Molecular Genetics and Functional Analysis of the Human Theta Class Glutathione Transferase, GSTT2-2

by

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To Yat Wah, for her love, understanding, and prayers.

STATEMENT

This thesis describes the research undertaken in the Molecular Genetics Group, Division of Molecular Medicine, of the John Curtin School of Medical Research, between February 1993 and January 1996. This work was accomplished under the supervision of Prof. Philip G. Board, Head of the Molecular Genetics Group.

The results presented in this thesis are my own original work, except where stated otherwise.

Kian Leong TAN

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First of all, I want to give thanks and praise to the Lord God Almighty that,

"For by him all things were created: things in heaven and on earth, visible and invisible, whether thrones or powers or rulers or authorities; all things were created by him and for him."

Colossians Chapter 1, verse 6.

From The Bible (NIV Version).

The findings of this thesis revealed only a small portion of His wondrous creation.

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I am grateful to my parents who supported my decision to pursue further study. Their love and care through out the past 3 years are greatly valued. My heartfelt gratitude goes to my fiancee Yat Wah, for without her support this endeavours would have been impossible. This thesis is dedicated to her for her understanding and courage to press on during times when I could not be there for her.

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ABSTRACT

The glutathione transferases (GSTs) represent a group of Phase II detoxication enzymes that catalyse the nucleophilic attack of reduced glutathione (GSH) on electrophilic centres of endogenous and xenobiotic compounds. Cytosolic GSTs from mammals can be divided into 4 distinct classes, Alpha, Mu, Pi and Theta. The Theta class GSTs in humans are unique from the other classes as they have low or lack of activity with the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) and they do not bind to immobilised glutathione affinity matrices. The absence of these two features has made the Theta class isoenzymes relatively difficult to study and as a result they have been largely overlooked. To gain a greater understanding of the human Theta class GSTs, the research carried out in this thesis described:-

1. **The molecular cloning of a cDNA encoding the human Theta class GSTT2-2.** Three different cDNA libraries were screened and four distinct clones were identified. One clone contains the full open reading frame of GSTT2-2. While the other three clones are all truncated transcripts which showed evidence of alternative splicing.

2. **The chromosomal localisation *GSTT2*.** *GSTT2* was mapped to chromosome 22 by somatic cell hybrid analysis and sublocalised to q11.2 by *in situ* hybridisation. There was no evidence for reverse transcribed pseudogenes of *GSTT2* dispersed through out the genome. Southern blot analysis of human genomic DNA indicated that the *GSTT2* gene is less than 4kb in length.

3. **The heterologous expression of GSTT2-2 from a cDNA clone.** GSTT2-2 was expressed in *Escherichia coli* as a ubiquitin fusion protein. The ubiquitin moiety was co-translationally cleaved by a co-expressed yeast ubiquitin specific protease (Ubp1), generating an enzymatically active GSTT2-2 without any additional N-terminal residues. The recombinant isoenzyme was purified to apparent homogeneity using DEAE anion exchange, gel filtration, dye ligand and high resolution anion exchange

(Mono Q f.p.l.c.) chromatography. The recombinant enzyme had significant activity with a range of substrates including cumene hydroperoxide, and 1-menapthyl sulphate (MS). The activity of GSTT2-2 with a range of secondary lipid peroxidation products such as the *trans,trans*-alka-2,4-dienals and *trans*-alk-2-enals as well as its glutathione peroxidase activity with organic hydroperoxides suggest that it may play a significant role in protection against the products of lipid peroxidation.

4. **The tissue expression of GSTT2-2.** Tissue expression of GSTT2-2 was studied using the cloned cDNA and also polyclonal antibodies raised against purified GSTT2-2. Northern and Western blots showed that GSTT2-2 appears to be predominantly expressed in liver and placenta although detectable levels were also observed in kidney, pancreas, lung, spleen, heart, and skeletal muscle but not in brain. The weak hybridisation signals from both studies indicate that, GSTT2-2 is not expressed abundantly in any of these tissues.

5. **The structure-function studies of GSTT2-2.** A conserved serine residue in the N-terminal domain of Theta GSTs has been implicated in catalysis by activating the enzyme-bound GSH. In GSTT2-2, the role of the equivalent serine (residue 11) in catalysis was examined by site-directed mutagenesis. Mutating Ser11 to Ala, Thr or Tyr abolished the catalytic properties of GSTT2-2 with cumene hydroperoxide and ethacrynic acid. However, two of the mutants, S11A and S11T, retained appreciable activities with MS. The mechanism of the reaction between MS and GSH was investigated in greater detail. It is proposed that the SO_4^{2-} group of MS is removed by GSTT2-2 in a mechanism similar to the action of a sulphatase. It is also proposed that this reaction is preceded by a conformational or charge modification to the enzyme upon the binding of glutathione or *S*-methylglutathione. This is followed by the binding of MS and the subsequent removal of the SO_4^{2-} group, giving rise to the carbonium ion of 1-methylnapthelene (the electrophile) to react with GSH or other thiols (the nucleophile). The reaction mechanism of GSTT2-2 with MS may represent a novel function of GSTT2-2 as a glutathione dependent sulphatase.

PUBLICATIONS

1. Tan, K. L., Webb, G.C., Baker, R.T., Board, P.G. (1995) Molecular Cloning of a cDNA and Chromosomal Localization of a Human Theta Class Glutathione *S*-Transferase (GSTT2) To Chromosome 22. *Genomics*, **25** : 381-387

2. Tan, K. L., and Board, P.G (1996). Purification and characterisation of a recombinant humanTheta class glutathione transferase (GSTT2-2). *Biochem. J.* (In Press)

3. Tan, K. L., Chelvanayagam, G., Parker, M. W. and Board, P.G. (1996). Mutagenesis of the active site of the human Theta class glutathione transferase GSTT2-2: Identification of a novel function as a glutathione dependent sulphatase. (Submitted)

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ABSTRACTS OF PAPERS PRESENTED AT CONFERENCES

1. Tan, K. L. and Board, P. G. (1993)."*Molecular Cloning of a Human Theta Class Glutathione S-transferase cDNA*". 17th Annual Scientific Meeting of the Human Genetics Society of Australasia. Canberra, Australia

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ABBREVIATIONS

CDNB	: 1-chloro-2-4-dinitrobenzene
Cu-OOH	: Cumene hydroperoxide
DCNB	: 1,2-dichloro-nitrobenzene
DCM	: Dichloromethane
EA	: Ethacrynic acid
EDTA	: Ethylenediaminetetra-acetic acid di-sodium salt
EPNP	: 1,2-epoxy-3-(*p*-nitro phenoxy)-propane
ddH_2O	: Double distilled water
IPTG	: Isopropyl-β-D-thiogalactoside
ME	: β-mercaptoethanol
MS	: 1-menapthyl sulphate
tPBO	: *trans*-4-phenyl-but-3-en-2-one
PCR	: Polymerase Chain Reaction

CHAPTER 1 : THE GLUTATHIONE TRANSFERASES --- AN OVERVIEW

1.1 INTRODUCTION

The well being of living cells is governed by their ability to combat the lethal effects of the cytotoxic or genotoxic compounds they are exposed to. These toxic compounds can be by-products of normal cell metabolism, or exogenous compounds which are introduced into the body through absorption, inhalation or ingestion. To protect against the harmful effects of these compounds, the detoxication, inactivation and elimination of these compounds are crucial. It is now understood that there are at least three different phases in the metabolism of toxic compounds from endogenous and exogenous sources.

Phase I metabolism involves oxidation, reduction and hydrolysis reactions as mediated by cytochrome P450 or flavin mixed-function oxidases (Cooper *et al.*, 1965; White and Coon, 1980; Pelkonen and Nebert, 1982). Many xenobiotics are lipophilic compounds which will remain in the cells indefinitely if they are not converted to more hydrophilic derivatives. During Phase I metabolism, foreign compounds are converted into reactive species by the introduction of an electrophilic centre. In Phase II metabolism, products from Phase I or other endogenous substrates undergo conjugation reactions. Recently, a Phase III metabolic system was proposed by Ishikawa (Ishikawa, 1992). Phase III metabolism is an elimination process where glutathione *S*-conjugates are exported out of the cells via an ATP-dependent glutathione *S*-conjugate export pump, originally described by Board (1981b).

Phase II enzymes are known for their capacity to catalyse conjugation reactions. This group of enzymes includes glutathione transferases (GSTs), sulfotransferases, UDP-glucuronosyl transferases and N-acetyl transferase, that catalyse the conjugation of glutathione (GSH), sulphate, glucuronate, or acetyl group respectively to a wide variety of second substrates. The GST isoenzymes are of particular interest because their role in

GSH conjugation has been proposed as one of the most important detoxication pathways for xenobiotics.

1.2 THE GLUTATHIONE TRANSFERASES

The glutathione transferases (GSTs, E.C. 2.5.1.18) are a group of Phase II conjugating enzymes which belong to a supergene family. They are ubiquitous in nature, existing in aerobic bacteria, fungi, yeast, plant, and all vertebrates. Mammalian cytosolic GSTs are divided into four different classes, namely, Alpha, Mu, Pi and Theta. The GSTs found in plants do not fall into any of these classes and are grouped into a special class termed, Phi (Blocki *et al.*, 1993). The amino acid sequence of plant GSTs indicate that they are most closely related to the Theta class in mammals. Another class of GSTs termed Sigma, is composed of the GST from squid (Harris *et al.*, 1991a; Tomarev *et al.*, 1993) and parasitic helminths (*Schistosoma*) (Henkle *et al.*, 1990; Liebau *et al.*, 1994a; Liebau *et al.*, 1994b) as well as the lens crystallins from the eyes of cephalopods (Tomarev and Zinovieva, 1988). The squid digestive gland GST is believed to be the ancestral gene of the lens crystallins, which have evolved to provide a structural role (Ji *et al.*, 1995). Recently, a rat spleen GST that exhibited prostaglandin H D-isomerase activity was found to be related to the Sigma class GSTs (Meyer and Thomas, 1995). This is the first example of the existence of a Sigma class GST in a vertebrate.

Additional GSTs, which can be purified from the microsomal fraction of cells, belong to a group of membrane bound GSTs. They include microsomal GST, which has a wide substrate specificity and leukotriene C4 synthase, which specifically catalyses the conversion of leukotriene A4 to leukotriene C4. There is little amino acid sequence identity between the membrane bound GSTs and the cytosolic GSTs, indicating that these two groups of GSTs probably arose from different ancestral genes.

GST research has progressed immensely since GSTs were discovered in 1961 (Booth *et al.*, 1961; Combes and Stakelum, 1961). A selective chronological overview of some significant events in GST research is presented in Table 1.1.

Table 1.1 : A Selective Chronological Overview of GST Research

Year	Events	Reference
1961	Discovery of GSH conjugating activity with DCNB on cytosolic fraction of homogenised rat liver	Booth *et al.* & Combes *et al.*
1965	Enzyme characterisation of GSTs	Boyland *et al.*
1974	Ligandin is a GST	Habig *et al.*
1975	Separation of various isoforms of GSTs from human liver	Kamisaka *et al.*
1976	Rat liver GST exhibits selenium independent glutathione peroxidase activity	Lawrence *et al.*
1976	Conversion of prostaglandin endoperoxides to prostaglandin is mediated by GST	Christ-Hazelhof *et al.*
1977	Cytosolic GSTs are dimeric proteins	Bass *et al.*
1977	Expression of GST is inducible by drugs	Hales and Neims
1979	Leukotriene C4 synthase is a GSH conjugating enzyme	Murphy *et al.*
1981	First genetic analysis of human GSTs and identification of polymorphism in the Mu class GSTs	Board
1982	Purification and characterisation of rat microsomal GST	Morgestern *et al.*
1985	Nomenclature of GSTs using the alpha, mu and pi designation	Mannervik *et al.*
1986	Isolation of first human Alpha class cDNA (GSTA1)	Tu *et al.*
1987	Expression of mammalian GST in prokaryotic host	Board and Pierce
1987	Isolation of first human Pi class cDNA (GSTP1)	Kano *et al.*
1988	Isolation of first human Mu class cDNA (GSTM1)	DeJong *et al.*
1990	Over expression of GSTs conferred resistance to anti-cancer drugs	Black *et al.*
1991	Characterisation of Theta class GSTs	Meyer *et al.*
1991	First three dimensional structure of Pi class GST (pGSTP1-1)	Reinemer *et al.*
1992	Consensus nomenclature for human GSTs	Mannervik *et al*
1992	First three dimensional structure of Mu class GST (rat 3-3)	Ji *et al.*
1993	First three dimensional structure of Alpha class GST (hGSTA1-1)	Sinning *et al.*,
1994	Isolation of first human Theta class cDNA (GSTT1) and identification of GSTT1 polymorphism	Pemble *et al.*
1995	Three dimensional structure resolution of a Theta-like GST from *Lucilia cuprina.*	Wilce *et al.*

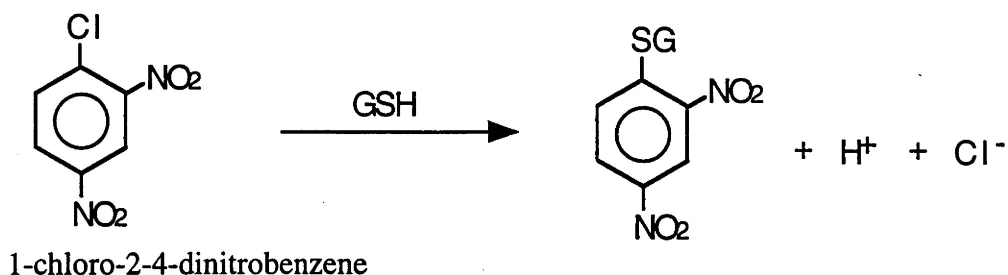
1.3 THE ROLE OF GLUTATHIONE TRANSFERASES

GSTs are involved in conjugating reduced glutathione (GSH, γ-glutamyl-cysteinyl-glycine) to a wide range of electrophilic compounds, alkylating agents and hydrophobic substrates (collectively termed as the second substrate). Besides the conjugating activity, several groups of GSTs also participate in the reduction of organic hydroperoxides (Lawrence and Burk, 1976; Prohaska and Ganther, 1977), thiolysis (Ohkawa and Casida, 1971; Keen and Jakoby, 1987) and reduction of organic nitrates (Heppel and Hilmore, 1950; Jensen and Stelman, 1987). These reactions represent examples of nucleophilic attack by the glutathione thiolate anion (GS^-) on electrophilic centres of oxygen, sulfur and nitrogen. GST is also known to exhibit isomerase activity, converting Δ^5-3-ketosteroids to α-β-unsaturated Δ^4-3-ketosteroids (Benson *et al.*, 1977) and maleylacetoacetic acid to fumarylacetoacetic acid (Keen and Jakoby, 1987). The isomerase activity may be due to the formation of a short-lived glutathione-conjugate that undergoes rearrangement to form the isomer and GSH (Beckett and Hayes, 1993). Figure 1.1 gives examples of reactions catalysed by GSTs. Apart from their catalytic properties, GSTs also bind to non-substrate ligands in a covalent or non-covalent manner.

Many excellent reviews have been written in recent years on the broad field of GST research. They focus on aspects such as enzymology (Mannervik, 1985a; Mannervik and Danielson, 1988); toxicology (Coles and Ketterer, 1990); gene structure and regulation (Pickett and Lu, 1989; Reed and Mann, 1985; Hayes and Pulford, 1995); protein structure and function (Dirr *et al.*, 1994; Wilce and Parker, 1994; Armstrong, 1994); molecular genetics (Board *et al.*, 1990; Board *et al.*, 1990), biomedical application (Beckett and Hayes, 1993), tissue distribution (Mannervik and Widersten, 1995) and GSTs contribution to cancer chemoprotection and drug resistance (Hayes and Pulford, 1995). The following sections will attempt to present a general overview of previous investigations on the function and structure of GSTs as they relate to the scope of this thesis.

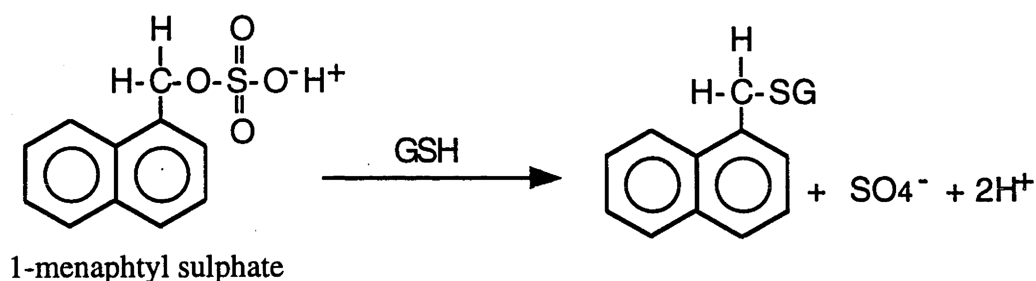
Figure 1.1 : Reaction Catalysed by GSTs

1. Aromatic carbon substitution



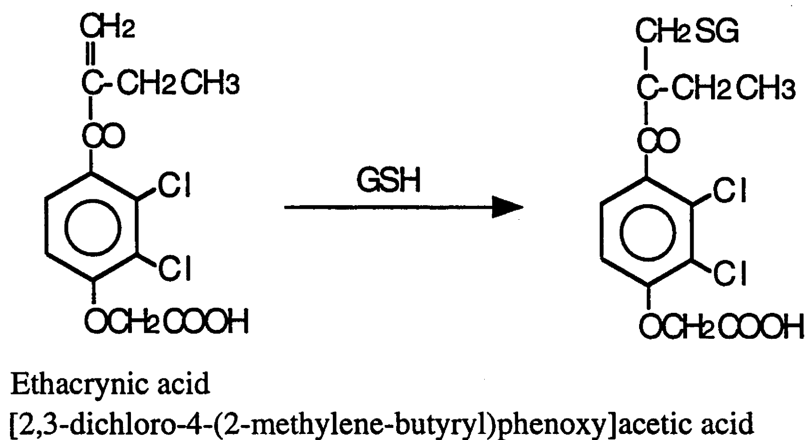
1-chloro-2-4-dinitrobenzene

2. Aromatic side chain substitution



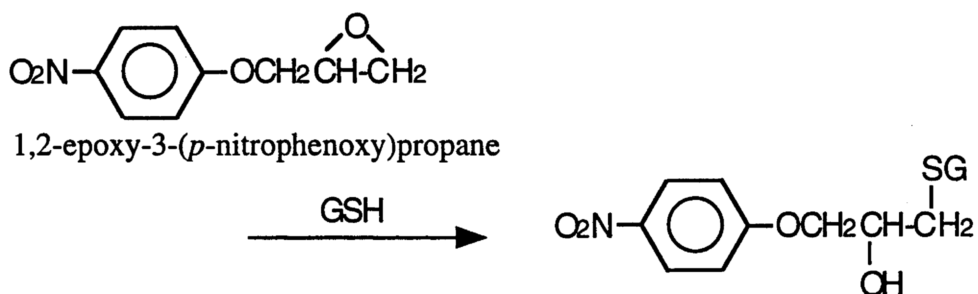
1-menaphtyl sulphate

3. α,β-unsaturated carbon addition : Michael addition



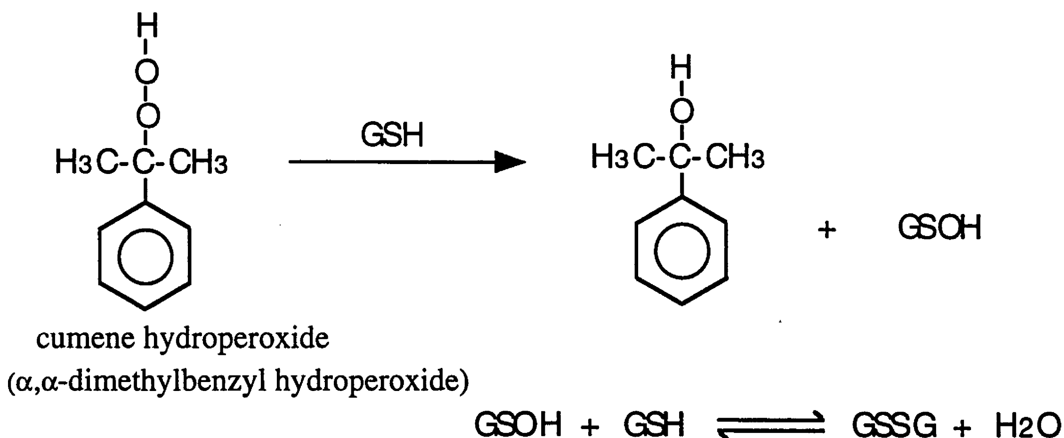
Ethacrynic acid
[2,3-dichloro-4-(2-methylene-butyryl)phenoxy]acetic acid

4. Epoxide ring opening : Addition



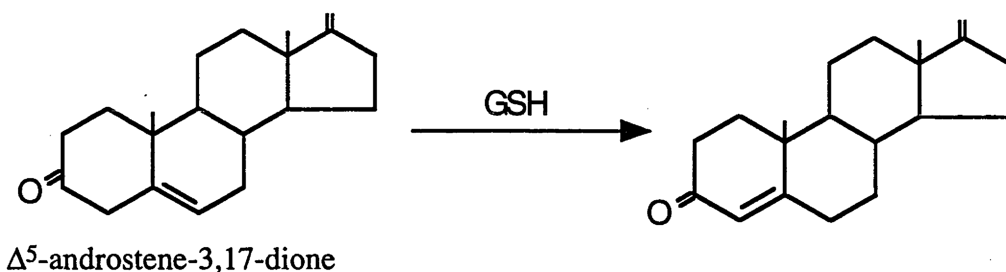
1,2-epoxy-3-(*p*-nitrophenoxy)propane

5. Reduction



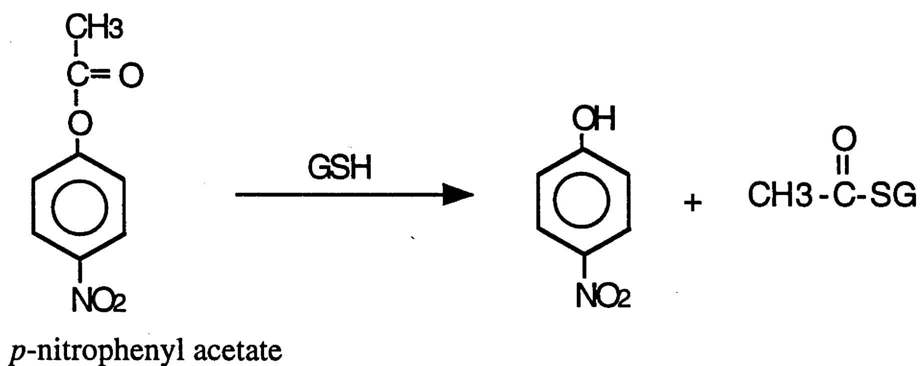
cumene hydroperoxide
(α,α-dimethylbenzyl hydroperoxide)

6. Positional Isomerisation



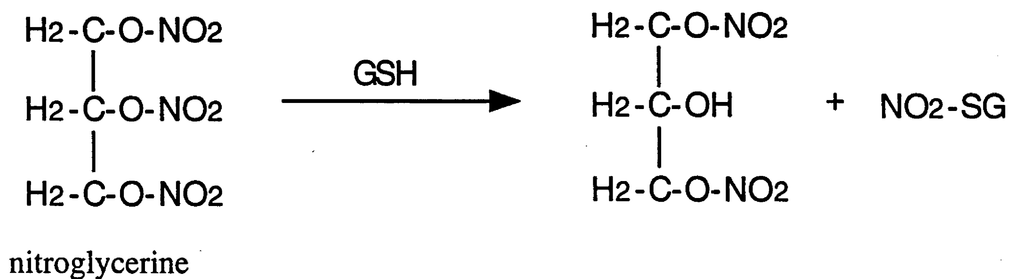
Δ^5-androstene-3,17-dione

7. Thiolysis



p-nitrophenyl acetate

8. Denitrosation



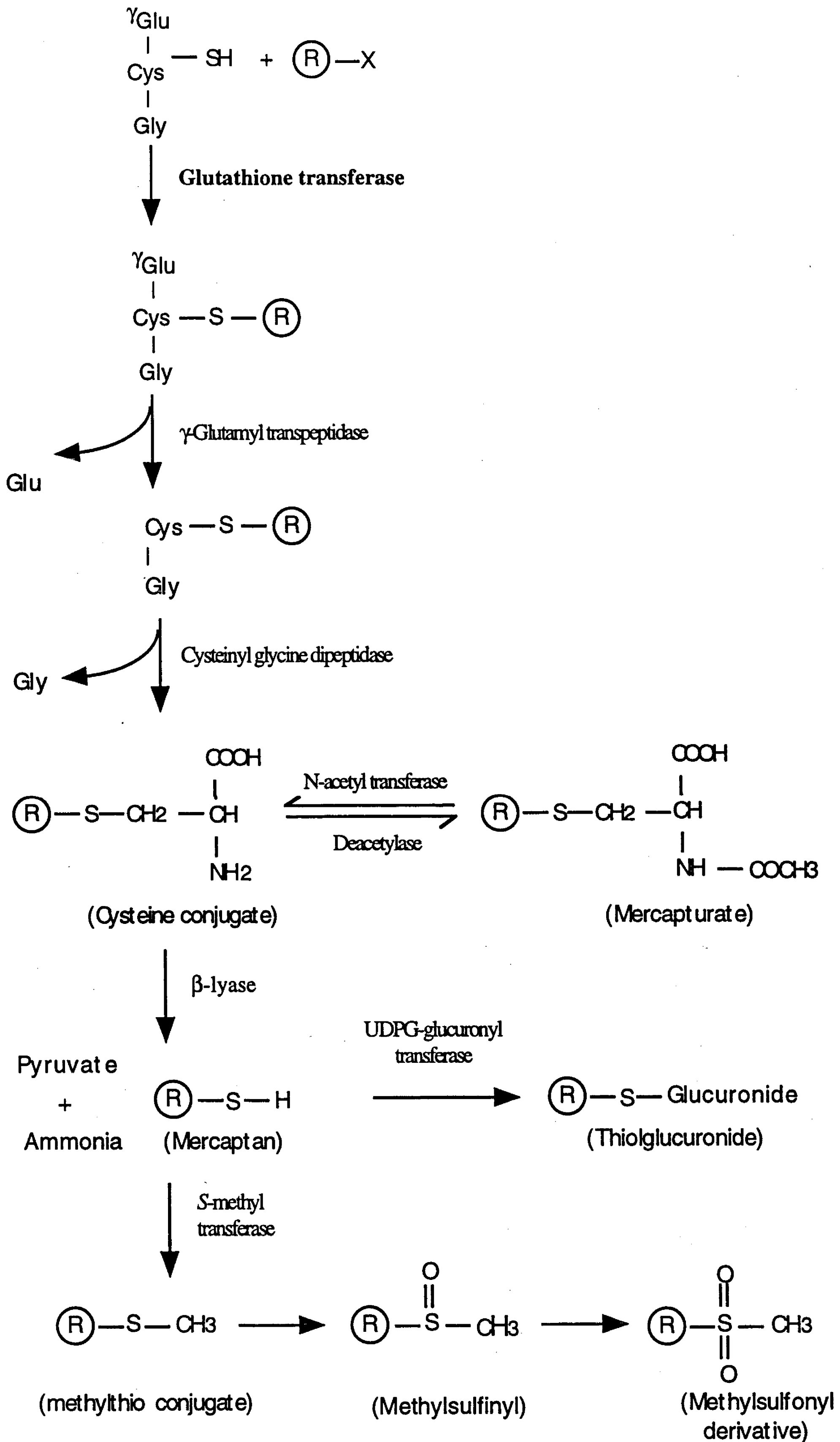
nitroglycerine

1.3.1 Conjugating Role of GSTs

The most important role of GSTs as a group of detoxication enzymes is catalysing the conjugation of reduced glutathione to a wide variety of second substrates containing an electrophilic carbon centre. Among these substrates are hydrophobic compounds, strong alkylating agents, potential carcinogens, therapeutic drugs and environmentally derived mutagens which include epoxides (Boyland and Williams, 1965), α-β-unsaturated compounds (Boyland and Chasseaud, 1967) and aromatic hydrocarbons (Habig *et al.*, 1974b; Jakoby, 1978; Coles and Ketterer, 1990).

