. This showed that the cells were showing some

expression of the zeocin resistance protein which could only come from transfected DNA.

It seemed more likely that the DNA was not stable once it was inside the cell. The length of survival of the cells was consistent with the survival of cells following a transient transfection, which is roughly 14 days. Transiently transfected cells cannot survive much longer because the episomal, SV40-based replication of the plasmid produces hundreds of copies that places the cell under increasing metabolic strain as the copy number of the plasmid increases. However, this should not be happening in these experiments because the careful linearisation of the construct with *NotI* should interrupt any episomal maintenance. After consulting with the suppliers, Invitrogen, they advised me that they had seen these results when they attempted to isolate stable transfectants from cell-lines that had been immortalised with SV40. Further investigation by them showed that the cells were able to ligate the linearised plasmid back together to form circles once selection pressure had been added. This meant that the plasmids were then replicated episomally. Removal of the SV40 origin of replication by restriction and re-ligation could not be carried out because the origin is found in the same DNA as the promoter and enhancer region; removing the origin also meant that the promoter that drove GST expression would also have to be removed.

The alternatives were to construct new, hygromycin B based expression vectors or to use the episomal replication that was likely to happen if BCMV1A2 cells were transfected with the existing expression vectors constructs. Time considerations in the project meant the construction of new expression vectors was not practical. The only drawbacks to using a transient transfection method were that it would result in a heterogeneous cell population, with cells that had been transfected with the GST constructs being indistinguishable from the those that had not, and that the cells would have to be transfected each time an experiment needed to be done.

4.3 Transient transfection of BCMV1A2 with pSVGST constructs.

As mentioned, in order for the transient transfection approach to work, cells expressing GST had to be distinguished from cells that did not. Analysis of mixed cell populations would mean that it would be impossible to say whether GST transfection led to increased protection from aflatoxin.

When mammalian cells are transfected, they take up large amounts of DNA from the environment and so can be easily transfected with two constructs simultaneously (Angelotti *et al*, 1993). Co-transfection in this manner with a pSVGST construct and a reporter gene could mean that the expression of an easily identifiable gene activity would coincide with the expression of a particular GST, providing that they are both given constitutive protein expression once inside the cell.

Transfection of mammalian cells is not very efficient. Therefore, co-transfection of pSVGST constructs with a β -galactosidase reporter or Luciferase construct would mean that many cells would have to be harvested and assayed to determine levels of reporter activity in the population. Also, the cells have to be ruptured to release the activity for assay and so cannot be used again. These assays would have to be completed after transfection and after treatment with aflatoxin B₁. They would be unlikely to work because the small amount of activity and the small number of cells involved would lead to large inaccuracies in the results. The activities would also have to be calibrated to transfection efficiencies and would lead to further inaccuracies.

What was needed was a reporter activity that could identify individual cells that had been transfected, avoiding transfection/activity calibrations. Ideally, the reporter gene activity should also be identified without the need for rupturing the cells. In this way, the number of cells transfected could be determined directly and the same population of cells could then be used to see how they responded to aflatoxin B₁. The reporter gene that satisfied all these criteria was that encoding Green Fluorescent Protein (GFP).

4.3.1 Green Fluorescent Protein.

Light is produced in several bioluminescent species as a result of energy transfer to Green Fluorescent Proteins (GFPs). The GFP from *Aequorea victoria* has been characterised (Shimomura *et al*, 1962), cloned (Prasher *et al*, 1992) and heterologously expressed (Chalfie *et al*, 1994). The protein fluoresces after receiving energy from a Ca²⁺ activated photoprotein aequorin *in vivo* and behaves similarly *in vitro*, absorbing blue light (λ_{max} =395nm, minor peak at 470nm) and emitting green light (λ_{max} =509nm). These spectral characteristics, particularly the minor absorption peak at 470nm, mean that its fluorescent characteristics are similar to fluorescein and can be detected using fluorescence microscopy, an ultra-violet light box or fluorescence activated cell sorting (FACS).

The advantage of using GFP as a reporter gene is that GFP fluorescence does not need any additional gene products or co-factors from *Aequorea victoria* and can be functionally expressed in both prokaryotic and eukaryotic systems. GFP has been successfully used as a reporter gene in yeast, mammalian cells, plants and living *Drosophila melanogaster* (Chalfie *et al*, 1994, Kain *et al*, 1995, Plautz *et al*, 1996) where protein expression could be monitored and localised to specific sub-cellular compartments by irradiating the cells with blue light.

Identification of transfected cells by co-transfecting expression constructs with GFP has not yet been reported but should have advantages over the method of Angelotti and co-workers who used fluorescein di- β -galactopyranoside substrate to see cells transfected with β -galactosidase (Angelotti *et al*, 1993). Using GFP as a transfection marker would mean that FACS could be used to analyse cell populations from individual transfections, specifically monitoring how transfected cells reacted to incubation with aflatoxin B₁. FACS has been used previously to identify individual cells transfected with GSTs (Pulchalski and Fahl, 1990), using a fluorescent GST substrate monochlorobimane but as cells have endogenous GST activity, the sensitivity would be reduced by a large background. Provided GFP fluorescence can be associated with GST after co-transfection, then it would prove much more sensitive.

However, wild type GFP is not suitable for these types of experiment. FACS machines are fitted with fluoroscein filters (488nm excitation and 509nm emission) and GFP fluorescence is not intense enough at these wavelengths for efficient sorting because of sub-optimal excitation of the minor absorption peak of GFP at 470nm. This has been overcome by understanding the biochemistry of the protein and its unique chromophore.

4.3.2 Engineering the GFP chromophore for use in FACS.

Green-fluorescent protein is an 11-stranded β -barrel wrapped around a central helix and forms a nearly perfect cylinder 42Å long and 24Å in diameter (Ormo *et al*, 1996) see fig 4.5. The chromophore sits right in the middle of the barrel and is protected from the bulk solvent which allows it to fluoresce. The chromophore is produced post-translationally by the peptide sequence alone. The only requirement for the formation of the chromophore is molecular oxygen (Heim *et al*, 1994), implying that oxidation of part of the peptide sequence was responsible. The peptide sequence had been previously been determined as Ser65, Thr66, Gly67 from papain digests of the protein (Cody *et al*, 1993) which on cyclisation gives the chromophore *para*-hydroxybenzylideneimidazolidinone, as shown in fig 4.6.

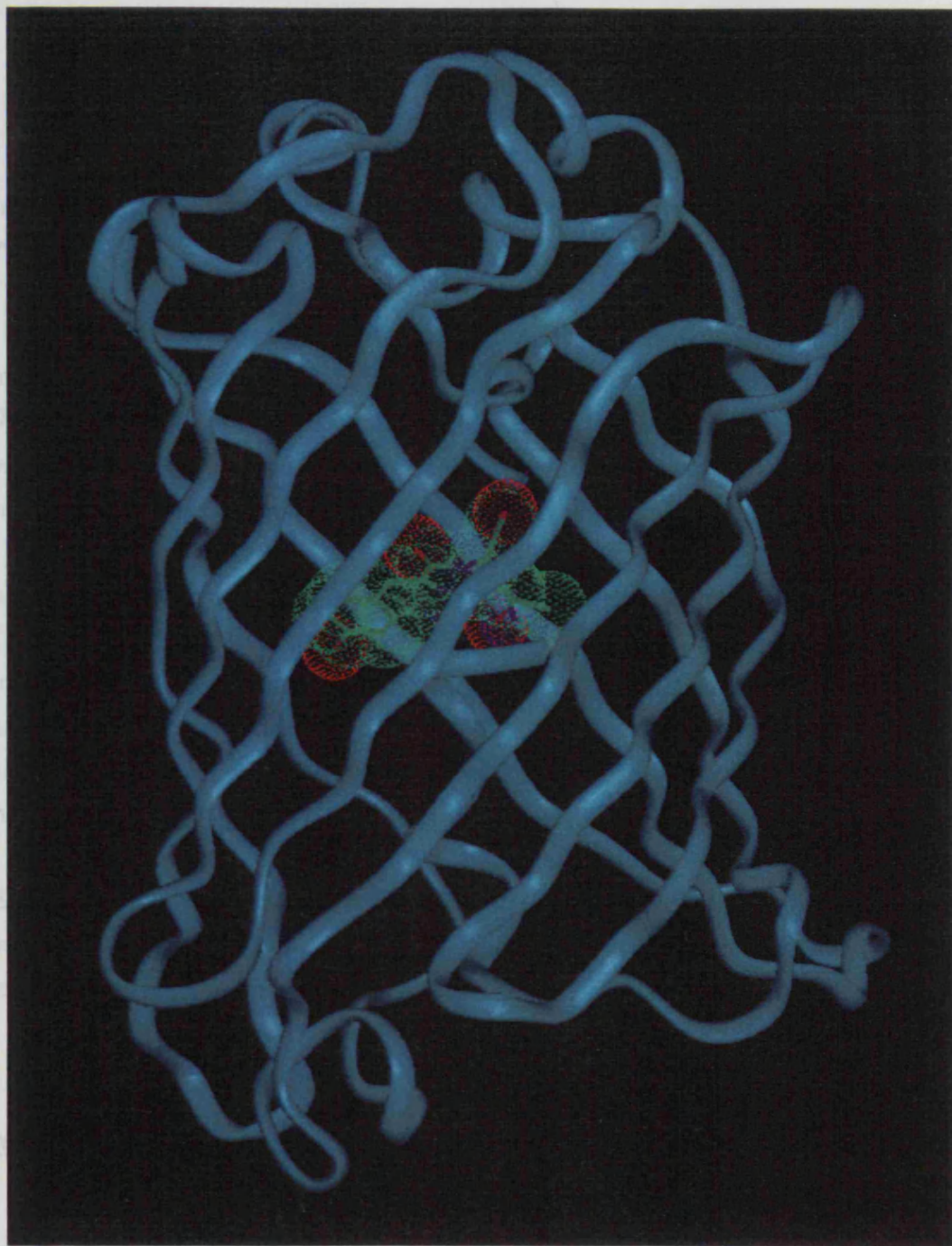
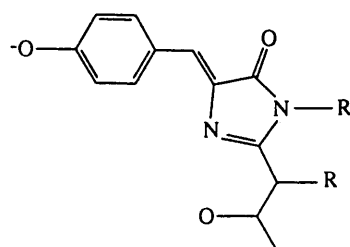


Fig 4.5 The three dimensional structure of Green Fluorescent Protein from *Aequorea victoria* (Ormo, et al., 1996). The almost perfectly cylindrical β -barrel is clearly seen. The chromophore is highlighted inside, protected from bulk solvent by the protein cage.



p-hydroxybenzylideneimidazolidinone.

Fig 4.6. Structure of the chromophore of GFP produced by oxidative residue cyclisation (Ormo *et al*, 1996).

With the chromophore structure determined, numerous random mutagenesis strategies were reported that gave altered fluorescence characteristics (Ehrig *et al*, 1995, Heim *et al*, 1994, Ormo *et al*, 1996). All reports showed that mutations were found either in the chromophore region, directly flanking the chromophore peptide sequence or in residues involved in electrostatic interactions with the chromophore.

Success in altering the protein's fluorescence gave rise to a random mutagenesis strategy to isolate mutants that could be used more efficiently in FACS (Cormack *et al*, 1996). Libraries of random mutants were expressed in *E.coli* and run through the FACS scanner. Cells with enhanced fluorescence were isolated and analysed. One clone isolated, GFPmut1, had an absorbance maximum which had been shifted to 488nm and consequently fluoresced 100-fold more intensely during FACS. The mutations were F64L and S65T (Cormack *et al*, 1996).

Throughout the experiments detailed in this chapter with GFP, the construct pEGFP was used. This construct contained the GFP cDNA with the mutations F64L and S65T and also more than 190 silent mutations that correspond to human codon preferences so that there was optimal expression levels in human cell-lines.

4.3.3 Correlating GFP fluorescence with GST expression.

(The FACS analysis in this chapter was kindly carried out by Dr R. Snowdon, MRC Toxicology Unit, University of Leicester, Lancaster Rd, Leicester.)

In order for the experimental design to be valid, co-transfection had to lead to co-expression of FACS-optimised GFP and GST. To demonstrate this, BCMV1A2 cells were co-transfected with 25 µg amounts of pEGFP and pSVYc₂ by electroporation, the populations expressing GFP were identified and isolated from the non-fluorescent cells using FACS. The resultant populations of cells could then be analysed by Western blotting total cell protein to see if there was any co-localisation of

GST with GFP. Highly specific monoclonal antibodies had been made to rGST Yc₂ by Dr. L. Weir and they could be used to identify recombinant protein expression in mammalian cells.

10⁷ BCMV1A2 cells were transfected with both of these constructs, the cells were left for 3 days to recover and express the proteins of interest. After 1 day the cells were examined under a confocal microscope with the filters set at 488nm excitation and 509nm emission. The transfected cells were easily identified by the green fluorescence produced by GFP and the transformation efficiency could be estimated at around 30%. Illustrations of the cells under phase contrast and fluorescence can be seen in fig 4.6, showing that individual, transfected cells can be easily identified. The fluorescence also showed that the electroporation protocol was working well and could be definitely ruled-out as a reason for failure of the stable transfection experiments.

The transfected cells were then treated with trypsin/EDTA to remove them from the extra-cellular matrix, concentrated by centrifugation and resuspended in 1ml of LHC-9 growth medium and sorted into fluorescent and non-fluorescent populations by FACS. The FACS optimised mutants (Cormack *et al*, 1996) worked well and the cells could easily be identified. However, because electroporation kills a great number of cells during the application of voltage across them, the number of cells that were finally sorted was quite low. A total of 1,054,107 cells were sorted. 362,664 cells were fluorescent and 691,443 cells were not fluorescent, giving a determined transfection efficiency of 34.4%.

Both populations of cells were collected by centrifugation in a bench-top microcentrifuge and resuspended in 60 µl of SDS-PAGE Loading Buffer before being boiled for 5 minutes to release the protein. The number of cells recovered was quite low, so to make sure that the total cellular protein could be detected on the gel, 30µl of the fluorescent cells were loaded on the gel and 16µl of the non-fluorescent cell population was loaded to keep the concentration of loaded protein similar. Duplicate gels were run and coomassie blue stained to see the concentration of total cell protein loaded.

The gels were Western blotted as described in *Materials and Methods* and the antigen detected with ECL kit (Amersham). The ECL kit is highly sensitive and is the method of choice when blotting small amounts of protein. The protein concentration on the membrane was quite low and the filter had to be developed for 50 minutes for the bands to be seen.

A band was detected in the Western blot (fig 4.7) for the fluorescent cells corresponding to the molecular weight of rGSTYc₂ which was absent in the non-fluorescent cells. This proved that GFP fluorescence could be used as a marker for

GST expression in co-transfected cells and that transient transfection of BCMV1A2 cells with the expression construct could give full length, antigenic rGSTYc₂ protein

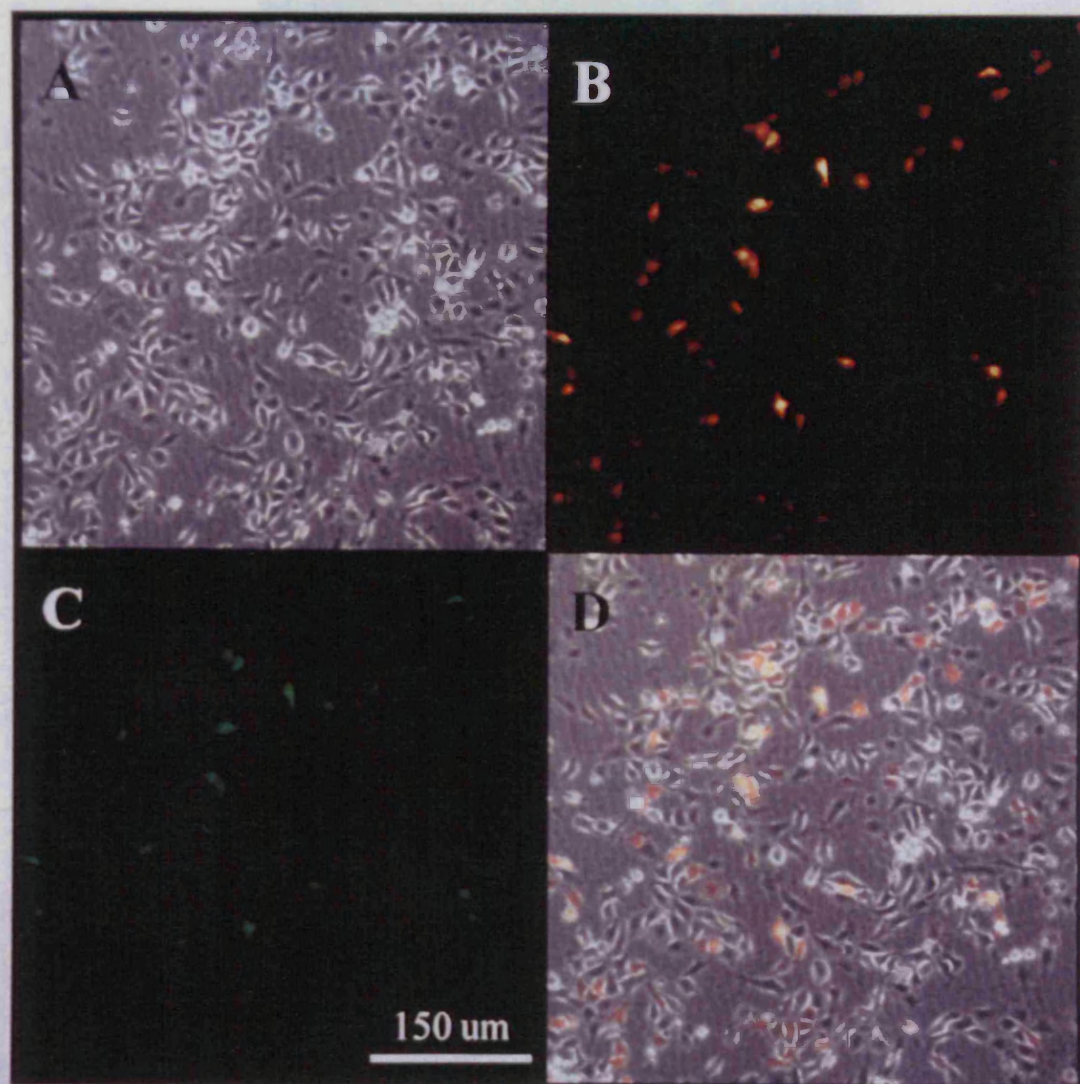


Fig 4.6 Illustrations of transfected cells. **A** BCMV1A2 cells transfected as viewed with phase contrast in a confocal microscope, scale included. **B** The same population cells viewed under fluorescence confocal microscopy, excitation at 488nm and emission at 590nm. While the cells appear a luminous green colour to the eye, the cells are shown here are artificially coloured to increase the contrast of the print. **C** Fluorescent cells as seen without enhancement. **D** Phase contrast and fluorescence images overlaid, allowing individual cells to be identified.

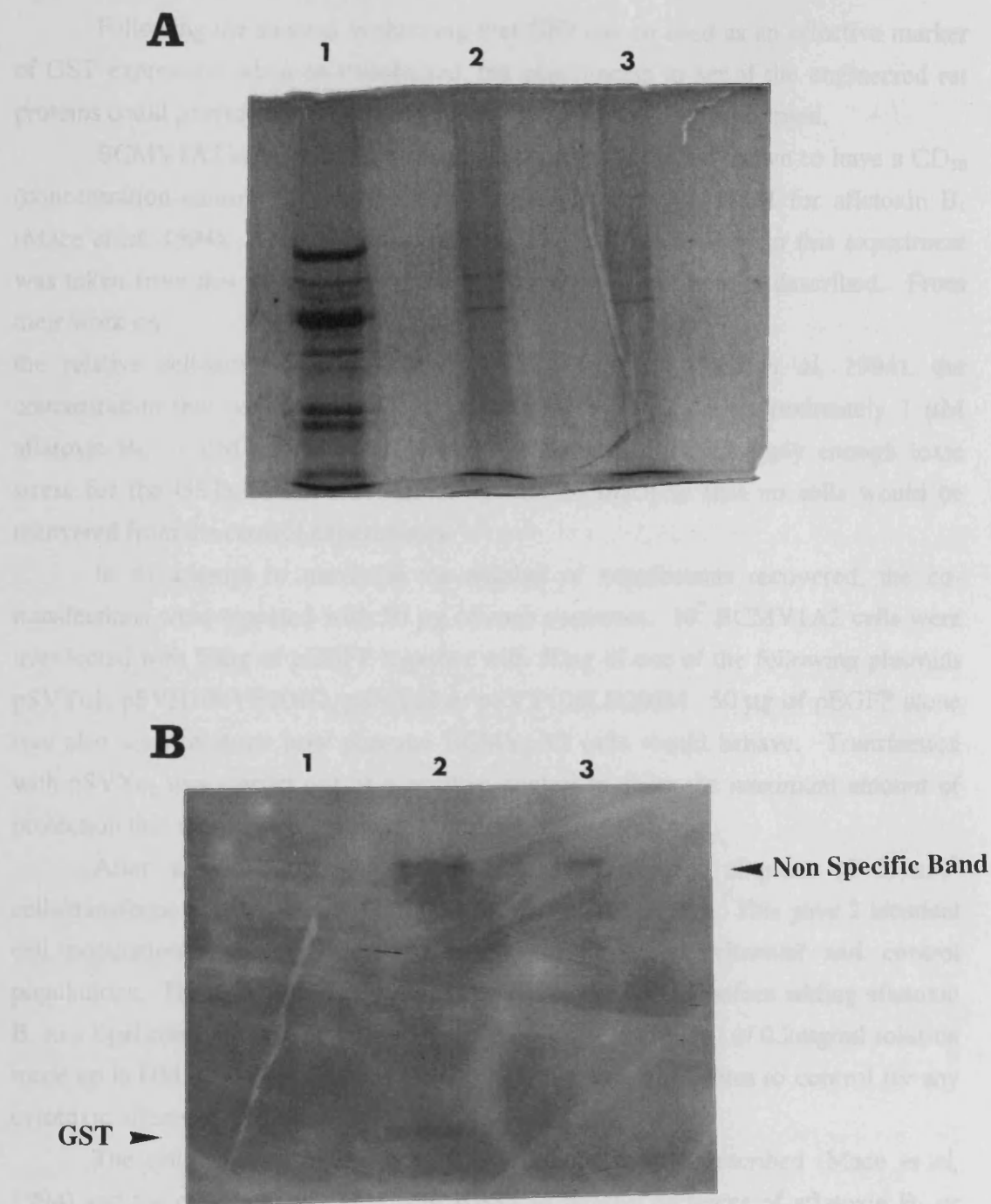


Fig 4.7 **A** Western blot to show that GST expression is associated only with fluorescent cells. **B** A duplicate coomassie blue stained gel is also shown for comparison. The antigen was detected using ECL (Amersham). **Lane 1** Molecular weight standards. **Lane 2** Fluorescent cells' total protein. **Lane 3** Non fluorescent cells' total protein.

4.3.4 Co-transfection and protection of cells from aflatoxin B₁.

Following the success in showing that GFP can be used as an effective marker of GST expression when co-transfected, the experiments to see if the engineered rat proteins could provide any protection against aflatoxin B₁ were attempted.

BCMV1A2 cells have been characterised previously and shown to have a CD₅₀ (concentration causing 50% cytotoxicity after 7-10 days) of 19nM for aflatoxin B₁ (Mace *et al*, 1994). The concentration of aflatoxin B₁ to be used in this experiment was taken from this paper because the exact cell line used here is described. From their work on

the relative cell-survival as a function of aflatoxin B₁ (Mace *et al*, 1994), the concentration that would be expected to give 90% toxicity is approximately 1 μ M aflatoxin B₁. 1 μ M aflatoxin B₁ was chosen because it would apply enough toxic stress for the GSTs to show an affect but not so much so that no cells would be recovered from the control experiments.

In an attempt to maximise the number of transfectants recovered, the co-transfections were repeated with 50 μ g of each construct. 10⁷ BCMV1A2 cells were transfected with 50 μ g of pEGFP together with 50 μ g of one of the following plasmids pSVYc1, pSVH108YE208D, pSVYc2 or pSVY108LD208M. 50 μ g of pEGFP alone was also used to show how parental BCMV1A2 cells would behave. Transfection with pSVYc₂ was carried out as a positive control to show the maximum amount of protection that should be possible.

After electroporation, the cells were split into 2 aliquots of 5 x 10⁶ cells/transfection before plating out into LHC-9 growth medium. This gave 2 identical cell populations from each co-transfection to act as experimental and control populations. The cells were left for 1 day to recover in LHC-9 before adding aflatoxin B₁ to a final concentration of 1 μ M to the experimental plates (3 μ l of 0.2mg/ml solution made up in DMSO). 3 μ l of DMSO was added to the control plates to control for any cytotoxic affects of DMSO.

The cells were incubated for 28 hours as previously described (Mace *et al*, 1994) and the cells washed twice with HBSS to remove all traces of aflatoxin B₁ or DMSO before replacing with fresh growth medium without aflatoxin. They were left for a further 3 days before evaluation. Mace and co-workers left them for 7-10 days before analysis but the cells appeared to be more sensitive to aflatoxin B₁ in my hands. Leaving them for 7 days resulted in 100% cytotoxicity.

The cells were then detached from the tissue culture plates by treating with trypsin/EDTA, concentrated by centrifugation and resuspended in 2mls LHC-9 before the control and experimental populations were analysed. FACS analysis gave an

accurate determination of the percentage of fluorescent cells in the experimental and control populations whereas the cell counter gave an accurate determination of the total number of cells in each population.

4.3.5 Results of protection studies of BCMV1A2 transfected with GSTs from aflatoxin B₁.

The results are collated in table 4.1. and show the percentage of cells that were identified as fluorescent during FACS analysis for both the aflatoxin B₁ treated and DMSO alone treated cells (% Fluorescent). The results also show the total number of cells surviving in each of the populations. Using these data together, it is possible to determine the number of cells that were likely to be transfected with GST expression vectors in each of the population, denoted as No. cells transfected. With the number of transfected cells determined for populations treated with aflatoxin B₁ and DMSO alone, it is possible to calculate the percentage of transfected cells surviving the aflatoxin B₁ toxic insult and to calculate the relative enrichment for each of transfections.

	rGSTYc1	rGSTYc2	H108YE208D	Y108LD208M	GFP
% Fluorescent (AFB+)	38	42	34	31	24
% Fluorescent (AFB-)	56	56	49	51	28
No. cells counted (AFB+)	148000	175000	161000	157000	137000
No. cells counted (AFB-)	1836000	1860000	1848000	2079000	1950000
No. cells transfected (AFB+)	56240	73500	48300	48670	32880
No. cells transfected (AFB-)	1028160	1041600	1177764	1060290	546000
% surviving treatment	5.19	6.59	5.70	4.39	5.68
Relative Enrichment*	0.913	1.160	1.004	0.773	1

Table 4.1. Table to show the results from FACS analysis of BCMV1A2 cells transfected with GST constructs and treated with aflatoxin. * Enrichment is shown relative to the negative control, GFP alone survival rates.

At first glance the FACS analysis results were quite surprising. If the experiment was to show that GST expression was to afford some protection against aflatoxin B₁, then it could be expected that there should be an increase in the percentage of fluorescent cells in the aflatoxin B₁ treated population as the cells expressing GST are selected for. As can be seen from the table, this is not what was

seen. In fact, there is a reduction in the percentage of fluorescent cells upon treatment with aflatoxin B₁.

The decrease in the number of fluorescent cells seems, in part, to be a function of treatment with aflatoxin B₁. The reduction in the percentage of GFP alone cells from 28% to 24% on treatment with aflatoxin B₁ leads to this conclusion. The reduction in fluorescent cells may occur because large amounts of aflatoxin 8,9-epoxide is produced in these cells generating a great potential for DNA adduct formation. The transfected plasmids are likely to have a high copy number because of SV40 episomal replication and so they will be very effective targets for aflatoxin 8,9-epoxide binding. Any adducts formed in the coding region of GFP will interrupt transcription and affect expression-levels in the cells leading to a decrease in the numbers of highly fluorescent cells the FACS machine needs for efficient sorting. However, a decrease in the proportion of fluorescent cells did not mean that the experiment was unable to show any evidence of protection.

If the numbers of transfected cells are examined, there were many more cells surviving as a result of transfection with pSVYc₂ than for any other construct. Survival was expressed as a percentage to ease comparison between experiments and showed that while the proportion of survivors was generally low <7%, it corresponded loosely with the previously determined specific activities for the purified recombinant proteins.

The low amount of surviving cells is most likely due to the increased sensitivity of the cells to aflatoxin B₁ than first characterised (Mace *et al*, 1994). There is a distinct possibility that there was some aflatoxin B₁ induced damage to the expression constructs and expressed protein which would reduce levels of transcription and active protein. This could, in turn, reduce the effectiveness of the transfection. It is also not known whether these cells express the right kind of export pumps to excrete any AFB-GSH conjugate that was formed. Accumulation of conjugate in the cytoplasm could lead to some product inhibition resulting in more aflatoxin 8,9-epoxide being formed than can be dealt with by GSTs. The expression levels of cytochrome P450 1A2 and GST are going to be high inside the cell which could lead to saturation of any relevant pump mechanisms that were present. Increased GST activity could also deplete of intra-cellular stores of glutathione reducing the cells' ability to effectively control intracellular redox potentials.

This kind of metabolic loading is suggested when the enrichment for transfected cells is measured relative to the results seen with the GFP alone control. The most active protein, rGST Yc₂ shows increased survival by a factor of 1.16 but the less active proteins such as rGST Yc₁ and the knock-out double mutant Y108LD208M have survival factors of 0.913 and 0.773 relative to GFP alone. This suggests that protein expression from these constructs was detrimental to cells when

under aflatoxin B₁ induced stress. The most likely explanation for this is the increasing metabolic load the cells were placed under in order to replicate the plasmids, transcribe the cDNA and translate the mRNA which ultimately led to accumulation of protein that could not metabolise aflatoxin 8,9-epoxide. Consequently, the cells did not survive as well as the parental cell line.

With a relative survival factor of 1.004, expression of the engineered rGST Yc₁ protein resulted in a cell line that did not show any measurable increase in survival against aflatoxin B₁. The increased activity seen in purified, recombinant protein *in vitro* was reflected in tissue culture because cells expressing E208DH108Y performed better than cells transfected with essentially inactive Glutathione S-Transferase.

4.4 **Future work.**

The results presented here could be further improved by work to make suitable stable cell lines, as originally attempted. To do this, a CMV transcription promoter could be cloned in place of the SV40 promoter. This would put in place another strong eukaryotic transcription promoter but would remove any SV40 origin of replication. The zeocin resistance marker could still be used to isolate clones, which would be more effective than cloning all the mutated cDNAs into a hygromycin B expression vector. This should overcome the difficulties encountered in isolating stable cell lines.

With stable transfected cell lines identified, the expression levels of heterologous GST in the cells could be properly analysed. With transient transfection experiments, it is assumed that the expression levels of GST is equal in each of the cell populations. While this is a fair assumption, it cannot be easily proved. Characterisation of stable cell lines by quantitative Western blot and by using the fluorescent substrate, monochlorobimane, (Pulchalski and Fahl, 1990) would give accurate determinations of the GST activity for each of the constructs and allow for standardisation between stable transfectant populations isolated.

The main advantage of characterised stable cell lines would be that time and material consuming transient transfections would not have to be repeated every time data was required. CD₅₀ values could be determined for each of the cell lines easily and these values could give a more accurate idea of how expression of individual engineered protein was changing cellular resistance to aflatoxin B₁ (Manohoran *et al*, 1987, Nakagawa *et al*, 1990, Schechter *et al*, 1993).

4.0 Protection of Cultured Cells by Transfection with GST

Analysis of the cytosol of aflatoxin B₁-treated cells could also be carried out. Measurements of intracellular glutathione concentrations and HPLC could be used to see how much conjugation was occurring and whether this correlated with survival.

Chapter 5

The C-terminal Helix of HuGSTA1-1

5.0 THE C-TERMINAL HELIX OF huGSTA1-1

5.1 Introduction.

In the superfamily of GSTs, alpha-class enzymes are unique in that, when bound to S-benzylglutathione, the C-terminal helix forms an ordered alpha-helix (Sinning *et al*, 1993). However, the exact nature of this structural element has been thrown into doubt because of the publication of the apo-protein huGSTA1-1 structure (Cameron *et al*, 1995). The electron densities for the residues in this helix were missing, although some could be detected at low contour levels, suggesting that the helix was disordered until the enzyme was bound to an inhibitor. Deletion studies have shown that the, while the helix is important, it is not essential for catalysis (Board and Mannervik, 1991).

In this chapter, the aim of the work was to examine the nature of the helix using mutagenesis, kinetics and NMR. The chapter also describes more fully how the helix may participate in catalysis. Oligonucleotide-directed mutagenesis is used to delete the helix and leave a truncated protein with no unwanted, secondary mutations as was seen using a restriction-site based method (Board and Mannervik, 1991). By adding a helix-sequence synthetic peptide to the truncated protein and comparing kinetic constants determined in the presence and absence of peptide, it is possible to propose a kinetic model for the action of the helix.

5.2 Solution NMR studies on the C-terminal helix.

This part of the work was done in collaboration with Dr. L-Y Lian and Ms C. Allardyce, NMR Centre, University of Leicester, MSB, University of Leicester, Leicester, who carried out the NMR spectroscopy.

5.2.1 ¹⁵N-labelled phenylalanine resonances in wild-type huGSTA1-1.

In the NMR studies of huGSTA1-1 in our laboratory we selectively labelled huGSTA1-1 using amide ¹⁵N-labelled amino acids and ¹H/¹⁵N Heteronuclear Multiple Quantum Correlation (HMQC) experiments to detect the amide resonances. By comparing the spectra of the protein in the free and the complexed form, it is possible to deduce the sites of interaction and conformational change due to ligand complexation. In the current studies, the ligand of interest is 1,3-(dinitro)-phenol glutathione (DNP-GSH), a product inhibitor. Among the most interesting

observations was that only 8 of the expected 10 resonances from ^{15}N -labelled phenylalanine could be seen in the apo-protein. However, on addition of DNP-GSH, all the resonances could be observed. This observation suggested that the signals from two phenylalanines were too broad to be detected in the apo-protein. The spectra are shown in fig 5.2.

There are two factors to consider when trying to establish why the phenylalanine ^{15}N signals are broader in the apo-protein spectrum. Firstly, as the experiment was carried out in aqueous solvent, the amide protons of the residues may be in chemical exchange with the solvent, as shown in fig 5.1.

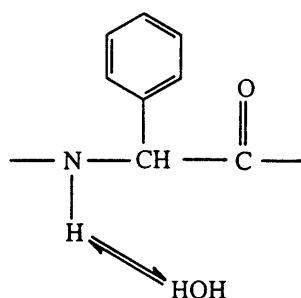


Fig 5.1. Chemical exchange between the amide group of phenylalanine and solvent.

This can lead to attenuation of the signal because the spectra were acquired using a water pre-saturation pulse to suppress the large water resonance in the spectrum. The method uses a continuous wave of radio frequency pulses to flip the spins of the water protons in the magnetic field such that they have no component of magnetisation in the direction of the detector at the end of the experiment, removing the water signal from the spectrum. Therefore, if the protons of interest are in rapid exchange with the solvent, then their resonances can be effectively removed from the spectrum along with the water signal.

The second factor is that the region of the protein that contains the phenylalanine residues could be in conformational exchange. If the protein structure visits several different environments and the interconversion between those states is slow, the chemical shifts of residues in the region will reflect those environments resulting in increasing line-width of the signal. The *total* signal intensity will not change but now has to be summed over the entire range of the chemical shifts that residues could take. Alternatively, if there are only two states, the lines can be markedly broadened if the rate of interconversion is comparable to the difference in chemical shifts between the two states. These factors when taken together can result in broadening of a signal to such an extent as to make it undetectable.

To discriminate between the two models for the undetectable phenylalanine resonances, a different method of water signal suppression was used called non-excitation. In this method, instead of removing any magnetisation associated with

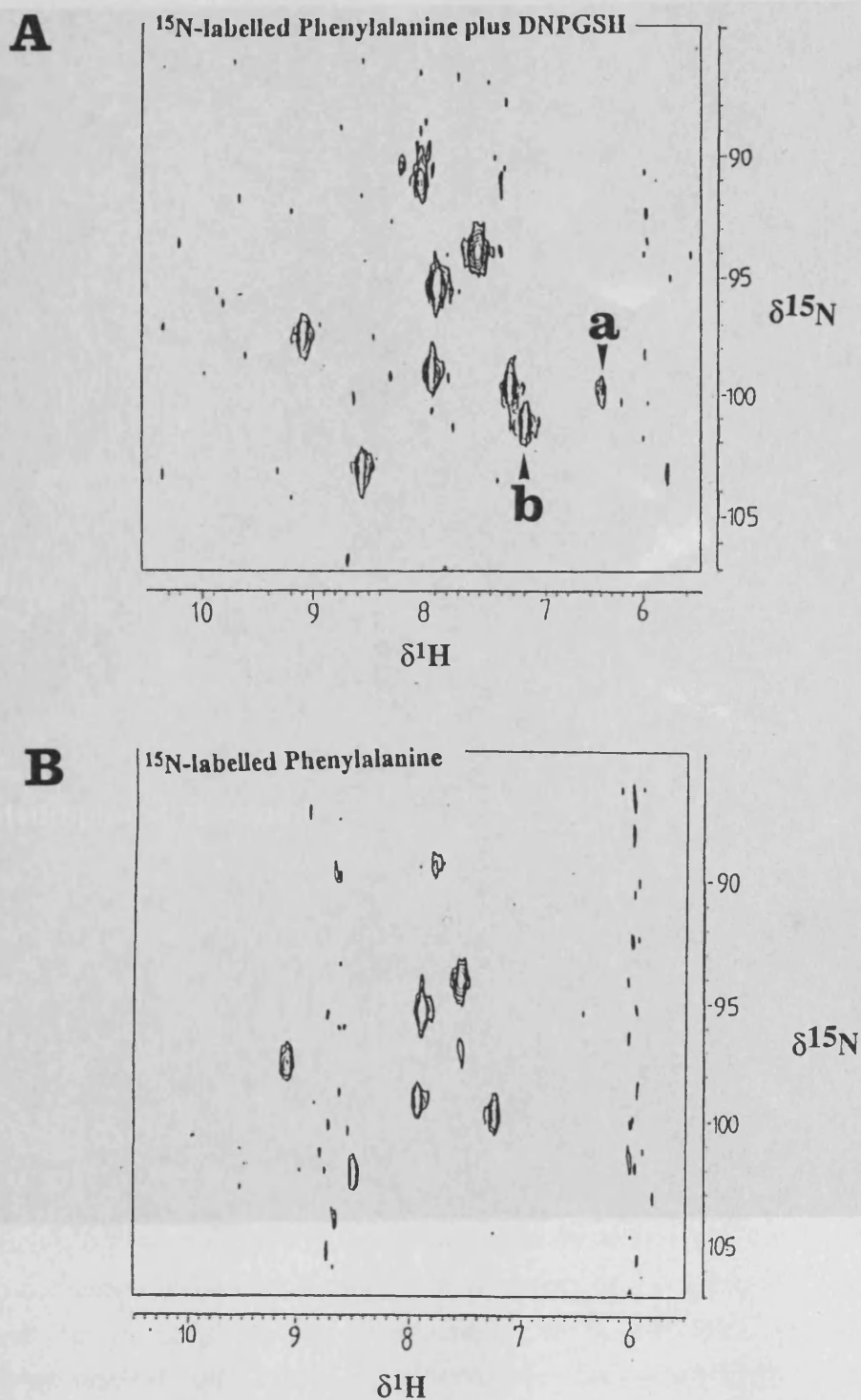


Fig 5.2. Heteronuclear Multiple Quantum Correlation Spectra showing the resonances for ¹⁵N-labelled protons for huGSTA1-1 in the presence (A) and absence (B) of DNP-GSH. The resonances appearing on addition of DNP-GSH are labelled (a) and (b). Spectra acquired at 298°K, pH7.0, 50mM NaPO₄ 1mM DTT

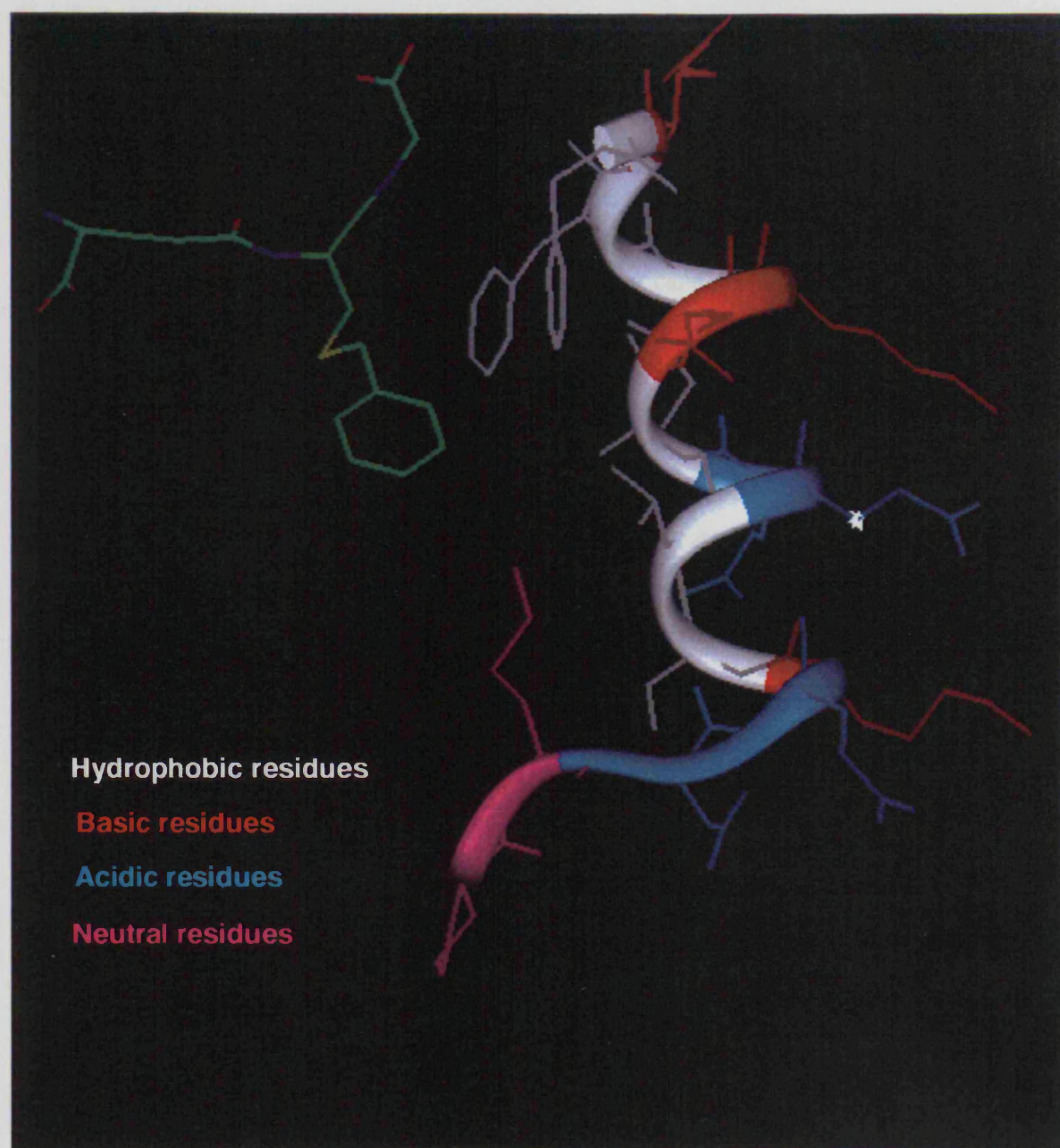


Fig 5.3. InsightII representation of the C-terminal alpha-helix. The C-terminal phenylalanine residues can easily be seen projecting into the active site. The hydrophobic residues are dark coloured and hydrophilic residues are light coloured, showing the amphipathic nature of the secondary structure.

water with a continuous pulse, the area of the spectrum that contains the water signal is not excited, leaving a gap in the spectrum; as the signals that are of interest are not in this region this method of water suppression is preferable as it eliminates the problem of chemical exchange with water. Results from the experiments using non-excitation show exactly the same results as with pre-saturation, proving that the region containing the interesting resonances was in conformational exchange. Had the residues been in chemical exchange with the solvent in the apo-protein, then their resonances would have been detected using this method.