The conjugation of GSH to a second substrate serves as the first step towards the formation of mercapturic acids (Habig *et al.*, 1974b, Boyland and Chasseaud, 1969). Mercapturic acids are the generic name of thioethers of N-acetyl-cysteine which are the metabolic products resulting from the sequential breakdown of glutathione-conjugates. It has been demonstrated that the N-acetylated cysteine moiety of mercapturic acid is derived from glutathione (Bray *et al.*, 1959), and the enzymes that catalyse the conjugation of GSH to the original compound are GSTs (Booth *et al.*, 1961). Glutathione-conjugates are either excreted from the cells by active transport (Board, 1981b) or undergo further hydrolysis (Bray *et al.*, 1959). In the latter case, the glutamate and glycine moieties are sequentially removed by γ-glutamyl transpeptidase and cysteinylglycine dipeptidase to form a cysteine-conjugate. Subsequent acetylation of the amino (NH_2) group of the cysteine moeity forms a mercapturic acid. Alternatively, the cysteine-conjugate can be cleaved by β-lyase to form a mercaptan. The thiol group of the mercaptan may undergo glucuronidation to form thioglucuronide or be methylated to form a methylthio-conjugate. Methylthio-conjugates may be oxidised to methylsulfinyl and methylsulfonyl derivatives. All these end products are in readily excreted forms. Figure 1.2 shows the involvement of GSTs in the biosynthetic pathway of mercapturic acid.

Figure 1.2 : Biosynthetic Pathway of Mercapturic Acid



1.3.2 Selenium Independent GSH Peroxidase Activity

The selenium-independent glutathione peroxidase activity is a unique characteristic of the Alpha (Mannervik and Danielson, 1988; Singhal *et al.*, 1994b) and Theta class GSTs (Meyer *et al.*, 1991; Hussey and Hayes, 1992). The non-selenium glutathione peroxidase activity was reported by Lawrence *et al.* (1976) and Prohaska *et al.* (1977) when an enzyme purified from rat liver exhibited activity towards cumene hydroperoxide but only negligible activity with hydrogen peroxide (H_2O_2). The fractions from ion exchange chromatography that showed non-selenium GSH peroxidase activity also had GST activity. Recently, Bartling *et al.* (1993) also purified a GST with GSH-peroxidase activity from *Arabidopsis thaliana* which is structurally and enzymatically unrelated to the selenium-dependent GSH-peroxidase, but was found to be closely related to the Theta class of the GST supergene family.

As GSTs catalyse the reduction of electrophilic oxygen in the presence of GSH, it is not surprising that similar reaction can take place with organic hydroperoxides. Under oxidative stress, lipid biomembranes and DNA can be peroxidised by reactive oxygen radicals such as superoxide anion, hydrogen peroxide and hydroxyl radicals. The peroxidation of polyunsaturated fatty acyl moieties can cause damage to the integrity of lipid biomembranes. It has been demonstrated that fatty acid hydroperoxides (Tan *et al.*, 1984; Hiratsuka *et al.*, 1994) and peroxidised DNA such as thymine hydroperoxides (Tan *et al.*, 1986; Tan *et al.*, 1988) are substrates for GSTs and these compounds may represent a category of endogenous substrates. GSTs are also known to detoxify toxic compounds that arise from lipid peroxidation (Berhane *et al.*, 1994). In view of their conjugating role and GSH-peroxidase activity, it is obvious that GSTs play an important role in detoxifying toxic compounds derived from lipid peroxidation (Alin *et al.*, 1985; Berhane *et al.*, 1994) as well as protecting cells from oxidative damage to lipids and DNA (Ketterer *et al.*, 1988; Ketterer and Meyer, 1989).

1.3.3 GSTs in Prostaglandin Biosynthesis

The conversion of prostaglandin endoperoxides (PGH_2) to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) in the presence of GSH (Christ-Hazelhof *et al.*, 1976; Burgess *et al.*, 1989) are additional examples of reduction reactions catalysed by GST. The proportion of $PGF_{2\alpha}$, PGE_2 and PGD_2 produced from PGH_2 is dependent on the type of GST isoenzymes catalysing the reaction (Burgess *et al.*, 1989). GSTs also catalyse the conjugation reaction between prostaglandin (PGA_1 and 15-keto-prostaglandin F_{2a}) and GSH (Cagen *et al.*, 1975; Chaudhari *et al.*, 1978). Although the physiological significance of these glutathione-conjugates is not clear, the conjugated products are rendered more water soluble. These observations suggest that GSTs play an important role in the biosynthesis of prostaglandins and may facilitate their intra/inter cellular transportation and/or excretion. A schematic diagram of arachidonic acid metabolism and the role of GSTs in prostaglandin conversion and conjugation is shown in Figure 1.3.

1.3.4 GST in Leukotriene Synthesis

The conversion of leukotriene A_4 (LTA_4) to leukotriene C_4 (LTC_4) involves a membrane-bound GSH conjugating enzyme called Leukotriene C_4 synthase (Murphy *et al.*, 1979; Soderstrom *et al.*, 1988; Nicholson *et al.*, 1993). LTC_4 is a glutathione-adduct and this suggests that LTC_4 synthase is an enzyme with GST activity. Cytosolic GSTs have also been reported to catalyse the conversion of LTA_4 to LTC_4 (Soderstrom *et al.*, 1985; Mannervik *et al.*, 1984). LTC_4, LTD_4 and LTE_4 are pro-inflammatory mediators in diseases such as bronchial asthma. They induce the contraction of smooth muscle, attraction and activation of leucocytes and increase vascular permeability. As LTC_4 is the precursor of LTD_4 and LTE_4, the role of LTC_4 synthase may have a profound effect on the aetiological factors in a number of inflammatory disorders. Figure 1.3 presents a schematic diagram of leukotriene biosynthesis from arachidonic acid and the involvement of GSTs in the pathway.

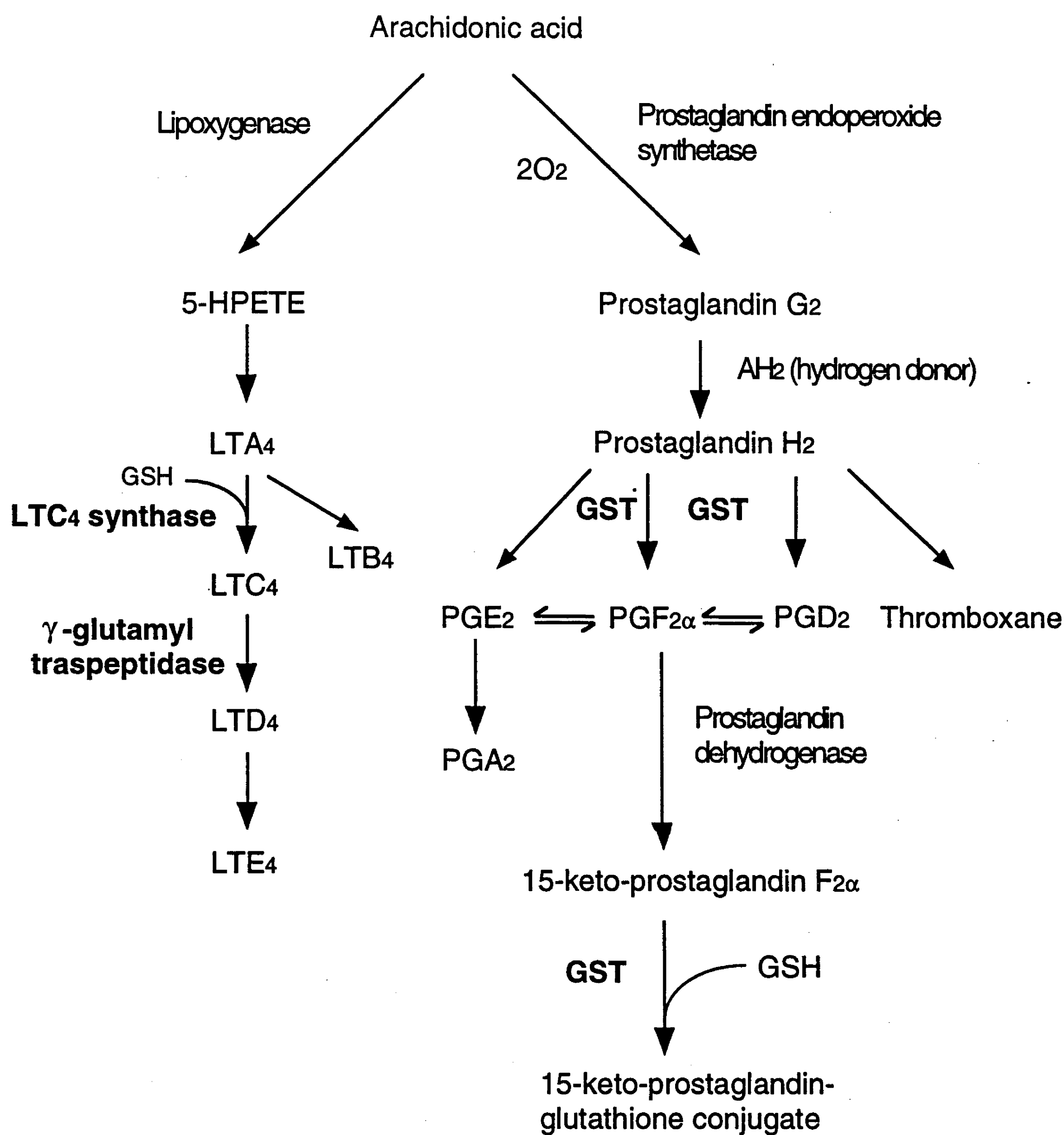


Figure 1.3 : Biosynthesis of Leukotrienes and Prostaglandins.
Involvement of GSTs or leukotriene C4 synthase are in bold. The involvement of γ-glutamyl transpeptidase in the conversion of LTC_4 to LTD_4 is highlighted. Abbreviations : LT : leukotriene; PG : prostaglandin; 5-HPETE : 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid.

1.3.5 Binding Role of GSTs

In addition to their catalytic roles, GSTs are also involved in the direct binding of non-substrates (see Table 1.2). The binding properties of GSTs have been identified in rat liver cytosol even before it was officially known to be a GST (Habig *et al.*, 1974a). In these early studies, GSTs were known by different names such as azo-dye carcinogen-binding proteins (Ketterer *et al.*, 1967; Ketterer and Christodoulides, 1968; Ketterer, 1971), corticosteroid binding proteins (Morey and Litwack, 1969), Y and Z proteins (Reyes *et al.*, 1971) and ligandin (Litwack *et al.*, 1971). Rat liver GST AA, A, B and C (Habig *et al.*, 1974a; Ketley *et al.*, 1975) and the basic human GSTs (Kamisaka *et al.*, 1975) bind non-covalently to a number of non-substrate ligands which include endogenous compounds such as bilirubin, haematin, bile acids, hormones, and neurotransmitters (Litwack *et al.*, 1971; Reyes *et al.*, 1971; Ketley *et al.*, 1975; Hayes *et al.*, 1979; Abramovitz *et al.*, 1988). It has been proposed that the binding of such compounds to GSTs may facilitate their transfer between intracellular compartments (Tipping and Ketterer, 1981; Senjo *et al.*, 1985; Harvey and Beutler, 1982) or provide temporary storage before their further metabolism and excretion (Wolkoff *et al.*, 1979).

In addition to their non-covalent binding, GSTs also bind covalently to some reactive xenobiotics. Xenobiotics such as the carcinogenic aminoazo dyes, polycyclic aromatic hydrocarbons and other reactive electrophiles can form covalent bonds with the cysteine residue of GSTs (Ketterer, 1971; Sarrif *et al.*, 1975). The human lung Pi class GST was found to bind ethacrynic acid (Awasthi *et al.*, 1993). The ethacrynic acid bound to the Pi class GST is a non-competitive inhibitor of the enzyme, suggesting that the covalent binding site is different from the catalytic site. Although GSTs may lose their catalytic activity as a result of covalent binding, this suicidal attempt may prove to be an additional protective mechanism for cells. Table 1.2 presents a list of non-substrate ligands for GSTs.

Table 1.2 : Compounds Bound by Rat GSTs

Heme and metabolites:
- Haematin
- Bilirubin

Bile acid and salts:
- Deoxycholate
- Taurodeoxycholate
- Chenodeoxycholate
- Cholate

Hormone, neurotransmitters and steroids:
- Corticosterone
- Dexamethasone
- Progesterone
- Androsterone
- Prednisolone
- Estrone
- Estradiol
- Testosterone
- Estrone sulphate
- Dihydroepiandrosterone sulphate
- Pregnenolone sulphate
- 2-hydroxyestradiol-glutathione
- Estrone glucuronide

Drugs:
- Apomorphine
- Caffeine
- Chlorpromazine
- Bromsulphthalein
- Tetracycline

Carcinogens and metabolites:
- *N, N*-dimethylaminoazobenzene
- *N, N*-dimethylaminoazobenzene metabolites
- *N*-methylaminoazobenzene-glutathione
- 3-methylcholanthrene
- 3-methylcholanthrene metabolite
- Diethylstilbestrol

Anions:
- Sulphate
- Glutathione
- Glucuronic acid

Source : Smith *et al*, 1977; Homma *et al*, 1986 and Abramovitz *et al.*, 1988.

1.3.6 Bioactivation of Glutathione-conjugates

It is generally believed that the conjugation of compounds to GSH results in their deactivation and detoxication. However, in several cases the conjugation reaction leads to bioactivation instead of deactivation (Andres *et al.*, 1989; Bakke and Gustafsson, 1984; Dekant and Vamvakas, 1993). Most of the compounds which become activated after conjugation to GSH form a reactive episulfonium ion which can form an adduct with the N^7 atom of guanine in DNA (Pickett and Lu, 1989). The adduct between DNA and the glutathione derivative cannot undergo DNA repair, thus causing mutations and DNA rearrangement. Another mode of bioactivation involves the formation of toxic cysteine *S*-conjugates after the sequential breakdown of glutathione-conjugates or the formation of reactive sulphur species when the corresponding cysteine *S*-conjugates of the glutathione-conjugate are cleaved by β-lyase (Elfarra and Anders, 1984; Anders, 1995).

1.4 NOMENCLATURE OF GST

There are numerous nomenclature systems for GSTs reported in the literature. They are based on different criteria as well as the preference of individual groups. Before the agreement of a unifying nomenclature for the human isoenzymes (Mannervik *et al.*, 1992), comparison of the GST isoenzymes identified in various laboratories was difficult and the discovery of "novel" GSTs could only be ascertained when their physical, immunochemical and enzymatic properties were fully characterised.

In 1969, Boyland and Chasseaud, classified the various forms of rat GSTs according to their substrate specificity. Hence, a series of names such as GSH *S*-aryltransferase, GSH *S*-epoxidetransferase, GSH *S*-alkyltransferase, GSH *S*-aralkyltransferase, GSH *S*-alkenetransferase were proposed to identify the GST isoforms. However, homogeneous preparations of several enzymes displayed broad and overlapping substrate specificities (Pabst *et al.*, 1973; Pabst *et al.*, 1974). For example, purified "epoxidetransferase" also exhibited alkyltransferase and aralkyltransferase activities. The nomenclature based on substrate specificity is unable to address this discrepancy and it also lacks biological significance because most of the substrates used

are not of endogenous origin. A similar nomenclature was also used to described several human GSTs (Grover and Sims, 1964; Johnson, 1966; Boyland and Chasseaud, 1967).

Kamisaka *et al.* (1975) originally described 5 basic GSTs from human liver cytosol. They were given Greek letter designations of α, β, γ, δ, and ε in the order of increasing isoelectric points. Transferases α-ε shared similar enzymatic and immunological properties. For this reason, they were often categorised as transferases α-ε or "basic transferases". An enzyme with a near-neutral isoelectric point called GST μ was purified from human liver (Warolm *et al.*, 1981) while acidic transferases were purified from human erythrocytes (GST ρ) (Marcus *et al.*, 1978), human lung (GST λ) (Stockman *et al.*, 1987) and human placenta (GST π) (Polidoro *et al.*, 1980). Guthenberg and Mannervik (1981) suggested that transferase π and transferase ρ were closely related, if not identical. Other GSTs with Greek letter designation include the acidic glutathione transferases ω (pI4.6), ζ (pI5.2), ψ (pI5.4). (Awasthi *et al.*, 1980; Singhal *et al.*, 1991; Singh *et al.*, 1987). Recently, Meyer *et al.* (1991) purified a novel class of GST from human liver cytosol termed GST θ. This class of GST has no significant N-terminal amino acid sequence similarity to the earlier reported GSTs and was believed to be a unique class of its own (see **Section 1.5.4** for more details).

Board (1981b) has taken a genetic approach to classify GSTs by their gene loci. By using a non-denaturing electrophoresis system, a direct comparison of GSTs expressed in different tissues and individuals became possible under the same conditions. This experiment suggested that the GST isoenzymes are products of 3 different loci designated GST 1, GST 2 and GST 3. Other laboratories have confirmed similar findings (Strange *et al.*, 1984). Under this nomenclature, allelic variants of GST 1 are designated as GST1 type 1 and GST1 type 2, each representing the homodimer of type 1 and type 2 subunit. Consequently, a heterodimer between type 1 and type 2 is termed as GST1 type 1-2. A study by Suzuki *et al.* (1987) on other human tissues revealed that there are at least another 3 separate gene loci termed, GST 4, 5 and 6, which encode GSTs. GST 4 was found to be expressed abundantly in skeletal muscle, with detectable

levels of expression also found in heart, liver kidney, lung, spleen, thymus and brain tissues (Laisney *et al.*, 1984). GST 5 is a brain specific GST, whereas GST 6, the most acidic one among the three, was purified from a number of tissues (Suzuki *et al.*, 1987).

Mannervik *et al.* (1985b) proposed a different classification system based on the comparison of the N-terminal amino acid sequences, immunological cross-reactivities, substrate specificities and enzyme inhibition studies. The proposed nomenclature divided mammalian cytosolic GSTs into 3 evolutionary classes termed alpha, mu and pi. A similar grouping of isoenzymes was also found in mouse and rat and the GSTs from the same class, though from different species, showed greater similarity than GSTs from the same species but different classes. Therefore, the alpha, mu and pi classification system is species-independent and can be applied to the GST isoenzymes in mouse, rat, human and probably other mammals. In this classification, the previously described GST 1, 4, 5, and 6 fall into the mu class; GST2, the alpha class and GST 3, the pi class.

Since mammalian GSTs are dimeric proteins composed of either identical (homodimer) or non-identical (heterodimer) subunits, the enzymes properties are largely determined by the combination of subunits. A nomenclature based on subunit composition has also been adopted. Under this nomenclature, the human alpha class GSTs were named as B_1B_1 or B_1B_2 based on the composition of subunit B_1 and B_2 (Stockman *et al.*, 1985; Stockman *et al.*, 1987) or subunits Ha_1/Ha_2 (Rhoads *et al.*, 1987; Rozen *et al.*, 1992) and subunits α_x/α_y (Ostlund-Farrants *et al.*, 1987) in other cases. The human Mu class GSTs in this nomenclature were named as GST *NN*, with different Roman number subscript under the letter "*N*", indicating the different subunits of the class (Hussey *et al.*, 1991) or was named subunit H_b by another laboratory (DeJong *et al.*, 1988a; DeJong *et al.*, 1991). Consequently, subunits N_1, N_2 correspond to GST μ and GST 4. The acidic GST π or GST 3 belonging to the pi class was called GST YfYf under the criteria of this nomenclature (Carmichael *et al.*, 1988).

Recently, a consensus nomenclature for the human GST isoenzymes has been agreed (Mannervik *et al.*, 1992). In this new nomenclature, the names of the enzymes reflect their evolutionary class, subunit composition and allelic variants. Furthermore, the product of a discrete gene has its own designation and no novel GST is to be included in this nomenclature until its complete primary structure is fully known. For example, GST 2 type 1 is now called GSTA1-1. "A" represents the Alpha class and "1-1" indicates it is a homodimer of subunit 1. GSTM1a-1b represents a GST from Mu class with an allelic variant of subunit 1 (the lower case letter indicates the allelic forms of the same gene loci). Consequently, the newly discovered theta class GSTs (Meyer *et al.*, 1991; Hussey and Hayes, 1992; Pemble *et al.*, 1994; see **Chapter 3** and **4**) were designated as GSTT1-1 and GSTT2-2. The same nomenclature can also be applied to other mammalian species by having a lower case letter prefix before the name of the enzyme. For example, a rat pi class GST can be termed rGSTP1-1. For a more comprehensive prefix, the Latin name of the species can be adopted. However, this nomenclature has not been fully implemented in other mammalian species (for previous nomenclature of rat and mouse GSTs see review by Mannervik and Danielson, 1988; Hayes and Pulford, 1995). Table 1.3 summarises the various nomenclatures described above.

Table 1.3 : Nomenclature of Glutathione Transferase Isoenzymes

Class	Nomenclature based on subunit	Greek letters nomenclature	Nomenclature based on gene locus	1992 unified nomenclature	pI	Gene locus designation	Chromosomal localization
Alpha	B1B1, Ha1, αx	ε	GST2 type1	A1-1	8.9	*GSTA1*	6p12
	B1B2	δ	GST2 type1-2	A1-2	8.75		
	B2B2, Ha2, αy	α,β,γ	GST2 type2	A2-2	8.4	*GSTA2*	6p12
Mu	$N1^aN1^a$, Hb	μ	GST1 type2	M1a-1a	6.1	*GSTM1*	1p13
	$N1^aN1^b$		GST1 type1-2	M1a-1b	n.d.		
	$N1^bN1^b$	ψ	GST1 type1	M1b-1b	5.5	*GSTM1*	1p13
	$N1^aN2$			M1a-2	n.d.		
	$N1^bN2$			M1b-2			
	N2N2	ζ	GST4	M2-2	5.2 - 5.4	*GSTM2*	1p13
	N2N3			M2-3	n.d.		
	N3N3		GST5	M3-3	5.9	*GSTM3*	1p13
				M4-4	5.2	*GSTM4*	1p13
				M5-5	n.d.	*GSTM5*	1p13
			GST6?				
Pi	YfYf	π, λ, ρ	GST3	P1-1	4.5 - 4.9	*GSTP1*	11q13
Theta		θ		T1-1	n.d.	*GSTT1*	22q11.2
				T2-2	5.2 - 5.3	*GSTT2*	22q11.2
Microsomal				Microsomal	10.1	*GST12*	12

n.d. : not determined. References for individual isoenzymes have been sited in the text.

1.5 GST ISOENZYMES

Mammalian cytosolic GSTs are dimeric proteins (either homo- or heterodimers) with functionally independent subunits. A heterodimer can be formed from members of the same class but inter-class dimers have not been identified (see Table 1.3). GSTs are subject to developmental (Hales and Neims, 1976; Strange *et al.*, 1985) and sex-dependent expression (Ryberg *et al.*, 1994; Egaas *et al.*, 1995), induced by different chemical compounds (Benson *et al.*, 1978; Pickett *et al.*, 1982; Clapper *et al.*, 1994) and expressed in different tissues (Board, 1981b; Strange *et al.*, 1984; Suzuki *et al.*, 1987); or cell types (Toffoli *et al.*, 1992; Green *et al.*, 1993; Anttila *et al.*, 1993).

With the adoption of a consensus nomenclature (Mannervik *et al.*, 1992), mammalian cytosolic GSTs have been grouped into 4 different evolutionary classes --- Alpha, Mu, Pi and Theta, each differing in their primary amino acid sequence. With the exception of the Theta class (see **Section 1.5.4**), amino acid sequence similarity within a class tends to be around 70% or greater while inter-class sequence similarity is usually less than 30%. The subunit size of human cytosolic GSTs ranges from 24,700 (GSTP1-1) to 27,000 Da (GSTT2-2).

On the other hand, the mammalian microsomal GSTs are a group of membrane-bound enzymes which have evolved separately to the cytosolic GSTs. The recent nomenclature designated the microsomal GSTs as a class of its own. Microsomal GSTs are believed to be a convergent evolutionary product, rather than a divergent product of the cytosolic GSTs.

For the remaining sections in this chapter, the 1992 nomenclature will not be strictly applied to describe the human Alpha, Mu and Pi class GSTs. Instead, the original names when they were first described will be used to reflect their originality. A comparison between the previous nomenclatures and the consensus nomenclature reached in 1992 is given in Table 1.3. Other GST classes have been suggested for non-mammalian species such as bacteria (Nishida *et al.*, 1994), fungus (Casey *et al.*, 1991),

yeast (Coschigano and Magasanik, 1991), plant (Moore *et al.*, 1986; Takahashi and Nagata, 1992), insect (Toung *et al.*, 1991; Wang *et al.*, 1991; Board *et al.*, 1994), cephalopods (Tomarev and Zinovieva, 1988; Tomarev *et al.*, 1993) and helminths (Brophy *et al.*, 1989). However, the present review is focused on the human GSTs.