In order to identify which phenylalanine residues were involved, the peptide sequence of the protein was examined to find likely candidates. These experiments were carried out before the publication of the apo-protein structure and it was felt that the phenylalanine residues, F220 and F222, in the C-terminal portion of the protein were the best candidates as they were in the most likely part of the protein to be flexible. The sequence of the helix is PGSPRKPPMDEKSLEEARKIFRF and inspection of the crystal structure shows that they form an integral part of the active site by contributing to the hydrophobicity. The secondary structure is shown in fig 5.3. In order to test the hypothesis the mutants F220Y, F222Y and Δ CT were constructed.

5.2.2 Construction of C-terminal mutants of huGSTA1-1.

The phenylalanine residues were mutated to tyrosine to make F220Y and F222Y using the oligonucleotides F220Y and F222Y shown in Table 8.1.4.2 (*Materials and Methods*). Mutants were identified by automated ABI sequencing using PGWLR as a sequencing primer and resulting plasmids were called pEMF220Y and pEMF222Y.

A C-terminal deletion mutant pEMGST Δ CT which had the residues 210-222 removed, was also constructed using the oligonucleotide-directed method, allowing the precise excision of the C-terminal DNA sequence from the cDNA using the oligonucleotide Δ CT, instead of a deletion method using appropriate restriction sites (Board and Mannervik, 1991). However, unlike the other mutations, the large single-stranded template-loop produced by the annealing of the mutagenic oligonucleotide meant that a highly efficient transformation of *E. coli* cells was necessary and electrocompetent cells were transformed by the mutagenesis reactions using electroporation.

Resultant colonies from the electroporation were screened for deletion using PCR and the primers PGWR and GSTSEQ4. Extension products from wild-type DNA gave products that were 231 bp and extension products from deleted DNA gave

shorter fragments of 195 bp which were identified by running the reactions through a 2% agarose gel (fig 5.4). Using this technique it was possible to identify putative Δ CT mutants which were subsequently confirmed using automated DNA sequencing to be correctly deleted with no secondary mutations. The expression of huGST Δ CT was checked in small scale preparations and could easily be distinguished from wild-type huGST by its different electrophoretic mobility through a 12% SDS-polyacrylamide gel (fig 5.5).

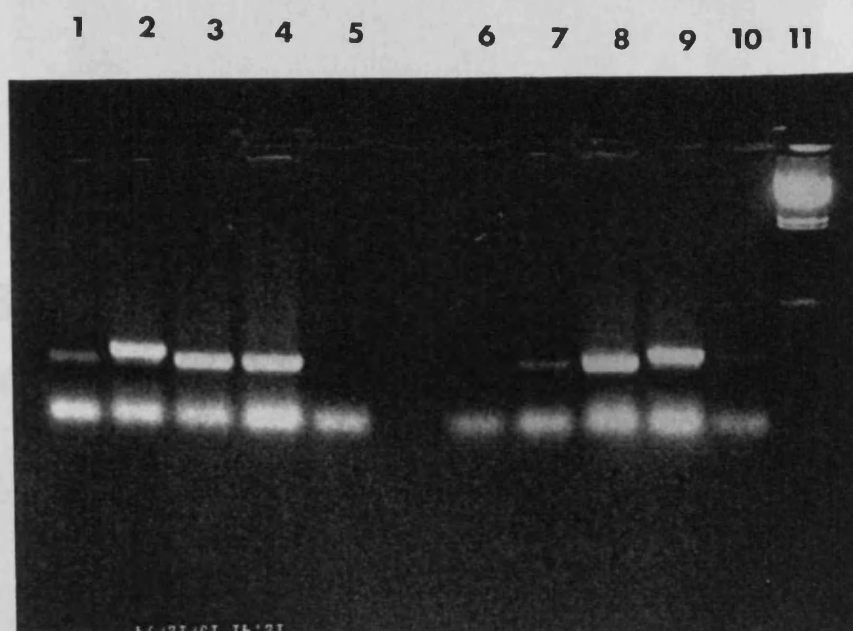


Fig 5.4 Results from the PCR screen to identify putative candidates for C-terminal deletion. Positive clones should contain PCR fragments 195bp and negative clones, 231bp. **Lanes 3, 4, 7 and 8** showed likely candidates for further sequencing. **Lanes 5 and 6** were the negative controls. **Lanes 1 and 10** were positive controls. **Lane 11** Molecular weight standards, λ Hind III.

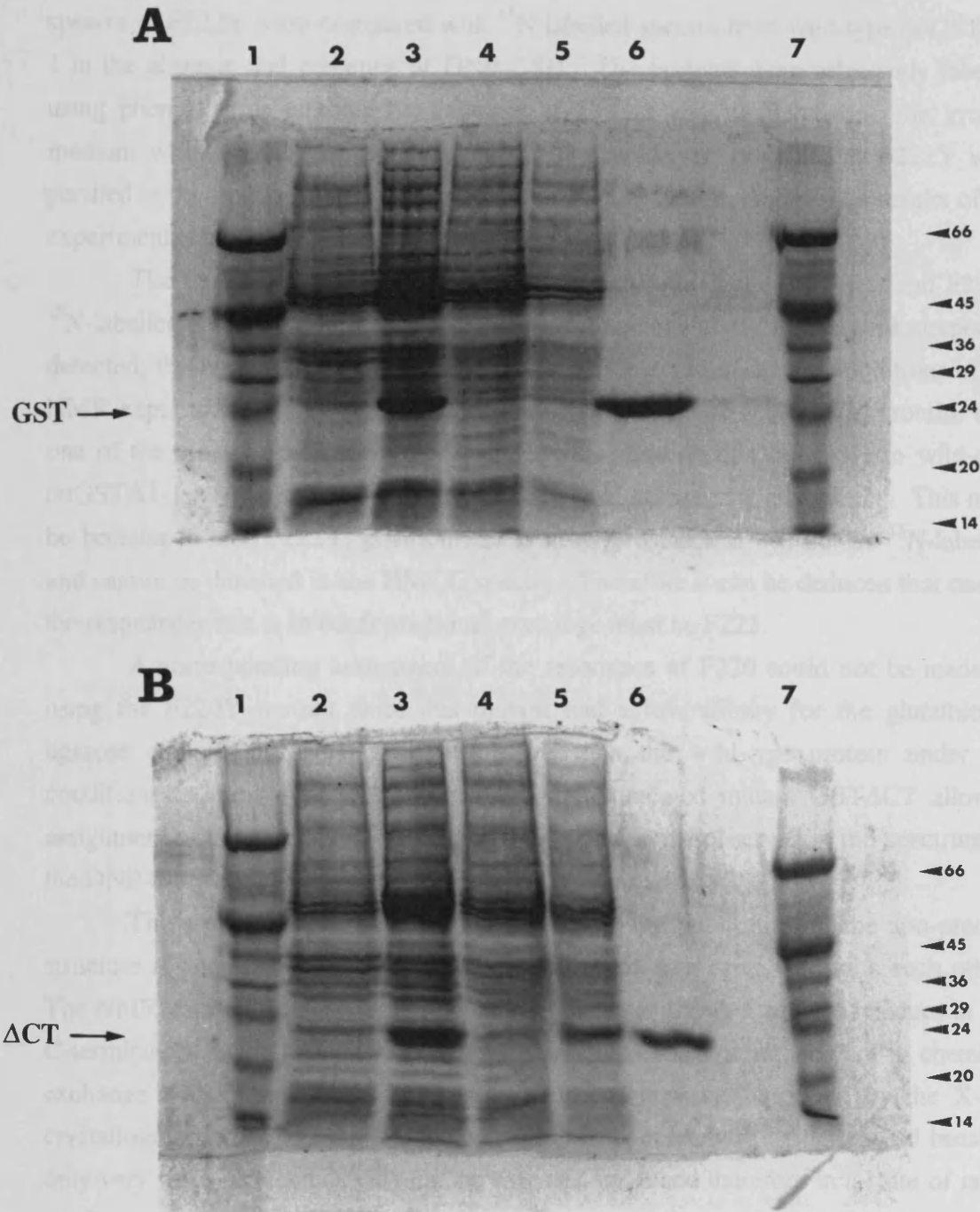


Fig 5.5. Coomassie Blue stained 12% SDS-Polyacrylamide gels showing expression of wild-type huGSTA1-1 (A) and huGST Δ CT (B) in *E. coli* JM109. **Lane 1** Molecular weight standards. **Lane 2** *E. coli* in absence of IPTG. **Lane 3** *E. coli* with pEMGST Δ CT(B) or pEMGST(A) + 1mM IPTG. **Lane 4** *E. coli* extract after column. **Lane 5** Column wash. **Lane 6** Purified protein. **Lane 7** Molecular weight standards.

5.2.3 Assignment of F222 in ^{15}N labelled HMQC spectra.

In order to assign the ^{15}N proton in the HMQC experiments, ^{15}N labelled spectra of F222Y were compared with ^{15}N labelled spectra from wild-type huGSTA1-1 in the absence and presence of DNP-GSH. The proteins were selectively labelled using phenylalanine auxotrophic mutants of *E. coli* and supplementing the growth medium with ^{15}N -labelled phenylalanine. The wild-type protein and F222Y were purified in the normal manner described in *Materials and Methods*. The results of the experiments are shown in fig 5.6.

The results show that in the absence of DNP-GSH, the wild-type and F222Y ^{15}N -labelled phenylalanine spectra are exactly the same, with eight crosspeaks expected detected; the two remaining crosspeaks were not detected under the conditions of the NMR experiments. However, on addition of DNP-GSH to the F222Y protein, only one of the crosspeaks is recovered, whereas the addition of DNP-GSH to wild-type huGSTA1-1 results in the re-appearance of both of the missing crosspeaks. This must be because in GSTF222Y, position 222 is now tyrosine and will not be ^{15}N -labelled and cannot be detected in the HMQC spectra. Therefore it can be deduced that one of the resonances that is in conformational exchange must be F222.

A corresponding assignment of the resonance of F220 could not be made by using the F220Y mutant since this mutant had a low affinity for the glutathione-agarose column and was much less stable than the wild-type protein under the conditions required for NMR. However, the truncated mutant GST Δ CT allowed assignment of the resonance of F220 as the second signal observed in the spectrum of the DNP-GSH complex but not in that of the apo-protein.

These experiments were carried out before the publication of the apo-protein structure (Cameron *et al*, 1995) and the two sets of data agree well with each other. The NMR data collected with non-excitation of water showed that the residues at the C-terminus of huGSTA1-1 were in conformational exchange rather than in chemical exchange with the solvent. This observation can now be supported by the X-ray crystallography data. The inference is that the helix is likely to be disordered because only very weak electron density can be detected for it and therefore in a state of rapid conformational exchange and is agreement with the NMR data presented here.

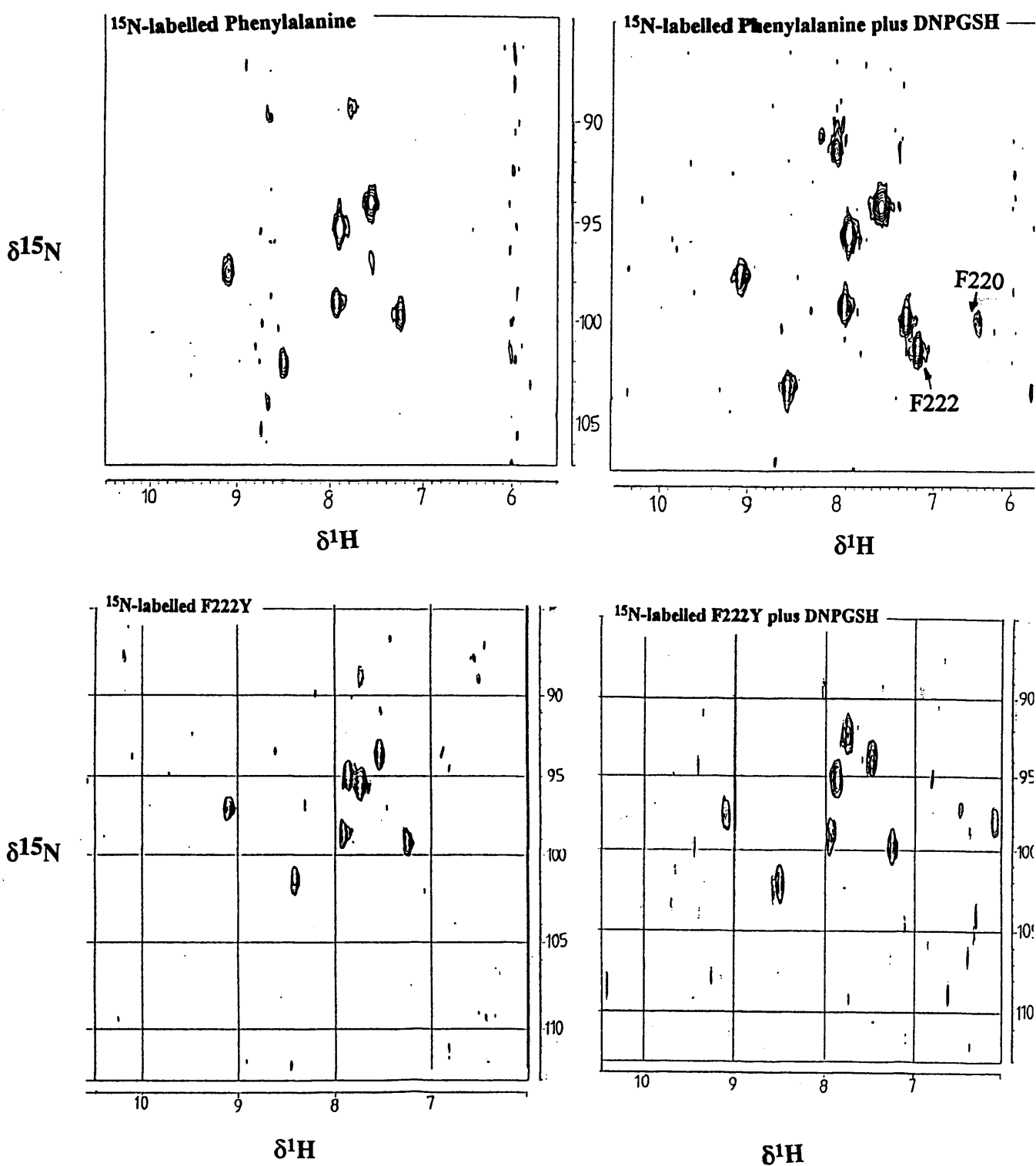


Fig 5.6 HMQC experiments showing ^{15}N labelled phenylalanine resonances in huGSTA1-1 and F222Y in the presence and absence of DNP-GSH showing that the missing resonances in the free protein spectra are F222 and F220. Spectra acquired at 298°K, pH7.0, 50mM NaPO_4 1mM DTT.

5.3 Kinetic studies on the C-terminal helix

The unusual mobility of the helix and the dramatic changes in its conformation when ligands bind (Cameron *et al*, 1995) point to some other role in ligand binding. In an attempt to understand what the helix may be doing, the Δ CT mutation was constructed, expressed and purified in order to more fully characterise the kinetics by seeing what effect adding a synthetic peptide (HEL1) with the same sequence as the helix that had been deleted to the truncated protein would have on the kinetic constants K_m and k_{cat} . The experiments were carried out at 1000 fold excess of peptide over protein. Table 5.1 shows the results that were obtained for the kinetic investigations.

From the results show in Table 5.1, it is clear that HEL1 is interacting in some way with GST Δ CT to change the kinetics of the protein. They show that there is a partial recovery of enzymatic activity when the peptide is present in the reaction mix, although, the partial restoration of the activity measured by k_{cat} , was accompanied by an increase in K_m such that k_{cat}/K_m is unchanged. Interestingly, the partial restoration of the activity of the protein only occurred when the peptide was added to the concentrated enzyme solution before adding the protein/HEL1 complex to the reaction. No change in activity of the truncated protein was seen by adding the peptide once the reaction was underway, suggesting that there was competition between substrate and peptide binding.

Protein	K_m^{CDNB} (mM)	k_{cat}^{CDNB} (s^{-1})	K_{cat}/K_m ($s^{-1}M^{-1}$)	$\Delta\Delta G$ (kJ/mol)
WT A1-1	0.59 $\pm$ 0.10	32.00 $\pm$ 2.90	5.42 $\times 10^4$	0.00
WT + HEL1	0.68 $\pm$ 0.10	38.50 $\pm$ 3.10	5.66 $\times 10^4$	-0.11
GST Δ CT	1.16 $\pm$ 0.29	0.06 $\pm$ 0.01	51.72	17.22
GST Δ CT + HEL1	3.62 $\pm$ 1.80	0.18 $\pm$ 0.09	49.72	17.32

Table 5.1. Results for huGSTA1-1 and GST Δ CT kinetics determined in the presence and absence of synthetic terminal peptide HEL1 in 1000-fold excess which was pre-bound before reaction was initiated.

To investigate the conformation of the peptide in solution, circular dichroism experiments were carried out to see if the peptide had a helical nature in solution which might explain why the peptide might interact at the active site to partially restore activity. The circular dichroism spectra of HEL1 in 100mM sodium phosphate buffer and 80% tri-fluoro-ethanol (TFE) are shown in fig 5.7.

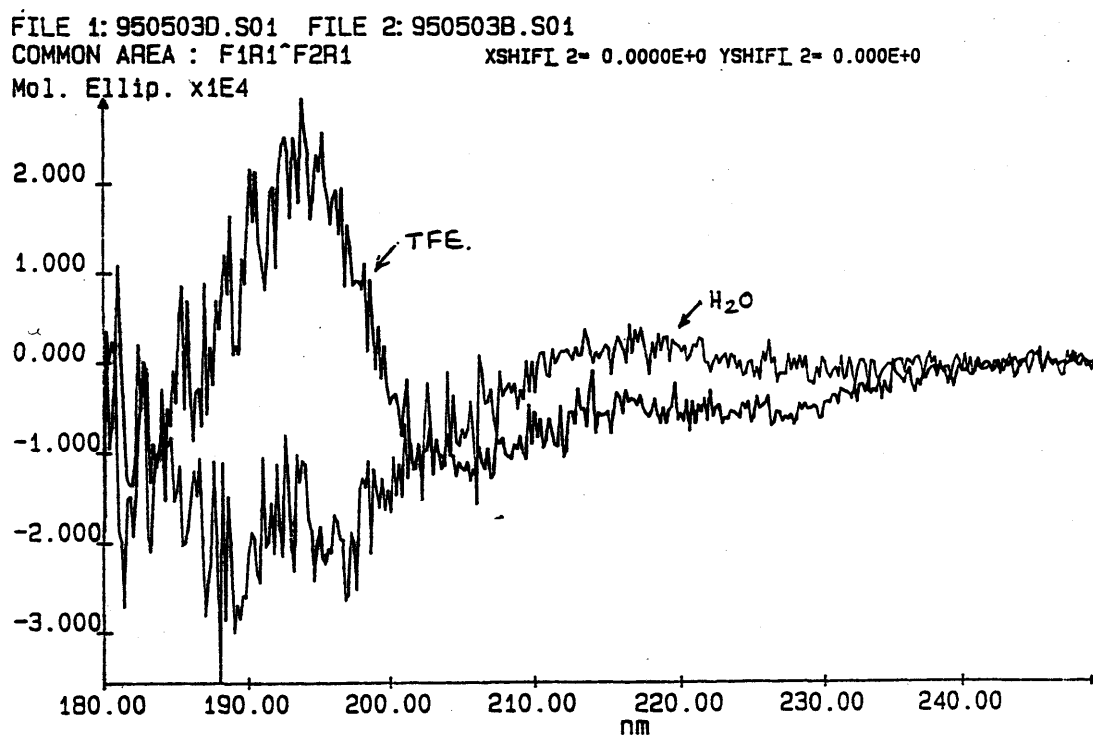


Fig 5.7. Circular Dichroism spectra of HEL1 in 100mM Na₂PO₄ buffer and 80% TFE

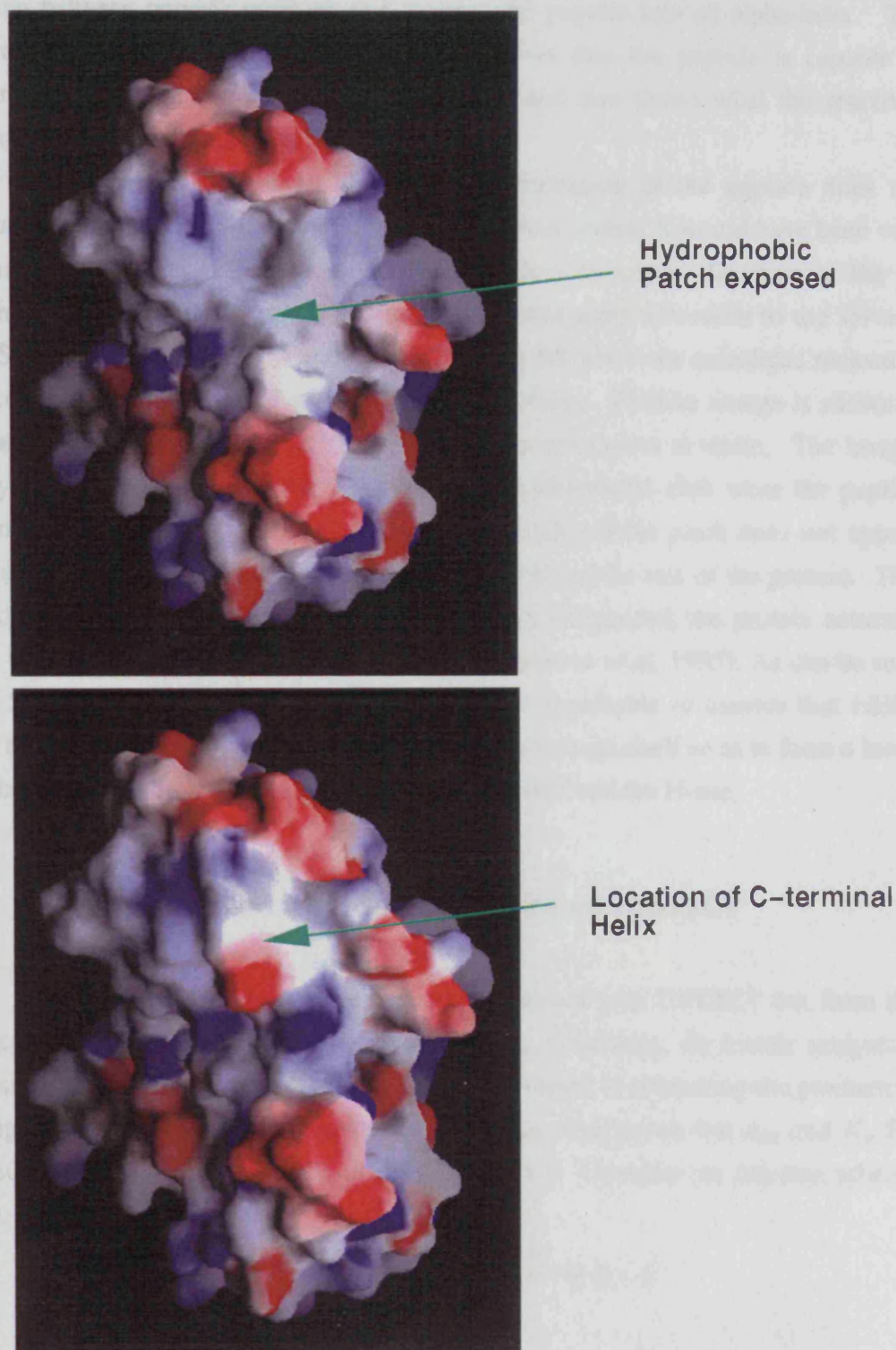


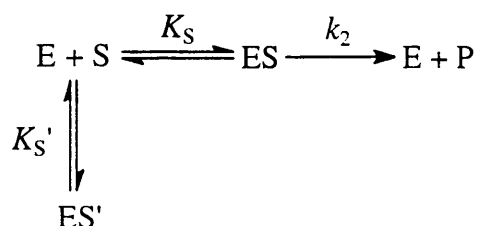
Fig 5.8. GRASP images of the calculated molecular surfaces and surface electrostatic charge of wild-type huGSTA1-1 (A) and GST Δ CT (B). The hydrophobic cleft exposed upon deletion can easily be seen in the upper right-hand quarter of the images.