1.5.1 Alpha Class GSTs

The human Alpha class GSTs encompass the basic isoenzymes α, β, γ, δ and ε isolated by Kamisaka *et al.* from human liver (Kamisaka *et al.*, 1975). These workers suggested that these isoforms were products of a single gene but may have risen as a result of *in vivo* deamination. However, Board (1981b) demonstrated that the 3 human liver Alpha class GSTs were homo- and heterodimers of two subunits, namely GST2 type 1 and GST2 type 2. Similar observations made by Stockman *et al.* showed that the basic GSTs were formed from two subunits, B_1 and B_2 which differ from one another catalytically (Stockman *et al.*, 1985; Stockman *et al.*, 1987). Based on their isoelectric points, it is evident that GST2 type 1 is equivalent to B_1 and GST 2 type 2 is the same as B_2 (Stockman *et al.*, 1985; Suzuki *et al.*, 1987).

Evidence that the multiple isoforms of the human Alpha class GSTs are products of separate genes came from the isolation of two human Alpha class cDNAs, termed GTH_1 and GTH_2 from a human liver cDNA library (Tu and Qian, 1986; Rhoads *et al.*, 1987). Independently, Board and Webb isolated an Alpha class cDNA clone, λGST2-3 (Board and Webb, 1987), which was the same cDNA as GTH_1. The deduced amino acid sequences of GTH_1 and GTH_2 are very similar and differ in only 11 amino acids. Direct amino acid sequencing showed that the amino acid sequence of subunits B_1 and B_2 were the same as those deduced from GTH_1 and GTH_2 respectively (Hayes *et al.*, 1989). From the present understanding, it can be concluded that the Alpha class subunits are products of 2 different gene loci.

The genes for the two human Alpha class GSTs have been isolated from different genomic libraries (Rozen *et al.*, 1992; Klone *et al.*, 1992; Suzuki *et al.*, 1993). The

genes span approximately 12kb, containing 7 exons, separated by 6 introns. A separate gene for a third Alpha class GST, *GSTA3* has also been reported (Suzuki *et al.*, 1993). However, the cDNA and protein derived from this gene has yet to be isolated and it is not clear where or when *GSTA3* is expressed. DNA sequence around the vicinity of the *GSTA1* gene reveals several pseudogenes belonging to the same class (Suzuki *et al.*, 1993).

The Alpha class genes appeared to be heterogenous from results of Southern blot analysis (Board *et al.*, 1990). Besides the cDNA cloning of GSTA1, GSTA2 and the identification of a putative GSTA3 gene and other Alpha class pseudogenes, it is notable that there have been reports of the isolation of additional human Alpha class isoenzymes from various tissues although their cDNAs have not been identified. They include the basic human skin GST with a pI of 9.9 (Del Boccio *et al.*, 1987) and an acidic GST (pI5.8), designated as hGST5.8, which is known to be expressed in several human tissues (Singhal *et al.*, 1994b; Singhal *et al.*, 1994a; Singhal *et al.*, 1995). hGST5.8 is closely related to the rat GST8-8 (Jensson *et al.*, 1986) and mouse mGSTA4-4 (Zimniak *et al.*, 1992) of the Alpha class in terms of amino acid sequence identity and substrate specificity. Singhal and co-workers proposed that these orthologous isoenzymes in mammalian species represent a subclass within the Alpha class isoenzymes (Singhal *et al.*, 1994b) which are characterised by their high activity towards 4-hydroxy-2-nonenal and probably other 4-hydroxyalk-2-enals.

Alpha class GSTs have several distinct characteristics. They all show selenium-independent GSH peroxidase activity towards hydroperoxide substrates (Mannervik and Danielson, 1988; Singhal *et al.*, 1994b). In rodents, the Alpha class GSTs are responsible for the detoxication of hepatocarcinogens, aflatoxin B_1-8,9-epoxide and several diol epoxides of polycyclic aromatic hydrocarbons (Ramsdell and Eaton, 1990; Hayes *et al.*, 1991; Hayes *et al.*, 1992). GSTs in human liver cytosol also conjugate aflatoxin B_1 exo- and endo-epoxides to GSH although the activities were much lower compared to that in rodents (Raney *et al.*, 1992).

1.5.2 Mu Class GSTs

The human Mu class of GSTs is the largest family among the mammalian cytosolic GSTs identified, with at least six distinct subunits that are the products of five different loci (Table 1.3). Many other Mu class GSTs have been characterised at the protein level, but their relationships with the other Mu class GSTs remain ambiguous until further information is available (Tsuchida *et al.*, 1990; Suzuki *et al.*, 1991).

Two hepatic Mu class GSTs, GST μ and GST ψ, are well characterised (Warholm *et al.*, 1981; Warholm *et al.*, 1983; Singh *et al.*, 1987). Both proteins are homologous and the amino acid sequences deduced from their cDNAs showed that they only differ at residue 173, which substitutes a lysine residue in GST μ for an asparagine in GST ψ. It is now apparent that GST μ and GST ψ are allelic variants of the *GSTM1* gene locus, and were initially characterized genetically by Board (1981b) as GST1 type 1 (GST ψ) and GST1 type 2 (GST μ).

Extrahepatic Mu class GSTs include GST 4, GST 5, GST 6 and GT-tSBO. GST 4 is a muscle specific GST purified from skeletal muscle (Suzuki *et al.*, 1987). A cDNA clone was isolated from a myoblast cDNA library (Vorachek *et al.*, 1991) and the deduced amino acid sequence was found to be similar to that of GST 4 (now known as GSTM2), confirming the gene product of *GST 4* locus. The cDNA encoding GSTM3 [formally known as GST 5 (Suzuki *et al.*, 1987)] have been isolated from testis and brain cDNA libraries (Campbell *et al.*, 1990). The deduced amino acid sequences from these cDNA clones have 72% amino acid sequence identity with the human liver GST μ, but unlike GST μ, GSTM3 has little activity towards *trans*-4-phenyl-3-buten-2-one (tPBO) and the N-terminus of the protein is blocked. A highly acidic Mu class isoenzyme GST 6 (pI 4.25), was purified from various human tissues (Suzuki *et al.*, 1987; Suzuki *et al.*, 1991). The first 24 N-terminal amino acid residues of GST 6 were found to be identical to GST 4 and differed in only one residue from GST 1, indicating a close relationship between these 3 isoenzymes (Suzuki *et al.*, 1991). As the cDNA encoding GST 6 has yet to be discovered, its genetic relationship with the other Mu class GSTs remains uncertain

at this stage. GT-tSBO which is identical to GST μ and GST1 type2, is expressed in human mononuclear leucocytes (Seidegard *et al.*, 1987; Seidegard *et al.*, 1989).

Various groups of workers have isolated and characterised a fourth member of the Mu class GST, GSTM4 (Ross and Board, 1993; Zhong *et al.*, 1993; Comstock *et al.*, 1993). Its gene structure and its heterologous expression have been described (Ross and Board, 1993; Zhong *et al.*, 1993; Comstock *et al.*, 1993). The recombinant GSTM4 protein appeared to have low activity with most substrates (Ross and Board, 1993). Partial cDNA clones of GSTM4 showed evidence of alternative splicing, which may represent a novel form of post-transcriptional regulation in the GST supergene family (Ross and Board, 1993). Recently, the cDNA of a fifth member of the Mu class GSTs, GSTM5, has been cloned from a human brain cDNA library (Takahashi *et al.*, 1993). GSTM5 was found to be expressed in brain, testis and lung but little is known about its protein at this stage. This may represent the GST 6 that was previously purified by Suzuki *et al.* (1991).

The Mu class GSTs is heterogeneous and polymorphism has been observed in the GSTM1-1 isoenzyme (Board, 1981b; Seidegard and Pero, 1985; Seidegard *et al.*, 1988). Non-denaturing gel electrophoresis, Southern blot analysis and GT-tSBO activity assays in blood monocytes have shown that about 40-50% of the European population are deficient in *GSTM1*. Board suggested this deficiency is due to a null allele at the *GSTM1* locus (Board, 1981b). The existence of a null allele corresponds to a deletion of the *GSTM1* gene (Seidegard *et al.*, 1988). This polymorphism appears to be widely spread among different ethnic groups (Board *et al.*, 1990). Several studies have been carried out to determine if the *GSTM1* polymorphism is related to an increased risk of cancer among smokers, but each giving variable results (Seidegard *et al.*, 1990; Nazar-Stewart *et al.*, 1993; Zhong *et al.*, 1991). Numerous other studies of the potential relationship between GSTM1 deficiency and susceptibility to various cancers have been undertaken but are beyond the scope of this review.

1.5.3 Pi Class GSTs

The human Pi class is the smallest family among the GST supergene family, with only one member, GSTP1-1, identified at the cDNA and protein level (Kano *et al.*, 1987; Moscow *et al.*, 1988). The Pi class GST has a low pI value and was identified as the cationic GST 3 in human erythrocytes (Board, 1981b). In humans, Pi class GST was purified from placenta (GST π), lung (GST λ) and erythrocytes (GST ρ) (Marcus *et al.*, 1978; Polidoro *et al.*, 1980; Stockman *et al.*, 1987). Although GST π is not expressed in adult liver, it is expressed abundantly in foetal liver (Strange *et al.*, 1985). Elevated levels of GST π were detected in certain preneoplastic tissues and its overexpression has been associated with resistance to chemotherapeutic drugs (Nakagawa *et al.*, 1990; Kuzmich *et al.*, 1992).

GST π, GST λ and GST ρ are closely related and thought to be the products of *GSTP1*. There is evidence that the *GSTP1* locus is polymorphic, with at least 2 allelic variants (Board *et al.*, 1989; Ahmad *et al.*, 1990). In fact the cDNA cloned by Kano *et al.*, differs from the partial cDNA clones isolated by Board *et al.* (1989), and Wang *et al.*, (1989) at two position, I105→V and A114→V respectively. It is most likely that these conservative substitutions represent allelic variation of the *GSTP1* gene locus. Recently, Bora *et al.* (1989; 1991) reported a protein which has fatty acid ethyl ester synthase III (FAEES III) and GST activity. Its cDNA clone has only 4 amino acid substitutions compared to that of GSTP1. This raised the speculation of novel role for the GST family. However, this speculation was proven to be unlikely when a FAEES III cDNA clone constructed by site-directed mutagenesis of a GSTP1 cDNA and expressed in *E. coli* was found to be inactive as a FAEES enzyme (Board *et al.*, 1993).

1.5.4 Theta Class GSTs

The Theta class GSTs were only recently recognised as a distinct GST class (Meyer *et al.*, 1991). The Theta class GSTs were largely overlooked due to their low or lack of activity with the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) and their failure to bind to immobilised glutathione affinity matrices.

However, the discovery of the Theta class GSTs can be traced back to the partial purification of rat transferase E (GST 5-5) (Pabst *et al.*, 1973) and a rat liver enzyme that catalysed the conjugation of GSH to 1-menapthyl sulphate (Gillham *et al.*, 1970). For a period of time, these two isoenzymes were not assigned to any class because there was insufficient knowledge of them. Recently, a novel class of GSTs with distinct characteristics were purified from rat and human liver (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991; Hussey and Hayes, 1992). They included the rat GST 5-5, rat GST 12-12, rat GST Yrs-Yrs (see **Section 1.5.4.2** below), as well as human GST θ and GSTT2*-2*. The primary structures, immunochemical properties and substrate specificities of these enzymes have little similarity with the existing Alpha, Mu and Pi class GSTs. In view of their distinct characteristics, they were assigned as a new class in the GST supergene family, termed Theta.

Subsequently, other mammalian Theta class GSTs were also characterised from mouse, dog and human (for references see Table 1.4). Several GSTs from insects (see review by Wilce and Parker, 1994) and plants (Moore *et al.*, 1986; Takahashi and Nagata, 1992) were also shown to have sequence similarity with the Theta class GST and Theta-like GSTs were also purified from subcellular compartment such as matrix of mitochondria and nucleus. Table 1.4 presents a list of GSTs which have been classified as Theta class or termed Theta-like in the literature.

Comparison of the characteristics of all the mammalian Theta class GSTs studied so far suggests that the Theta class may be subdivided into two subfamilies based on their overall amino acid sequence identity (Ogura *et al.*, 1991; Pemble and Taylor, 1992; Pemble *et al.*, 1994; Tan *et al.*, 1995) and substrate specificities (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991). In spite of their amino acid sequence identity in the N-terminal domain, their C-terminal sequences show greater diversity. Within the same subfamily, overall amino acid sequence identity is greater than 78%, e.g. rat GST Yrs-Yrs and human GSTT2-2. However, overall amino acid sequence identity is less than 52% between a subfamily even from the same species, e.g. rat GST 5-5 and rat GST Yrs-Yrs.

The percentage of sequence identities between subfamily 1 and subfamily 2 members are shown in Table 1.5.

1.5.4.1 Subfamily 1

Subfamily 1 comprises the rat GST 5-5 (Meyer *et al.*, 1984; Meyer *et al.*, 1991; Pemble and Taylor, 1992) [previously known as rat transferase E (Habig *et al.*, 1974b; Meyer *et al.*, 1984)] and its human orthologue GSTT1-1 (Pemble *et al.*, 1994) [previously termed GST θ (Meyer *et al.*, 1991)]. Recently, a mouse Theta class GST belonging to this subfamily was detected in cytosol of mouse liver (Hiratsuka *et al.*, 1995). Unfortunately, due to its instability during purification, the protein was not available for further characterisation.

1.5.4.1.1 Substrate specificity for subfamily 1

GST 5-5 has significant activities towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), *p*-nitrobenzyl chloride (PNBC), *p*-nitrophenethyl bromide (PNPB), dichloromethane (DCM), cumene hydroperoxide (Cu-OOH) and DNA hydroperoxide (Tan *et al.*, 1988; Meyer *et al.*, 1991). As for GSTT1-1, the partially purified sample (90% pure) lost significant activity during purification. Nevertheless, in the crude cytosolic fraction which was not retained by GSH affinity matrix, detectable activity was observed with CDNB, EPNP, PNBC and PNPB. It was proposed that the specific activity of GSTT1-1 would be of the same order as that of rat GST 5-5 (Meyer *et al.*, 1991).

In vivo expression of GST 5-5 in *Salmonella typhimurium* TA1535 has been implicated in the production of base-pair revertants upon exposure to ethylene dihalides or dihalomethanes (Thier *et al.*, 1993). This mutagenic effect is caused by the *S*-(1-haloalkyl)GSH intermediates, formed through the GST catalysed conjugation of GSH with dihalomethanes. These substances are genotoxic and are known to form DNA

Table 1.4 : List of Theta Class GSTs and the Information Available on Them

Species	Tissue	Enzyme name	Protein characterization	cDNA information	Gene structure	References
Human	Liver, erythrocytes	GSTT1-1	+	+	-	Meyer *et al.*, 1991 Pemble *et al.*, 1994
	Liver	GSTT2-2	+	+	-	Hussey *et al.*, 1992 Tan *et al.*, 1995
Rat	Liver	rat 5-5 (rat transferase E)	partial	+	-	Meyer *et al.*, 1984 Pemble *et al.*, 1992
	Liver	rat Yrs-Yrs	+	+	+	Hiratsuka *et al.*, 1990 Ogura *et al.*, 1991 Ogura *et al.*, 1994
	Liver	rat Yrs'-Yrs'	+	-	-	Hiratsuka *et al.*, 1994
	Liver	rat 12-12	+	-	-	Meyer *et al.*, 1991
	Mitochondria matrix of rat liver	rat 13-13	+	-	-	Harris *et al.*, 1991
	Liver nucleous	rat 5*-5*	+	-	-	Tan *et al.*, 1988
Mouse	Liver	mYrs-Yrs (or mGSTT2)	+	+	+	Whittington *et al.*, 1996. Hiratsuka *et al.*, 1995
Dog	Liver	Ydf-Ydf	+	-	-	Igarashi *et al.*, 1991
Fish (*Pleuronectes platessa*)	Liver	GST-A	+	+	-	Leaver *et al.*, 1993
Blowfly (*Lucilia cuprina*)	*L. cuprina* pupae	Lu GST1	+	+	-	Board *et al.*, 1994
Plant (*Arabidopsis thaliana*)	Leaves	GST 1527-1	+	+	-	Bartling *et al.*, 1993

Note : (+): Information available. (-): Information not available at time of writing.

Table 1.5 : Amino Acid Sequence Comparison of the Mammalian Theta Class GSTs. Amino acid sequence are deduced from cDNA clones. GSTT1-1 : Pemble *et al.*, 1994; GSTT2-2: Tan *et al.*, 1995 (see Chapter 3); rat 5-5 : Pemble *et al.*, 1992; rat Yrs-Yrs : Ogura *et al.*, 1991. mGSTT2-2 (mYrs-Yrs) : Whittington *et al.*, 1996.

	Subfamily 1		**Subfamily 2**		
	GSTT1-1	**rat 5-5**	**GSTT2-2**	**rYrs-Yrs**	**mYrs-Yrs**
GSTT1-1	100%	82.0%	55.0%	55.4%	55.2%
rat 5-5		100%	50.2%	51.1%	51.1%
GSTT2-2			100%	78.3%	77.0%
rYrs-Yrs				100%	91.8%
mGSTT2-2					100%

adducts with 2'-deoxyguanosine (Pickett and Lu, 1989). Mice, but not rats, exposed to dihalomethanes were found to develop liver tumours (Wong *et al.*, 1982). In view of this, the risk assessment of dihalomethanes to humans becomes important (Ramsey *et al.*, 1979).

1.5.4.1.2 Dihalomethane metabolism and *GSTT1* polymorphism

In humans, there is an inter-individual variation on the conjugation of dihalomethane with GSH (Schroder *et al.*, 1992). About 60-85% of the individuals studied were termed "conjugators" while the other are "non-conjugators". The enzyme responsible for this conjugation is a GST present in erythrocytes as well as liver (Schroder *et al.*, 1992; Bogaards *et al.*, 1993). It was only recently, that this polymorphism was clearly attributed to the Theta class GSTT1-1 isoenzyme (Pemble *et al.*, 1994; Hallier *et al.*, 1994). The *GSTT1* polymorphism is due to a null allele caused by a gene deletion as indicated by PCR-based genotyping and Southern blot analysis (Pemble *et al.*, 1994). In a recent study, the *GSTT1* polymorphism was shown to vary substantially across different ethnic groups (Nelson *et al.*, 1995). By a PCR-based genotyping assay, the homozygous deletion of *GSTT1* was found to have the highest prevalence in the Chinese (64.4%) and Korean (60.2%), followed by African-Americans (21.8%), Caucasians (20.4%) and Mexican-Americans (9.7%). It seems likely that the *GSTT1* null genotype is associated with gene-environment interaction. Since GSTT1-1 is involved in the dihalomethane metabolism, individuals with the *GSTT1* normal allele may have a greater risk of cancer when exposed to dihalomethanes while individuals homozygous for the null alleles may have a lower risk.

However, whether this polymorphism is deleterious or beneficial is still debatable. For example, sister chromatid exchanges in lymphocytes of "non-conjugators" were markedly increased upon exposure to methyl bromide, ethylene oxide and dichloromethane (Hallier *et al.*, 1993). In a separate study, individuals carrying a homozygous deletion of the *GSTT1* gene showed a marked increase in spontaneous (background) as well as diepoxybutane induced sister chromatid excahnges (Wiencke *et*

al., 1995). It appears that the expression of GSTT1-1 in the "conjugator" group grants protection against this cytogenetic damage (Hallier *et al.*, 1993).

Studies on the importance of *GSTT1* allele on the risk assessment of cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma (SCC) did not indicate a role for GSTT1-1 in the aetiology of these diseases, nor was there a correlation between the interactive effect of *GSTM1* null and *GSTT1* null in the incidence of CIN and SCC (Warwick *et al.*, 1994). Risk assessment of the *GSTT1* null genotype and colorectal cancer did not show any significant difference with the control group (Chenevix-Trench *et al.*, 1995). However, the homozygous *GSTT1* null was more common in individuals diagnosed with colorectal cancer before age 70. Other studies on the *GSTT1* genotype show that the *GSTT1* positive genotype conferred some protective effect against total ulcerative colitis (Duncan *et al.*, 1995), while in another study, the positive genotype was associated with an increased risk of astrocytoma (Elexpuru-Camiruaga *et al.*, 1995). These mixed results suggest that it may be important to evaluate *GSTT1* polymorphism as a risk factor in other malignancies.

1.5.4.2 Subfamily 2

The subfamily 2 of the mammalian Theta class consists of rat GST Yrs-Yrs (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991), mouse GST mYrs-mYrs (or mGSTT2-2) (Hiratsuka *et al.*, 1995; Whittington *et al.*, 1996) and their human orthologue GSTT2-2 (Hussey and Hayes, 1992; see **Chapter 3**). Several additional closely related isoenzymes have been purified from this subfamily in rats (Meyer *et al.*, 1991; Hiratsuka *et al.*, 1994) and there is evidence at the DNA level of heterogeneity of this subfamily in mice (Whittington *et al.*, 1996).

1.5.4.2.1 Heterogeneity within the subfamily 2

In recent publications, the rat GST 12-12 is commonly equated to rat GST Yrs-Yrs. However, Hiratsuka *et al.* (1995) noted that although rat GST 12-12 and rat GST Yrs-Yrs are structurally similar, they are not identical and represent two distinct

members of subfamily 2 in the rat. Indeed, comparison of the partial amino acid sequence of rat GST 12-12 (Meyer *et al.*, 1991) with the amino acid sequence deduced from a cDNA clone of rat GST Yrs-Yrs (Ogura *et al.*, 1991) showed the following substitutions: Cys13→Ser, Arg35→Leu, Cys36→Lys and a glutamine deletion in rat GST 12-12 at position 43 of rat GST Yrs-Yrs. In fact, heterogeneity in the rat GST 12-12 was first observed when N-terminal sequencing of rat GST 12-12 yielded both serine and cysteine at residue 13 suggesting two proteins were being sequenced (Meyer *et al.*, 1991). Cyanogen bromide cleavage of rat GST 12-12 (Meyer *et al.*, 1991) produced 2 similar but distinct fragments that also differed from the deduced amino acid sequence of rat subunit Yrs cDNA (Ogura *et al.*, 1991). These results suggest that there are at least 2 members of subfamily 2 in rats.

Recently, Hiratsuka *et al.* (1994) purified another rat Theta class GST which is closely related to subunit Yrs. This subunit, termed Yrs', has comparable substrate specificities to subunit Yrs, with the exception of lower activity towards mono-hydroperoxides of endogenous polyunsaturated fatty acids. Although amino acid sequencing of the first 40 residues of subunit Yrs' showed that it is identical to subunit Yrs, the two subunits have different pI values and have different retention time on reverse phase HPLC. Subunit Yrs and Yrs' can exist as homodimers, as well as the heterodimer Yrs-Yrs'. This observation suggests that Yrs and Yrs' are possibly post-translational modified products from the same gene locus (Hiratsuka *et al.*, 1994).

In the process of isolating the rat GST 5-5 cDNA, Pemble and Taylor (1992) reported a partial clone of a Theta family member which shares high N-terminal amino acid sequence similarity with cDNAs of subunit Yrs and subunit 5. It is not known if the 3' region of this partial cDNA shares greater identity to subunit Yrs or subunit 5. However, from the deduced N-terminal sequence, this cDNA clone is likely to be a member of subfamily 2.

Recently, the cDNA and gene for a mouse Theta class GST belonging to the subfamily 2 has been cloned (Whittington *et al.*, 1996). Heterogeneity in this mGSTT2 has also been observed. Comparison of the amino acid sequence deduced from genomic sequence and cDNA sequence revealed a single amino acid change at position 206 which substituted a cysteine for a tyrosine. This substitution may be the result of allelic variation of the locus. The heterogeneity occurring in the subfamily 2 of Theta class GST in rats and mice suggests that similar heterogeneity may also exist in human and other mammals.

1.5.4.2.2 Substrate specificity for subfamily 2

The rat GST Yrs-Yrs was originally discovered because of its GSH-conjugating activity towards reactive sulphate esters formed from carcinogenic polycyclic arylmethanols (Hiratsuka *et al.*, 1990). A less mutagenic aralkyl sulphate ester, 1-menapthyl sulphate was the best substrate among the arylmethyl sulphates tested. Recently, Hiratsuka *et al.* (1995) purified and characterised the equivalent of rat GST Yrs-Yrs in mouse, termed GST mYrs-mYrs (Hiratsuka *et al.*, 1995). Of particular interest with GST mYrs-mYrs is its high specific activity towards MS (4.24 μmol/mg protein/min), the highest known value among the members of subfamily 2. The high activity of GST mYrs-mYrs towards reactive sulphate esters may grant protection against the carcinogenic effects of 5-hydroxymethylchrysene (5-HCR) which when converted to reactive sulphate, is highly carcinogenic in mouse skin where the activity of GST mYrs-mYrs is low but is not carcinogenic in mouse liver where GST mYrs-mYrs activity is relatively high (Hiratsuka *et al.*, 1995).

The human GSTT2-2 was first characterised by Hussey and Hayes (1992). As a member of the subfamily 2, GSTT2-2 utilises MS as a substrate but has no detectable activity with CDNB, EPNP and DCM (see **Chapter 5**). Apart from this initial characterisation, nothing is known about the genetics, structure and function of GSTT2-2.

1.5.4.2.3 The cDNAs and gene structure of subfamily 2

cDNAs encoding members of the rat, mouse and human subfamily 2 have been cloned (Ogura *et al.*, 1991; Whittington *et al.*, 1996, see **Chapter 3**). They all contain an open reading frame of 732bp, encoding a polypeptide of 244 amino acids (subfamily 1 members have a polypeptide of 240 amino acids), which is the longest mammalian GST characterised so far. The rat gene spans approximately 4kb (Ogura *et al.*, 1994) while the mouse gene is approximately 3.1kb (Whittington *et al.*, 1996). Nevertheless, both genes have similar gene structure, consisting of 5 exons and 4 introns. This structural identity suggests that the gene organisation of the subfamily 2 is conserved in mammalian species and the human *GSTT2* gene can be expected to share the same structure as its rat and mouse orthologue.

1.5.4.3 Summary

The current understanding of the Theta class GSTs groups them into two subfamilies. Heterogeneity within the subfamilies has been observed. From the enzymological point of view, subfamily 1 and subfamily 2 can be distinguished by subfamily-specific substrates. Members of subfamily 1 are characterised by their activity towards EPNP and DCM while members of subfamily 2 can be characterised by their activity towards MS. Substantial differences in the amino acid sequence in exons 4 and 5 may explain the different substrate preferences between subfamily 1 and subfamily 2. The secondary structural elements encoded by exons 4 and 5 form the C-terminal half of α-helix 4, α-helices 5, 6, 7 and the remaining C-terminal structure of the enzyme (see **Section 1.10.2**, Figure 1.6). Differences in this region will significantly affect the substrate specificities as these structural elements contribute to the architecture of the hydrophobic binding site (H-site) (see **Section 1.8.5**). Members of subfamily 1 and subfamily 2 have some common characteristics, in that they are not retained by GSH-affinity matrices, exhibit low activity towards CDNB and both demonstrate selenium-independent GSH peroxidase activity.

Polyclonal antibodies raised against one subfamily member cross-react with members of the same subfamily (Meyer *et al.*, 1991; Hiratsuka *et al.*, 1995) but not with members of the other subfamily (Watabe *et al.*, 1995). Members of subfamily 1 are generally more labile and lose activity upon storage (Meyer *et al.*, 1991), while the members of subfamily 2 are more robust during purification and retain appreciable activity during storage (Hiratsuka *et al.*, 1995).

1.5.5 Membrane-Bound GSTs

Membrane-bound GSTs can be divided into the general microsomal GST which has a broad substrate specificities, and leukotriene C_4 synthase which is specific for the addition of GSH to leukotriene A_4. Although they are both involved in GSH conjugation, they share little amino acid sequence similarity to the cytosolic GSTs.

Most of the initial investigations of microsomal GST were conducted by Morgenstern and co-workers on enzyme from rat-liver microsomes (Morgenstern *et al.*, 1979; Morgenstern *et al.*, 1980; Morgenstern *et al.*, 1982). The microsomal GST is distinct from the cytosolic GSTs in its primary amino acid sequences (Morgenstern *et al.*, 1985; DeJong *et al.*, 1988b), kinetic parameters and substrate specificities (Morgenstern and DePierre, 1983). It can be activated by sulfhydryl reagents (Morgenstern *et al.*, 1980; Morgenstern and DePierre, 1983), heat (Aniya and Anders, 1989), radiation (Boyer *et al.*, 1986) and partial trypsin digestion (Morgenstern *et al.*, 1989), and exhibit detergent-dependent activity (Morgenstern and DePierre, 1983). Microsomal GST exists as a trimer in its native form and the integrity of the trimer is essential for enzymatic activity (Boyer *et al.*, 1986; Lundqvist *et al.*, 1992). In rat liver, around 80% of the total microsomal GST is localised in the endoplasmic reticulum. The outer membrane of mitochondria contains another 5% of the total microsomal GST (Morgenstern *et al.*, 1984.

McLellan *et al.* first described the purification of human liver microsomal GST (McLellan *et al.*, 1989). The subunit of the purified protein co-migrates with the rat

microsomal GST on SDS/PAGE with an Mr value of approximately 17,300. Both proteins are immunologically related and show activity towards the neph rotoxin and hexachlorobuta-1,3-diene (Wolf *et al.*, 1984; McLellan *et al.*, 1989). Understanding of the rat and human microsomal GSTs at the molecular level was made possible when cDNA clones from both species were isolated from liver cDNA libraries (DeJong *et al.*, 1988b). The cDNAs share 77% nucleic acid identity and encode polypeptides of 154 amino acids with 83% amino acid identity. It is confirmed that only one cysteine residue is present in the mature protein and this cysteine residue is thought to be involved in the activation of the protein by sulfyldryl reagents (Morgenstern *et al.*, 1980; Morgenstern and DePierre, 1983).

The second membrane-bound GST, leukotriene C4 synthase, is involved in the biosynthetic pathway of this pro-inflammatory mediator (see **Section 1.3.4**). Leukotriene C4 synthase has been purified to homogeneity and shown to be a dimeric protein composed of two identical 18kDa subunits (Nicholson *et al.*, 1993). The cDNA encoding leukotriene C4 synthase has been subsequently cloned and expressed (Lam *et al.*, 1994; Welsch *et al.*, 1994). The deduced amino acid sequence shares no obvious similarity with the cytosolic GSTs.

1.6 CHROMOSOMAL LOCALISATION

Chromosomal localisation of the GST genes has been studied using rodent-human somatic cell hybrid analysis, gene-specific PCR and *in situ* hybridisation approaches. However, the precise gene loci were determined using the latter approach. The human Alpha class gene, *GSTA1*, has been mapped to the short arm of chromosome 6 at band 12 (6p12). As there was 95% identity between cDNAs of GSTA1 and GSTA2 (Rhoads *et al.*, 1987) and no significant hybridisation signal was observed elsewhere in the genome, it can be concluded that human Alpha class GSTs genes are clustered on a single chromosome. These results rule out the possibility of reverse transcribed pseudogenes on other chromosomes, although several pseudogenes have been found upstream and downstream of the *GSTA1* gene (Suzuki *et al.*, 1993).

Prior to the current understanding of the Mu class GST gene cluster, the mapping of the Mu class genes was complicated by their heterogeneity and the existence of a *GSTM1* null allele. The mapping of Mu class GSTs by somatic cell hybridisation approach have yielded mixed results (chromosome 3, by Islam *et al.*, 1989 and chromosome 1p31, 6 and 13 by DeJong *et al.*, 1991). *In situ* hybridisation of a GSTM4 cDNA clone to prophasic human chromosomes mapped the Mu class genes to 1p13 in individuals with or without the *GSTM1* gene (Ross *et al.*, 1993). In a separate *in situ* hybridisation study, Pearson *et al.* (Pearson *et al.*, 1993) demonstrated that the Mu class genes cluster around the 1p13.3 region by using a yeast artificial chromosome clone which contained the *GSTM1-GSTM5* genes as a probe. Taken together, these results indicate that the Mu class GSTs are not dispersed throughout the human genome but exist as a gene cluster on chromosome 1 at 1p13.3.

Previous studies using rodent-human somatic hybrid cell lines and electrophoretic isoenzyme fractionation have assigned the *GSTP1* gene to chromosome 11 (Laisney *et al.*, 1984; Islam *et al.*, 1989; Suzuki and Board, 1984). Subsequently, the precise gene location of *GSTP1* was refined to band 11q13 by *in situ* hybridisation (Board *et al.*, 1989; Moscow *et al.*, 1988). A strong secondary peaks of grains was also observed over chromosome band 12q13-14 (Board *et al.*, 1989). This hybridising sequence was later confirmed to be a partial reverse-transcribed pseudogene of *GSTP1* (Board *et al.*, 1992).

The gene loci of the human Theta class GSTs, *GSTT1* and *GSTT2*, have been mapped to the long arm of chromosome 22 at subband q11.2 (see **Chapter 4** and Webb *et al.*, 1996). The chromosomal localisation of *GSTT2* by somatic cell hybridisation analysis and *in situ* hybridisation will be discussed in greater detail in **Chapter 4** of this thesis.

Lastly, the gene which encodes the human microsomal GST (DeJong *et al.*, 1988b) has been assigned to chromosome 12 using somatic cell hybridisation analysis (DeJong *et al.*, 1990). Its gene locus has been designated as *GST12*.

In summary the human GSTs exist as gene clusters which reside on five different chromosomes: 6p12 (*GSTA*); 1p13 (*GSTM*), 11q13 (*GSTP*), 22q11.2 (*GSTT*) and 12 (*GST12*, microsomal GST). Although pseudogenes have been identified in the Alpha and Pi classes, no pseudogenes have been unambiguously identified in the Mu and Theta classes.

1.7 TISSUE DISTRIBUTION OF HUMAN GSTs

The expression of human GSTs are subject to variation between tissues (Strange *et al.*, 1984; Suzuki *et al.*, 1987), cell types (Toffoli *et al.*, 1992; Green *et al.*, 1993; Anttila *et al.*, 1993) and stages of development (Strange *et al.*, 1985). Studies on the tissue distribution of human GSTs were carried out using specific staining of electrophoretic gels (Board, 1981b; Strange *et al.*, 1984; Suzuki *et al.*, 1987; Laisney *et al.*, 1984), Northern blotting with the respective cDNA clones (Takahashi *et al.*, 1993; DeJong *et al.*, 1988a), Western blotting with polyclonal antiserum (Campbell *et al.*, 1990; Takahashi *et al.*, 1993), monoclonal (Kantor *et al.*, 1991) or polyclonal immunohistochemical staining (Anttila *et al.*, 1993) and also direct purification and characterisation of GST proteins from natural tissues (Tsuchida *et al.*, 1990; Hussey and Hayes, 1993. For comprehensive review, see Table 8 of Hayes and Pulford, 1995). Due to the close relationship between members of the same class, cross hybridisation of cDNA probes or antibodies can occur within a given class and the interpretation of results are to be treated with great caution.

The differential expression of multiple GST isoforms in tissues may alter the levels of protection against some carcinogens. For example, Hiratsuka *et al.* (1995) has attributed the differential carcinogenicity of 5-HCR in skin and liver of mouse to the different levels of expression of GST mYrs-Yrs in those tissues. On the other hand, the high level of expression of Alpha class GSTs in liver and kidney, provides a useful biomarker to evaluate liver and kidney damage by assaying Alpha class GST released into the plasma (Beckett and Hayes, 1993). The tissue distribution of human GSTs has been studied extensively by various groups; an excellent review on this subject has been compiled by Mannervik and Widersten recently (Mannervik and Widersten, 1995).

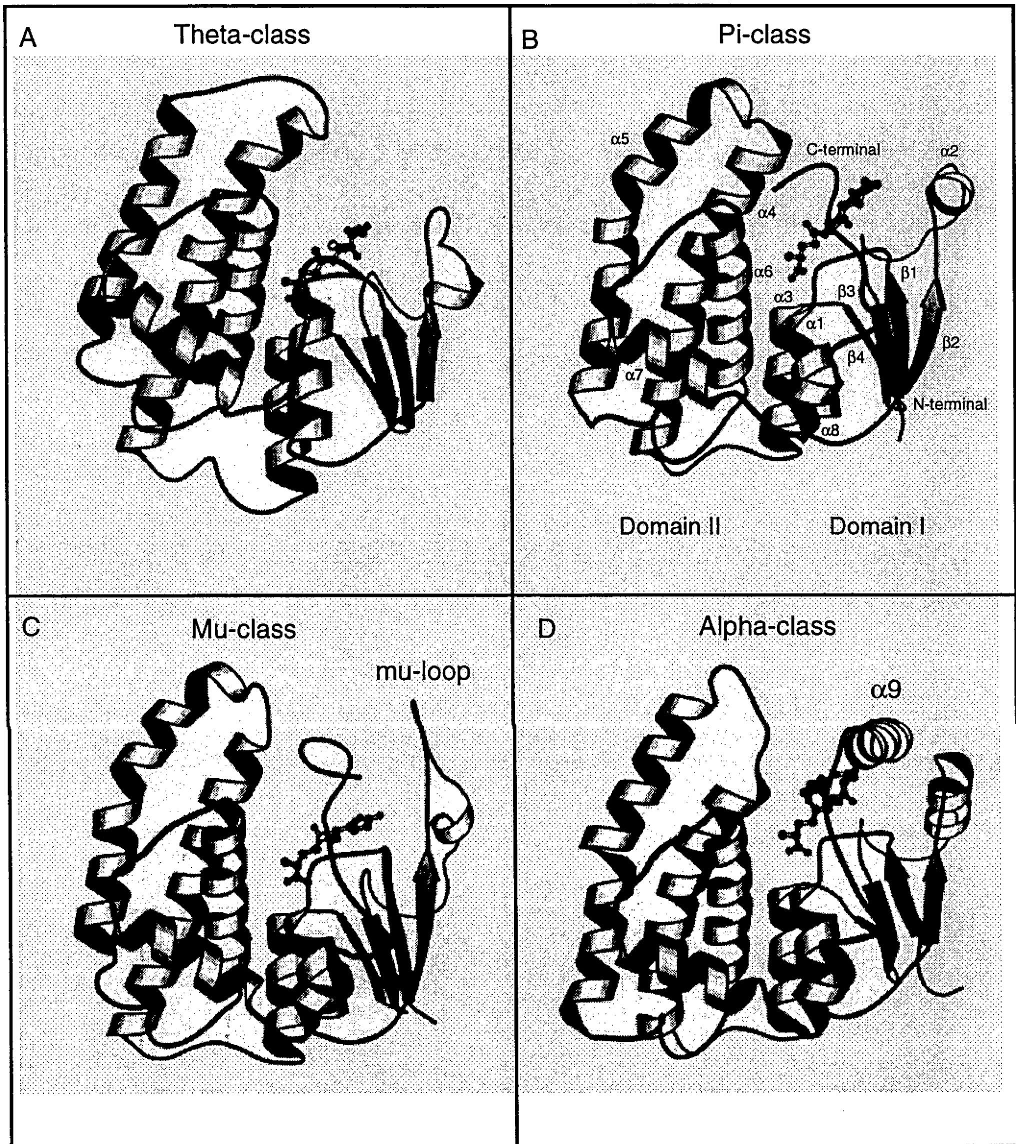
1.8 PROTEIN ARCHITECTURE

The structural resolution of GSTs at the atomic level was made possible when high quality protein crystals were analysed by X-ray diffraction. The crystal structures of GSTs either in complex with substrate, substrate analogues, products or in the apo-form, revealed the general protein architecture and the interaction between enzyme and substrate-products. In concert with site-specific mutagenesis, modular mutagenesis and construction of chimeric enzymes, Structural studies are a powerful tool in understanding the the catalytic properties of these enzymes.

The first three-dimensional GST structure solved was that of a porcine Pi class GST in complex with the substrate analogue, glutathione sulphonate (Reinemer *et al.*, 1991). Subsequently, the three dimensional structure of rat Mu class GST3-3 in complex with the physiological substrate, GSH, was also resolved (Ji *et al.*, 1992). The homogenous preparation of GSTs from human tissue or heterologous expression systems enabled the crystallisation and solution of the structures of the human Alpha (Sinning *et al.*, 1993), Mu (Raghunathan *et al.*, 1994) and Pi (Reinemer *et al.*, 1992) class GSTs. The first three dimensional structure of an invertebrate GST comes from the crystal structure of a Theta-like representative from the Australian sheep blowfly, *Lucilia cuprina* (Wilce *et al.*, 1994; Wilce *et al.*, 1995). The three dimensional structure of *L. cuprina* GST shall provide some structural information about the Theta class family until the crystal structure of mammalian Theta isoenzyme is available. The three dimensional structure of a Sigma class GST from squid has also been solved recently (Ji *et al.*, 1995).

From the structures solved so far it is evident that the mammalian cytosolic GSTs are composed of two subunits which interact with one another to form a dimer. Each subunit functions independently in catalysis and the topology of the subunits can be divided into two domains. The protein architectures of the cytosolic GSTs are shown in Figure 1.4.

Figure 1.4 : Protein Architectures of Cytosoloc GSTs



Modified from Wilce et al. (1995)

1.8.1 Domain I

Domain I comprises the first third of the polypeptide (except for the α9 helix in the Alpha class GSTs). It contains four strands of mixed β-sheets (β1 to β4) flanked on one side by two α-helices (α1 and α3) and another α-helix (α2) on the solvent-facing side. The topology of the secondary structure in Domain I is in a βαβαββα arrangement. Domain I, which is often referred to as the G-site, contributes to a larger extent to the binding of GSH. Sinning *et al.* (1993) noted that its secondary structure mimics other GSH binding proteins such as *E. coli* thioredoxin (Holmgren *et al.*, 1975), T4 glutaredoxin (Soderberg *et al.*, 1978) and glutathione peroxidase (Epp *et al.*, 1983). A distinctive characteristic of the Mu class GSTs in this domain, is the presence of an extended and mobile loop between β2 and α2, termed the Mu loop (Figure 1.4). The Mu loop occurs as a result of a short peptide insertion between β2 and α2. A structural characteristic which is unique to the Alpha class GSTs is also found in this domain. The extra amino acids at the C-terminus of the Alpha class GSTs fold into an additional alpha helix (α9) and form a lid over the active site when a hydrophobic substrate occupies the active site (Sinning *et al.*, 1993).

1.8.2 Domain II

Domain II comprises the C-terminal two thirds of the polypeptide and is joined to Domain I by about 6 amino acids. The topology of the secondary structure in Domain II consists of five right-handed α-helices (α4 to α8). The interface between these two domains forms a V-shape cleft (or a more parallel interface for the *L. cuprina* GST) which make up the wall of the active site subunit (Wilce *et al.*, 1995).

Domain II of the Alpha, Mu and Pi class GSTs shares a very similar folding topology. However, detailed comparison of root mean square (r.m.s.) deviations between protein units, r.m.s. deviations between the ligands and rotations between structural units has shown significant differences in the Alpha, Mu and Pi enzymes (Sinning *et al.*, 1993). A dominant feature of Domain II is the two long helical segments α4 and α5 which form the wall of the central channel. The crescent-shape of the α5 helix

is due to the presence of *cis*-proline residue(s) in the helix. In the Alpha class, the α5 helix is slightly longer than that in the Pi and Mu class due to an insertion near the middle of this helix. However, in the case of the *L. cuprina* GST, the α5 helix is shorter and is not bent. The *L. cuprina* model may only reflect the general structure of the Theta class GSTs as there is only 25% of overall amino acid identity between GSTT2 and the Theta-like GST from *L. cuprina* .

Based on amino acid sequence alignment, Wilce *et al.* (1995) suggested that the insertion of 8 residues in the α5 helix in the mammalian Theta class GSTs would result in a longer α5 helix. While C-terminal of the *L. cuprina* GST is disordered in the crystal structure, secondary structure prediction of the extended C-terminus of the mammalian Theta class GSTT2 suggests that it might adopt an amphipathic helical structure similar to the α9-helix of the Alpha class (Wilce *et al.*, 1995; Chelvanayagam *et al.*, 1996).

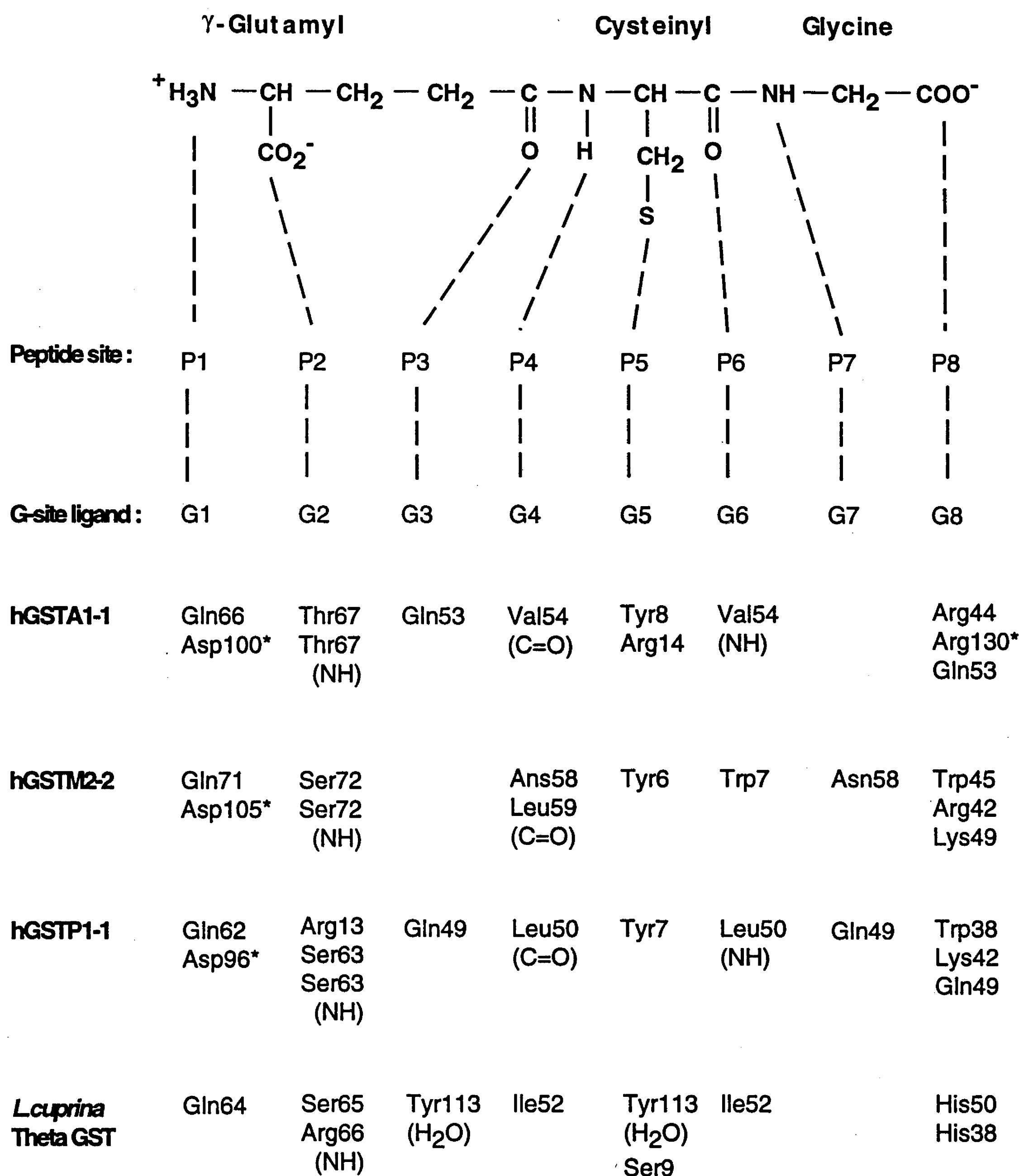
1.8.3 Subunit Dimerisation

Subunit interactions stabilise the dimeric structure of the enzyme and the tertiary structures of individual subunits. The mammalian enzymes are only fully functional as dimers. Although the active sites of each subunit are kinetically independent in the dimer (Mannervik and Jensson, 1982; Tahir and Mannervik, 1986), there are contributions to the binding of GSH from residues in Domain II of the other subunit (see **Section 1.8.4**). The subunits come into contact with each other through interaction between Domain I of one subunit (peptide loop connecting α2 and β3; one side each of α3 and β4) and Domain II of the other subunit (one face of α4 and one side of α5) via electrostatic and hydrophobic interactions. As the result of dimerisation, around 14% of the total hydrophobic surface is buried at the subunit interface. The dimeric protein is globular in shape with approximate dimensions of 6.2nm x 5.1nm x 4.6nm (Dirr *et al.*, 1994) and the distance between the active sites of the subunits is approximately 1.4nm in the human GSTP1-1, based on the closest distance between the GSH molecules (Wilce and Parker, 1994).

1.8.4 G-Site

The G-site refers to the GSH binding domain of GST. Secondary structures forming the G-site consist of the α1 and α3 helices (which assist in attracting and orientating the GSH through helix dipoles); the β1-α1 peptide loop (forming part of the architecture of the G-site as well as the H-site, consists of the non-polar residues surrounding the conserved tyrosine residue at the N-terminal domain) and the sharp bend in the peptide loop connecting α2 and β3 (which contributes to the proper folding and packing of G-site substructure). For review see Dirr *et al.*, (1994) and Wilce and Parker (1994).

The tripeptide GSH binds in an extended conformation through electrostatic interactions with various residues of the enzyme (Figure 1.5). Domain I of the protein contributes most of these interactions with the exception of one (Mu and Pi class) or two (Alpha class) interactions from Domain II of the other subunit in the dimer. In the Mu and Pi classes, the α-amino group of the glutamyl residue of GSH interacts with the carboxylate of an aspartate from the adjacent subunit (Mu: Asp105; Pi: Asp96). Mutating this aspartate to alanine or asparagine has been shown to reduce the affinity of the enzyme for GSH significantly (Wang *et al.*, 1992; Kong *et al.*, 1993). In the Alpha class, besides Asp100 which interacts with the γ-Glu moiety of GSH, the glycyl's α-carboxyl group of GSH also interacts with Arg130 from the adjacent subunit. Therefore, the contribution of the neighbouring subunit on GSH binding is important in conferring the full functional properties of the dimeric enzyme.



	G1	G2	G3	G4	G5	G6	G7	G8
hGSTA1-1	Gln66 Asp100*	Thr67 Thr67 (NH)	Gln53	Val54 (C=O)	Tyr8 Arg14	Val54 (NH)		Arg44 Arg130* Gln53
hGSTM2-2	Gln71 Asp105*	Ser72 Ser72 (NH)		Ans58 Leu59 (C=O)	Tyr6	Trp7	Asn58	Trp45 Arg42 Lys49
hGSTP1-1	Gln62 Asp96*	Arg13 Ser63 Ser63 (NH)	Gln49	Leu50 (C=O)	Tyr7	Leu50 (NH)	Gln49	Trp38 Lys42 Gln49
L.cuprina Theta GST	Gln64	Ser65 Arg66 (NH)	Tyr113 (H_2O)	Ile52	Tyr113 (H_2O) Ser9	Ile52		His50 His38

Figure 1.5 : Schematic Diagram of Electrostatic Interaction Between Glutathione and Amino Acid Residues in the G-site of Human Glutathione Transferases. Peptide sites of GSH are represented by P1, P2...P8 etc.. and the corresponding G-site ligands interacting with the peptide sites are represented by G1, G2...G8 etc.. Carbonyl and amide groups in parenthesis are ligands from the main-chain polypeptide. H_2O in parenthesis are water molecules which mediate hydrogen bond with the corresponding residues. Residues marked with an asterisk are G-site ligands from the other subunit in the dimer. The numbering of amino acid does not include the initiator methionine (Source taken from Dirr *et al.*, 1994 and Wilce *et al.*, 1995).