The CD spectra clearly show that the HEL1 has, essentially, a random coil conformation when free in solution. TFE is an organic solvent that promotes hydrogen bonding between peptide residues and 'forces' the peptide into an alpha-helix. The experiment in TFE are important because it shows that the peptide is capable of forming an alpha-helix under the right conditions and also shows what the spectrum should be like if the peptide was a free alpha-helix.

However, the lack of an alpha-helix conformation of the peptide does not preclude the involvement of the peptide in the reaction, indeed it would have been very unusual to see a free 12-mer peptide with secondary structure. Deletion of the C-terminus from huGSTA1-1 leaves a large hydrophobic patch accessible to the solvent. GRASP images of huGSTA1-1 and GSTΔCT in fig 5.8. show the calculated molecular surfaces of the proteins and the charges on the surfaces. Positive charge is shown in red, negative charge in blue and hydrophobic areas are shown in white. The images clearly show that removal of the helix creates a hydrophobic cleft were the peptide should sit. Interestingly, exposure of the internal hydrophobic patch does not appear to result in denaturation (Board and Mannervik, 1991) of the rest of the protein. This should not be so unexpected now given that, when unliganded, the protein naturally exists with the C-terminus in a disordered state (Cameron *et al*, 1995). As can be seen in fig 5.2. the peptide is very amphipathic and it is reasonable to assume that HEL1 could bind to the enzyme's exposed 'oily patch' and arrange itself so as to form a loose helix because of hydrophobic interactions between itself and the H-site.

5.3.1 Kinetic model for the action of HEL1 with GSTΔCT

It is not yet clear how the peptide might interact with GSTΔCT but from the kinetic data it is possible to speculate on the function of the helix. By kinetic analysis it is possible to show that the peptide is likely to be involved in promoting the productive binding of the substrate to the enzyme by using the observation that k_{cat} and K_m for GSTΔCT are both increased in the presence of HEL1. Consider the reaction scheme below.



Where E is free enzyme, ES is the productive enzyme-substrate complex that goes on to produce products P and free enzyme E; and ES' is a non-productive enzyme-substrate complex, where the substrate has bound in a way in which the

reaction cannot occur. The reaction scheme can be expressed in standard, steady-state terms as equation (1).

$$v = \frac{[E]_0[S]k_2}{K_2 + [S](1 + K_s / K'_s)} \quad (1)$$

In the case of GST Δ CT, the deletion should reduce the ability of the enzyme to bind its substrates because mutation removes a major defining region of the active site and opens it up to the bulk solvent. This increases the possibility that the formation of ES is likely to be rapid and highly reversible meaning that Michealis Menten assumptions should hold. If equation (1) is compared with the Michealis Menten equation (2), interesting comparisons can be drawn that help in determining what kinetic constants characterise non-productive binding (3)

$$v = \frac{[E]_0[S] k_{cat}}{K_m + [S]} \quad (2)$$

By inspection of the two equations, expressions for k_{cat} and K_m defined in equation (iii) in terms of K'_s (i) can determined as shown in equations (iii).

$$k_{cat} = \frac{k_2}{(1 + K_s / K'_s)} \quad K_m = \frac{K_s}{(1 + K_s / K'_s)} \quad (3)$$

Using these derivatives it can be seen what consequences K'_s , the equilibrium constant for the formation of the non-productive enzyme-substrate complex, has on the values for K_m and k_{cat} . It can be seen from equation 3 that as non-productive binding increases (K'_s decreases), both k_{cat} and K_m will increase appropriately assuming that K'_s is larger than K_s . An increase in K_m associated with increasing amounts of productive binding may seem paradoxical but it must be remembered that K_m values also reflect additional binding modes thus leading to apparently tighter binding with non-productive binding.

The relationship between the increase in K_m and k_{cat} when involved in changes in the productivity of binding are very interesting and can be compared by deriving k_{cat}/K_m values. From equations (3) this parameter can be expressed as equation (4)

$$\frac{k_{cat}}{K_m} = \frac{k_2}{K_s} \quad (4)$$

Equation (4) predicts that K'_s will have no effect on k_{cat}/K_m , so that when comparing k_{cat}/K_m values there should be no difference regardless of how much non-productive binding is occurring.

The predictions from equations (3) and (4) can be used to describe what happens to GST Δ CT kinetics in the presence and absence of HEL1 peptide. Both K_m

and k_{cat} for GST Δ CT increase in the presence of peptide compared to those determined in its absence, implying that there is an increase in the amount of productive binding i.e. K 's decreases. Additionally, in the presence of peptide, the values for k_{cat} and K_{m} both increase but the values for $k_{\text{cat}}/K_{\text{m}}$ stay the same which again supports the proposed role of the peptide in promoting productive substrate binding.

5.3.2 GST from *Arabidopsis thaliana* supports the kinetic model for productive binding.

The role of the C-terminal helix is further supported by studies of a GST isolated from *Arabidopsis thaliana* (Zettl *et al*, 1994, Zhou and Goldsbrough, 1993). The protein was cloned and characterised and shown to be 211 amino-acids long but did not show any great sequence homology to any previously structurally determined GSTs. The protein was subsequently crystallised and the structure solved in complex with S-hexyl-glutathione (Reinemer *et al*, 1996).

The X-ray data showed that the *Arabidopsis thaliana* structure showed clear topographical relationships and significant structural homology with the other structurally determined GSTs. In particular, the protein showed the greatest amount of structural homology with the alpha-class protein huGSTA1-1. Sequence alignments based on structure (Reinemer *et al*, 1996) shows identities of 25% and 19.9% for G-site domain and the H-site domain respectively between alpha and *Arabidopsis*, while only 19.0% and 13.3% for the *Arabidopsis*-mu pair and 17.7% and 15.5% for the *Arabidopsis*-pi pair.

It is clear that the H-site is more like an alpha-class enzyme than any thing else but the *Arabidopsis* protein is only 211 amino-acids long compared to the 222 in huGSTA1-1. Inspection of the structure reveals that the protein does not have any C-terminal element, that is to say it can be considered as a natural analogue of the huGSTA1-1 C-terminal mutant. (Indeed the absence of the C-terminal helix is responsible for an artificially low value for the structural alignments).

The crystal structure was determined in the presence of S-hexyl-glutathione and the electron density of the inhibitor in the active site of the *Arabidopsis* protein clearly shows that there are two molecules of inhibitor bound simultaneously. One molecule appears to be bound at the expected site, with the glutathione moiety present in the G-site and represents a productively bound mode. However, the other S-hexyl-glutathione molecule also appears to be bound in the active site but in a position next to the G-site, suggesting that it is bound in an unproductive mode (Reinemer *et al*, 1996).

This is therefore direct, structural evidence that an alpha-class-like protein with no C-terminal helix has two distinct modes of binding ligands, one of which can only form an unproductive enzyme-substrate complex. The structural evidence reported agrees with the kinetic determinations presented here.

5.3.3 Comparison of ΔG values shows differences in binding.

However, if the figures for k_{cat} and K_m for GST Δ CT are compared to the wild-type enzyme, and the non-productive binding predictions followed through, it may be expected that as more peptide is added, K_s will become increasingly small thus, K_m and k_{cat} will continue to rise until equation (1) becomes (2) but this clearly cannot be possible here as K_m for GST Δ CT in the presence of HEL1 is 3.62mM. The addition of more peptide will lead to increased values for K_m , even though wild-type K_m is 0.59mM. Indeed, experiments carried out at 2000-fold excess of HEL1 to protein have given data that cannot be used for curve-fitting. This is likely to be because CDNB is only soluble to a maximum of 1.2mM and the K_m value at this concentration of peptide K_m becomes too high for the curve fitting program to cope with. To understand why equation (1) will not become equation (2) in this case, $\Delta\Delta G$ values should be considered.

$\Delta\Delta G$ is the incremental Gibbs free-energy of transfer and is calculated using equation (5) below.

$$\Delta\Delta G = -RT \ln \frac{K_{\text{cat}}}{K_m} \quad (5)$$

Using k_{cat}/K_m values in the calculations of ΔG gives values that include contributions from both the activation energy and binding energy and avoids underestimations of ΔG that occur through use of dissociation constants alone. $\Delta\Delta G$ values are not instructive for absolute estimates for ΔG values but are significant when comparing ΔG values. The $\Delta\Delta G$ values determined show why the kinetics of GST Δ CT cannot be directly compared with WT GSTA1-1. There is a difference of approximately 3 kcal/mol, regardless of whether the peptide is present or not, showing that WT GSTA1-1 has more free energy to stabilise unfavourable charge formation in the transition state. It suggests that WT GSTA1-1 has a different catalytic mechanism which involves more transition state stabilisation, or at least that K_s for GST Δ CT is not equal to K_s for wild-type and so comparisons of GSTA1-1 and GST Δ CT are difficult.

This difference in catalytic mechanism suggested is supported by recent work centred on site-directed mutagenesis on residues in the C-terminal helix of rat GSTA1-1 (Ya₁) (Dietze *et al*, 1996). In this paper, parallels are drawn with mu-class enzymes

where an aromatic hydrogen bond to Tyr6 modulates the pK_a of bound glutathione. The H-bond plays a role in catalysis by transmitting its effect through the electron cloud of Tyr6. Dietze and co-workers use F220 on the C-terminal alpha helix as a scaffold to engineer an on-face hydrogen-bond to Tyr9, similar to that in mu-class, by mutating it to phenylalanine. They show that glutathione, when bound to F220Y, has a lower pK_a than that when bound to wild-type huGSTA1-1. While it is surprising that the wild-type enzyme does not have this adaptation, *ab initio* calculations based on comparisons of the proton affinity of methanethiol hydrogen bonded to *p*-cresol (a Tyr220 mimic) and in the presence and absence of methyl-benzene (a Phe220 mimic) show that Phe220 is likely to play the same sort of role (Dietze *et al*, 1996). While phenylalanine is not as efficient as tyrosine, indications are that the presence of phenylalanine at position 220 lowers the pK_a of bound glutathione relative to a protein with 'nothing' at that position.

Therefore, there is accumulating evidence that the C-terminal helix of alpha-class enzymes is important in the catalytic mechanism. Not only does the helix influence productive enzyme-substrate complex formation but it can also influence the charge state of bound glutathione. The charge state of bound glutathione and the pK_a value of Tyr9 are discussed in the next chapter.

Chapter 6

Glutathione Activation in Catalysis

6.0 GLUTATHIONE ACTIVATION IN CATALYSIS

6.1 Introduction.

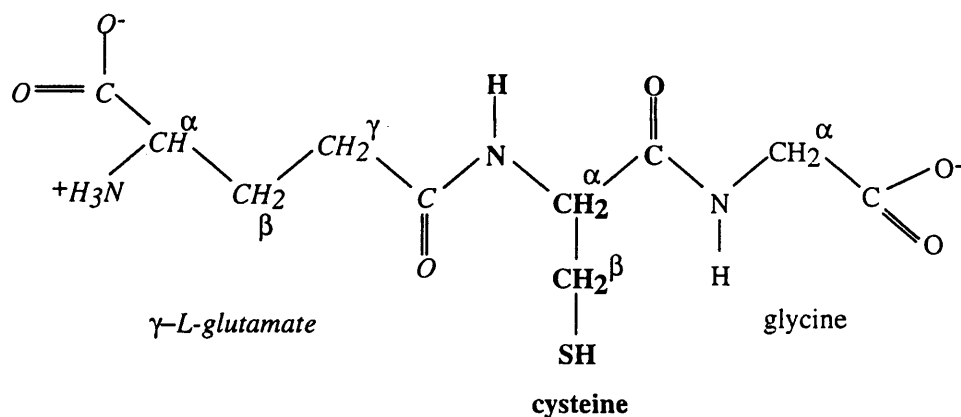
Activation of glutathione by deprotonation of the sulphydryl group is believed to be central to the reaction mechanism of GST. As evidence for this, most references cite ultra-violet difference spectroscopy experiments carried out with glutathione and γ -L-glutamyl-L-serylglycine (Graminski *et al*, 1989). These form the E.GSH and E.GOH complexes respectively, thus allowing the titration of the glutathione sulphydryl group by differential observation. However, these experiments showed that there were conformational changes between the different enzyme complexes which were also reflected in the absorption difference spectra. It could not be conclusively proved that the absorption differences in the region between 235nm and 240nm titrated were due entirely to the ionisation of the sulphydryl group of glutathione. In the first part of this chapter, the ionisation state of glutathione at physiological pH is investigated using ^{13}C - glutathione, labelled at the β position of cysteine, and ^{13}C -edited NMR spectroscopy.

The role of Tyr9 in the catalytic activity is intimately linked to the ionisation of the sulphydryl group of glutathione. There is evidence that Tyr9 has an usually low pK_a (Atkins *et al*, 1993, Dietze *et al*, 1996, Meyer *et al*, 1993). However these experiments used difference fluorescence measurements from mutants and inferences from biochemical modifications, again there has been little published on the direct observation of Tyr9 in wild-type protein. In the second part of this chapter, the C^ϵ proton of Tyr9 is assigned using selective protonation of tyrosine residues in deuterated wild type and Y9F proteins. Using the assignment, the pK_a of Tyr9 can be investigated directly by titration.

6.2 NMR Studies of Glutathione in complex with huGSTA1-1.

The ^1H NMR spectrum of glutathione has been assigned (Kubal *et al*, 1995, Rabenstein, 1975) and the chemical shifts and assignments are shown in fig 6.1. Once assigned, the spectrum was titrated to see how the chemical shifts changed with pH (Corrazza *et al*, 1996, Rabenstein, 1975). The α proton from γ -glutamate showed the most sensitivity to pH and the shifts reflect two ionisation events with pK_a values of 2.3 and 9.65 corresponding to the deprotonation of the carboxylate group and the amino group of γ -glutamate respectively. The pH dependent chemical shifts of the α

and β protons of cysteine reflect the ionisation of the sulphohide group with a pK_a of 9.0.



Proton	Chemical Shift ppm	pK_a
Cys α	4.57	9.07 ± 0.01
Cys β	2.93	9.07 ± 0.03
Cys β	2.98	
Glu α	3.79	2.3 ± 0.1 , 9.65 ± 0.02
Glu β	2.17	2.2 ± 0.4 , 9.69 ± 0.04
Glu β	2.17	
Glu γ	2.55	9.57 ± 0.05
Glu γ	2.58	
Gly α	3.78	3.48 ± 0.05

Fig 6.1. Chemical structure of glutathione identifying the protons observed in ^1H NMR. The chemical shifts of the assigned protons (Kubal *et al*, 1995) and pK_a values (Corrazza *et al*, 1996, Rabenstein, 1973) are shown

6.2.1 ^{13}C Labelling of glutathione

Intuitively we chose the β position to be labelled with ^{13}C , assuming that this position would give the largest change in chemical shift when bound to GST.

^{13}C labelled glutathione was necessary because the experiment depended on being able to detect the signals from glutathione in a E.GSH complex. If unlabelled glutathione was used in the experiments the β - proton signal of glutathione would be swamped by the proton signals from the protein. However, the labelled glutathione signals can be extracted from the spectra because the one-bond coupling constant between ^1H and ^{13}C is known to be 140 Hz and can be used as a filter to observe only

protons directly bonded to ^{13}C . ^{13}C will be present in the protein backbone at natural abundance and in order to ensure that the only signal detected corresponded to labelled glutathione, the protein used in the experiments was 98% deuterated.

6.2.2 Production of C112S mutant of huGSTA1-1

In order to carry out the NMR experiments, as well as using labelled glutathione, the protein has to be very stable at room temperature for long periods of time. Wild-type huGSTA1-1 is not stable enough to get good signal to noise ratios because the protein tends to aggregate and precipitate as the experiment progresses, producing a white precipitate in the NMR tube. The structure (Sinning *et al*, 1993) showed a cysteine residue at position 112 on the protein surface. There are many natural GSTs in which the cysteine is replaced by serine. It was postulated that the surface cysteine could form inter-protein disulphide bridges that leading to aggregation in the NMR tube.

The mutant C112S was therefore made using Method 1 in *Materials and Methods*. Initially, the cDNA for huGSTA1-1 was present in the construct pEGST, which was based on the pKK223 series of vectors from Pharmacia, and did not contain the M13 origin of replication to produce the single stranded template for the mutagenesis reactions. Firstly, the gene was cloned into pBluescript SK+ in order to use the M13 origin to drive single-stranded plasmid DNA production to give the plasmid pbsGST. The cDNA was lifted out of pEGST and cloned into pBluescript SK+ with *EcoRI*. The mutation was introduced using the oligonucleotide C112S and once the presence of the mutation was confirmed by automated DNA sequencing using GSTSEQ1, 2, 3 and 4, the gene was recloned into the expression vector using *EcoRI* to produce pEC112S and the correct orientation was selected using a diagnostic *PvuII* digest. The primer sequences are detailed in *Materials and Methods*.

The mutant protein was expressed in *E.coli* and purified as usual and showed similar kinetics and ^1H NMR spectra to the wild-type protein. The protein also proved to be extremely stable in the NMR tube. Good signal to noise ratios were obtained, and there was no precipitate seen in the tube after the experiments showing that aggregation had been retarded. Consequently, there was an improvement in the line-width of the spectra with sharper signals being seen. Thus the mutant GST C112S was used to see what effect binding glutathione to GST had on the ionisation of the sulphhydryl group of glutathione.

6.2.3 NMR experiments with C112S and ^{13}C -labelled glutathione

Initial experiments with labelled glutathione were not instructive because although the bound glutathione signals could be seen, titration did not show whether the glutathione was ionised or not because no change in chemical shift of the glutathione $^{13}\text{C}_\beta\text{-H}$ protons was not observed. The titration of free glutathione (Corrazza, et al., 1996, Rabenstein, 1973) shows that the chemical shift of Cys $\text{C}_\beta\text{-H}$ protons is not very sensitive to pH, the position most sensitive to sulphydryl ionisation being the Cys $\text{C}_\alpha\text{-H}$ position, fig 6.3.

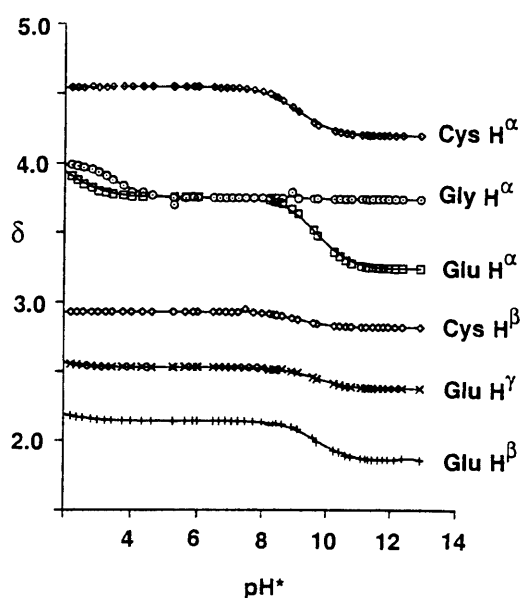


Fig 6.3. Effect of pH on the variation of ^1H NMR chemical shifts of glutathione at 298K at 400MHz. (Corrazza, et al., 1996)

Since the Cys $\text{C}_\alpha\text{-H}$ proton resonates near the water position in the spectrum and the bound resonances are broad, it is often difficult to detect them. Thus a transferred NOE method (20 mode) was used to identify the position of the bound species. In this method, due to chemical exchange between the free and bound signals, magnetisation is transferred between these two forms and a cross-peak is observed connecting the signals of the free and bound species. Such a connection allows the position of the bound species to be detected and identified.

Now that the bound glutathione signals can be observed, it is possible to determine the ionisation state of glutathione when bound to GST by measuring the

6.2.4 2-D NOESY experiments with GST and glutathione.