1.8.5 H-Site

The H-site which is known as the hydrophobic binding site or xenobiotic binding site, situated adjacent to the G-site of the same subunit. Structural elements which contribute to the architecture of the H-site come from both Domain I and Domain II of the same subunit. They consist of the peptide loop connecting β1 to α1; the C-terminal half of α4 and the C-terminal tail of the polypeptide. In the Mu and Alpha class GSTs additional secondary structures such as the Mu loop (Thr33 to Arg42) (Ji *et al.*, 1992; Raghunathan *et al.*, 1994), as well as the α9 helix of the Alpha class (Sinning *et al.*, 1993), also form part of the structural components of the H-site. The C-terminal sequence of GSTs which are generally more diverse, account for the variability of the H-site topology and could explain the broad substrate specificity of GSTs. Moreover, the binding of the second substrate involves hydrophobic interactions and therefore is less constrained to any particular residues in the H-site. The understanding of the H-site topology mostly came from the three dimensional structure of substrate-product analogues in the crystal structures of the GSTs (Reinemer *et al.*, 1992; Ji *et al.*, 1992; Sinning *et al.*, 1993; Raghunathan *et al.*, 1994). Site directed mutagenesis and deletional mutagenesis further confirmed the involvement of essential residues or region of the H-site in catalysis (Board and Mannervik, 1991; Johnson *et al.*, 1993).

1.9 CATALYTIC MECHANISM

The role of GST in GSH dependent catalysis depends on the activation and the stabilisation of the thiol group of enzyme-bound GSH. Site directed mutagenesis, chemical modification, fluorescence emission, fluorescence excitation, UV difference spectral studies and also three dimensional structure studies of GSTs indicate that a conserved tyrosine residue at the N-terminal domain is involved in activating the thiol group of GSH by lowering its pKa from about 9.0 in free aqueous solution (Jung *et al.*, 1972) to 5.7-6.9 for class Mu (Graminski *et al.*, 1989b; Liu *et al.*, 1992; Graminski *et al.*, 1989a), 6.7-7.0 for class Alpha (Wang *et al.*, 1992; Huskey *et al.*, 1991) and 6.3 for class Pi (Kong *et al.*, 1992) in the enzyme-bound GSH. The lowering of pKa by about 2 units corresponds to a stabilisation energy of at least 2.7kcal/mol (Armstrong, 1994). In

the Alpha class, besides the conserved Tyr8, additional electrostatic interaction between the sulfhydryl group of enzyme-bound GSH with Arg14 also plays an important role in the activation of GSH (Bjornestedt *et al.*, 1995).

The activation mechanism of the Theta class GSTs seems to involve residues other than the conserved N-terminal tyrosine. Although there is an N-terminal tyrosine residue in most of the Theta class GSTs, a similar residue is absent in the human GSTT2 (Hussey and Hayes, 1992, see **Chapter 3**). In fact, a recent three dimensional structure of a Theta-like GST from *L. cuprina* suggests that the topologically equivalent tyrosine (Tyr5) is 13.9Å away from the sulfhydryl group of GSH and is unlikely to participate in hydrogen bonding with GSH (Wilce *et al.*, 1995). On the other hand, amino acid sequence comparison of the Theta class GSTs showed a conserved serine residue in the N-terminal domain which could possibly act as a general base to abstract the sulfhydryl proton from GSH (Wilce *et al.*, 1995). Site directed mutagenesis of Ser9 in *L. cuprina* GST resulted in mutants with severely impaired activity with CDNB (Board *et al.*, 1995). The involvement of the conserved serine residue in the catalysis of the Theta class GSTs is a subject of interest in this thesis and shall be discussed in greater detail in **Chapter 7,** using GSTT2-2 as an example.

Other contributing factors which have been taken into consideration in the analysis of GST catalytic activity include solvation energy, effect of positive electrostatic field generated by the helix dipole and second-sphere electrostatic interactions between amino acids (Karshikoff *et al.*, 1993; Dirr *et al.*, 1994; Armstrong, 1994).

1.10 EVOLUTIONARY PERSPECTIVE OF GSTs

In the broad spectrum of living organisms, GSH exists only in aerobes. Likewise, other enzymes which utilise GSH as their substrate or co-enzyme are also found only in aerobic organisms. This suggests that GSH and GSTs play an important role in the survival of living cells in an aerobic environment. This is supported by the fact that GSTs of the Alpha, Pi and Theta class GSTs are involved in conjugating GSH to the

products of lipid peroxidation as well as the reduction of fatty acid hydroperoxides (Alin *et al.*, 1985; Berhane *et al.*, 1994; Hiratsuka *et al.*, 1994). Consequently, GSH and GSTs are believed to have evolved in prokaryotes as a protection mechanism against oxygen toxicity (Pemble and Taylor, 1992). Recently, it has been proposed that the Theta class GSTs represent the ancestral gene of the GST supergene family (Pemble and Taylor, 1992). This proposal suggests that the Alpha, Mu and Pi class GSTs are the results of gene duplications from a GST progenitor gene which in the course of evolution, retained its function while allowing the duplicated gene to develop novel functions. Two views are presented below in relation to the evolution of the GST supergene family of GSTs.

1.10.1 Inference From Amino Acid Sequence

Amino acid sequence comparisons between homologous proteins provide a useful means to investigate their phylogenetic relationships. Comparison of available amino acid sequence from existing databases has shown that the Pi and Alpha classes share greater sequence identity than the Pi and Mu or Alpha and Mu classes (Pemble and Taylor, 1992; Blocki *et al.*, 1993). On the other hand, the Theta class GSTs have little amino acid identity with the Alpha, Mu and Pi class but share relatively high sequence identities with the *Methylobacterium*, *D. melanogaster* and *L. cuprina* GSTs, several plant GSTs as well as GSTs purified from mitochondria matrix (Harris *et al.*, 1991b) and nucleus (Tan *et al.*, 1988) of rat liver. However, the 3' non-coding sequence of the rat subunit 5 cDNA shares a high level of sequence identity with the 3' non-coding sequence of three human Mu class cDNA. This suggests that the Mu class is more closely related to the Theta class and may have diverged from an ancestral GST gene after the Alpha/Pi lineage (Pemble and Taylor, 1992). While the Alpha, Mu and Pi class GSTs are restricted to only fungi and mammals, Theta-like GSTs are found distributed in insects, plants, yeast, archaebacteria, cyanobacteria, purple bacteria and mitochondria. In this regard, the Theta class appears more like the ancestral GST and the Alpha, Mu and Pi class could have arisen from the Theta class GSTs through gene duplication events (Pemble and Taylor, 1992)

1.10.2 Inference From Exon/Intron Boundaries

The evolutionary perspective of proteins deduced from comparing the exon/intron boundaries of homologous proteins is based on the theory that proteins were built from structural (Gilbert, 1978) or functional (Blake, 1978) blocks, corresponding to the exons of proteins. Therefore, novel proteins arise from the event of exon shuffling through homologous recombination and the structural/functional modules were brought together by alternative splicing of heterogenous nuclear RNA. The question whether exons code for structural or functional units in proteins has been discussed by Traut (1988). It appears that certain proteins follow this rule while others show no such correspondence [for review see Traut (1988) and references therein] and inference of protein structural or functional units from exons are not conclusive at this stage.

The relationship between gene and protein structures of the mammalian GSTs has been reviewed recently (Armstrong, 1994). It is obvious from the gene structures, that the Mu and Pi classes are more closely related than the Mu and Alpha or Pi and Alpha. The exon/intron boundaries of the Mu and Pi classes differ only in two positions. Firstly, it appears that the loss of intron 2 and part of exon 3 (which encodes the μ loop of the Mu class) have resulted in a larger exon 3 in the Pi class. Secondly, intron 7 of the Mu class is missing in the Pi class and a larger exon 7 is found in the Pi class (equivalent to exon 7 and 8 of Mu class) (see Figure 1.6). Therefore, the Pi class appears to have evolved from the Mu class as a result of intron loss. In the Alpha class, the gene structure is rather different from that of the Mu and Pi classes. Except for the exon/intron boundaries between exon 3 and 4, the other boundaries do not coincide with those found in the Mu and Pi class. More distinctly, the α4, α5 and α7 helices of the Alpha class are interrupted by introns 3, 4 and 5, perhaps through junction sliding or intron loss/addition events.

Based on the mouse Theta class gene structure (*mGSTT2)* (Whittington *et al.*, 1996) and a homology model of the human GSTT2 structure (Chelvanayagam *et al.*, 1996) constructed based on the structure of the *L. cuprina* GST (Wilce *et al.*, 1995), the

relationship between protein secondary structure and gene organisation of a Theta class GST is presented in Figure 1.6. Compared to other GST classes, the *mGSTT2* gene has fewer exons and introns though it encodes the longest polypeptide among the GST classes. The α4 helix is interrupted by intron 3 at the last helical turn and the α6 helix is interrupted by intron 4 (Chelvanayagam, personal communication). The gene organisation encoding secondary structural elements of the Theta class GST are very different from the Alpha, Mu and Pi classes. None of the exon/intron boundaries of *mGSTT2* gene coincide with those of the Alpha, Mu and Pi genes. The only similarity is the boundary between Domain I and Domain II of the Theta, Mu and Pi isoenzymes, which correspond to the end of exon 4 (Chelvanayagam, personal communication). It is not obvious from the inference of exon/intron boundaries that the Theta class is the progenitor gene of the Alpha, Mu and Pi class genes.

The evolution of GSTs supergene family had commonly been studied by comparing their amino acid sequences (Tan et al., 1988; Harris et al., 1991; Pemble and Taylor, 1992; Blocki et al., 1993). Recently, the evolution of GSTs was also evaluated from the position of their exon/intron boundaries and protein structures (Armstrong, 1994). This hypothesis is based on the assumption that proteins were built from structural (Gilbert, 1978) or functional (Blake, 1978) blocks, corresponding to the exons of proteins. It appears, while some proteins abide to this rule, others show no such correspondence. At this stage, the evolution of proteins inferred from the exon/intron are not conclusive (Traut, 1988 and references therein).

Based on amino acid sequence identity, the Theta class GST have been proposed as the progenitor of other classes (see Pemble and Taylor, 1992 for its argument). Comparison of the GSTT2 sequence, which is the cDNA isolated in the course of this study, found many similarities in amino acid sequence with other Theta class GSTs (see Table 1.5, page 28) Thus by inference the Theta class enzyme studied here can be related to the GST super family. However, when the homology model of GSTT2 and the mouse gene structure were compared with the Alpha, Mu and Pi GSTs, the exon/intron boundaries of mGSTT2 showed little correspondence with the other 3 classes. Therefore the intron/exon boundaries do not reflect the same evolutionary relationships as are apparent from sequence alignments Furthermore it is now apparent that the members of the Alpha, Mu, Pi and Theta classes all have a similar structure despite significant differences in their intron/exon organization. Thus it seems that sequence alignment methods may provide a more reliable strategy for deriving an evolutionary history of the GSTs

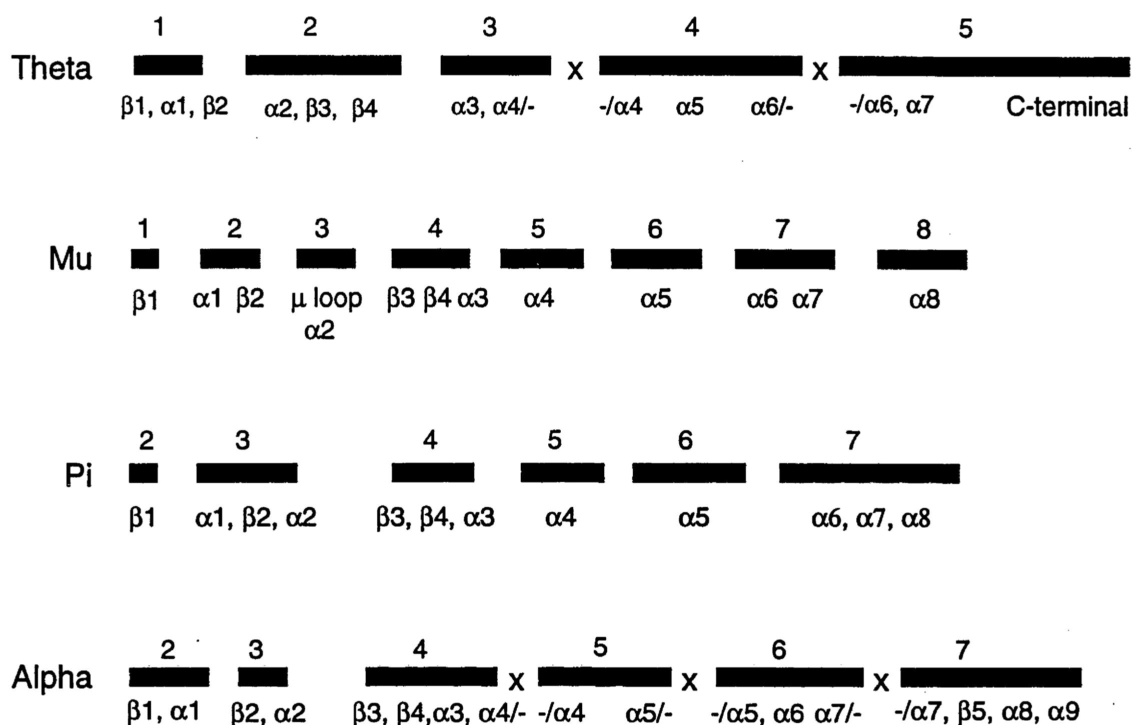


Figure 1.6 : Schematic Diagram of Exons and the Corresponding Secondary Structural Elements of GSTs. Exons are roughly drawn to scale. The exon/intron boundaries are deduced from gene structure of (Whittington *et al.*, 1996; Telakowski-Hopkins *et al.*, 1986; Okuda *et al.* 1987; Lai *et al.*, 1988). Secondary structural elements are derived from three dimensional structure of (Reinemer *et al.*, 1991; Ji *et al.*, 1992; Sinning *et al.*, 1993), except for the Theta class GST which was derived from a homology model of GSTT2 (Chelvanayagam, *et al.*, 1996) based on the structure of a *L. cuprina* GST (Wilce *et al.*, 1995) and the gene structure of mGSTT2-2 (Whittington, *et al.*, 1996). Intron interuption of secondary structural elements are marked by X.

1.11 OBJECTIVES AND SCOPE OF THESIS

The multifunctional role of GSTs is a subject of great interest in view of their involvement in the Phase II detoxication pathway and the existence of polymorphism in the supergene family. As a group of detoxication enzymes, understanding of their substrate specificities will help to identify the categories of compounds that are substrates of GSTs. Considerable effort has been made in the studies of the Alpha, Mu and Pi class GSTs at the protein and genetic levels. However, knowledge of mammalian Theta class GSTs is still in its infancy in comparison to the other GST classes, especially for the Theta class GSTs in humans. The objective of the research reported in this thesis is to attain a greater understanding of the human Theta class GSTs through the approaches of molecular genetics and enzymology .

Chapter 3 describes the molecular cloning of a cDNA encoding a human Theta class GST, GSTT2. It also describes the cloning of several truncated cDNA transcripts which correspond to the exon/intron boundaries of its orthologous gene in mouse (Whittington *et al.*, 1996). The possibility that *GSTT2* is regulated at the post-trascriptional level by alternative splicing is discussed.

To gain a greater understanding on the chromosomal location of *GSTT2* in relation to the other GST classes, mapping of *GSTT2* was carried out using rodent-human somatic cell hybrid analysis as well as *in situ* hybridisation techniques. **Chapter 4** is devoted to the description and discussion of these studies in detail.

Chapter 5 describes the heterologous expression and purification of GSTT2-2 as a ubiquitin-fused protein and the co-translational removal of the ubiquitin moiety by a cloned ubiquitin-specific protease, Ubp1. The enzymatically active recombinant GSTT2-2 was used for protein characterisation. Polyclonal antibodies were raised against purified GSTT2-2 and the tissue expression of GSTT2-2, determined using the antiserum as well as Northern blot analysis, is detailed in **Chapter 6**.

In **Chapter 7**, the catalytic function of GSTT2-2 as studied by site-directed mutagenesis is discussed. A conserved serine residue (Ser11) at the N-terminal domain is mutated to alanine, threonine, or tyrosine and the activity of the wild type and mutant proteins are compared using cumene hydroperoxide, ethacrynic acid and 1-menapthyl sulphate as substrates. The role of Ser11 in catalysis is discussed and a novel functional role of GSTT2-2 as a glutathione-dependent sulphatase is proposed.

CHAPTER 2 : ROUTINE MATERIALS AND METHODS

The materials and methods described in this chapter are those commonly used throughout this research. Specific research materials, chemicals and procedures will be described in the respective chapters.

2.1 MATERIALS

All routine chemical compounds used in the preparation of culture media, buffers and reagents were of analytical grade. They were purchased from Difco laboratories (USA), Sigma Chemical Co. (USA), Ajax Chemical Co. (Australia), Aldrich Chemical Co. (USA), BDH Chemicals Ltd. (England), Merck (Germany), Progen (Australia). Other source of materials were : radioisotopes [α-^{32}P]dATP and [α-^{33}P]dATP, from Bresatec (Australia); X-ray film Rx or NCII, from Fuji Photo Film Co. (Japan); black and white polaroid film (T667), from Polaroid Corp. (USA); nitrocellulose and Hybond-N$^+$ nylon membrane, from Bio-Rad Laboratories (USA) and Amersham (UK) respectively; Sequenase™ Version 2.0 DNA sequencing kit, from USB (USA); Rainbow™ coloured protein molecular weight markers, from Amersham (UK); Lambda DNA-*Hin*dIII digest DNA size markers, from Pharmacia (Australia) and dNTPs, from Boehringer-Mannheim (Australia).

Oligonucleotides were synthesised on an Applied Biosystems 380B DNA synthesiser, Applied Biosystems Inc. (USA) by the Australian National University Biomolecular Resource Facility. Sources for cloning vectors, restriction endonucleases, cloning enzymes and their buffers are summarised in Table 2.1.

2.2 METHODS

2.2.1 Preparation of Competent Cells

Competent *Escherichia coli* TG1 cells were prepared by the calcium/maganese/potasium chloride method as described by Sambrook *et al.* (Sambrook *et al.*, 1989). They were dispensed into 100µl aliquots and frozen at -70°C if not used immediately.

2.2.2 Transformation and Selection

Transformation of competent cells by plasmids or RF M13 phage were achieved using the heat shock method. Competent cells were mixed with the ligated DNA and incubated on ice for 30 minutes. The cells were heat shocked for 50 seconds at 42°C and returned to ice. For *E.coli* transformed with plasmids containing the β-lactamase gene, 500µl of Luria Broth (1% tryptone, 0.5% yeast extract, 1% NaCl) was added to the heat shocked cells and incubated at 37°C for 30-60 minutes before plating on Luria Broth agar media (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, pH 7.4) supplemented with ampicillin (100µg/ml). In the case of blue/white colony selection utilising the β-galactosidase gene, IPTG (10µl of 100mM stock) and X-Gal (50µl of 2% stock) were spread on the agar surface. For M13 phage transformation, *E. coli* transformed with M13 phage were added to a test tube containing 3ml of Top Agar (1% tryptone, 0.5% yeast extract, 0.8% NaCl, 1% agar, pH7.4); IPTG (10µl of 100mM stock); X-Gal (35µl of 2% stock) and 200µl of exponentially growing TG1 cells. The components were mixed and poured on a prewarmed minimal agar plate. [Minimal plates were prepared using 50ml of 10xM9 salts (60g Na_2HPO_4, 30g KH_2PO_4, 10g,NH_4Cl, 5g NaCl in 1L of ddH_20), 0.5ml of 1M $MgSO_4$, 0.5ml 0.1M $CaCl_2$, 5.0ml 20% glucose, 0.5ml 1M thiamine, 7.5g Bacto agar in 450ml of ddH_2O and autoclave]. Plates were incubated at 37°C overnight and transformed host cells containing non-recombinant or recombinant plasmids or M13 phage can be distinguished by a blue/white colour selection. Transformed host cells containing recombinant plasmid or M13 were identified by white colonies or

opaque plaques as a result of disruption of the β-galactosidase gene by the insertion of cloned DNA.

2.2.3 DNA Sequencing

Single-stranded DNA was prepared and sequenced by the dideoxy termination method (Sanger *et al.*, 1977; Sanger *et al.*, 1980) and separated on a 6% polyacrylamide gel containing 7.4M urea in 1x TBE (0.09M Tris-borate, 0.002M EDTA, pH8.0) buffer.

2.2.4 Agarose Gel Electrophoresis

Routine DNA separations were performed by electrophoresis in 0.8% or 1.5% agarose gels in either TAE (0.04M Tris-acetate, 0.001M EDTA, pH8.0) or TBE buffers (0.09M Tris-borate, 0.002M EDTA, pH8.0). Gels were stained with ethidium bromide and viewed under fluorescent UV light at 254nm or 312nm wavelength. DNA fragments excised from gels were purified using Geneclean™ II reagents supplied by Bio101 (USA).

2.2.5 Capillary Transfer and Hybridisation of Nucleic Acid (Southern blot)

DNA transfer from agarose gels to nylon membranes utilised the capillary transfer method as described by Southern (Southern, 1975) and modified by Reed and Mann (1985). Following agarose gel electrophoresis and ethidium bromide staining, the gel was soaked in 0.25M HCl (results in partial depurination) with gentle agitation until the bromophenol blue tracking dye turned yellow (approximately 8 min) and then rinsed briefly in distilled water. After depurination, the gel was placed on a blotting paper, prewet with Transfer Solution (0.4M NaOH, 0.6M NaCl) and covered with the Hybond™ N^+ nylon membrane (Amersham, UK). The membrane was covered sequentially with 2 sheets of wet blotting paper, 6 sheets of dry blotting paper, a 3 inch stack of paper towels and finally with a 0.5kg weight on the top. Capillary transfer was carried out with Transfer Solution. Transfer of DNA was allowed to proceed for at least 2 hours in the case of plasmid DNAs, or overnight for human genomic DNA digests.

Following capillary transfer, the membrane was placed in Neutralising Solution (0.5M Tris-HCl, pH7, 1M NaCl) for 15 minutes, with occasional shaking. Membranes were blotted dry and DNA was cross-linked to the membrane under long-wave UV (365nm) for 5 minutes. Hybridisation probes were labelled with [$\alpha^{32}P$] dATP by random priming using a Megaprime DNA labelling kit (Amersham, UK) following the manufacturer's protocol. Hybridisation was carried out in Nasmyth's Solution (Nasmyth, 1982) at 65°C for 18 hours. Filters were washed sequentially with 2xSSC and 2xSSC, 0.1% SDS at room temperature (A 20x SSC stock is made of 3M sodium cloride, 0.3M sodium citrate). For more stringent washes, filters were washed in 2xSSC , 0.1% SDS at 65°C and/or the concentration of SSC was reduced to 1x or 0.2x. Filters were then exposed to X-ray film at -70°C.

2.2.6 Protein Analysis by SDS/PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) of protein (Laemmli, 1970) was performed with a 12% gel on a Mini-PROTEAN II Dual Slab Cell electrophoresis unit, Bio-Rad Laboratories (USA). Alternatively, the Pharmacia PhastSystem™ was used for routine screening. Gels were stained using Coomassie Brilliant Blue R250 or a Silver Staining Plus kit (Bio-Rad, CA. USA), following the manufacturer's protocol. Pierce Albumin standard was used as a protein standard and protein concentrations were determined using the method described by Bradford (Bradford, 1976).

2.2.7 Computer Software for DNA Analysis

DNA and protein sequence alignment and comparison was carried out using the GCG software (Version 8, 1994, Genetics Computer Group, Madison WI, USA) provided through the Australian National Genomic Information Service (ANGIS). Generation of DNA restriction map and cDNA sequence translations were performed by DNA Strider™ version 1.0, Commissariat al'Energie Atomique (France).

Table 2.1 : Sources for cloning vectors, restriction endonucleases, cloning enzymes and their buffers

Enzyme	**Source**
Restriction endonucleases	Boehringer-Mannheim (Australia) Pharmacia (Australia) Progen (Australia)
Alkaline phosphatase	Boehringer-Mannheim (Australia)
Polynucleotide kinase	Pharmacia (Australia)
Klenow fragment	Pharmacia (Australia)
T4 DNA ligase	Pharmacia (Australia)
Taq polymerase	Promega (Australia)
Ribonuclease A type 1- As (RNase)	Sigma (USA)
M13 vectors	Boehringer-Mannheim (Australia)
pUC vectors	Pharmacia (Australia)
pGEM™-T vector	Promega (Australia)

CHAPTER 3 : cDNA CLONING AND SEQUENCING OF A HUMAN THETA CLASS GSTT2

3.1 INTRODUCTION

Little is known about the molecular genetics of the Theta class GSTs in human. From an enzymological point of view, their existence was largely overlooked due to their low activity with the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) and their failure to bind to immobilised glutathione affinity matrices which are common characteristics of the Alpha, Mu and Pi class GSTs. The cDNAs encoding Theta class GSTs from rat and mouse have been cloned recently and further information on their gene structures has also been reported during the course of this present study (see Table 1.4). However, at the start of this investigation, the human Theta class GSTs were still largely unexplored.

3.2 OBJECTIVES

Evidence from protein characterisation suggests that the human Theta class GSTs are the products of at least 2 different gene loci (Meyer *et al.*, 1992; Hussey and Hayes 1992). During the course of this study, Pemble *et al.* (1994) reported the cDNA cloning of a human Theta class GST, termed GSTT1, which appeared to be the cDNA encoding GSTθ (Meyer *et al.*, 1991). The objective of this chapter is to gain a greater understanding of the second human Theta class isoenzyme GSTT2*-2* (Hussey and Hayes, 1992) through cDNA cloning.

3.3 MATERIALS

The source of chemical compounds and materials for routine use were provided in **Chapter 2.** The λgt10 and λgt11 human liver cDNA libraries were provided by Dr G. Howlett (University of Melbourne, Australia). The λgt 11 human liver 5' stretch cDNA library was purchased from Clonetech Laboratories (Palo Alto, CA, USA). PCR primers which were designed to amplify across putative exon/intron boundaries were :-

HTA3 (sense primer) : 5' TTAGACGTGCGCACCGTG 3'

HTB2 (antisense primer) : 5' ATCCTTGAGCGTCGGCAG 3'

HTIPF (sense primer) : 5' GGGGACAGGCCCTTCCTC 3'

HTIPR (antisense primer) : 5' CATCAGCTCCTCCAGGGC 3'

3.4 METHODS

3.4.1 Development of a Theta Class GST Probe

Total RNA was isolated from rat liver as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987), and poly(A)$^+$ RNA was prepared by oligo(dT) affinity chromatography using a mRNA Purification Kit (Pharmacia, Australia). Two primers :-
RGTTH1 (sense primer) : 5' GGAATTCACCATGGGTTTGGAGCTCTAC 3'.
RGTTH2 (antisense primer) : 5' ACAAGCTTCAGGGAATCCTGGCAATTCG 3', based on the cDNA sequence of rat subunit Yrs (Ogura *et al.*, 1991), were synthesised. RGTTH1 contains an additional *Eco*RI site while RGTTH2 has a *Hin*dIII site to facilitate cloning. These primers were used to amplify the coding region of the rat subunit Yrs from rat liver cDNA prepared by reverse transcription of rat liver poly(A)$^+$ RNA using a Riboclone® cDNA Synthesis Kit (Promega, Australia), procedure as described by the manufacturer's protocol.

A 50µl reaction mix containing 500ng of first-strand cDNA, 200µM dNTPs, 20pmol of each primer, 1.5u of *Taq* polymerase, and 1x*Taq* polymerase buffer were subjected to DNA amplification by the Polymerase Chain Reaction (PCR) in an oil-bath thermal cycler (Bartelt Instruments, Australia). PCR conditions were 5 cycles of initial amplification (95°C, 1min; 45°C, 1min; 72°C, 1min) followed by 30 cycles of (95°C, 1min; 64°C, 1min; 72°C, 1min). The resulting PCR product was 740bp in length and was cloned into the *Eco*RI/*Sma*I sites of pUC18 to give the plasmid pRTH1. The pRTH1 cDNA fragment was sequenced to confirm its identity as a copy of the rat subunit Yrs cDNA (Ogura *et al.*, 1991). The insert in pRTH1 was excised by *Eco*RI-*Bam*HI digestion and used as a probe to screen human cDNA libraries.

3.4.2 cDNA Library Screening by Filter Hybridisation

A λgt10 human liver cDNA library and a λgt11 human liver 5'-stretch cDNA library were screened for Theta class clones by filter-lift hybridisation. The libraries were plated out at a density of approximately 27,000 pfu per 150mm LBM plate (1% tryptone, 0.5% yeast extract, 1% NaCl, 10mM $MgSO_4$ and 1.5% Bacto agar) on the *Esherichia coli* host strain Y1090. The resulting plaques were screened using the filter hybridisation method of Benton and Davis (Benton and Davis, 1977). The cDNA insert in pRTH1 was labelled with [α^{32}P]dATP by the use of a Megaprime random primer labelling kit (Amersham, UK). Hybridisation was carried out at 65°C for 18h in hybridisation solution containing 10x Denhardt's solution (Sambrook *et al.*, 1989), 4x SSC, 0.1% SDS, 10% dextran sulphate and 500μl (in every 25ml of hybridisation solution) of boiled salmon sperm DNA (10mg/ml). Filters were washed sequentially in 2xSSC; 2xSSC, 0.1% SDS and a final wash of 2xSSC, 0.1% SDS at 65°C. Filters were exposed to X-ray film over night at -70°C. Plaque purification was achieved by replating positive clones and rescreening with the rat Theta class cDNA probe. The cDNA inserts in cross-hybridising clones were excised from the λ phage by *Eco*RI digestion and cloned into pUC18 and M13mp18. DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977; 1980).

3.4.3 cDNA Library Screening by PCR

Human liver cDNA libraries in λgt 10 and λgt11 were screened by a PCR based method. Total DNA from the amplified library lysate was subjected to PCR with the rat primer RGTTH2 (RGTTH2 sequence was sufficiently similar to the corresponding region of the human cDNA clone λHT1 to use it as a PCR primer) and a primer based on the sequence from the left arm of either λgt10 or λgt11:-

λgt11 (5' GTGTTAACGGTGGCGACGACTCCTGGAGCCCG 3') or

λgt10 (5' CCCGGGATCCAGCAAGTTCAGCCTGGTTAAG 3').

The amplified products were cloned and sequenced as described in **Chapter 2.**

3.5 RESULTS

3.5.1 Characterisation of Four Human Theta Class GST cDNAs

Four distinct cDNA clones were identified by either direct hybridisation to cDNA libraries or by a PCR approach. λHT1 was originally isolated from a λgt10 human liver cDNA library by hybridisation with a rat subunit Yrs cDNA sequence. This clone contains an open reading frame of 621bp encoding a peptide which shares homology with rat subunit Yrs after the first 37 residues of the rat sequence. The 5' sequence upstream of the start of homology with rat subunit Yrs does not contain any recognisable sequence and contains an in frame termination codon (TGA*) that would cause a premature termination of translation. The 3' non-coding region of λHT1 extends for 222bp and lacks a poly-A tail. The basic structure of λHT1 is shown schematically in Figure 3.1.

Because λHT1 was clearly missing the 5' end of the cDNA that encodes the N-terminal amino acid sequence, an attempt was made to use an oligonucleotide primer RGTTH2, and primers located in the λ vector arms, to amplify cDNAs that encode the full length 5' sequence. pHT2 was generated by this PCR approach from a λgt11 human liver cDNA library lysate and contains a considerable region of coding sequence that is identical to λHT1. However, within the 3' end of the coding region there is an apparent deletion of 42bp that does not alter the reading frame but results in a deletion of 14 residues. The 5' non-coding sequence of pHT2 extends for approximately 800bp before it enters an open reading frame that corresponds to the coding sequence of λHT1 (Figure 3.1). The open reading frame of pHT2 starts at residue 67 of the rat subunit Yrs. Therefore, in terms of its coding region, pHT2 is also a truncated clone which is even shorter than λHT1. The sequence of pHT2 ends after a TGA termination codon which was incorporated in the PCR primer.

Another clone pHT3, was generated from a λgt10 human liver cDNA library using the same PCR approach. pHT3 is very similar in structure to pHT2 (Figure 3.1).

Although the 5' non-coding sequence of pHT3 is shorter (165bp), it is identical to the 5' non-coding sequence of pHT2. Similarly, the open reading frame corresponding to the rat subunit Yrs sequence starts at residue 67. Unlike pHT2, pHT3 does not contain a 42bp deletion. Like pHT2, pHT3 ends after a termination codon (TGA) which was incorporated in the reverse PCR primer.

A human Theta class GST cDNA clone encoding the complete amino acid sequence was isolated from a λgt11 human liver 5'-stretch cDNA library by hybridisation with the pRTH1 cDNA. This clone λHT4 is 3.5kb in length and is a fusion of at least 3 different cDNA species. Sequencing from each end of λHT4 revealed that the Theta class GST cDNA was flanked by identifiable and unrelated cDNA clones (*H. Sapiens* TL25 mRNA from LNCaP cell line and human group-specific component Vitamin D-binding protein), presumably as a result of a cloning artefact. The complete sequence of the human Theta class GST cDNA within λHT4 was determined by the use of several internal oligonuleotide primers, based on the sequence of λHT1, and further restriction enzyme digestion. The structure of λHT4 and the sequencing strategy on both strands is shown in Figure 3.1. The sequence of the cDNA was determined on both strands and is shown in Figure 3.2. The human Theta class GST cDNA clone, here termed pHT4, has a coding sequence of 732bp. The exact length of the 5' non-coding region is unclear because of the presence of other flanking cDNAs and in Figure 3.2 only 132bp is presented. The 3' non-coding region of pHT4 is the same as λHT1 except for a poly-A tail that is clearly evident in pHT4.

3.5.2 Evidence for Alternate Splicing

The divergence of λHT1, pHT2 and pHT3 from the coding sequence of λHT4 clearly suggests that these clones may represent examples of alternate splicing. Examination of the three clones suggests the position of three potential exon/intron boundaries. The first one is at the start of the recognisable coding sequence in λHT1. The second is at the start of the recognisable coding sequence in pHT2 and pHT3. It is notable that although pHT2 and pHT3 diverge from the expected coding sequence at the

Figure 3.1 : Characterisation of four human Theta class GST cDNAs
Four distinct cDNA clones encoding GSTT2 were identified either by direct hybridisation to cDNA libraries or by a Polymerase Chain Reaction (PCR) approach. λHT1, pHT2 and pHT3 are all incomplete transcripts which showed evidence of alternate splicing. The complete open reading frame of GSTT2 was found in the clone λHT4. The open boxes indicate the open reading frame in each clone. The hatched region in pHT2 and pHT3 indicates identical 5' non-coding sequence that extends from approximately 800bp in pHT2 and 165bp in pHT3. The arrows above and below λHT4 indicate the sequencing strategy on each strand.

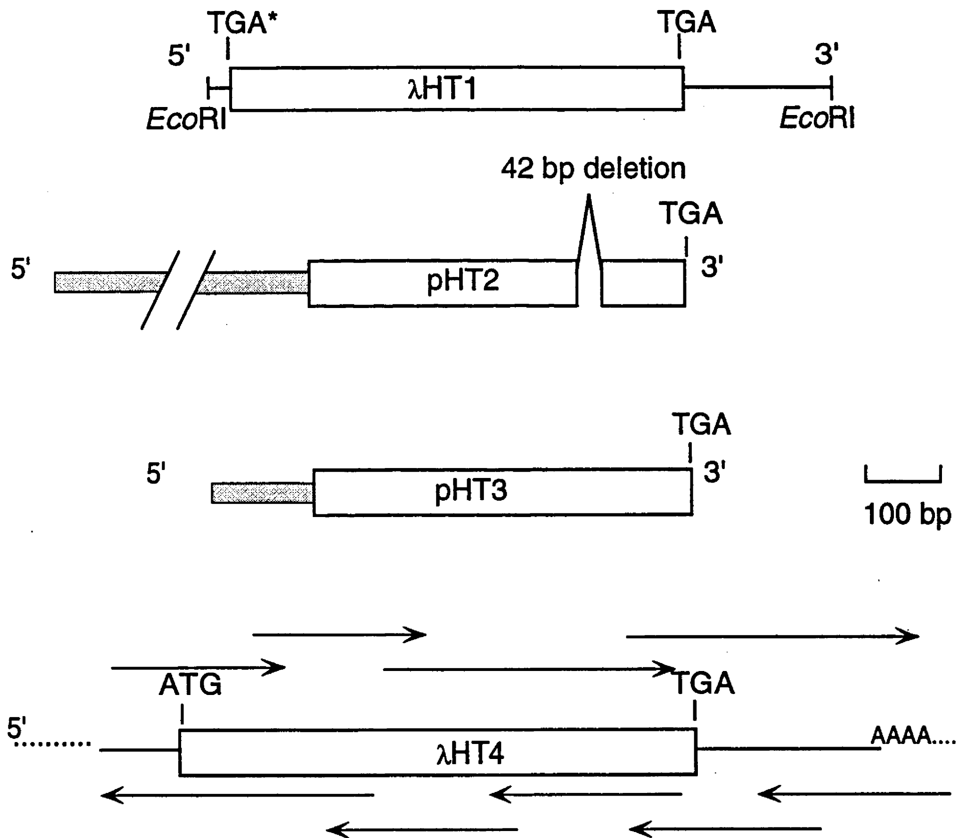


Figure 3.2: DNA Sequence of pHT4 and Its Deduced Amino Acid Sequence

The inverted arrows mark the positions of putative introns derived from λHT1, pHT2 and pHT3. The vertical arrows indicate the positions of introns in the mouse GSTT2 gene (Whittington *et al*., 1996). The termination codon of pHT4 is marked by three asterisks, and the poly(A) addition signal is in bold. The position of the 42bp deletion in pHT2 is in bold and italics. Position of PCR primers HTA3/HTB2 and HTIPF/HTIPR are underlined. The DNA sequence of pHT4 has been deposited in GenBank (Accession No. L38503).

```
                                                gcggcaagctga -121
ctggctgctaggctgctggctgcttggccacagacgggcgcacggagacactcgacgccg -61
ctcccgccgccgccgcgctcagcgccgtctgccatccccgctgtccttgccgcccccgcc -1

ATGGGCCTAGAGCTGTTTCTTGACCTGGTGTCCCAGCCCAGCCGCGCCGTCTACATCTTC  60
M  G  L  E  L  F  L  D  L  V  S  Q  P  S  R  A  V  Y  I  F   20
                                                     ↓

                    HTA3 →                           ><
GCCAAGAAGAATGGCATCCCCTTAGAGCTGCGCACCGTGGATTTGGTCAAAGGGCAGCAC 120
A  K  K  N  G  I  P  L  E  L  R  T  V  D  L  V  K  G  Q  H   40

                                                     ←    HTB2
AAGAGCAAGGAGTTCTTGCAGATCAACAGCCTGGGAAACTGCCGACGCTCAAGGATGGT 180
K  S  K  E  F  L  Q  I  N  S  L  G  K  L  P  T  L  K  D  G   60
                  ↓
                  ><
GATTTCATCTTGACCGAAAGCTCGGCCATCCTGATTTACCTGAGCTGTAAGTACCAGACG 240
D  F  I  L  T  E  S  S  A  I  L  I  Y  L  S  C  K  Y  Q  T   80

CCGGACCACTGGTATCCATCTGACCTGCAGGCTCGTGCCCGTGTTCATGAGTACCTGGGC 300
P  D  H  W  Y  P  S  D  L  Q  A  R  A  R  V  H  E  Y  L  G   100
                                                     ↓
TGGCATGCCGACTGCATCCGTGGCACCTTTGGTATACCCCTGTGGGTCCAGGTGTTGGGG 360
W  H  A  D  C  I  R  G  T  F  G  I  P  L  W  V  Q  V  L  G   120

CCACTCATTGGGGTCCAGGTGCCCGAGGAGAAGGTGGAACGCAACAGGACTGCCATGGAC 420
P  L  I  G  V  Q  V  P  E  E  K  V  E  R  N  R  T  A  M  D   140

                                  HTIPF →
CAGGCCCTGCAATGGCTGGAGGACAAGTTCCTGGGGGACAGGCCCTTCCTCGCTGGCCAG 480
Q  A  L  Q  W  L  E  D  K  F  L  G  D  R  P  F  L  A  G  Q   160
                                               ↓

  >                                ←    HTIPR    <
CAGGTGACACTGGCTGATCTCATGGCCCTGGAGGAGCTGATGCAGCCGGTGGCTCTCGGC 540
Q  V  T  L  A  D  L  M  A  L  E  E  L  M  Q  P  V  A  L  G   180

TACGAACTGTTTGAGGGACGGCCACGACTGGCAGCATGGCGTGGACGAGTGGAGGCTTTC 600
Y  E  L  F  E  G  R  P  R  L  A  A  W  R  G  R  V  E  A  F   200

CTGGGTGCTGAGCTATGCCAGGAGGCCCACAGCATCATCTTGAGCATCCTGGAACAGGCG 660
L  G  A  E  L  C  Q  E  A  H  S  I  I  L  S  I  L  E  Q  A   220

GCCAAGAAAACCCTCCCAACACCCTCACCAGAGGCCTATCAGGCTATGCTGCTTCGAATC 720
A  K  K  T  L  P  T  P  S  P  E  A  Y  Q  A  M  L  L  R  I   240

GCCAGGATCCCCTGAagggtctgggatgggggccaggagattagcaacaaggattcattc 780
A  R  I  P  ***

tgttacttacttgcccctttttatctttccctcttgccccagtcccttctctccagcttc 840
atgtgaagctctgcacagacaagacactcagtgtccttggcagtgctgctactcctcagg 900
tgcagcatacataaccagtaagagactaaatctgcaatatataaagagctcctacaaatc 960
agtaacatgaagaacactcaaaaattggcaaatgtcatcagtgttttaaacagaataaag 1020
attccaaacactttgaaaaaaaaaaaaaaaaa 1052
```

same point, they were amplified from separate libraries and represent distinct cloning events. The third potential exon/intron junction is at the site of the 42bp deletion in the 3' end of the coding region of pHT2. This same 42bp deletion has been discovered independently by Mannervik *et al.* in a partial GSTT2 cDNA clone (unpublished data, see review by Mannervik and Widersten, 1995). Taken together, the 42bp deletion is not an isolated event arising from an artefact of cDNA library construction or introduced during the course of the PCR amplification.

PCR primers, HTA3 and HTB2, based on the λHT4 sequence, flanking the putative exon/intron junction at the 5' end of the coding sequence of λHT1 were used to amplify human genomic DNA (Figure 3.3) (PCR conditions are described in **Section 4.4.2**). DNA sequencing indicated that this fragment of about 600bp, spanned an intron of approximately 520bp (Figure 3.4). The sequence of this intron has not been fully determined. This result showed that the 5' termination of coding sequence of λHT1 corresponds exactly with the position of an exon/intron junction. Comparison with the equivalent mouse gene revealed the existence of an intron at the same position (Whittington *et al.*, 1996). Similarly, the structure of the mouse gene confirmed the presence of an intron at the 5' end of coding sequence in pHT2 and pHT3.

The potential third exon/intron junction could be located at the 5' or 3' end of the 42bp deletion in the pHT2 clone. Oligonucleotide PCR primers, HTIPF and HTIPR, flanking the 5' junction of the 42bp deletion in pHT2 were used in an attempt to amplify a possible intron in this position (Figure 3.2). No amplified product was obtained, suggesting that there is no intron at this position. As shown in Figure 3.2, the position of PCR primers, HTIPF and HTIPR, did not flank an intron, when compared to the mouse gene. However, the 3' end of the 42bp deletion corresponds to the 3' splice site of intron 4 in the mouse gene (Whittington *et al.*, 1996). As a whole, these results suggest that the GSTT2 gene structure is very similar to the mGSTT2 gene with regards to the positions of exons/introns.

Figure 3.3 : DNA Amplification of a Putative Intron at the 5' End of λHT1. An inframe termination codon at the 5' end of λHT1 is marked TGA* and the existence of a putative intron at this position was suspected. PCR primers, HTA3 and HTB2, based on the sequence of λHT4 were used to investigate this possibility by amplifying human genomic DNA.

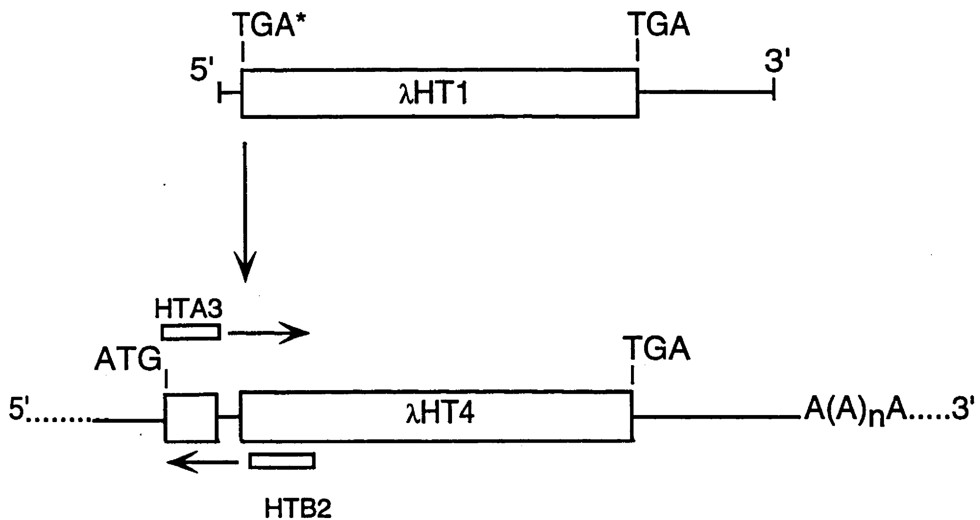
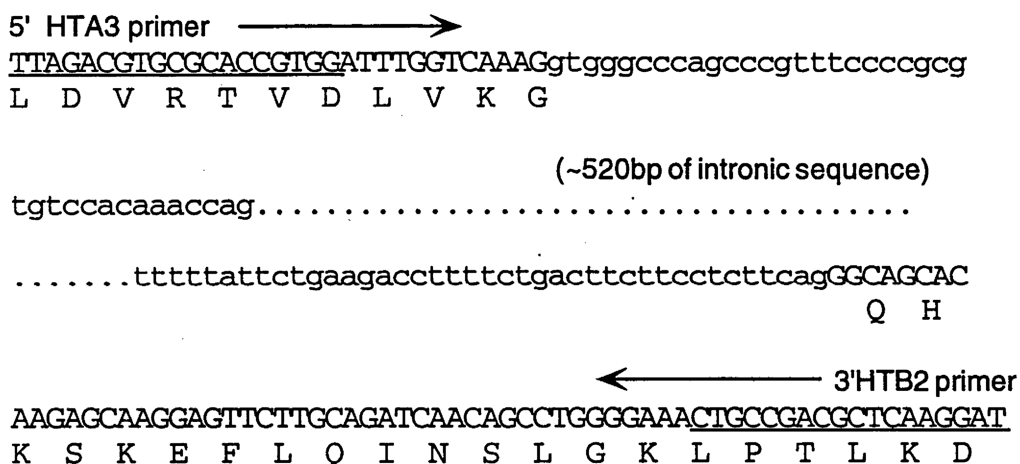


Figure 3.4 : Evidence for Alternate Splicing
The existence of an intron at the 5' end of the coding sequence of λHT1 was confirmed by PCR, using primers HTA3/HTB2 that flanked the putative exon/intron junction. The existence of an intron at this position was also confirmed by comparison with the equivalent mouse gene (Whittington *et al.*, 1996).



3.5.3 Properties of the Encoded Protein

The complete amino acid sequence deduced from clone pHT4 is shown in Figure 3.2. The open reading frame of pHT4 encodes a polypeptide of 244 amino acids. The first 21 amino acid residues of this peptide share almost complete identity with the amino terminal sequence of GSTT2*-2* described by Hussey and Hayes (1992), except for a valine to glycine substitution at position number 2. As discussed in detail in the **Discussion** section, it was concluded that the peptide encoded by pHT4 is the same as the one purified by Hussey and Hayes (1992). For clarity we therefore refer to the subunit encoded in pHT4 as GSTT2.

GSTT2 is the longest human GST subunit when compared to the known Alpha, Mu and Pi class GSTs and a human Theta class GSTT1 subunit which was reported recently by Pemble *et al.* (1994). A comparison of the amino acid sequences of 5 mammalian Theta class GSTs is shown in Figure 3.5. These Theta class GSTs which can be divided into 2 subfamilies (see **Section 1.5.4**) have well conserved N-terminal sequences and show greater divergence in the C-terminal half. The percentage of amino acid sequence identity between subfamily 1 and subfamily 2 has been presented in Table 1.5 of **Chapter 1.**

The 244 amino acid residues of GSTT2 have a calculated molecular mass of 27,489 Daltons. Although Hussey and Hayes (1992) estimated the molecular mass of GSTT2*-2* subunit at 25,100 Daltons, the discrepancy may reflect the non-ideal migration of the subunits in SDS/PAGE and is within the expected experimental range

Figure 3.5: Amino acid sequence comparison of mammalian Theta class GSTs

Five Theta class GSTs from subfamily 1 and subfamily 2 have well conserved N-terminal sequences and show greater divergence in the C-terminal half. The 244 amino acid residues of GSTT2 have a calculated molecular mass of 27,489 Da. The conserved serine residue at position 11 is highlighted. Identical amino acids are marked with a period.

```
GSTT2    MGLELFLDLVSQPSRAVYIFAKKNGIPLELRTVDLVKGQHKSKEFLQINS   50
ratYrs   .....Y...L................FQ......L....L.EQ.S.V.C   50
mGSTT2   .....Y...L................FQT....IL....L.EQ.S.V.C   50
GSTT1    .....Y...L...C..........D..F...I...I....L.DA.A.V.P   50
rat 5    .V...Y...L...C..I.......N..FQMH..E.R..E.L.DA.A.V.P   50

GSTT2    LGKLPTLKDGDFILTESSAILIYLSCKYQTPDHWYPSDLQARARVHEYLG  100
ratYrs   .K.V.V....S.V....T.......S...VA.....A......Q......  100
mGSTT2   .N.V.V....S.V....T.......S...VA.....A......Q......  100
GSTT1    .K.V.A......T....V...L..TR..KV..Y...Q........D...A  100
rat 5    MK.V.AM...G.T.C..V...L..AH..KV......Q........D...A  100

GSTT2    WHADCIRGTFGIPLWVQVLGPL-IGVQVPEEKVERNRTAMDQALQWLEDK  149
ratYrs   ....N......VL..TK....-..............NS.VL...R....  149
mGSTT2   ....N......VL..TK....-......Q......DRS.VLV..Q....  149
GSTT1    .QHTTL.RSCLRA..HK.MF.VFL.GP.SPQTLAATLAEL.VT..L....  150
rat 5    .QHTTL.RSCLRT..HK.MF.VFL.EQIRP.MLAATLADL.VNV.V...Q  150

GSTT2    FLGDRPFLAGQQVTLADLMALEELMQPVALGYELFEGRPRLAAWRGRVEA  199
ratYrs   ..R..A.I...........S....I......CN......Q.T...E....  199
mGSTT2   ..R..A..V..........S............N......Q.T...E....  199
GSTT1    ..QNKA..T.PHIS....V.IT...H..GA.CQV.....K..T..Q....  200
rat 5    ..Q.KD..V.PHIS...VV.IT...H..GG.CPV........A.YR....  200

GSTT2    FLGAELCQEAHSIILSILEQAAKKTLPTPSPEAYQAMLLRI-ARIP  244
ratYrs   ...........NP.M.V.G... ....V.P...HAS.M...-....  244
mGSTT2   ......Y.....T.....G.....M..V.P..VHAS.Q...-....  244
GSTT1    AV.ED.F....EV..KA------.DF.PAD.TIK.KLMPWVL.M.R  240
rat 5    AV.KD.FL...EV..KV------RDC.PAD.VIK.KLMP.VLTM.Q  240
```

3.6 DISCUSSION

The mammalian Theta class GSTs do not utilise 1-chloro-2,4-dinitrobenzene as a substrate and do not bind to immobilised glutathione affinity matrices. The absence of these two key properties that are such useful characteristics of the Alpha, Mu, and Pi classes resulted in the existence of the Theta class GSTs being largely overlooked. Although two distinct Theta class cDNA clones have been isolated from rat liver cDNA libraries (Ogura *et al.*, 1991; Pemble and Taylor, 1992), the first clear evidence for the Theta class in humans has come from the purification of two apparently distinct proteins (Meyer *et al.*, 1991; Hussey and Hayes, 1992). During the course of this thesis, Pemble *et al.*, (1994) reported the cloning of a cDNA encoding a human Theta-class GST subunit termed GSTT1. This subunit appears to be the orthologue of the rat subunit 5 and encodes the protein GSTθ purified earlier by Meyer *et al.* (1991). In this study, the molecular cloning of a cDNA encoding the second human Theta class GST, GSTT2-2 is reported.