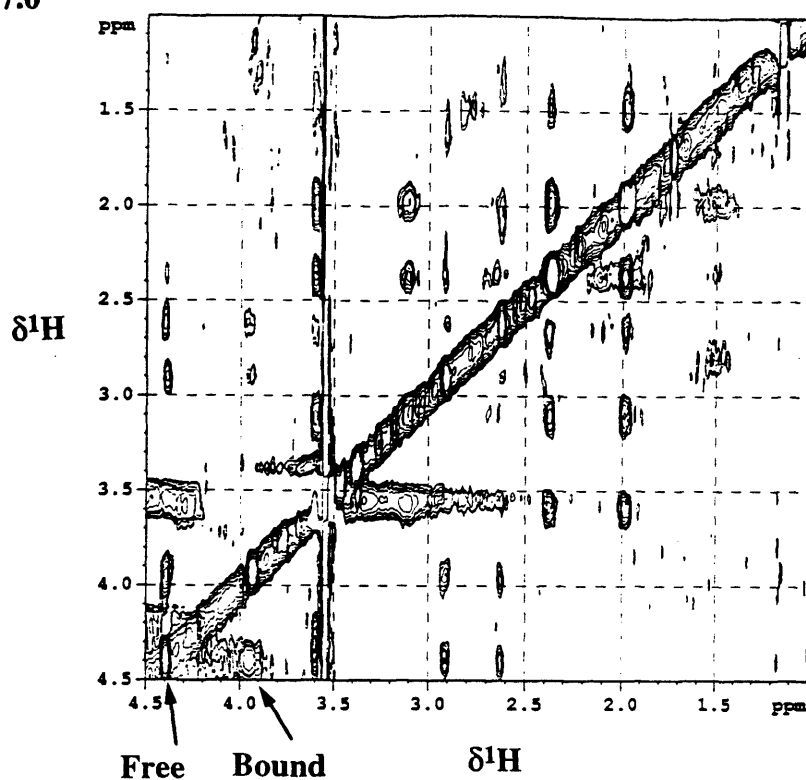
Glutathione was added to C112S in a 1:0.3 ligand to protein ratio. The preparation of the complex was completed under anaerobic conditions with all solutions used flushed with argon in order to prevent glutathione oxidising to form GSSG. The complex was then examined using two dimensional NOESY spectra at 298K at pH 7.0 and pH 7.8. The spectra are shown in figure 6.4.

In a NOESY experiment, cross-peaks arise from magnetisation transfer either by dipolar relaxation between two spins which are close together in space or by chemical exchange of one spin between two states in which it has different chemical shifts. In this experiment, one might expect to see chemical exchange cross-peaks between the resonances of the free and bound glutathione, provided that the rate of exchange between these states is at least of the same order as the two spin-lattice relaxation rate

In the experiment in fig 6.4, an exchange cross-peak can be seen between resonances at 3.91 ppm and 4.45 ppm from the Cys C α -H of glutathione. It can be identified as an exchange crosspeak because there is characteristic broadening in one dimension (the cross-peaks on each side of the diagonal having different shapes) and secondly the chemical shift of 4.45 ppm corresponds to that of the Cys C α -H of free glutathione. The other glutathione protons that would give signals in this region of the spectrum, namely Gly C α -H and Glu C α -H would not be expected to give NOEs to the Cys C α -H because they are too far away.

The chemical shift of the Cys C α -H of bound glutathione, 3.91 ppm corresponds much more closely to the thiolate form rather than the thiol form of glutathione. This chemical shift is the same at pH 7.0 and pH 7.8. Similarly, the C β -H proton of γ -glutamate in the bound glutathione appears at the position of a deprotonated species and is not affected by pH. In free glutathione, it can be seen that the chemical shift changes slightly between pH 7.0 and 7.8 as the sulphydryl group begins to become deprotonated. Therefore the invariance of the chemical shifts between these pHs indicates that the pK $_a$ of sulphydryl group of bound glutathione must be below pH 7.0, consistent with previously published estimates (Graminski *et al*, 1989).

pH=7.0



pH=7.8

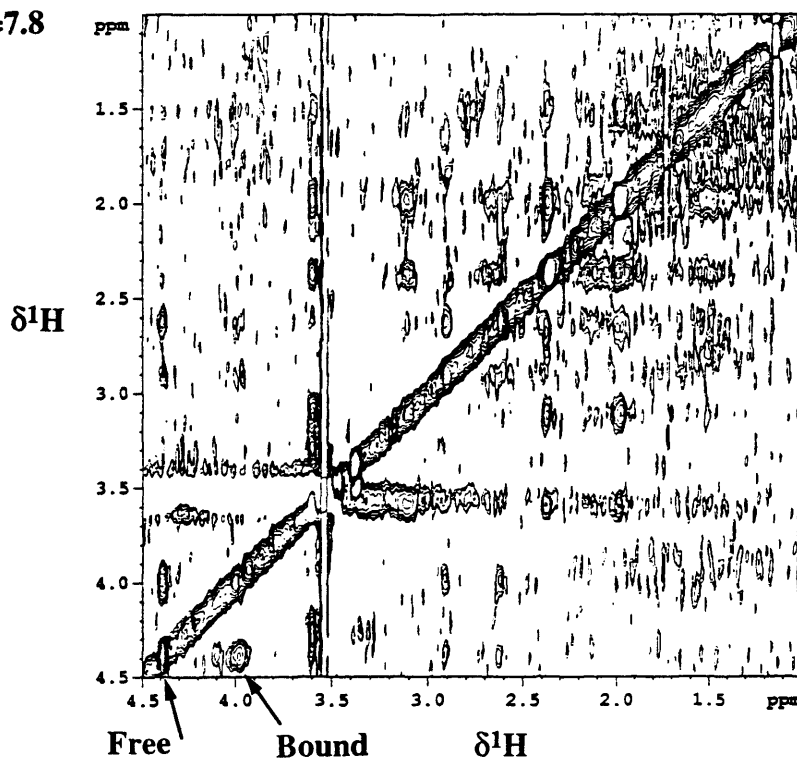


Fig 6.4. 2-D NOESY spectra of perdeuterated GST with ^{13}C -C $^{\beta}$ -glutathione. Exchange crosspeaks connect the resonances from the free and bound ligand. The crosspeaks between the protons at 3.91 and 4.45 ppm are from bound and free Cys C $_{\alpha}$ -H protons. Spectra were acquired at 285°K, 0.3mM protein, 1mM GSH and variable pH, 50mM NaPO $_4$.

6.3 Role of Tyr 9 in the catalytic activity of huGSTA1-1.

Previous biochemical analyses of GSTs have suggested that the deprotonation of bound glutathione is most likely coupled to the presence of an important tyrosine residue, found in crystal structures hydrogen bonded to the sulphur atom of bound glutathione conjugates. However, for a tyrosine residue to be important in the catalytic mechanism, its pK_a would have to be considerably lower than that of tyrosine in free solution in order to be a proton acceptor for glutathione.

Ultra-violet absorption difference spectroscopy (Atkins *et al*, 1993, Bjornestedt *et al*, 1995) has been used to examine the properties of active site tyrosine. These experiments involve the comparison of the ultra-violet spectra of wild-type and mutant protein and are thus open to possible artefacts arising from structural perturbations.

Using NMR it is possible to use site-directed mutagenesis to assign signals from the tyrosine residue and then return to the wild-type protein to observe the behaviour of tyrosine directly. In this experiment, C^ϵ proton resonance of tyrosine 9 is assigned and the protein titrated to establish the pK_a directly and probe the hypothesis that a tyrosine with an usual pK_a may be important in catalysis (Atkins *et al*, 1993, Bjornestedt *et al*, 1995).

6.3.1 NMR Assignment of Tyr 9 C^ϵ proton.

In order to assign the proton resonance of tyrosine 9, the mutant Y9F was made. The mutant was made using the oligonucleotide Y9F and *Method 1* in *Materials and Methods*. pBSGST was used in order to produce suitable single stranded template for the mutagenesis reaction, just as in the production of C112S, and the mutation was confirmed by automated DNA sequencing with GSTSEQ1, 2, 3 and 4. Once the mutation was established, the mutant gene was cloned back into pE using *Eco RI* to replace the wild-type gene. The orientation of insertion was confirmed again by digestion with *PvuII* to give bands of 286 bp, 962 bp and 4365 bp as illustrated in fig 6.2.

The C^ϵ proton of Tyr 9 was assigned by expressing Y9F and wild type (C112S) protein in deuterated CellTone medium. Using this medium, the only undeuterated sites in the protein are C^ϵ -H protons of tyrosine. Examination of the 1H NMR spectra at pH 6.65 (fig 6.5), revealed there was significant overlap of the tyrosine proton resonances in both the Y9F and C112S spectra, meaning a positive assignment was not possible.

The overlap of the signals was resolved by repeating the experiment at different pH values. The signal from Tyr 9 is clear at pH 9.55 and pH 9.65 and comparison of the spectra from Y9F and C112S shows that the tyrosine resonance is at 6.65 ppm at pH 6.65 and shifts to 6.55 ppm at pH 9.65. There are other changes seen which complicate the spectrum and these are likely to be mutational effects. While the activity of the C112S mutant is not changed significantly and the spectrum is very similar to wild-type protein, the mutation of an active site residue involved in a hydrogen bonding network (Sinning, et al., 1993) may cause differences in the environments of the other tyrosine residues. While these changes are undesirable, they did not preclude the assignment of the C ϵ -H proton of Tyr9 which was completed by inspection of the number of peaks which could be resolved at different pH values.

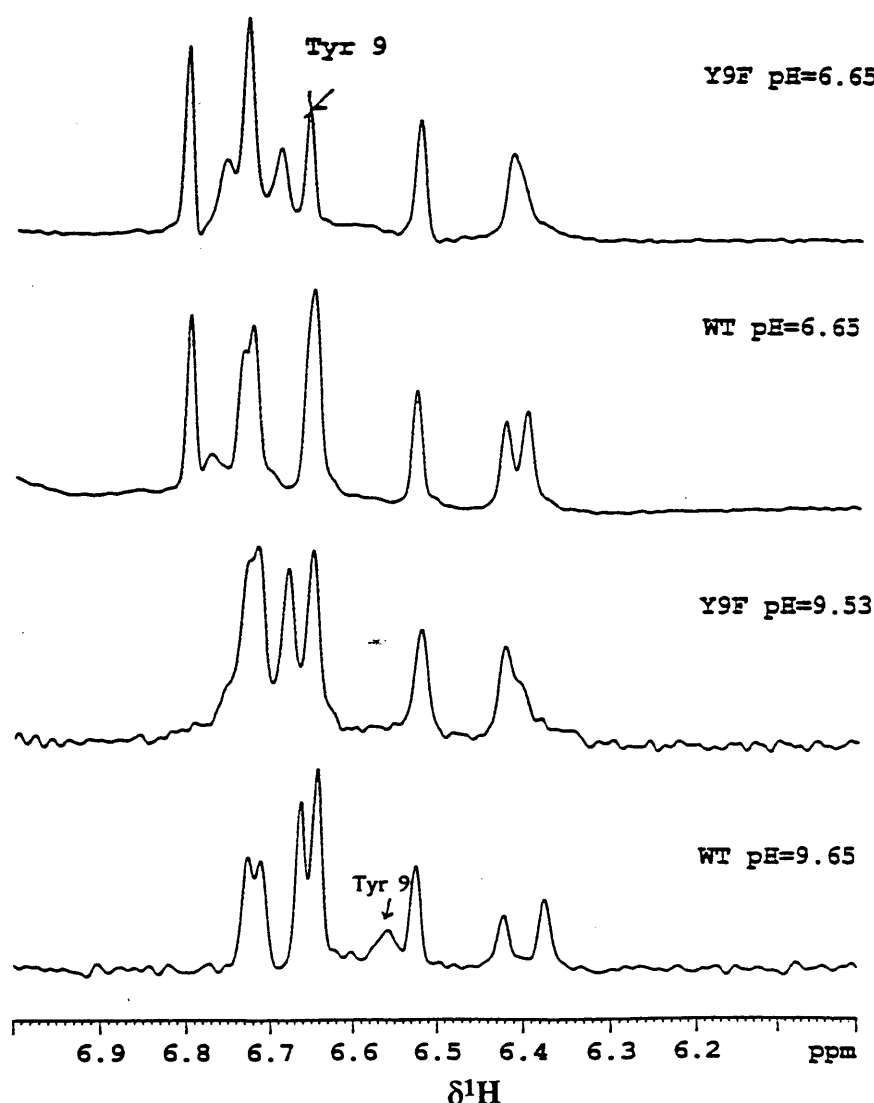


Fig 6.5. ^1H NMR spectra of a perdeuterated sample with selectively protonated tyrosine of wild-type huGSTA1-1 and Y9F at different pH values. The assignment of the resonance is made from these spectra to be at 6.65 ppm at pH 6.65 and shifted to 6.55 ppm at pH 9.65. Spectra acquired at 0.3mM protein, 283°K, variable pH, 50mM NaPO_4 and 0.3mM DTT.

Resolving the overlap of the spectra by changing the pH was encouraging because it meant that the chemical shift of Tyr C ϵ proton was sensitive to titration, hinting that the residue may indeed have an usual pK_a value. To determine the pK_a value, the chemical shift was determined at various pH values, plotted against pH and a curve fitted to the data to give an accurate estimation of the pK_a , the pH where the residue is 50% deprotonated. The spectra determined at the different pH values are shown in fig 6.6.

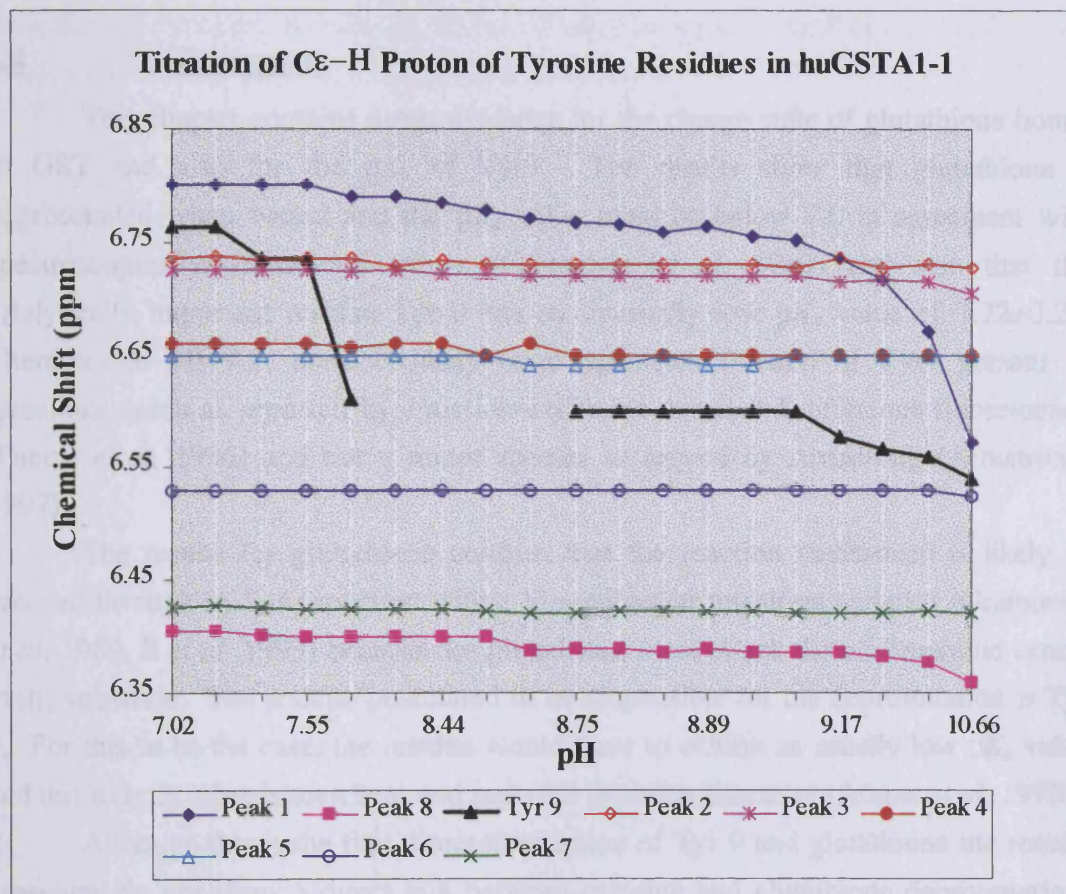


Fig 6.6. ^1H NMR spectra showing the pH titration of C112S. Clearly, Tyr 9 has a lower pK_a value than tyrosine in free solution <10.5. There also appears to be a second tyrosine that has an unusual pK_a value.

From the titration data it is clear that the resonance assigned as Tyr 9 has a much lower pK_a than the rest of the tyrosines in the protein. Chemical shifts across the entire pH range for this residue was not possible. This is common in NMR titrations of residues because the signal of the proton of interest is often at its broadest at the midpoint of its titration. This is clearly the case with Tyr 9, however curve fitting of the available data gives a pK_a of 7.72 ± 0.21 which is lower than data previously reported showing that the pK_a of Tyr9 is 8.3 ± 0.2 (Atkins *et al*, 1993,

1995, Dietze *et al*, 1996b, Dietze *et al*, 1996). However, it is also apparent that there is another tyrosine residue that has an unusually low pK_a value, Peak 1. The results from curve fitting show that the pK_a value for this tyrosine is 9.60 ± 0.02 . The identity of the second tyrosine residue is unknown at this time and there is no reason this second tyrosine should be involved in the mechanism. All difference fluorescence determinations have discounted any other tyrosine residues titrating over the pH range 6.5-10.5. The contribution of the other titrating tyrosine residue could be the main factor in the determination of a higher pK_a value by fluorescence than by NMR.

6.4 Discussion

This chapter contains direct evidence for the charge state of glutathione bound to GST and also for the pK_a of Tyr9. The results show that glutathione is deprotonated when bound and the pK_a value must be below 7.0 in agreement with spectroscopically determined values (Graminski *et al*, 1989) and also that the catalytically important residue Tyr 9 has an unusually low pK_a value of 7.72 ± 0.21 . Therefore at pH 7.5, there is likely to be a significant fraction of Tyr9 present as tyrosinate anion as reported by emission-excitation correlated difference fluorescence (Dietze *et al*, 1996) and not a minor species as argued by Armstrong (Armstrong, 1997).

The results for glutathione confirm that the reaction mechanism is likely to proceed through an S_NAr reaction with a Meisenheimer transition complex (Graminski *et al*, 1989, Ji *et al*, 1993) because the glutathione must attack the electrophilic centre in the substrate. The residue postulated to be responsible for the deprotonation is Tyr 9. For this to be the case, the residue would have to exhibit an unusually low pK_a value and this is exactly what is seen here and reported in earlier literature (Atkins *et al*, 1993).

Although this is the first direct description of Tyr 9 and glutathione the results presented do not show a direct link between tyrosine and glutathione deprotonation. In order to show whether Tyr 9 really was responsible for the low pK_a of glutathione, it would be necessary to repeat the glutathione experiments with the Y9F mutant protein. Unfortunately this was not possible, the single Y9F mutant was not stable enough in the NMR tube due to aggregation problems explained earlier and there was not sufficient time to make the double mutant C112S Y9F which should have been stable enough to complete the experiments.

However, with the pK_a value of Tyr9 determined to be lower than those previously reported, it is interesting to think about how this may influence the catalytic mechanism and whether a general base mechanism occurs in alpha class enzymes. Despite the low pK_a of tyrosine, deuterium isotope effects for the enzyme catalysed reaction with CDNB show that solvent proton transfer from glutathione to tyrosine are

unlikely, $k^H/k^D = 0.8-0.9$ which is very similar to the reaction of $\text{GS}^-_{(\text{aq})}$ with CDNB (Huskey *et al*, 1991). As such, a general base mechanism must be ruled out.

The work with excitation-emission correlated difference fluorescence showed that there was significant tyrosinate character of Tyr9 at pH 7.5 (Dietze *et al*, 1996) which agrees with pK_a value determined here. They propose that there is water molecule present in the structure that lies between Arg15 and Tyr9 with some evidence for a disordered water molecule near Arg15 coming from the apo-protein structure (Cameron *et al*, 1995). They speculate the water molecule is able to H-bond to the tyrosinate and stabilise the charge. As the pH of the experiments is close the pK_a value of Tyr9, there must be an equilibrium as shown below.

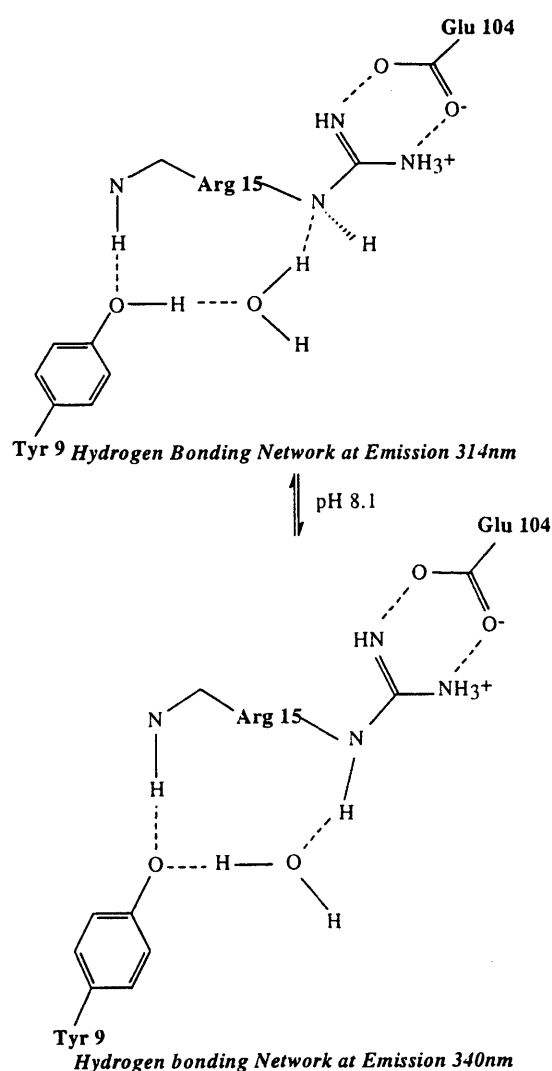


Fig 6.7 Proposed Hydrogen bond network in unliganded alpha-class GST (Dietze *et al*, 1996)

Addition of glutathione abolishes the component of the spectrum with emission at 340nm and the majority of Tyr9 appears as 'normal' tyrosine. It is tempting to consider how the proposed H-bonding network would re-organise as bound

glutathione ionises. If the proton released from glutathione resides in an electrostatic well between Tyr9 and the sulphur group as previously suggested (Ji *et al*, 1992, Meyer *et al*, 1993), then inspection of the pK_a values suggests that the proton must lie much closer to Tyr9 as glutathione as a pK_a of <7.0 and Tyr9=7.72 and thus makes the formation of Tyr9-OH much more likely. However, this must also be consistent with the evidence that direct proton transfer from glutathione to Tyr9 does not take place as with a general base mechanism (Huskey *et al*, 1991).

This could be accommodated by modifying the H-bonding scheme proposed for the emission component at 314nm to include bound glutathione.

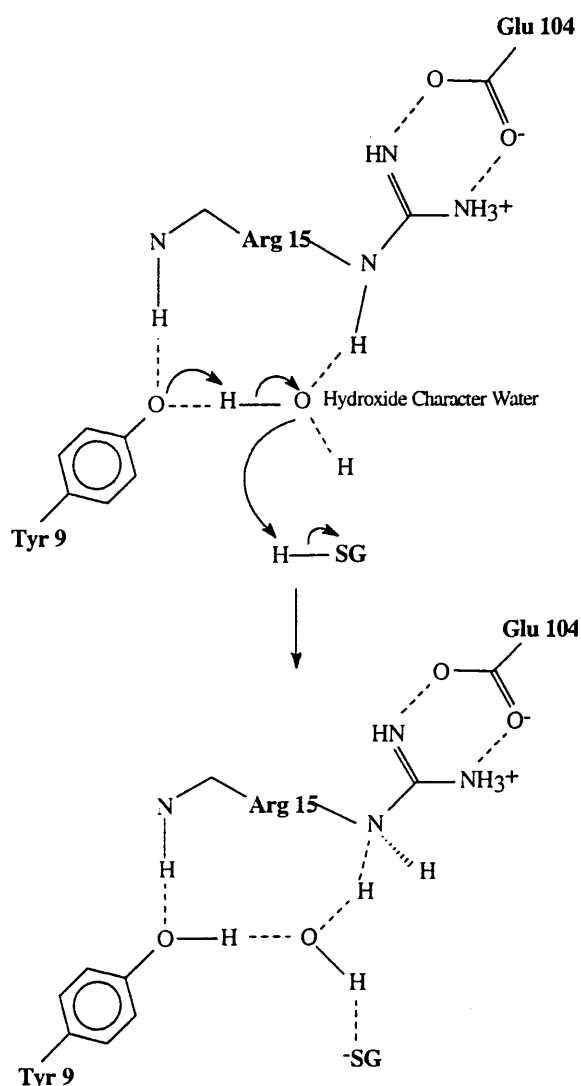


Fig 6.8 Possible Hydrogen bond network for glutathione bound to the active site of an alpha class GST.

Dietze and co-workers speculate about the presence of a hydroxide character water being present, activated by the electrostatic field of Arg15. This would provide a good candidate for the proton acceptor from glutathione. However, they are dismissive of the idea as there is no ordered water molecule found in either the apo-

protein crystal structure (Cameron *et al*, 1995) or the S-benzyl glutathione structure (Sinning *et al*, 1993). However, the water molecule only needs to be ordered when glutathione is bound; a crystal structure for a binary complex with glutathione alone is not yet available. An equilibrium of states may then exist at neutral pH around the water molecule but due to the pK_a values of Tyr9 and bound glutathione, the proton is likely to spend most of its time in proximity to the tyrosine residue. This could explain the unusual reactivity of catalytic Tyr with DEPC even in the presence of glutathione (Meyer *et al*, 1993). The crystal structure of glutathione alone bound to GST is not yet available and the solution of such a structure is likely to be a very important step in discovering the catalytic mechanism and understanding the conformational change in the H-site.

Further interesting NMR experiments would involve closer investigation of Tyr 9 to determine why the residue exhibits such a low pK_a value. Helix dipoles have been suggested to be involved in stabilisation of the tyrosinate ion but there are no reports to date that give evidence for this and examination of the crystal structure does not provide clear evidence. It seems much more likely that Arg 15 is involved in some kind of hydrogen bonding network to stabilise the tyrosinate ion. This has been explored by making R15L and R15K mutations and titrating ultra-violet difference absorption spectra (Bjornestedt *et al*, 1995) of wild-type and Y9F proteins. They showed that both these mutations increased the pK_a of Tyr9. It would be interesting to make these mutations along with C112S and look at the pK_a of Tyr 9 to directly observe the stabilisation of the tyrosinate ion. Perturbing the pK_a of Tyr 9 should logically affect the pK_a of bound glutathione and NMR should be a sensitive enough technique to see the effect of Arg 15 mutations on glutathione. NMR experiments could also be carried out to investigate results that residues present on the C-terminal alpha-helix, particularly Phe220, are able to form on-face hydrogen bonds that are able to influence the pK_a value of Tyr 9 (Dietze *et al*, 1996).

This chapter also contains the first direct evidence that the thiol group of glutathione is deprotonated when bound to GST. However, the dynamics of the proposed network of hydrogen bonds resonance forms (Dietze *et al*, 1996) cannot be explored because they occur too quickly on the NMR timescale.

While the charge state of glutathione is clearly important in the catalytic mechanism, there is evidence that the substrate bound at the H-site is not 'passive' and may be also activated during catalysis. Kinetic analyses have been carried out using β -mercaptoethanol as an alternative thiol compound to glutathione (Principato *et al*, 1988). They show that GST can catalyse the reaction of β -mercaptoethanol to 1-chloro, 2,4-dinitrobenzene (CDNB) but only in the presence of a glutathione analogue, S-methyl glutathione. The inference is that the binding of S-methyl glutathione induces a conformational change in the protein which is required to allow relaxed thiol

specificity. This initial observation has been confirmed in part by the publication of the apo-protein crystal structure (Cameron *et al*, 1995). Unlike glutathione, it is difficult to see how the enzyme could activate β -mercaptoethanol as there is no defined binding site for it suggesting that the enzyme is promoting catalysis by activating bound CDNB. This type of interaction could proceed by stabilising transition-state of CDNB as it reacts with β -mercaptoethanol. There is some evidence from panels of random mutants that the charge state of the residue at position 208 influences the activity of the enzyme towards CDNB by interacting with the negative charge that accumulates on the *para*-NO₂ group during the transition state (Widersten *et al*, 1994). The binding of S-methyl glutathione may move the residue at position 208 into the correct position for this to occur.

Chapter 7

Discussion

7.0 DISCUSSION

To conclude, how do the results presented here contribute to understanding how GSTs, and in particular alpha-class GSTs, recognise their substrates and catalyse the nucleophilic addition of glutathione to those substrates.

From the engineering experiments with huGSTA1-1, rGST Yc₁ and rGST Yc₂ (Chapters 2 and 3) it seems clear that there are subtle differences in the residues that line the H-site which can have an effect on the activity of the enzyme with particular classes of substrates. However, there appears to be a very important residue located at the end of the H-site, just before the C-terminal alpha-helix at position 208 in the protein sequence. From molecular models presented here, the size of the residue present at this position appears to be important in determining activity towards aflatoxin 8,9-epoxide. Substitution of Glu208 to the less bulky Asp208 in the inactive rGST Yc₁ results increased activity of the protein.

The models predict that the reaction product AFB-GSH will not be properly accommodated in the active site if the glutamate is present at position 208 but this is unlikely to occur with aspartate. The change in the activity of the enzyme must be due mainly to steric considerations as the charge on Asp208 is likely to be similar to Glu208 as the pK_a values in free solution are both 4.4. Position 208 has also been shown to be important in substrate selection as mutants at that position were identified in random mutagenesis phage display libraries for their abilities to confer novel binding properties (Widersten and Mannervik, 1995).

The charge at this position has also been shown to be important in the catalytic mechanism (Widersten *et al*, 1994) and this has also been reflected in the mutations at position 208 with huGSTA1-1. Mutation Met208 to Asp decreased the specific activity of the enzyme for CDNB, with the negative charge at the position postulated to interfere with the negative charge that accumulates on the *para*-nitro group of CDNB during the transition state due to their close proximity.

While the charge may be important, I feel that the steric nature of the residue at position 208 is more important. The publication of the apo-protein structure of huGSTA1-1 (Cameron *et al*, 1995) was very instructive. It showed that binding of an ethacrynic-glutathione conjugate to the protein causes some displacement of Met208 out of the H-site when compared to the crystal structure of huGSTA1-1 complexed with S-benzyl-glutathione which is a much smaller molecule. This displacement in turn causes some disruption to the C-terminal alpha helix making it less ordered.

Further evidence for position 208 moving and changing its environment comes from NMR experiments carried out in our laboratory (L-Y Lian and C.S. Allardyce). ¹⁵N methionine labelled samples were investigated using 2-D HMQC NMR and the

M208 resonance was identified comparing the spectrum to ^{15}N -labelled M208D protein from the engineering experiments. The cross-peak was investigated when wild-type protein was complexed with glutathione and with 2,4 dinitrophenol glutathione (2,4 DNP-GSH). The cross-peak is visible when ligand-free but becomes very broad when the protein is just in complex with glutathione and finally is found in a greatly shifted position when bound to 2,4 DNP-GSH indicating that the residue is in a different environment in the enzyme-inhibitor complex.

The difference in the sizes of S-benzyl-glutathione and 2,4-DNP-GSH are large enough to cause some distortion of the C-terminal alpha helix in the case of the latter, so what is likely to be the effect of aflatoxin 8,9-epoxide binding? Aflatoxin B₁ is a larger molecule than CDNB or ethacrynic acid and accommodation of the molecule, particularly by position 208, in the active site seems to be an important determinant in the activity (Chapter 3). The proposed interplay with the secondary structure in the helix is hinted at when the sequences of huGSTA1-1 and rGST Yc₂ are considered along with the 3-dimensional structures. The residues that are different on these protein's C-terminal alpha helices do not appear to be confined to the H-site side of the helix. Rather, the residues are substituted in huGSTA1-1 so that they could be lined up to face the H-site by unwinding the helix slightly. Therefore, activity may be increased in the human enzyme if the mutations at 208 and 108 are coupled with a helix swap mutation.

The influence that position 208 may have on the secondary structure in the C-terminal helix could be crucial to the correct functioning of the enzyme. The C-terminal helix is a dynamic part of the protein structure. This is clear from the electron density of the apo-protein crystal structure being present only at low contour levels (Cameron *et al*, 1995) and also from the ^{15}N labelled phenylalanine samples of wild-type huGSTA1-1 and the mutant F222Y which show the changes in the cross-peaks in response to the binding of 2,4 DNP-GSH (Chapter 5). It also appears that the C-terminal helix is involved in promoting the formation of a productive enzyme-substrate complex. This can be deduced from experiments in chapter 5 where huGSTA1-1 was mutated to delete the C-terminal helix and the kinetics were investigated in the presence and absence of a synthetic peptide of the same sequence and also from the crystal structure of a GST from *Arabidopsis thaliana* which does not have a C-terminal helix and shows inhibitors bound in both productive and non-productive complexes (Reinemer *et al*, 1996). The non-productive complex bound the inhibitor further inside the enzymes so that it partially occupied the G-site. It is also interesting to note that the enzymes that were inactive with aflatoxin epoxide also appeared to bind aflatoxin more tightly than the active enzymes. This could be due to the formation of a non-productive enzyme-substrate complex.

I would like to suggest that the residue at position 208 acts as a 'steric sensor' in the protein. If a substrate cannot be accommodated in the H-site of the enzyme, either because of size or because the other residues that line the H-site subtly affect the binding position, then displacement of 208 occurs which in turn interferes with the formation of secondary structure in the C-terminal helix. It is likely that GSTs are already loaded with glutathione in the cell and the C-terminal helix is probably in the disordered state described. However, when it encounters a potential, hydrophobic substrate, the substrate interacts with the enzyme firstly through hydrophobic interactions, as the disorder of the helix will expose the hydrophobic H-site. Correct interaction of the substrate with H-site residues and accommodation of the side-chain of the residue at position 208 allows the helix to become ordered over the H-site. This, in turn, allows the substrate to be bound in a productive enzyme-substrate complex with residues in the C-terminal helix placed in the correct places to allow catalysis to occur.

This is particularly important for position 220. It has been suggested that mutating Phe220 to tyrosine in rGSTA1-1 creates an on-face H-bond with Tyr9 (Dietze *et al*, 1996). The construction of this hydrogen bond has been shown to affect the pK_a of Tyr9, actually lowering it from 8.3 ± 0.2 to 7.8 ± 0.2 (Dietze *et al*, 1996). This change in pK_a value is also reflected in the pK_a of glutathione when bound to F220Y, it is further lowered from 7.46 ± 0.06 to 7.02 ± 0.05 . *Ab initio* calculations suggest also that Phe220 present in the wild-type carries out a similar role and could help in stabilising the low pK_a of Tyr9 reported and supported by our NMR data (Chapter 6). Thus, the correct formation of the helix could have a real impact on the catalytic mechanism by helping to stabilise the low pK_a of Tyr9.

The low pK_a of Tyr9 can now be confirmed by the NMR data and has been found to be lower than that previously reported. The data in chapter 6 show that the pK_a of Tyr9 is 7.72 ± 0.2 whereas difference fluorescence measurements put the value at 8.3 ± 0.2 . The reason for the rather higher pK_a values previously determined by difference fluorescence techniques is that there is another tyrosine residue that has a lower than expected pK_a value which will influence curve-fitting parameters of these types of experiment if not taken into account (Atkins *et al*, 1993, Dietze *et al*, 1996, Dietze *et al*, 1996). What is clear is that the pK_a value of Tyr9 is lowered compared to the pK_a in free solution. The stabilisation of Tyr9 is due to likely 'second sphere' interactions of other residues with Tyr9. They are called second sphere interactions because they also contribute the lowering of the pK_a of bound glutathione and evidence suggests that Arg15 (Bjornestedt *et al*, 1995) and Phe220 (Dietze *et al*, 1996) contribute to the electrostatic field around Tyr9 and subsequently glutathione.

The NMR data also support other evidence that the pK_a of bound glutathione is also lowered compared to that in free solution (Graminski *et al*, 1989). While the

NMR data cannot give a determination of the pK_a of bound glutathione because the cysteine C_β proton did not give a sufficiently large change in chemical shift they do show that the pK_a of bound glutathione is <7.0 . The data generated however are consistent with current ideas of how alpha-class enzymes may catalyse their reactions.

Despite the low pK_a value of Tyr9, and the existence of a significant fraction of Tyr9 present as phenolate anion, it seems unlikely that alpha-class enzymes catalyse the conjugation of electrophilic hydrophobes via a general base mechanism where proton transfer occurs between glutathione and Tyr9 because of investigations with deuterium isotope solvent effects (Huskey *et al*, 1991). However, the proton from glutathione is lost when glutathione binds and this can be seen by the magnitude of the chemical shift of glutathione. Therefore the real problem in GST biochemistry presently is where is the proton from glutathione during catalysis? Dietze and co-workers suggest from their emission-excitation correlation fluorescence experiments that there may a hydroxide-character water molecule involved in catalysis (Dietze *et al*, 1996). While the hypothesis is tenable, there is no further evidence to support it and there are no reports of ordered water molecules in the active site. Perhaps the solution of a crystal structure of GST in complex with glutathione alone will be able to shed some light on what happens to the proton of glutathione.

Elucidation of the mechanisms of substrate recognition and catalysis of GSTs are potentially important clinically. Evidence exists to show that treatment of clinical tumours with alkylating agents results in sub-populations of the cells that are resistant and have increased expression levels of GSTs (Lewis *et al*, 1988). The resistance of the cells is not surprising as the drug used will be activated intracellularly, trapping of the reactive intermediate of the drug in a glutathione conjugate will effectively deactivate it, after all that is the job that GSTs are there to perform. To guard against drug resistant populations, clinicians treating cancer with chemotherapy often have to increase the dose of the drug to compensate for this effect but this is generally limited by toxicity to the patient.

Understanding the mechanisms of GSTs may allow the design of potent inhibitors to the specific GSTs that are up-regulated in tumour cells. These inhibitors could be given along with chemotherapy to increase the efficacy. More adventurous applications could be the use of gene therapy to express engineered GSTs in bone marrow cells (Deisseroth, 1993, Gulick and Fahl, 1995, Pulchalski and Fahl, 1990, Sorrentino *et al*, 1992). Engineering resistance to chemotherapy drugs by gene therapy could increase the efficacy of chemotherapy administered, lowering the toxicity and indeed combinations of inhibitor and gene therapy treatments may prove to be extremely effective. The results presented in this show that these types of engineering experiments are certainly possible with enzyme activity being rationally altered and

heterologous expression of the mutant protein in cultured human cells altering their resistance to model compounds.

Chapter 8

Materials and Methods

8.0 MATERIALS AND METHODS.

8.1 Materials.

8.1.1 Chemicals.

General laboratory chemicals were supplied by Fisons, BDH or Sigma. Unless otherwise stated and were of analytical grade or equivalent. Bacterial culture medium was obtained from Oxoid (Unipath). Radio-labelled nucleotides were supplied by Amersham International. Millipore Super-Q System treated water was used for all aqueous solutions. All tissue culture growth media was purchased from Biofluids Inc., Rockville, U.S.A, Hanks's Buffered Saline solution and Trypsin/EDTA were supplied by Gibco BRL.

8.1.4 Bacterial Strains.

E. coli RZ1032. Derived from *E. coli* BW313 partial genotype only available; *lacZ*_{bd-279::Tn10} Hfr *dut⁻ ung⁻*.

E. coli JM109. F' *traD36 lacI^q D(lacZ) M15 proA⁺B⁺ e14 (McrA⁻) D(lac-proAB) thi gyrA96 (Nal^r) endA1 hsdR17 (r_k⁻ m_k⁺) relA1 supE44 recA1*.

E. coli BL21 (DE3). F' *ompT hsdS^B (r_B⁻ m_B⁻) gal dcm* (DE3) (Studier, 1986)

8.1.2 Enzymes and Proteins.

Restriction endonucleases were purchased from Gibco BRL, Pharmacia, Boehringer Mannheim, or New England Biolabs. DNA polymerase I Klenow Fragment, *Taq* DNA polymerase, T4 DNA Ligase and T4 Polynucleotide kinase were supplied by Boehringer Mannheim. Deep Vent DNA polymerase was supplied by New England Biolabs. BSA (enzyme grade) was obtained from Gibco (BRL).

8.1.3 Synthetic Oligonucleotides and Peptides.

All Synthetic Oligonucleotides and peptides were synthesised by the Protein and Nucleic Acid Laboratory, University of Leicester using an ABI Synthesiser Model 394-08 for the oligonucleotides and an Applied Biosystems 431 Solid Based Synthesis machine for the peptide.

8.1.3.2 Table of Sequencing primers.

Oligonucleotide	DNA sequence 5' to 3'
GSTSEQ1	GCACTTGCTGGAACATCAAATATCC
GSTSEQ2	CCTTGATCAAGGCAAGCTTGGCATC
GSTSEQ3	GGTTGCTGATTCTGGTTTTTCAGGGC
GSTSEQ4	GCCCTGAAAACCAGAATCAGCAACC
PGWLF	GTGTGGAATGTGAGCGG
PGWLR	GTTCTGATTTAATCTGTATCAG
RODSEQ	ATGGTAGAGAAGTTCAACCAGGGAAACATC

8.1.3.1 Table of Mutagenic primers.

Oligonucleotide	DNA Sequence 5' to 3'
Y8F	CCAAGCTCCACTTCTTCAATGCACG
M208D	GGAAGCCTCCCGTGGATGAGAAATCTTTAG
L108Y	GGTGAAATGATCCTCTATCTGCCCGTATGTCC
F220Y	GCAAGGAAGATTTACAGGTTTTAATAACG
F222Y	GGTATTAATAACGCAGTC
DCT	GCCTCCCATGGATGAGTAATAACGCAGTCATGGAGG
H108Y	GATGAAATAGTTCTCTATTACCCTTACATTCCC
E208D	CAGAGGAAGCCATTAGATGATGAGAAATGTGTAG
Y108L	GAGTTGATGGTTCTCCTTTACCCCTACATGCCC
D208M	CAGAGGAAGCCTTTTATGGATGAGAAATGTGTAG

8.1.3.3 Table for PCR primers

Oligonucleotide	DNA sequence 5' to 3'
YCF	GGAATTCTATGCCGGGGAAGCCAGTCCTTCAC
YC1R	ATGCCCGGGTTATTAAGTGAAGATCTTAAGTGCAGATTC
YC2R	ATGCCCGGGTTATTAAGTGAAGATCTTCTTCGCTGATTC
YCFPET	ATGCATCGTTACGCATATGCCGGGGAAGCCAGTCCTTC
YC1RPET	CTGACTACGCGGATCCTTATTAAGTGAAGATCTTAAGTGCAGATTC
YC2RPET	CTGACTACGCGGATCCTTATTAAGTGAAGATCTTCTTCGCTGATTC
YCFZEO	CATGCTTACGGAATCCCAGCCATGCCGGGGAAGCCAGTCCTTC
YC1RZEO	GCACTACGCACGCGTTTATTAAGTGAAGATCTTAAGTGCAGATTC
YC2RZEO	GCACTACGCACGCGTTTATTAAGTGAAGATCTTCTTCGCTGATTC
F1F	AGCTTCGACAGCATGCGTAAACGTTAATATTTTGTTAAATTCG
F1R	CAGCATTTCGAGCATGCACGCGCCCTGTAGCGGCGCATTAAAG

Synthetic Peptide Sequence

Hel 1 Lys Ser Leu Glu Glu Ala Arg Lys Ile Phe Arg Phe.

8.1.5 Bacterial Culture Media.

Bacterial strains were grown using the following solid and liquid culture media.

Solid Media

Luria Agar (LA). 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar

Low Salt Agar (LSA). 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.25% (w/v) NaCl, 1.5% (w/v) agar.

Liquid Media.

Luria Broth (LB) 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.25% (w/v) NaCl.

Low salt Luria Broth (LSLB) 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.25% (w/v) NaCl

2xYT. 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl.

8.1.5 DNA Vectors

Bacterial expression vectors used were pKK128 (Pharmacia) and pET12(a) (Invitrogen). The mammalian expression vector used was pSVZeo (Invitrogen).

8.2 Methods of Sterilisation.

Autoclaving. Large scale autoclaving was carried out with a Cabburn 8 cubic feet capacity autoclave (Cabburn Sterilisers, Shoeburyness) with the programme set to reach 121°C for 30 minutes. Small scale sterilisation was carried out in the lab using a Dixon ST19 portable autoclave (Dixon's Surgical Instruments); again the autoclave was set to reach 121°C for 30 minutes.