3.6.1 Characterisation of a cDNA Clone Encoding GSTT2

The complete cDNA clone encoding GSTT2 was isolated from a λgt11 human liver 5'-stretch cDNA library, subcloned into pUC18 plasmid, and termed pHT4. The amino acid sequence identity between rat subunit Yrs (78.3%) and mouse GSTT2-2 (77.0%) with the protein encoded in pHT4 suggests that they may be orthologous. The deduced amino acid sequence from pHT4 shares almost complete identity with GSTT2*-2* (Hussey and Hayes, 1992) over the first 21 amino acid, except for the substitution of valine for glycine at position 2. This disparity is probably explained by the reported ambiguity of valine in the amino acid sequence analysis (Hussey and Hayes, 1992). Since both the rat GST Yrs-Yrs and human GSTT2*-2* have activity with 1- menaphthyl sulphate (Hiratsuka *et al.*, 1990; Hussey and Hayes, 1992; and **Chapter 4** of this thesis) and both have close similarities to the sequence encoded in pHT4, it seems highly likely that pHT4 encodes the GSTT2*-2* isoenzyme described by Hussey and Hayes (1992). Therefore, in line with the GST nomenclature guidelines (Mannervik

et al., 1992), the protein encoded by pHT4 will be referred to as GSTT2-2, and its gene locus as *GSTT2*.

It is noteworthy that the residue at position 6 in GSTT2 is phenylalanine instead of tyrosine, as compared with the other mammalian Theta class GSTs (Figure 3.5). Structural studies of all mammalian Alpha, Mu and Pi class GSTs have suggested that a tyrosine near the N-terminal domain is the most significant residue involved in the activation of glutathione by the formation of a hydrogen bond between its phenolic hydroxyl and the cysteinyl sulphur of glutathione (Dirr *et al.*, 1994; Wilce and Parker, 1994). Site directed mutagenesis of this tyrosine residue in the Alpha, Mu and Pi class GSTs resulted in mutant enzymes which are severely impaired in CDNB conjugating activity (Stenburg *et al.*, 1991; Liu *et al.*, 1992; Kolm *et al.*, 1992). Due to the fact that GSTT2 lacks this tyrosine residue, it is probable that non-tyrosine residues may play a more important role in the catalytic function of GSTT2. This possibility is explored in greater detail in **Chapter 7** of this thesis.

3.6.2 Implications of Alternative Splicing

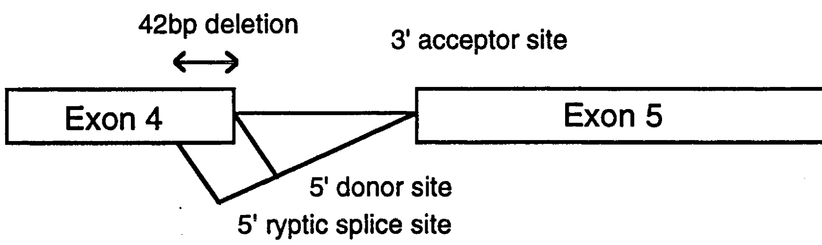
In the present study, evidence of alternative splicing was found in at least 3 sites in transcripts of *GSTT2*. The presence of exon/intron junctions at these sites was confirmed by PCR analysis and comparison with the structure of an equivalent mouse gene (Whittington *et al.*, 1996). It is unlikely that either of the alternatively spliced transcripts encodes a catalytically active enzyme as the amino terminal residues that are important in catalysis in all the other cytosolic GSTs are missing from both forms. In addition, there is a termination codon (TGA*) within the 5' non-coding sequence of λHT1 which falls in the same reading frame as the protein coding region of the cDNA. This in-frame termination codon would be expected to either prohibit or greatly reduce the efficiency of translation of the truncated transcript if there is an upstream translation start codon in the same reading frame.

When the position of the 42bp deletion was compared with the mouse gene, the 3' end of the 42bp deletion corresponds to the 3' splice site (acceptor site) of intron 4. However, the 5' splice site (donor site) selection falls on a cryptic donor site in exon 4 (42bp upstream of the actual donor site). This cryptic donor site fulfils the consensus sequences of the general 5' splice junction of an intron as compiled by Mount (Mount, 1982) and Iida and Sasaki (Iida and Sasaki, 1983). Therefore, the observed 42bp deletion appears to result from an alternate 5' splice site selection (Figure 3.6). Ohshima and Gotoh (1987) suggest that the selection of donor and acceptor sites based on consensus sequence might be an over simplified model to explain the mechanism of RNA splicing. The existence of numerous of 5' donor-like sequences within the introns of a gene, makes the proposal of a simple scanning mechanism in splice sites selection difficult to reconcile (Ohshima and Gotoh, 1987). Caceres *et al.* (1994) proposed that the selection of 5' splice sites is governed by the intracellular levels of antagonistic splicing factors. The results of their studies suggest that the SF2 alternative splicing factor, which belongs to the SR family of non-snRNP splicing factors promotes the use of proximal 5' splicing sites, hence preventing exon skipping and preserve splicing fidelity *in vivo*. On the other hand, the hnRNP A1 splicing factor acts in an antagonistic manner to promote distal 5' splice sites. The 42bp deletion at the 3' end of exon 4 may represent an event where antagonistic splicing factors play an important role in determining the 5' splice site.

It has been suggested that the *GSTP1* gene may be regulated by a post-transcriptional mechanism (Morrow *et al.*, 1992). In a recent study, Ross and Board (1993) reported the first evidence of alternative splicing in a human Mu class GST, GSTM4. The discovery of alternatively spliced transcripts in different GST classes suggests that alternative splicing may play a regulatory role in the expression and function of GSTs. In this study, the discovery of alternatively spliced transcripts of *GSTT2* from more than one cDNA library as well as from independent laboratories suggests that this is not an uncommon event and may reflect a more important process than the chance mistaken splicing of illegitimate transcripts (Chelly *et al.*, 1989). Andreadis *et al.* (1987) proposed that alternative splicing can give rise to new gene products without changing the

nucleotide sequence of the original gene. This process could possibly play an important role in the evolution of novel enzyme functions in detoxication enzymes such as the GSTs and cytochrome P450s (Ding *et al.*, 1995). It is not clear at this stage if the presence of λHT1, pHT2 and pHT3 represent such experimental processing or as suggested above, represent another form of post-transcriptional gene regulation.

Figure 3.6 : Alternative 5' splice site selection on pHT2



3.7 CONCLUSION

A cDNA encoding the Theta class isoenzyme GSTT2-2 which was originally purified by Hussey and Hayes (1992), was cloned from a human liver cDNA library. The full length cDNA clone, λHT4, with an open reading frame of 732bp, encodes a polypeptide of 244 amino acids with a deduced molecular mass of 27,489 Da. Comparison of amino acid sequence deduced from the cDNAs of GSTT2, mGSTT2 (Whittington *et al.*, 1996) and rat subunit Yrs (Ogura *et al.*, 1991) showed that they are orthologous and belong to subfamily 2 of the Theta class GST.

As compared with the other members in the supergene family of GSTs, one distinct feature of the GSTT2-2 is the absence of a conserved N-terminal tyrosine. In the Alpha class GSTs, non-tyrosine residue (Arg14) Bjornstedt *et al.*, (1995) has been shown to act in concert with the conserved tyrosine residue (Tyr8) to activate the enzyme bound GSH. However, the present characterisation of the GSTT2 cDNA provides the first example in the GST supergene family, where catalytic mechanism can be independent from the conserved tyrosine. This possibility is explored in greater detail in **Chapter 7** of this thesis.

The isolation of 3 other partial cDNA clones of GSTT2-2 showed evidence of alternative splicing. The existence of introns in these truncated cDNA clones was confirmed by comparison to the equivalent mouse gene (Whittington *et al.*, 1996) and also elucidated by a PCR approach. This is the second reported case of alternative splicing in the GST supergene family. The possibility of *GSTT2* gene regulation at post-transcriptional level by alternative splicing is proposed. It can also be considered from these results that the human *GSTT2* gene organisation is similar to the equivalent mouse gene.

CHAPTER 4 : CHROMOSOMAL LOCALISATION OF *GSTT2*

4.1 INTRODUCTION

The gene loci for human cytosolic GSTs have been studied using PCR based screening, human-rodent somatic hybrid analysis or *in situ* hybridisation techniques. The genes encoding the Alpha, Mu and Pi class GSTs have been mapped and localised to chromosomes 6p12, 1p13 and 11q13 (see **Section 1.6** for review). It appears that human cytosolic GST genes occur in class specific gene clusters which are dispersed through out the human genome.

4.2 OBJECTIVES

To gain a greater understanding of the chromosomal location of the *GSTT2* gene, its relationship with the gene loci of other human cytosolic GSTs and the possible existence of related pseudogenes, by using somatic cell hybrid and *in situ* hybridisation mapping. This study also seeks to investigate the extent of heterogeneity of the Theta class gene family through Southern blot analysis of human genomic DNA.

4.3 MATERIALS

The source of chemical compounds and materials for routine use were as stated in **Chapter 2**. The tritiated bases [^{3}H]dATP, [^{3}H]dCTP, [^{3}H]dTTP, were purchased from and Amersham (UK). Human-rodent somatic cell hybrid cell lines containing individual human chromosome were obtained from the NIGMS human mutant cell repository: GM07299 (Chr 1, Chr X), GM10826B (Chr 2), GM10253 (Chr 3), GM10115 (Chr 4), GM10114 (Chr 5), GM10629 (Chr 6), GM10791 (Chr 7), GM10156C (Chr8), GM10611 (Chr 9), GM10926B (Chr 10), GM10927A (Chr 11), GM10868 (Chr 12), GM110926B (Chr 13), GM10479 (Chr 14), GM11418 (Chr 15), GM10567 (Chr 16), GM10498 (Chr 17), GM11010 (Chr 18), GM10449 (Chr 19), GM10478 (Chr 20), GM10323 (Chr 21), GM10888 (Chr 22), GM06318C (Chr X), and

GM06317 (Chr Y). DNA from these cell lines were prepared by the methods of Grunebaum *et al* (1984).

4.4 METHODS

4.4.1 Southern Blot Analysis

Human genomic DNA samples prepared from peripheral blood leucocytes were digested using *Eco*RI, *Bam*HI, and *Hin*dIII. Digested DNAs from 7 individuals were separated by agarose gel electrophoresis and blotted to Hybond™ N+ nylon membrane (Reed and Mann, 1985). A 767bp cDNA fragment extending from the initiation codon of pHT4 to 35bp 3' to the translation stop signal was labelled with [α^{32}P]dATP by random priming and used as a hybridisation probe. Detailed procedures for Southern blotting have been described in **Chapter 2.**

4.4.2 Human-Rodent Somatic Cell Hybrid Analysis

A 700bp fragment was amplified from human DNA using primers based on the λHT4 cDNA sequence. The sense primer, HTA3 (5'TTAGACGTGCGCACCGTG3') and antisense primer, HTB2 (5'ATCCTTGAGCGTCGGCAG3') were selected to amplify across a putative exon/intron boundary, the position of which was predicted from the 5' termination of coding sequence in λHT1 (see Figure 3.4 **Chapter 3**). The DNA amplification (PCR) was performed using an oil-bath thermal cycler (Bartelt Instruments, Australia) in a 50μl reaction mix containing 250ng of genomic DNA, 200μM dNTPs, 20pmol of each primer, 1.5u of *Taq* polymerase and 1x*Taq* polymerase buffer. PCR conditions were 30 cycles of (94°C, 1min; 56°C, 1min; 72°C, 80sec). The PCR product was cloned into pGEM-T vector (Promega, Australia) according to the manufacturer's protocol and subcloned into the *Sph*I and *Sac*I sites of M13mp18 and mp19 to generate single-stranded DNA template for sequencing. DNA sequencing confirmed that the fragment encodes an intron of approximately 600bp (Figure 3.4). The amplified intron was used as a specific hybridisation probe on Southern blots of *Bam*HI-

digested human-rodent somatic cell hybrid cell lines that retain a single human chromosome.

4.4.3 *In situ* Hybridisation

A 872bp cDNA insert was excised from pHT1 by *Eco*RI digestion and after nick translation with [^{3}H]dATP, [^{3}H]dCTP and [^{3}H]dTTP to a specific activity of 9 x 10^7 cpm/μg, the fragment was used as a probe for *in situ* hybridisation. Chromosome slides were prepared from four individuals of both sexes and each gave essentially identical results. The slides were treated with RNase, denatured, probed and stringently washed. They were then dipped in Ilford L4 emulsion diluted 1:1 and exposed for 12-16 days. Following the processing of emulsion and staining to give G-banded chromosomes (Buckle and Craig, 1986), silver grains in the emulsion were scored onto computer drawn 550 band ideograms of the G-banded chromosomes (Chromo Map, P. Board unpublished program).

The precise chromosomal location assignment of *GSTT2* using *in situ* hybridisation was carried out in collaboration with Dr. G. C. Webb of the Genetics Department, Queen Elizabeth Hospital, Woodville, South Australia where all the experiments were performed. My contribution in this collaboration was limited to the construction of the recombinant plasmid (pHT1) and preparation of the *Eco*RI insert from pHT1 for use as a probe. Detailed procedures for the preparation of chromosomes and *in situ* hybridisation have been described previously (Board and Webb, 1987; Board *et al.*, 1989).

4.5 RESULTS

4.5.1 Southern Blot Analysis

Southern blots of human genomic DNA digested with *Eco*RI, *Hin*dIII, or *Bam*HI showed a single fragment that hybridised with the coding region of the pHT4 cDNA. The smallest hybridising band is a 3.6kb fragment from the *Bam*HI-digested DNA, while

other hybridising bands in the *Eco*RI- and *Hin*dIII-digested DNA are approximately 11.7kb and 19.9kb respectively (Figure 4.1). These data suggest that the *GSTT2* gene may be relatively small, as the exons containing the coding sequence fall within the 3.6kb *Bam*HI fragment.

4.5.2 Somatic Cell Hybridisation Analysis

An intron probe (Figure 3.4) was used to hybridise Southern blots of DNA prepared from 24 somatic cell hybrid cell lines predominantly containing single human chromosomes (Figure 4.2A, 4.3A). In this study all human chromosomes were examined and only one cell line containing chromosome 22 provided evidence of hybridisation (Figure 4.2B). The intron probe hybridised to a 3.6kb *Bam*HI fragment from the CHO-human cell line GM10888 bearing chromosome 22. No hybridisation was observed from CHO cells or cell lines bearing other human chromosomes (Figure 4.3B). The hybridising fragment from the cell line containing chromosome 22 is the same size as the single hybridising fragment observed in *Bam*HI digested human genomic DNA (Figure 4.1). These data therefore suggest that the *GSTT2* gene is located on chromosome 22.

4.5.3 *In situ* Hybridisation

Figure 4.4 features the G-banded chromosome spreads probed with the cDNA insert in λHT1. Initial scoring of metaphase chromosomes showed the highest accumulation of silver grains over the proximal region of the long arm of chromosome 22 and only minor background levels on other chromosomes (Figure 4.5). The tallest peak of grains was over the subband 22q11.2, suggesting that this band is the most likely position of the *GSTT2* gene. Therefore, prophasic chromosomes 22 were examined and detailed plotting of grains confirmed the overall result (Figure 4.6). In this study, 27 grains were scored over the proximal half and 21 grains over the distal half of a divided 22q11.2; the difference between the two scores is not significant ($\chi^2 = 0.75$). Therefore, the *GSTT2* gene cannot be sublocalised within the broad subband 22q11.2.

Figure 4.1 : Southern blot of human genomic DNA digested with *Bam*HI, *Hin*dIII, or *Eco*RI and hybridised with the coding region of *GSTT2* derived from pHT4.

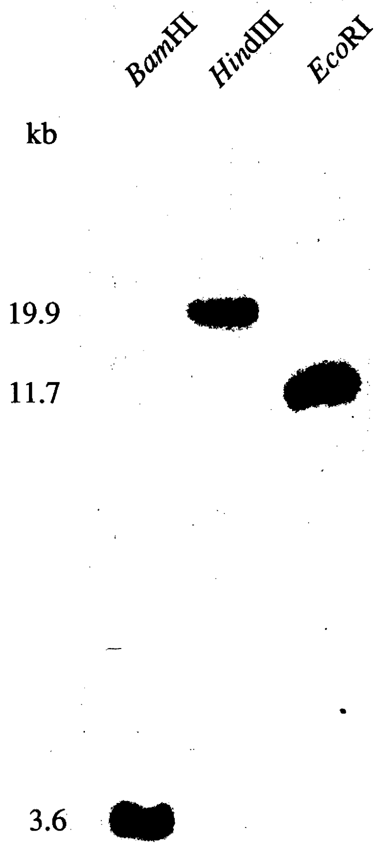
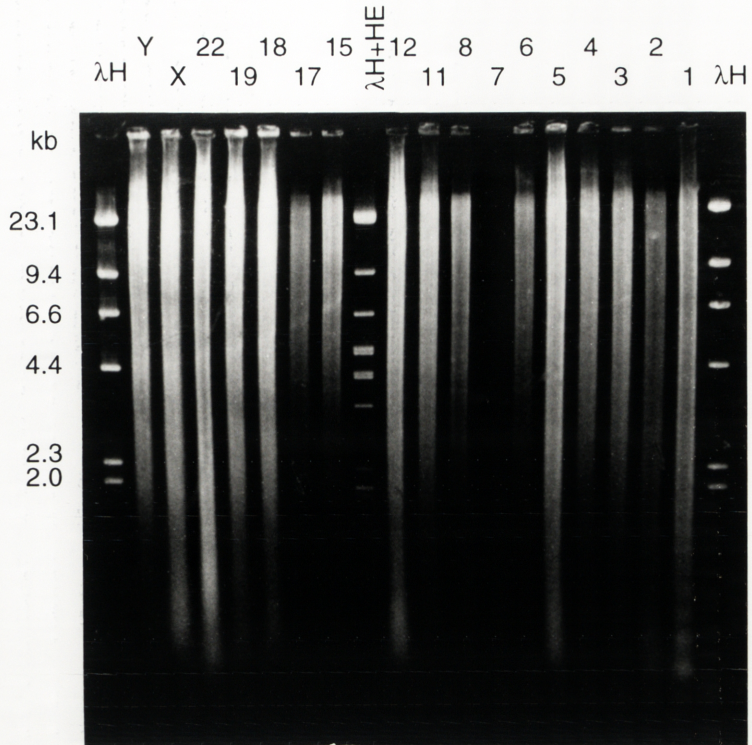

BamHI
HindIII
EcoRI
kb
19.9
11.7
3.6

Figure 4.2 : (A) : Southern blot of DNA prepared from somatic cell hybrid cell lines was digested with *Bam*HI and separated on a 0.8% agarose gel. The human chromosome in each cell lines is numbered above the lanes. Cell line GM10791 containing chromosome 7 which did not appear in the lane was reloaded on accompanying gel in Figure 4.3A. λ*Hin*dIII (λH) and λ*Hin*dIII/*Eco*RI (λH+HE) molecular weight markers are shown. (B) : Cell line 10888 bearing human chromosome 22 showed a single hybridising band of approximately 3.6kb (indicated by arrow).


A
λH
Y
X
22
19
18
17
15
λH+HE
12
11
8
7
6
5
4
3
2
1
λH
kb
23.1
9.4
6.6
4.4
2.3
2.0

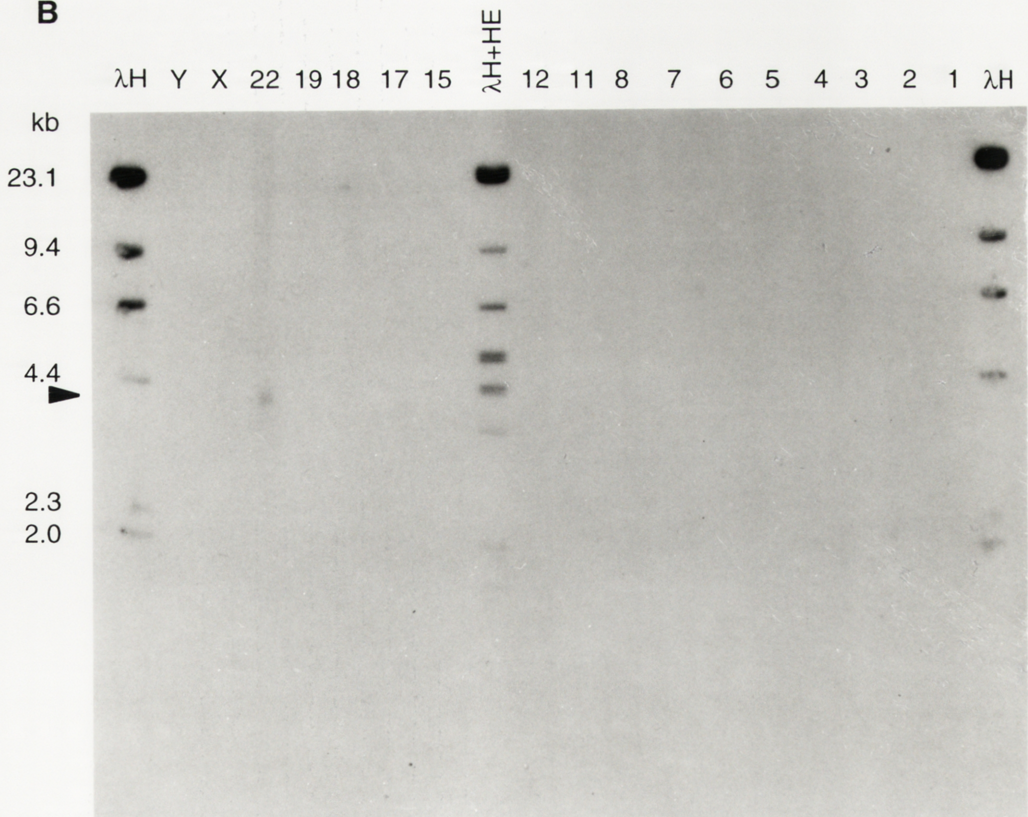
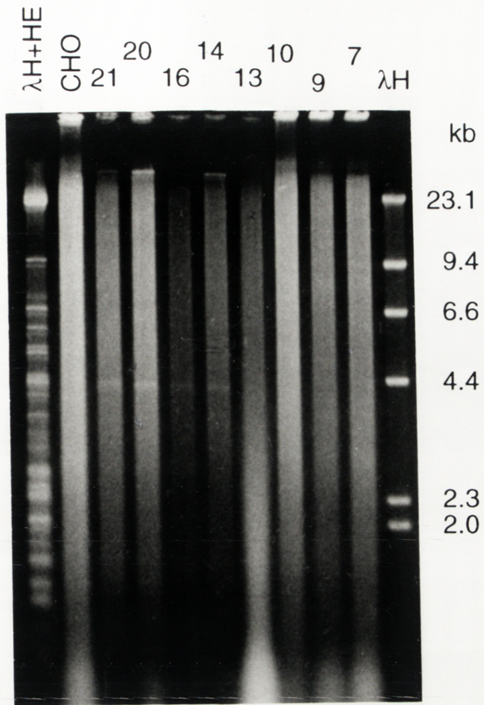

B
λH
Y
X
22
19
18
17
15
λH+HE
12
11
8
7
6
5
4
3
2
1
λH
kb
23.1
9.4
6.6
4.4
2.3
2.0

Figure 4.3 : (A) : Southern blot of the remaining somatic cell hybrid cell lines which was not shown in Figure 4.2A. The DNA prepared from these cell lines was digested with *Bam*HI and separated on a 0.8% agarose gel. (B) : No hybridisation was observed from the CHO cell line as well as other CHO-human cell lines.