Dry Sterilisation. To sterilise glassware, a B&T 'Unitemp' sterilising cabinet was used at a temperature of 160°C for 6 hours.

Filter Sterilisation. Small quantities of solutions (up to 5ml) were sterilised using sterile pipettes and 0.2mm Acrodiscs (Gelman Sciences, Ann Arbor). Larger volumes were sterilised using Nalgene 0.2mm filter sterilising units under vacuum. (Nalgene, Rochester, New York).

8.3. Nucleic Acid Methods.

8.3.1 Definition of Standard Solutions used in Preparation of DNA.

50mg/ml Ampicillin Sodium Salt. An ethanol-flamed metal spatula was used to weigh out the required amount of ampicillin which was dissolved in sterile water and filter sterilised using a 0.2mm acrodisc before aliquotting and storage at -20°C.

1M Calcium chloride. 29.4g of CaCl_2 were dissolved in 50mls of distilled water and made up to final volume of 200ml before sterilising by autoclaving. The solution was stored at room temperature until use.

24:1 Chloroform/Isoamyl alcohol. Chloroform (96% v/v) and Isoamyl alcohol (4%) was prepared by mixing and stored at room temperature.

2mM dNTP solutions. A 2mM dNTP stock solution was prepared by adding 2ml each of 100mM dATP, dCTP, dGTP, dTTP and 92ml of sterile water to make up the volume. The solution was stored at -20°C for not more than 1 month..

10mg/ml Ethidium bromide. Ethidium bromide was dissolved at 10mg/ml in deionised water and stored in light-resistant polypropylene bottles at room temperature.

80% Ethanol. Absolute Ethanol was diluted to 80% with deionised water and stored at room temperature.

50% Glycerol. Glycerol was diluted with deionised water 50% (v/v) and autoclaved before storage at room temperature.

0.2M HCl. A stock solution of 0.2M was prepared by adding 17.24ml of concentrated HCl to 982.76ml of deionised water. The solution was stored at room temperature.

200mg/ml IPTG. 2g of IPTG (Nova Biolabs) was dissolved in 10ml deionised water and stored at -20°C in 5ml aliquots after filter-sterilising through 0.2mm acrodiscs.

700mM Magnesium chloride. 28.46g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were dissolved in 50ml of distilled water and made up to 200ml. The solution was sterilised by autoclaving before use.

20% PEG/2.5M NaCl. 20g of polyethylene glycol and 11.7g of NaCl were dissolved in 100ml of deionised water. The polyethylene glycol dissolved upon autoclaving and the solution was stored at room temperature.

Phenol (liquefied). Liquefied phenol containing 0.1% (w/v) 8-hydroxy-quinoline was equilibrated against 100mM Tris (pH 7.6) and stored in the dark at 4°C .

3M Sodium acetate. 40.8g of sodium acetate.3H₂O was dissolved in 90ml deionised water and adjusted to pH 5.6 with glacial acetic acid before making up the volume to 100ml and autoclaving. The solution was stored at room temperature.

5M Sodium Chloride. 29.25g of Sodium Chloride were dissolved in 95ml of deionised water and the volume made up to 100ml before autoclaving and storing at room temperature

10M Sodium Hydroxide. 200g of Sodium Hydroxide were added to 400 ml deionised water slowly with cooling. Once dissolved the solution was made up to 500 ml and stored at room temperature.

Denaturing Solution. 0.5M NaOH 1.5 NaCl mixed from stock solutions and autoclaved.

Neutralising Solution. 0.5M Tris.HCl (pH 7.4) 3M NaCl made up from stock solutions and autoclaved.

SSC (20x). SSC was made as 20x stock solution and then used at various concentrations during hybridisation experiments. 3M NaCl 300mM tri-Sodium citrate adjusted to pH 7.0 with 10M NaOH, autoclaved and stored at room temperature.

Tris.EDTA.Acetate (TAE). 10x stock solution was made by dissolving 48.4g of Trizma and 0.5M EDTA were dissolved in 950ml deionised water. The pH was adjusted to 7.5 with glacial acetic acid and the volume made up to 1l.

8.3.2 Preparation of Competent *E. coli* strains.

Trituration Method.

This method was carried out as directed in (Titus, 1989). 5ml of LB was inoculated with a single colony of *E. coli* and grown overnight at 37°C with continuous shaking. The overnight culture was used to inoculate 200ml of LB in a 2l conical flask, pre-warmed at 37°C. The culture was incubated with continuous shaking at 37°C until A₆₀₀ was approximately 0.5. When the cells had reached the required density, they were poured into two 250ml Beckman polypropylene centrifuge pots, which had been rinsed with copious deionised water to remove traces of detergent, and

left to chill on ice for 1 hour. The cells were collected by centrifugation using a Sorval J2-ME centrifuge in a JA-10A rotor, spinning at 2500g for 20 minutes at 4°C. After aspirating the supernatant, the cells were gently resuspended in a total of 20ml ice-cold Trituration Buffer (100mM CaCl₂, 70mM MgCl₂, 40mM Sodium acetate pH 5.5) and then diluted to a total of 200mls. This was then left to incubate on ice for 45 minutes before finally collecting the cells in a low speed spin, 1800g for 10 minutes at 4°C. The buffer was decanted and cells resuspended in a total of 20ml of trituration buffer by gently swirling the cells around the pot before adding 2.5ml of 80% Glycerol. The cells were then snap-frozen in a salt/water/dry-ice bath in aliquots of 200ml in pre-chilled eppendorfs and stored at -80°C.

Electrocompetent cells.

The method was essentially that described in (Dower *et al*, 1988, Taketo, 1988). 10ml of LB was inoculated with a single colony and grown overnight at 37°C with continuous shaking. 10ml was used to inoculate 1l of pre-warmed LB at 37°C in two 2l flasks and grown until A₆₀₀ was 0.4>0.5. The flasks were chilled on ice for 30 minutes and the cells harvested by centrifugation at 4000g for 5 minutes at 4°C in 4 Beckman 250ml polypropylene centrifuge pots in a Sorvall J2-ME centrifuge using a JA-10 rotor. Once harvested the cells were washed with water to remove all traces of electrolytes and progressively concentrated. Initially they were resuspended in a total of 1l ice-cold, sterile, deionised water with gentle swirling. The cells were harvested again at 4000g for 5 minutes at 4°C as before and the water decanted. They were resuspended in 500ml of ice-cold, sterile, deionised water with gentle swirling before further centrifugation at 4000g for 5 minutes at 4°C. The supernatant was decanted and the cells resuspended in 20 ml of sterile 10% Glycerol. The cells were centrifuged again at 4000g for 5 minutes at 4°C in a bench top centrifuge and the cell pellet resuspended in 2 ml of ice cold sterile 10% Glycerol. The cells were finally snap-frozen in a dry-ice/salt/water bath in 40ml aliquots and stored at -80°C.

8.3.3. Transformation of Competent *E. coli* with plasmid DNA.

For trituration method cells (Titus, 1989), competent *E. coli* were removed from -80°C storage and thawed on ice for 10 minutes. The plasmid DNA was added to the cells and incubated on ice for 20-30 minutes. The cells were then heat-shocked by placing the eppendorfs into a 42°C water-bath for exactly 1 minute. Immediately afterwards they were cooled for 1 minute on ice before adding 800ml of LB. The mixture was incubated in a 37°C water-bath to allow expression of the resistance

mechanism for 1 hour before plating 200ml aliquots on LA containing the required antibiotic. The plates were incubated overnight at 37°C to allow colony formation.

For electrocompetent cells (Dower *et al*, 1988, Taketo, 1988), an aliquot was taken from -80°C storage and thawed gently on ice. 10-100ng of DNA was added in a volume no less than 10ml. For DNA used in enzymatic reactions, the salt from the reactions was removed using Millipore microdialysis discs and the reactions dialysed against deionised water for 30 minutes prior to introduction to the cells. The cell/DNA mixture was added immediately to an ice-cold 0.2cm gap cuvette (BIO-RAD). The cells were electroporated using a Gene-Pulser connected to a Pulse controller (BIO-RAD) set at 25mF, 2.5 kV and 200W. Immediately after the pulse was delivered, the cells were transferred to 1ml of prewarmed 2xYT medium and incubated for 1 hour before plating onto selective agar plates and incubated overnight at 37°C until discrete colonies were seen.

8.3.4. Small-scale preparation of plasmid DNA.

Small-scale preparation of plasmid DNA (Birnboim, 1983, Birnboim and Doly, 1979) was carried out using Promega Wizard minipreps as directed by the manufacturer's instructions. A single colony was used to inoculate 5ml of LB in a sterile universal with appropriate antibiotic and grown overnight at 37°C with continuous shaking. 1.5ml of the fresh overnight culture was spun down in a sterile eppendorf at 13000rpm for 2 minutes in a bench-top microcentrifuge. The pellet was resuspended in 200µl Promega Wizard miniprep cell resuspension solution (50µM Tris-HCl, 10mM EDTA, 100mg/ml RNase A) and the DNA released in to the solution by alkaline lysis with 200µl of cell lysis solution (0.2M NaOH 1% SDS). Finally the solution was neutralised by the addition of 150µl neutralising solution (3M Potassium acetate). Cell debris was spun down in a microcentrifuge for 10 minutes to leave a cleared supernatant containing the plasmid DNA. This was purified by adding Promega Wizard minipreps resin that selectively bound the plasmid DNA. The resin was removed from the solution with the supplied column and washed using column wash solution (200mM NaCl, 20mM Tris-HCl pH 7.5, 5mM EDTA; diluted 1:1 with 95% ethanol). The DNA was finally eluted from the resin using 50µl sterile water. The quantity and purity of the preparation was then determined by agarose gel electrophoresis.

8.3.5. Large-scale preparation of plasmid DNA.

Large scale preparation of plasmid DNA was carried for transfection of mammalian cells was carried out using Qiagen Tip-100 plasmid kits. Alkaline lysis (Birnboim, 1983, Birnboim and Doly, 1979) was used to produce a cleared bacterial lysate that contained soluble proteins and plasmid DNA. A single colony was used to inoculate 5mls of LB containing the appropriate antibiotic and grown overnight at 37°C with continuous shaking. The fresh overnight culture was used as a starter culture to inoculate 100mls of LB with the appropriate antibiotic which was grown overnight. The cells were harvested by spinning at 6000g for 15 minutes at 4°C in JA-14 rotor in a Sorvall J2-ME. The bacterial pellets were resuspended in 2ml of P1 buffer (100mg RNase A, 10mM EDTA, pH 8.0, 50mM Tris-HCl) and lysed by adding 2ml of P2 buffer (200mM NaOH, 1% SDS). To ensure complete lysis, cells were incubated at room temperature for 5 minutes with P2. To neutralise the P2, 2ml of ice-cold P3 (3M Potassium acetate pH 5.5) were added and incubated on ice for 20 minutes. The precipitated cellular and chromosomal debris was pelleted by centrifuging for 30 minutes at 30000g in a JA-20 rotor at 4°C. The cleared lysate was decanted and kept on ice while a Qiagen Tip-100 column was equilibrated with 2ml QBT buffer (750mM NaCl, 50mM MOPS, 15% (v/v) absolute ethanol, 0.15% Triton X-100, pH7.0). After equilibration the cleared lysate was added, where the plasmid DNA was selectively bound. The column was washed twice with 7ml QC buffer (1M NaCl, 50mM MOPS, 15% (v/v) absolute ethanol, pH 7.0) to remove impurities and the DNA finally eluted off the column with 3ml QF buffer (50mM Tris-HCl, 1.25mM NaCl, 15% (v/v) absolute ethanol, pH 8.5). The eluted DNA was precipitated with 2.1ml iso-propyl alcohol. The DNA was recovered by centrifuging immediately at 15000g for 30 minutes at 4°C in a JS-13.1 rotor. The pellet was washed with 3ml 70% ethanol and resuspended in 500µl sterile water. The concentration and purity were measured by comparing the ratio of A_{260} and A_{280} .

8.3.6. Agarose gel Electrophoresis.

Electrophoresis was carried out using agarose gels cast in appropriate gel trays (McDonnell *et al*, 1977). The concentration of agarose used was 0.8% to 1.5% (w/v) depending on the size of DNA fragments to separate made up with TAE as defined in 7.3.1. Samples for analysis were prepared by adding 1/10th loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and loaded by pipetting into the cast wells. The fragments were separated by applying a voltage across the gel no greater than 150V. DNA fragments were visualised by staining with

Ethidium bromide (Sharp *et al*, 1973) and illuminating on an ultraviolet transilluminator at 254nm.

8.3.6. Restriction Enzyme Digests.

Restriction digests were carried out as instructed by the manufacturer using the buffer supplied. Incubation times were varied in order to digest to completion and monitored using agarose gel electrophoresis. Restriction digests where two enzymes were used were completed by incubating with the enzyme that had the least salt requirement first, denaturing by heating at 70°C for 10 minutes and then adjusting the salt concentration according to the second enzyme's requirements. (Sharp *et al*, 1973)

8.3.7. 5' Dephosphorylation of Polynucleotides.

5' dephosphorylation of polynucleotides was carried out using Calf Intestinal Alkaline Phosphatase (CIAP) to increase the efficiency of ligation reactions where a single restriction site is used (Maniatis *et al*, 1989). After completing the restriction digest, the enzyme was denatured by heating at 80°C for 15 minutes in water-bath to stop any star-activity. The restriction enzyme buffer was supplemented by adding an appropriate amount of the 10x *Dephos buffer* supplied and 1 Unit of Calf Intestinal Alkaline Phosphatase/mg DNA. The reaction was left at 37°C for 30 minutes and terminated by heat denaturation at 80°C for 10 minutes. The reaction volume was adjusted to 50µl with sterile water and the restriction enzyme and CIAP was extracted by adding 50µl of TE/phenol and vortexing for 30 seconds to partition the protein to the organic phase. Excess phenol was extracted with 24:1 chloroform/IAA. The DNA was recovered by ethanol precipitation.

8.3.8 Purification of DNA Fragments from Agarose Gels.

DNA fragments, separated by gel electrophoresis, were purified by electroelution (Maniatis *et al*, 1989). The ethidium bromide-stained band was cut from the gel and placed in a small piece of dialysis tubing sealed with dialysis clips. TAE was added until the level surrounded the gel slice. The DNA was then electrophoresed from the fragment by applying a voltage of 180V across the gel slice. The progress of the elution could be followed by placing the dialysis bag on a transilluminator at 254nm. Once the DNA had been completely removed from the slice, the voltage was

reversed to release the DNA from the side of the bag. The TAE containing the DNA was removed from the bag and the DNA was precipitated by adding 1/10th volume 3M Sodium Acetate and 2 volumes absolute ethanol. The fragment was resuspended in an appropriate volume of sterile water.

8.3.9 PCR Amplification Reactions.

Polymerase Chain Reactions were used in a variety of applications to manipulate DNA sequences to give appropriate restriction sites flanking the genes so that they could be cloned into the appropriate vectors. Amplifications were carried out using a Biometra TrioBlock thermocycler. Reactions were standardised at a volume of 100µl containing 2.5 units of *Taq* polymerase, 250µM dNTPs, 0.2 pM primers, 1x manufacturer's reaction buffer and the volume finally overlaid with 50µl of mineral oil to stop volume loss due to evaporation during the amplifications. The cycles were carried out using a denaturation temperature of 95°C for 1 minute, an annealing temperature of 5°C below the lower calculated T_m for the primer pair for 1 minute and an extension temperature of 72°C for 1 minute. The final extension was carried out for 3 minutes. The cycle was repeated 30-35 times depending on the template concentration.

8.3.10. Purification of PCR Amplified DNA.

After PCR amplification, the products were examined using agarose gel electrophoresis. If the amplification gave a single reaction product, then this was purified away from contaminating primers, *Taq* polymerase and reaction buffer using Wizard PCR Clean-Ups as directed by the manufacturer directly from the reaction buffer without separating the reaction mixture on an agarose gel. 400µl of sterile water was added to the reaction and the extended DNA selectively bound by adding Wizard PCR Clean-Ups resin. The resin was filtered from the buffer using a Wizard column and washed using wash buffer (200mM NaCl, 20mM Tris-HCl pH 7.5, 5mM EDTA; diluted 1:1 with 95% ethanol) and dried by centrifugation. The DNA was eluted from the resin by adding 50µl of sterile water and collected by centrifugation. The DNA was ready to be used in further applications. Where discrete amplification products were not seen, the band that was of the expected size was cut from the gel and the DNA purified using electroelution.

8.3.11. DNA Ligation Reactions.

DNA ligation reactions were carried out using recombinant T4 DNA Ligase (Weiss *et al*, 1968) and the manufacturer's buffer. Concentrations of DNA in the reaction varied from 20-100ng with an approximate molar ratio of 2:1 (insert:vector). The reactions were incubated at 16°C overnight and circular DNA reaction products were recovered by transformation of *E. coli*.

8.3.12 Screening bacterial colonies for recombinant plasmids using PCR.

Bacterial colonies were screened for recombinant plasmids straight from the plate without preparing plasmid DNA using PCR. The LA plates containing the colonies to be screened were incubated until the colonies were approximately the same diameter as a Gilson P200 tip end. The colonies were then lifted using a Gilson P200 tip by placing the tip directly on the colony so that the cell mass was contained inside the tip. The colony was gridded on a replica LA plate for later reference and assigned a number. The tip was then placed on a Gilson Pipetteman and the colony resuspended in 10µl of sterile water. The suspension was boiled at 100°C for 10 minutes to release the DNA. The 10µl was then used in a 50µl PCR reaction as described in 2.3.9 with appropriate primers but amplified for 40 cycles. The reaction was then separated using agarose gel electrophoresis. The presence of an extension product of the predicted size showed that the colony arose from transformation with a recombinant plasmid. The replica plate was used as a reference and the colony was grown for further analysis.

8.3.13 Screening Bacterial Colonies for Recombinant Plasmids using Colony Hybridisation.

Large numbers of bacterial colonies were screened concurrently for recombinant plasmids using colony hybridisation (Maniatis *et al*, 1989). Circular, gridded nylon filters were placed onto LA plates containing the appropriate antibiotic and allowed to become wet. Putative colonies were streaked onto the membrane, and streaked on a reference LA plate that was gridded in the similarly, using sterile toothpicks. The membranes and plates were incubated overnight at 37°C to allow colony growth. The next day, the membranes were removed from the plates and the cell growth lysed by placing the membranes on Wattman 3MM filter papersoaked with 10% SDS for 10 minutes. The membranes were removed and transferred to filter

paper soaked in denaturation solution (defined in 8.3.1) for 5 minutes to denature the released DNA and finally neutralised by placing the membranes on filter paper soaked in neutralisation solution (defined in 8.3.1) for 5 minutes. Finally the filter was washed with SSC to remove cellular debris and the DNA fixed to the membrane using a Stratagene UV Stratalinker set to 'auto crosslink'. The membrane was then ready for hybridisation with a radio-labelled probe.

8.3.14 Radio-labelling DNA using ^{32}P dCTP and Random Priming.

The method used was that described by (Feinberg and Volkenstein, 1983). 10ng of the double stranded DNA to act as the primer was added to 5 μl oligo-labelling buffer (250mM Tris.HCl, 25mM MgCl_2 , 0.3%(v/v) β -Mercaptoethanol, 1M HEPES pH 6.6, 0.5 μM hexanucleotides, 100mM dATP, 100mM dGTP and 100mM dTTP) and the volume made up to 22 μl . The mixture was boiled at 100°C for 10 minutes to denature the DNA and allowed to cool annealing the random hexanucleotides. The DNA was finally labelled by adding 1 μl of ^{32}P -labelled dCTP (0.37MBq/ml), 1 μl 10mg/ml BSA and 10 units DNA polymerase I Klenow fragment and incubating at 37°C for 30 minutes. The reaction was used immediately.

8.3.15 Hybridisation of Membrane-bound DNA.

The nylon membranes with the DNA fixed were prehybridised for a minimum of 10 minutes at 58°C in 20ml Church and Gilbert buffer (Church and Gilbert, 1984) (7% (w/v) SDS, 1% (w/v) BSA, 0.5M NaPO_4 pH7.0 and 1mM EDTA) in sealed Hybaid oven hybridisation bottles. After prehybridising 0.5ng/ml of ^{32}P radio-labelled probe was added and hybridised for 16 hours at 58°C. After hybridisation, the filters were washed initially twice with 2x SSC, 0.1% (w/v) SDS at 60°C and then with 1x SSC, 0.1% SDS at 60°C for 15 minutes. The filters were blotted dry and autoradiographed at -70°C with intensifying screens overnight. X-ray film used was Kodak X-OMATTM (Eastman Kodak Company, New York). If the signal to background ratio was low then the filters were further washed with 0.5x SSC 0.1% SDS at 60°C for a further 15 minutes.

8.3.16 5' Phosphorylation of Synthetic DNA primers.

Synthetic mutagenic oligonucleotides were 5' phosphorylated by using T4 polynucleotide kinase. The primers were ethanol precipitated because the NH_4^+ ions in which the primer was supplied inhibit the action of T4 polynucleotide kinase. The concentration was determined spectrophotometrically at 260nm. 48pmoles of the DNA was taken and added to 1x reaction buffer, 1mM dATP and 5 units T4 polynucleotide kinase in a total volume of 10 μ l. The reaction was incubated at 37°C for 30 minutes before being terminated by heating at 80°C for 20 minutes. The primers were then stored at -20°C until required. The reaction was kept brief and terminated by heat denaturation because T4 PNK is able to catalyse the reverse reaction.

8.3.17 Isolation of Single-Stranded Phagemid DNA.

5 ml of LB containing the appropriate antibiotic was inoculated with a single colony from a, M9 minimal medium plate. Growth on M9 is essential to ensure the presence of the F' plasmid that is required for effective helper 'phage infection. The culture was incubated overnight at 37°C with constant shaking. The next afternoon, 50 μ l of the overnight culture was used to inoculate a further 5ml of LB. The culture was grown until A_{600} was approximately 0.5 when VCSM13 helper phage (Stratagene) was added at a ratio of phage:cells of 10:1 or a final concentration of 2×10^8 pfu/ml. The culture was then left on the bench for 5 minutes to allow the helper 'phage to adsorb to the bacterial membranes before incubation overnight in a 37°C incubator with agitation. The following day 2 x 1.5ml aliquots were taken and the cells killed by heating at 80°C for 5 minutes before centrifugation at 13000 rpm at room temperature for 5 minutes in a benchtop microcentrifuge. The supernatant was collected and the centrifugation repeated to ensure that there was no bacterial contamination. The 'phage particles in the supernatant were recovered by precipitating with 20% polyethylene glycol (PEG 8000) 2.5M NaCl and recovered by centrifugation at 13000rpm for 5 minutes as before. The phage pellets were each resuspended in 50 μ l of sterile water and combined to give 100 μ l. The protein coats were extracted with 100 μ l phenol/TE followed by 100ml of chloroform/IAA. The DNA released into the aqueous phase was concentrated by precipitating with 10 μ l 3M Sodium acetate and 250 μ l of 100% ethanol and centrifuging at 13000rpm. The salt was removed from the DNA pellet by washing with 50 μ l of 80% ethanol before resuspending in 20 μ l of sterile water.

8.3.18 Site-Directed Mutagenesis.

Method 1.

Site-directed mutagenesis was, in the first instance, carried out using the Oligonucleotide-directed Mutagenesis System Version 2.1 from Amersham. The kit uses a single strand template and mismatched oligonucleotides to produce the desired mutations. The kit instructions advised that an M13 template be used but in this case a pBluescriptSK+ template was used following the manufacturer's instructions. The 5' phosphorylated mutagenic oligonucleotide was annealed to the single-stranded template and 3' OH group extended by DNA polymerase I Klenow fragment in the presence of T4 DNA Ligase to generate a mutant heteroduplex. Selective removal of the non-mutant strand was made possible by the incorporation of thionucleotides into the mutant strand during *in vitro* synthesis. Certain enzymes cannot cleave phosphorothioate DNA and as a result, single strand breaks can be generated in the non-phosphorothioate-containing DNA strand. Thus nicks were generated by digesting the mutant heteroduplex with *NciI*, which allowed exonuclease III to digest away the majority of the non-mutant, non-thiolated strand. Since pBluescriptSK+ has 2 more *NciI* sites than M13, the exonuclease III digestion was incubated for 20 minutes instead of the 30 minutes recommended to ensure that there was sufficient duplex DNA to act as a primer for the next step. The mutant strand was used as a template to reconstruct a double-stranded closed-circular molecule using the proof-reading enzyme DNA polymerase I and T4 DNA Ligase, creating a homoduplex, mutant molecule. *E. coli* TG1 was transformed with the reaction and the colonies recovered were analysed by isolating plasmid DNA and checking the size of the plasmid to check that there had been no deletion mutations. Plasmids of the correct size were then sequenced to ensure that they had the correct mutation in the DNA sequence.

Method 2

Mutagenesis was carried out using a variation of the method of (Kunkel, 1985) pEMGST was transformed into *E.coli* RZ1032. Single stranded DNA was produced as described above. 4 pmoles of phosphorylated oligonucleotide was annealed to 800ng of single stranded DNA by incubating them together with 1µl annealing buffer (1.4M MOPS pH8.0, 1.4MNaCl) in a total volume of 9µl. Annealing was completed by incubating at 70°C for 5 minutes, 37°C for 30 minutes and finally on ice for 5 minutes. The annealed primer was extended using 2 units of DNA polymerase I Klenow fragment (Boehringer Mannheim) 500µM dNTPs, 1mM MgCl₂ and the

plasmid concurrently closed using 1 unit of DNA Ligase (New England Biolabs) and the volume made-up to 20µl with sterile water. The reaction was incubated at 37°C for 30 minutes before terminating by heating at 70°C for 5 minutes prior to transformation into *E.coli* JM109.

8.3.19 Automated DNA Sequencing.

DNA sequencing was carried out on an Applied Biosystems Model 373A DNA Sequencer. The reactions to sequence the DNA were carried out according to the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit manufacturer's instructions. The kit uses four ABI dye-labelled dideoxy nucleotides; G, A, T and C. When these terminators replace standard dideoxy nucleotides in enzymatic sequencing, the fluorescent dye-label is incorporated into the DNA along with the terminating base. The chemistry of the reactions completely eliminates labelled false stops. The mix of terminators also includes dITP and the thermally stable enzyme Amplitaq DNA Polymerase to increase the length of the sequence that can be read. All four terminator reactions are carried out in the same tube and cycled on a PCR machine. Once the reactions are complete, any unincorporated dye-labelled terminators are removed by phenol extraction and the sequencing fragments recovered by ethanol precipitation. The DNA is resuspended and heated to denature the fragments and loaded on a customised polyacrylamide gel. A specialised machine scans the gel and locates the order of the fluorescent tags to determine the actual sequence.

8.3.20 Manual DNA Sequencing.

The method used was that first reported in (Sanger *et al*, 1977) using a Sequenase kit (USB). The method was used when automated sequencing was unavailable or unreliable. To begin with 3µg of template per track was denatured and precipitated prior to adding 3.2 pmoles of sequencing primer. The reaction was heated at 65°C and the primer allowed to anneal by cooling slowly. The annealed primer was then extended with dNTPs and Sequenase 2.0 (Tabor and Richardson, 1989) for 2 minutes at room temperature before adding ³⁵S α-dATP and ddNTPs to the reactions and extending for a further five minutes at 37°C. ddNTPs were added to discrete templates thus each template had four individual reactions. The extensions were terminated by adding 2µl of loading dye (containing formamide) and heated at 80°C to denature the fragments from the template. The reactions were loaded on a urea/polyacrylamide gel and run at 120V at 45°C using a Bio-Rad electrophoresis kit. Once the dye front had reached the bottom, the gel was removed from the plates and

fixed in methanol/acetate and transferred onto 3MM paper cut to the appropriate size. The mounted gel was dried using a Bio-Rad gel dryer at 75°C for 30 minutes. The radioactive bands on the dried gel were detected by exposing Kodak XOMAT film overnight at room temperature overnight. The film was developed and the sequence read from the gel.

8.4 Protein Methods.

8.4.1 Expression of Glutathione S-Transferases from a *ptacII* promoter.

Expression from pEM-based vectors was carried out in *E.coli* JM109. A single colony was used to inoculate a 50ml overnight culture and used to inoculate 1l prewarmed 2xYT (20mg/ml ampicillin). The culture was then induced when $A_{280}=0.6$ by adding 10ml 100mM IPTG and left to grow for a further 3 hours before harvesting the cells.

8.4.2 Expression of Glutathione S-Transferase from a *T7* promoter.

Protein expression driven from the *T7* promoter was very strong and had to be slowed down to stop the heterologous protein expression overwhelming the cells' folding pathways. The protein was expressed in *E. coli* BL21(DE3) (Studier, 1986), a strain of *E. coli* that has the DE3 prophage which expresses *T7* RNA polymerase allowing it to transcribe a heterologous gene, under the control of the *lac* operator. However, in this strain the expression of *T7* RNA polymerase is not tightly controlled so there is a fair amount of 'leaky' expression before full induction of the prophage with IPTG. This type of expression with out induction was used with these vectors. A single colony was used to inoculate a 50ml overnight culture was then used to inoculate 1l of prewarmed LB. The culture was then grown for 6 hours and the bacteria harvested by centrifugation.

8.4.3 Purification of Glutathione S-Transferases from *E. coli* cells.

The bacteria were harvested by centrifugation at 4420g for 10 minutes at 4°C in 4 centrifuge pots in a Beckman JA10A rotor. The bacterial pellets were washed with 20mls Loading Buffer (20mM Tris, 20mM NaCl, Dithiothreitol 0.3g/l, pH7.2) and spun again at 4420g. The pellets were finally resuspended in 25ml Loading Buffer and the cells disrupted by French pressing at 10,000-12,000psi. The cell debris was

pelleted by centrifugation at 17600g for 10 minutes at 4°C. The soluble protein was then run through a 1.5ml Glutathione-Agarose resin column that had been equilibrated with 5 column volumes of Loading Buffer. The column was washed with a further 5 volumes of Loading Buffer to remove non-specific, unbound protein. The polypeptides were eluted using 2ml Elution Buffer (25mM Na₂CO₃, 20mM NaCl, Dithiothreitol 0.3g/l, pH10.5) which was dripped into 20mM NaH₂PO₄ to neutralise the alkali. The purity of the protein was tested by SDS-PAGE and the concentration determined by absorbance at 280nm. For aflatoxin 8,9-epoxide assays, the protein were microdialysed (using Pierce and Warriner Slide-A-Lyzer dialysis kits) against 200mM Sodium phosphate buffer pH 6.5 for 2 hours before being vacuum dried and resuspended in 100mM Potassium phosphate buffer pH 7.2.

8.4.4 Determination of Molar Extinction Coefficients for GSTs.

The molar extinction coefficients (ϵ) were determined by calculation (Gill and Von Hippel, 1989). The method uses the theoretical contribution to A_{280} of tryptophan, phenylalanine and tyrosine residues in the proteins.

8.4.5 SDS Polyacrylamide Gel Electrophoresis.

Proteins were separated by electrophoresis through SDS-containing discontinuous polyacrylamide gels SDS PAGE according to the method of (Laemmli, 1970). The gels were poured in Mini-Protean II cells (Bio-Rad) were used according to the manufacturer's instructions employing 10% separating gels. The Pharmacia high molecular weight calibration kit was used to estimate protein sizes. Samples of *E. coli* cytosol were prepared by taking the equivalent of 1ml of $A_{600}=0.55$ culture and spinning down the cells. The cells were then resuspended in 100 μ l Laemmli buffer (50mM Tris.Cl, 100mM dithiothreitol 2% SDS, 0.1% bromophenol blue and 10% glycerol) and boiled in a water bath for 10 minutes, 15 μ l was then loaded on the gel. The gel was run until the bromophenol blue dye front was seen at the bottom of the gel to ensure maximum separation. The protein within the gel was visualised by soaking with Coomassie Brilliant Blue protein stain (0.25% (w/v) Coomassie Brilliant Blue R250, 45% (v/v) methanol, 45% (v/v) water and 10% (v/v) glacial acetic acid) followed by destaining with 30% (v/v) methanol and 10% (v/v) glacial acetic acid. The gels were then soaked in water to remove any acetate and dried onto 3MM paper on a gel drier.

8.4.6 Kinetic Characterisation of GSTs Using Spectrophotometry.

Specific activities and the kinetic constants K_m and k_{cat} were determined with the substrates 1-Chloro-(2,4)-dinitro-benzene (CDNB) and ethacrynic acid (EA) in isotonic 100mM phosphate buffers (Falkner and Clark, 1992). The reaction between; glutathione and CDNB was monitored at 340nm and at 25°C; between glutathione and EA was monitored at 240nm. All solutions used (except glutathione and enzyme stock solutions which were kept on ice) were preincubated at 25°C. The concentrations of the substrate and cofactor were varied between 0.1mM and 1mM. All rates were measured in duplicate and corrected for the background rate of the spontaneous reaction by simultaneous measurement in a Beckman A260 Spectrophotometer.

8.4.7 Determination of K_d by Fluorescence Spectroscopy Titration.

The dissociation constant K_d for aflatoxin B₁ was determined by titrating aflatoxin B₁ (200µg/ml) into the enzyme (>5µM) by exciting at 285nm and measuring the quench of protein fluorescence at 345nm. The inner-filter effect was corrected for by concurrently titrating aflatoxin B₁ into a tryptophan solution that had been set at the same fluorescence intensity as the protein sample. The titration was repeated until the enzyme was saturated and there was no more fluorescence change compared to the tryptophan blank. The resulting fluorescence difference was analysed by fitting the data to a quadratic function to estimate K_d .

8.4.8 Calculation of Inter-molecular Distances Using Fluorescence Resonance Energy Transfer.

Intermolecular distances between aflatoxin B₁ and Trp21 were measured using FRET. All emission spectra were corrected using quinine sulphate and collected by excitation at 295nm (Demas and Crosby, 1971). Quantum yields were calculated by comparison of corrected protein emission spectra with the emission spectra of *p*-terphenyl using equation 1 (Demas and Crosby, 1971).

$$\phi_D = \phi_S \left(\frac{A_S \int F_D dF}{A_D \int F_S dF} \right) \quad (1)$$

Where A_S is the absorbance of *p*-terphenyl at 295nm, A_D is the absorbance of the protein at 295nm, F_S is the area under the fluorescent peak of *p*-terphenyl, quoted

as 0.93. The quantum yields was used to define the Förster distance, R_0 given by equation 2

$$R_0 = 979(J\kappa^2\phi_D n^{-4})^{\frac{1}{6}} \quad (2)$$

Where κ^2 is the orientation factor and given the value 2/3 (Dos Remedios and Moens, 1995) n is the refractive index of water and taken as 1.35, and J is the integral of the spectral overlap given by equation 3.

$$J = \frac{\int \epsilon_A(\lambda)F_D(\lambda)\lambda^4 d\lambda}{\int F_D(\lambda)d\lambda} \quad (3)$$

The Förster distance was substituted into equation 4 to calculate intermolecular distances

$$R = R_0 \left(\frac{1-E}{E} \right)^{\frac{1}{6}} \quad (4)$$

Where E is the efficiency of energy transfer, calculated as described in equation 5

$$E = \left(\frac{I_{AD}}{I_A} \right) \left(\frac{\epsilon_A}{\epsilon_D} \right) \quad (5)$$

I_{AD} is the area under the corrected fluorescent peak from the protein:aflatoxin complex between 300 and 400nm, I_A is the area under the corrected fluorescent peak of aflatoxin B_1 alone between 300nm and 400nm, ϵ_A is the extinction coefficient for aflatoxin B_1 at 295nm and ϵ_D is the extinction coefficient of the protein at 295nm.

8.4.9 Western Blotting.

Western Blotting was carried out essentially as described in (Burnette, 1981). Total bacterial cell protein was separated on a 10% SDS-PAGE in duplicate to give a Coomassie-Blue stained gel to complement the blotted gel. Briefly, the separated proteins were 'blotted' onto nitro-cellulose filters (Hybond-C, Amersham) by electrophoretic transfer (Towbin *et al*, 1979). A Trans-blot electro-transfer kit (Bio-Rad) was used with transfer buffer (160mM glycine, 12mM Tris, 10%(v/v) methanol). The apparatus was set at 100V and kept on ice for 3 hours. Complete transfer was monitored by using coloured Rainbow markers which could easily be seen on the membrane.

8.4.10 Antigen Detection on Nylon Membranes.

Detection of protein antigens on nitro-cellulose filters was achieved by using ECL Western Blotting system from Amersham. The membranes were incubated in 5% (w/v) Marvel-TBST (10mM Tris.HCl pH 8.0, 0.9% (w/v) NaCl, 0.05% Tween) for 1 hour at room temperature. The milk protein was added to block non-specific sites on the membrane. The blocked membrane was then washed in fresh Marvel TBST containing the primary-antibody ascetic fluid at a dilution of 1:1000 for 1 hour at room temperature with constant agitation. The primary antibody was washed from the membrane by 3 x 5min washes in Marvel-free TBST. The secondary, horseradish peroxidase-conjugated antibody was added at a dilution of 1:1000 in 5% Marvel-TBST and incubated for 1 hour at room temperature with constant agitation. The membrane was transferred to ECL Detection solution containing Luminol and incubated for a further minute at room temperature. The membrane was wrapped quickly in SaranWrap and secured in an autoradiography cassette and exposed to Hyperfilm for 15 seconds. The film was developed and the exposure times were varied accordingly.

8.5 Tissue Culture Methods.

8.5.1 Solutions used for culture of BCMV-1A2 cells.

LHC-9 Tissue Culture Medium. The medium was purchased from Biofluids Inc, Rockville USA. Aliquots of 500ml were supplemented with 250µl of adrenaline (Epi), 50µl of 3.3µM Retinoic Acid (RA) and 150µg/ml G418.

PBS (tissue culture): Dulbecco's modified PBS (without Mg^{2+} or Ca^{2+}) was prepared by dissolving ten tablets (ICN Flow) in 1000ml of Q water. The PBS was dispensed (250ml aliquots) into 500ml tissue culture bottles (Gibco BRL). Following sterilisation by autoclaving, the solution was stored at room temperature.

Hanks's Balanced Salts Solution (HBSS). The solution was purchased from Gibco BRL and contained phenol red indicator.

Electroshock buffer for transfection: Electroshock buffer for the electroporation of BCMV-1A2 cells contained 140mM NaCl, 25mM HEPES pH 7.5 (pH corrected with

4M NaOH) and 0.75mM Na₂HPO₄. 500ml aliquots of the buffer were filter sterilised. The final pH was pH 7.0.

Gene Pulser cuvettes: Gene Pulser cuvettes with a 0.4cm electrode gap, for the electroporation of MEL cells, were obtained from Flowgen.

Geneticin (G418): Geneticin (G418) was bought as a ready sterilised liquid from Gibco BRL and stored at 4°C.

Trypsin/EDTA: A solution containing 0.5g Trypsin and 0.2g EDTA in 1l H₂O was used to remove the adherent cells from the floor of the tissue culture vessels

Fibronectin. A 1mg/ml solution was bought from Sigma and diluted 50 times in PBS. The solution was stored at 4°C and reused for 3 months before discarding.

8.5.2. General Cell Culture.

Cell manipulations were performed in a class II microbiological safety cabinet (Medical Air Technology Ltd., Manchester). Cells were routinely cultured in 15ml of medium in 260ml Nunclon[®] (Gibco BRL) flasks that had been previously coated in fibronectin/PBS for 5 minutes at 37°C. All mammalian cells were incubated at 37°C (3.5% CO₂ atmosphere) in a humidified incubator, and killed using Chlorox (Mace *et al*, 1994).

8.5.3 Sub-culturing BCMV-1A2 cells.

Cells were routinely sub-cultured when growth in the flasks was approximately 80% confluent to minimise the chances of the cells changing their behaviour. The culture medium was aspirated and 5mls Trypsin/EDTA at 37°C was added. No washing with PBS was necessary because LHC-9 medium is serum-free (Mace *et al*, 1994). The Trypsin/EDTA was incubated at 37°C for a maximum of 10 minutes before agitating the flasks to dislodge the cells. The digestion was stopped by adding 20mls 2.5% Foetal Calf Serum in HBSS. Cells were concentrated by centrifugation at 3000g for 5 minutes at 20°C and Trypsin/EDTA was aspirated. The cells were resuspended in a suitable volume of LHC-9 medium and used to seed fibronectin-coated flasks.

8.5.4 Cryogenic Storage of Cells.

Cells were frozen in aliquots of 2×10^6 cells per vial and were prepared by treating growing cells with Trypsin/EDTA. The digestion medium was aspirated and replaced with 500 μ l Freezing Solution A (Biofluids, Rockville) and 500 μ l of Freezing Solution B (Biofluids, Rockville). The vial containing the cells were put in a polystyrene box and placed in a -80°C freezer for 24 hours before being transferred to liquid nitrogen for long-term storage (Mace *et al*, 1994). To revive the cells after being stored in liquid nitrogen, the cells were thawed in a 37°C water-bath and added directly to a flask containing LHC-9 medium. The use of the freezing solutions meant there was no need to wash the cells to remove DMSO.

8.5.5 Transfection of Cells using Electroporation.

1×10^7 cells that were in logarithmic growth were used per transformation as described in (Chu *et al*, 1987). The cells were washed and centrifuged twice with HBSS at 3000g for 5 minutes at 20°C to remove all traces of foetal calf serum. The cells were resuspended in 900 μ l of electroschock buffer and 25 μ g of DNA added. The total volume was kept under 1ml. The mixture was transferred to a sterile electroporation cuvette and electroporated using a Biorad Gene-Pulser with a pulse controller. The machine was set at 250V and 960 μ F and the pulse delivered to the cells. Immediately, the cells were added to 2ml of LHC-9 medium and plated onto 6 well plates (Nunc). pEGFP was used to co-transfect the cells in order to identify any transiently expressed cells. The construct expressed Green Fluorescent Protein and allowed the transfected cells to be identified under a fluorescent microscope by exciting at 495nm (Angelotti *et al*, 1993).

8.6 Computer methods.

8.6.1 Sample Script and input into MODELLER for rGST Yc₁

Modeller requires the compilation of a macro program to run the site of programs. The program was run in UNIX on a Silicon Graphic INDY machine. The input files ycl.mod and ycl.seg were written using the standard SGI text editor *jot*. The scripts and input for the generation of the model of rGSt Yc₁ is given. Three files were needed to execute the program; (a) ycl.seg which gave Yc₁ polypeptide sequence and the resolution and R-factor for the crystal structure of huGSTA1-1; (b)

lguha.pdb which gave the co-ordinates for huGSTA1-1; (c) ycl.mod which executed the modeller program. All these files were saved in the directory /users/people/pdml

ycl.mod

INCLUDE

directory with input atom files

SET ATOM_FILES_DIRECTORY = './:../atom-files'

name of the input file specifying templates and the target sequence

SET SEGFILE = '/users/people/pdml/gst.seg'

templates' pdb codes

SET KNOWN = '/users/people/pdml/lguha'

target code

SET SEQUENCE = 'ycl'

optimisation executable

SET FINISH_METHOD = 'refine'

fires up the modeller algorithms

CALL ROUTINE = 'full_homol'

ycl.seg

>p1;lguha

structureX:lguha: : : : human GSTA1-1 : 2.6 : 22.9

MAEKPKLHYFNARGRMESTRWLLAAAGVEFEEKFIKSAEDLDKLRNDGYLMFQQVPMVEIDGMKLVQTR

AILNYIASKYNLYGKDIKERALIDMYIEGIADLGEMILLLPVCPPEEKDAKLALIKEKIKNRYFPFAFEK

VLKSHGQDYLVGNKLSRADIHVELLYYVEELDSSLISSFPLLKALKTRISNLPVKKFLQPGSPRKPP

MDEKSLEEARKIFRF*

>p1;ycl

structureN:ycl

MPGKPVLYHYFDGRGRMEPIRWLLAAAGVEFEEQFLKTRDDLARLRNDGSLMFQQVPMVEIDGMKLVQTR

AILNYIATKYNLYGKDMKERALIDMYAEGVADLDEMILHYPYIPPGKEASLAKIKDKARNRYFPFAFEK

VLKSHGQDYLVGNRSLRADVYLVQVLYHVEELDPSALANFPLLKALRTRVSNLPVKKFLQPGSQRKPL

EDEKCVESAVKIFRF*

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