A



B

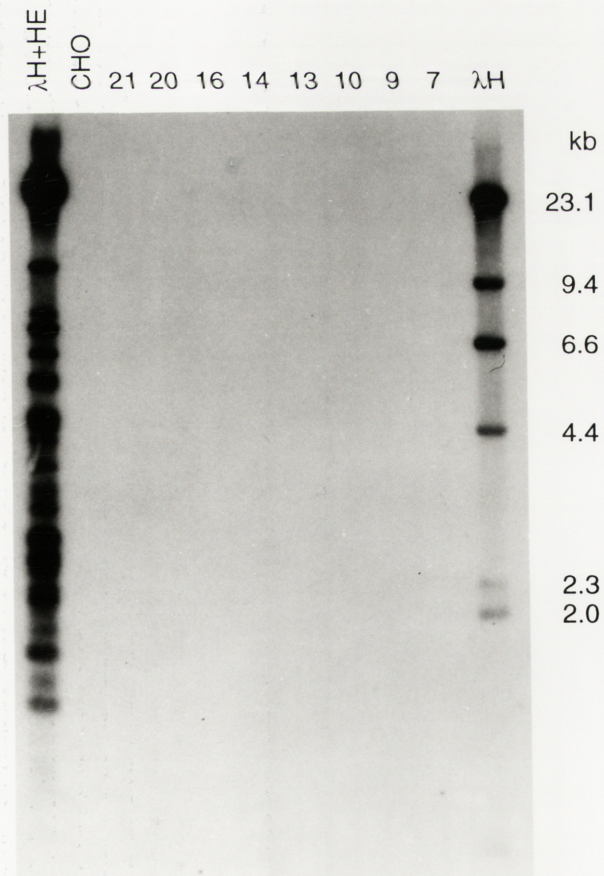
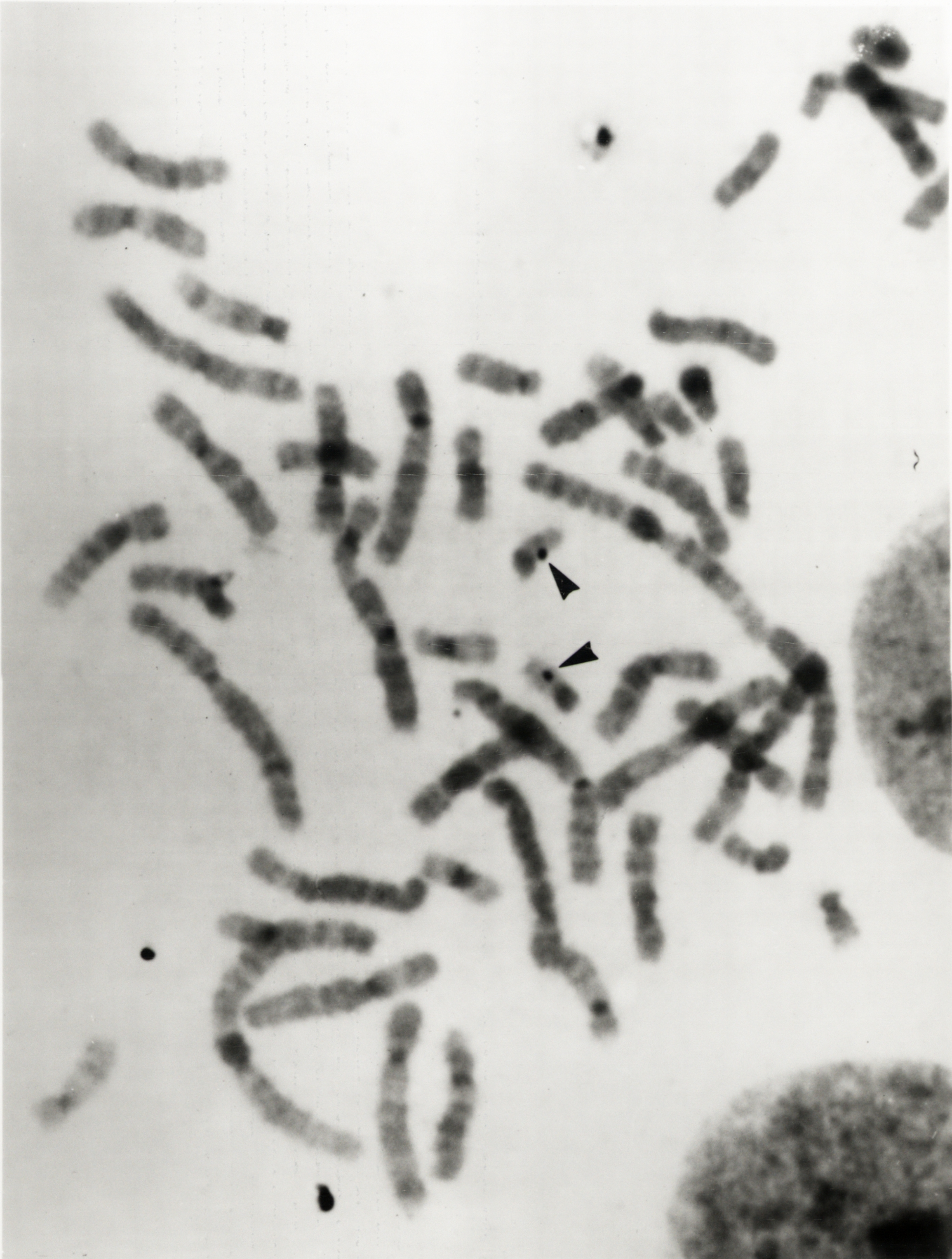


Figure 4.4 : A 872bp cDNA insert of pHT1 was nick translated with three tritiated bases and hybridised to G-banded metaphase chromosome spreads. The arrows indicate silver grains accumulated on chromosome 22 at subband q11.2.



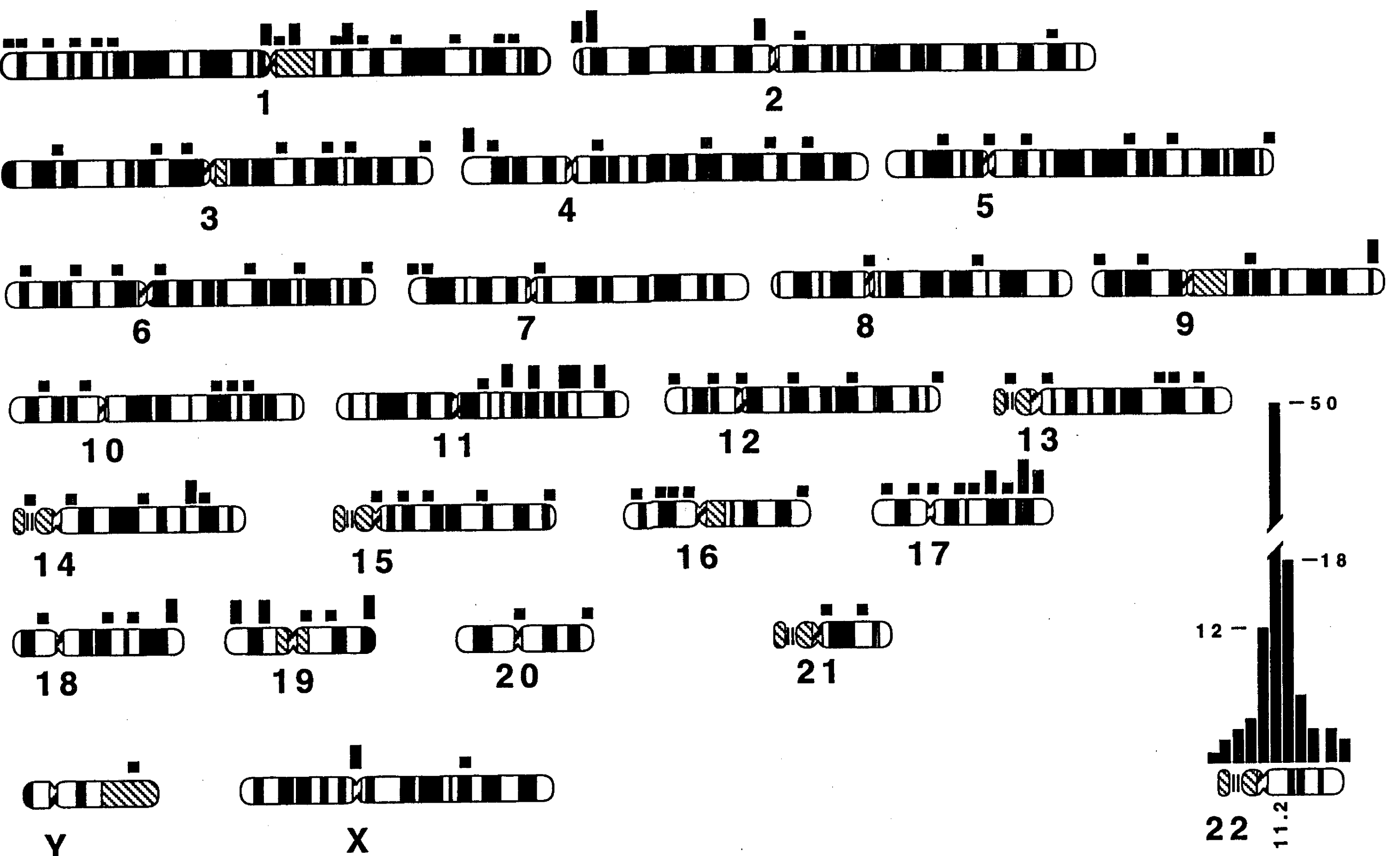


Figure 4.5 : *In situ* hybridization of metaphase arrest chromosomes from 4 individuals probed with the cDNA insert in λHT1. The highest concentration of grains is over the long arm of chromosome 22 with the tallest column over the faint sub-band 22q11.2 with only minor background levels on other chromosomes.

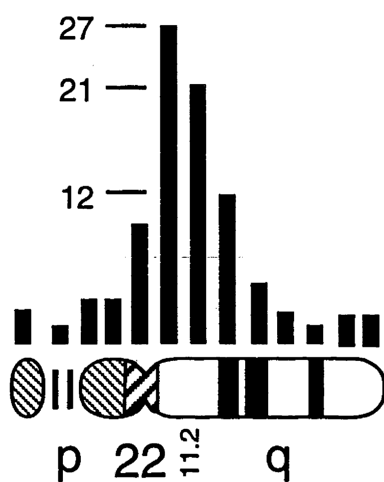


Figure 4.6 : Scores of silver grains over prophasic chromosomes 22, probed with the cDNA insert in λHT1. The highest concentration of grains is over the faint subband 22q11.2.

4.6 DISCUSSION

4.6.1 Southern Blot Analysis

Southern blots of human genomic DNA digested with *Eco*RI, *Bam*HI, or *Hin*dIII showed a single fragment that hybridised with the coding region of the GSTT2 cDNA, suggesting the existence of only one closely related gene. It seems unlikely that the 767bp GSTT2 cDNA hybridises to GSTT1 sequences or other possible members in the Theta class gene family under the stringency conditions of the present experiment. Recently, Pemble *et al.* (1994) showed that a GSTT1 cDNA hybridises only to a single 7.5kb *Hin*dIII or 18kb *Eco*RI fragments and neither of these were observed in the 7 individuals evaluated in this study. This again demonstrated the differences within the Theta class GSTs and confirmed the observation that the DNA sequences of individual Theta class members do not cross-hybridise with other member(s) under the experimental conditions.

4.6.2 Gene Mapping

The chromosomal location of GSTT2 has been investigated by two approaches. In studies using somatic hybrid cell lines containing single human chromosomes, a DNA fragment from a CHO-human cell line bearing chromosome 22 was found to hybridise with an intron probe derived from the *GSTT2* gene. The use of an intron probe largely avoids cross-hybridisation between the cDNAs and the orthologous rodent gene(s). In addition, intron probes do not hybridise with reverse transcribed pseudogenes (Webb *et al.*, 1990; Webb *et al.*, 1994). The second approach using *in situ* hybridisation with the *GSTT2* cDNA clone, confirmed the chromosome assignment and mapped the precise location of the *GSTT2* gene to subband 22q11.2. The fact that there were no other significant peaks of silver grains on other chromosomes indicated that it is unlikely that there are reverse transcribed pseudogenes derived from *GSTT2* dispersed throughout the genome (Webb *et al.*, 1990). Furthermore, the data suggest that if there are other cross-hybridising genes, they must be relatively closely clustered on chromosome 22q.

4.6.3 Other Gene Loci on Chromosome 22q11

Chromosome 22q11 contains several interesting gene loci, among them, genes encoding the immunoglobulin lambda constant regions (McBride *et al.*, 1982; Erikson *et al.*, 1981), beta-crystallins (Bijlsma *et al.*, 1991), Hulsebos *et al.*, 1991), cytochrome P450 subfamily IID gene cluster and related pseudogenes (Kimura *et al.*, 1989), γ-glutamyl transpeptidase (Bulle *et al.*, 1987), transposable element (Dombroski *et al.*, 1991) and genes associated with cancer such as breakpoint cluster region (BCR) gene family (Heisterkamp and Groffen, 1988). Chromosome 22 is also the site of translocation found in Ewing's sarcoma (Aurias *et al.*, 1984), neuroepithelioma (Whang *et al.*, 1986) and Burkitt lymphoma (Croce *et al.*, 1983).

4.6.3.1 The Gene Loci for γ-Glutamyl Transpeptidases

Like the GSTs, γ-glutamyl transpeptidases (GGT, EC 2.3.2.2) are involved in glutathione metabolism (Meister *et al.*, 1981). The GGT multigene family consists of at least 4 different genes and several pseudogenes (Pawlak *et al.*, 1988; Rajpert-De Meyts *et al.*, 1988; Morris *et al.*, 1993). Interestingly, the GGT1 gene was mapped to chromosome 22 by *in situ* hybridisation, at the interface of q11.1-11.2 with a minor peak in q13.1 (Bulle *et al.*, 1987). Somatic cell hybrid analysis also mapped the GGT genes to chromosome 22 and found other GGT-related sequences on chromosomes 18, 19, and 20 (Figlewicz *et al.*, 1993). GGT plays an important role in the γ-glutamyl cycle. It catalyses the transfer of the γ-glutamyl moiety of GSH to an appropriate acceptor, such as amino acids, peptides or water (to form glutamate) (Meister *et al.*, 1981; Tate and Meister, 1981). In this process, amino acids are thought to be transported across cell membranes by the transpeptidation of plasma amino acids to form intracellular γ-glutamyl amino acids. GGT is also involved in converting leukotriene C_4 to leukotriene D_4 (Hammarstrom, 1983) by hydrolysing the γ-glutamyl moiety of LTC_4 (see Figure 1.3 of **Chapter 1**). Individuals with GGT deficiency were found to excrete large amounts of GSH (glutathionuria), γ-glutamylcysteine, and cysteine in their urine (Griffith and Meister, 1980). Despite the common association with glutathione metabolism, the lack of

homology between GGTs and GSTs does not suggest a common genetic origin and it is likely that their localisation to the same chromosomal band is a matter of chance.

4.6.3.2 The Gene Loci for beta-Crystallins

The beta-crystallins genes form another interesting gene cluster near the GSTT2 locus and have been sublocalised to 22q11.2-q12. In earlier studies, lens crystallins from cephalopods are found to be closely related to cephalopod GSTs as well as GSTs from invertebrates (Tomarev *et al.*, 1993; Chiou *et al.*, 1995) and recently a vertebrate GST (Meyer and Thomas, 1995). In view of their amino acid similarity, the cephalopod lens crystallins and other closely related GSTs have been called the Sigma class GSTs (see **Section 1.2**). It has been demonstrated that many major proteins in the lenses have their homologues in metabolic enzymes (Wistow and Piatigorsky, 1987; Cuthbertson *et al.*, 1992) and they might have been recruited for a structural purpose from their enzymatic functions. Recently, the three-dimensional structure of a Sigma class GST from squid digestive gland has been solved (Ji *et al.*, 1995). In this study, Ji and co-workers proposed that the Sigma class GST diverged from the ancestral Theta class gene before the divergence of the Alpha/Mu/Pi precursor presumably by a gene duplication event. Although there is a close evolutionary relationship between the squid lens crystallins and the Theta class GSTs, the amino acid sequences of human lens crystallins are very different from the cytosolic GSTs. They do not appear to have been recruited from cytosolic GSTs for a structural purpose. As in the case of the GGT genes, the co-location of the beta-crystallin genes and the Theta class GST genes appears to be a matter of chance.

4.7 CONCLUSION

Previous studies of the human Alpha and Mu class genes have shown that they are grouped in class specific clusters on distinct chromosomes (Board and Webb, 1987; Ross *et al.*, 1993). The apparent absence of cross-hybridisation between the GSTT1 and the GSTT2 sequences prevents the drawing of any conclusions about the chromosomal location of *GSTT1* from the present data. However in a recent study, Webb *et al.*,

(Webb *et al.*, 1996) confirmed the chromosomal location of the *GSTT1* gene to subband 22q11.2. This confirms that the human cytosolic GSTs exist as class specific gene clusters which are dispersed through out the human genome. Although the gene loci of *GSTT1* and *GSTT2* are sublocalised on the same chromosomal band, the lack of cross-hybridisation and differences in DNA sequence between the two, suggest that they have not been homogenised by gene conversion. This is in contrast to the Mu class GST gene cluster at chromosome 1p13, where gene conversion has played an important evolutionary role (Ross *et al.*, 1993; Pearson *et al.*, 1993). The lack of significant silver grain accumulation on other chromosomes and the results from Southern blot analysis both suggest that it is unlikely that there are reverse transcribed pseudogenes of *GSTT2* dispersed through out the genome. The *GSTT2* gene is less that 4kb in length as indicated by Southern blot analysis of human genomic DNA.

CHAPTER 5 : HETEROLOGOUS EXPRESSION, PURIFICATION AND CHARACTERISATION OF GSTT2-2

5.1 INTRODUCTION

The heterologous expression of cDNAs in prokaryotic or eukaryotic hosts has been widely used to produce large amounts of recombinant protein for characterisation as well as for commercial purposes. These techniques have been applied extensively to express mammalian GSTs (Board and Pierce, 1987; Vorachek *et al.*, 1991; Kong *et al.*, 1993; Leaver *et al.*, 1993; Ross and Board, 1993). In the case of the human Theta class GSTs, knowledge of their substrate specificities has been largely obtained by the characterisation of small amounts of protein purified from liver sources (Meyer *et al.*, 1991; Hussey and Hayes, 1992). Distinct from the Alpha, Mu and Pi class GSTs, the mammalian Theta class GSTs are not retained by glutathione or *S*-hexylglutathione affinity matrices. This particular feature of the Theta class GSTs has limited the use of a single step GSH-affinity purification and multi-step purification protocols have been required (Hiratsuka *et al.*, 1990, Hussey and Hayes, 1992). As a result, the recovery of purified proteins from natural sources was usually low. cDNAs encoding the human Theta class GSTs have been cloned (Pemble *et al.*, 1994; see **Chapter 3**) and the heterologous expression of their cDNAs will allow more extensive studies of the structure and function of the Theta class proteins.

5.2 OBJECTIVES

Little is known about the enzymological properties of GSTT2-2. The objective of this chapter is to express recombinant GSTT2-2 in sufficient quantity to enable a more extensive characterisation than has been possible previously with enzyme purified from human tissue.

5.3 MATERIALS

Details of common materials used in this study have been outlined in **Chapter 2.** Chemicals used in enzyme assays were purchased from Aldrich Chemical Co., Wisconsin (USA), Sigma Chemical Co. (St. Louis, MO), and Boehringer Mannheim (Australia). Glutathione reductase is a product of Sigma Chemical. Co. (St. Louis, MO). 1-menapthyl sulphate was synthesised by the method of Clapp and Young (1970) and was generously provided by Dr. G. Barlin and Mr S. Ireland (Division of Neuroscience, JCSMR, ANU, Canberra, Australia).

5.4 METHODS

5.4.1 Enzyme Assays

GST assays using *trans, trans*-alka-2,4-dienals and *trans*-alk-2-enals were described by Brophy *et al.* (1989). The determination of GST activity with 1-menapthyl sulphate was essentially the same as described by Gillham (1971) and glutathione peroxidase activity was determined by the procedure described by Beutler (1975). GST activity with dichloromethane (DCM) as a substrate was determined at 37°C in a 1.5ml eppendorf tube containing 40mM DCM, 100mM GSH, 0.5M Sodium Phosphate Buffer, pH6.5 and 2μg of purified enzyme. Formaldehyde released after 60 minutes was determined spectrophotometrically at 415nm, by the method of Nash (1953), using known concentrations of formaldehyde as standards. Glutathione transferase assays using 1-chloro-2-4-dinitrobenzene (CDNB), 1,2-dichloro-nitrobenzene (DCNB), ethacrynic acid (EA), 1,2-epoxy-3-(*p*-nitro phenoxy)-propane (EPNP), *trans*-4-phenyl-but-3-en-2-one (tPBO) and 2-cyano-1,3-dimethyl-1-nitrosoguanidine as substrates were previously described in detail by Mannervik and Widersten (1995). Activity with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole was determined spectrophotometrically at 419nm in sodium acetate buffer, pH 5.0 as described by Ricci *et al.* (1994).

5.4.2 Heterologous Expression of GSTT2-2

GSTT2-2 was expressed in *Escherichia coli* as a ubiquitin fusion protein and co-translationally cleaved from the ubiquitin moiety by a co-expressed yeast ubiquitin specific protease (Ubp1) by methods previously described in detail by Baker *et al.* (Baker *et al.*, 1994). Two oligonucleotide primers:-

HT2ExA (5' GTGCCGCGGTGGTATGGGCCTAGAGCTGT 3') and

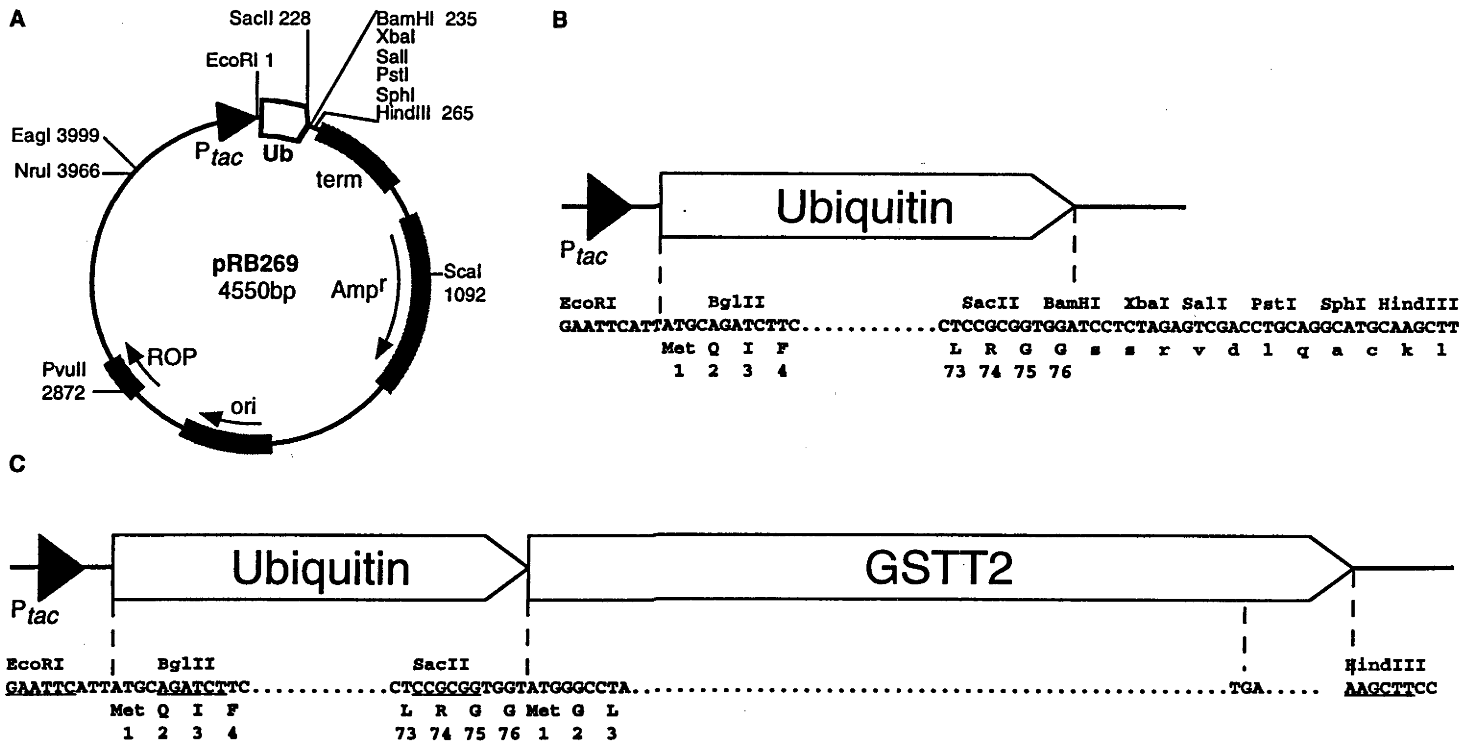
HTExB (5' GGAAGCTTTTGTTGCTAATCTCCTGG 3'), were designed to amplify the coding region of a GSTT2 cDNA clone, pHT4 (See **Chapter 3**) and create *Sac*II and *Hin*dIII sites at the 5' and 3' ends respectively. PCR was performed on a capillary thermal cycler (Corbett Research, Australia) for 30 cycles (95°C 10sec; 50°C 10sec; 72°C 60sec) in a 20μl reaction mix containing 200ng of pHT4, 200μM dNTPs, 10pmol of each primer, 1.5u *Taq* polymerase (Promega) and 1x *Taq* polymerase buffer (Promega). The 790bp PCR product was digested with *Sac*II and *Hin*dIII and cloned into a ubiquitin fusion expression vector pRB269 (Baker *et al.*, 1994). The resulting plasmid pRBKL3, produced a ubiquitin-GSTT2 fusion protein. The cDNA cloned in pRBKL3 was sequenced entirely to confirm that there were no errors introduced during the amplification step. When pRBKL3 was transfected into a strain of *E. coli* expressing the yeast ubiquitin specific protease Ubp1 (Tobias and Varshavsky, 1991), from the plasmid pCG001 (Gilchrist and Baker, personal communication), the amino terminal ubiquitin moiety was co-translationally removed from the fusion protein generating recombinant GSTT2-2 without any additional N-terminal residues. A schematic diagram showing the detailed ubiquitin-GSTT2 construct is presented in Figure 5.1.

5.4.3 Enzyme Purification

A 40L culture of *E. coli* containing pRBKL3 and pCG001, was grown in a large scale fermenter. Growth media contained standard Luria Broth, supplemented with glucose to a final concentration of 6.3g/L. Ampicillin and chloramphenicol were added for selection to a final concentration of 100μg/ml and 34μg/ml respectively. The culture was allowed to grow until log phase (OD_{600} between 0.7 to 0.9) when IPTG was added to a final concentration of 0.1mM and the culture was allowed to grow for another 3

Figure 5.1 : Detail construct of the ubiquitin-GSTT2 expression vector

A: The expression vector pRB269 contains a ubiquitin protein downstream of a P*tac* promoter
B: The ubiquitin fusion expression vetor has the ability to enhance levels of protein expression in a prokaryotic system.
C: The coding region of GSTT2 was amplified from λHT4 clone using a set of primers which contain a *Sac*II and *Hin*dIII sites respectively. These restriction sites enable the construction of ubiquitin-GSTT2 fusion vector.



hours. Bacterial cells were harvested by centrifugation and resuspended in Buffer A (10mM Tris, 1mM EDTA, 0.5mM β-mercaptoethanol, pH 8.25). Cells were lysed by passing through a Ribi cell disruptor and stored frozen in 50ml aliquot at -20°C if not used immediately.

A 50ml sample of cell lysate was diluted with Buffer A to 150ml and centrifuged at 26,000g for 20 minutes. The supernatant was applied to a 29 x 2.5cm DEAE-cellulose column equilibrated in Buffer A. The column was developed using a NaCl gradient from 0-150mM. Fractions containing activity towards cumene hydroperoxide were pooled and concentrated on an Amicon concentrator with a Diaflo PM10 ultrafilter (Amicon Corp., Lexington, MA, USA). The concentrated material was loaded onto a 60 x 3cm Sephacryl S-200 gel filtration column pre-equilibrated in Buffer B (10mM sodium phosphate, 1mM EDTA, 0.5mM β-mercaptoethanol, pH 6.8). Fractions containing activity towards cumene hydroperoxide were again pooled and loaded onto a 8 x 1.5cm Orange A Matrex gel column (Amicon Corp., Lexington, MA, USA) pre-equilibrated in the same buffer. The column was developed using a KCl gradient from 0 to 1M in Buffer B. Fractions possessing activity towards cumene hydroperoxide were pooled and concentrated using a Diaflo PM10 ultrafilter. While concentrating, the sample was desalted and the buffer was changed to Buffer A. The concentrated material was loaded on to a high resolution Mono Q HR 5/5 f.p.l.c. column and developed using two linear salt gradients of 0-20mM and 20-200mM NaCl in Buffer A. Fractions containing activity towards cumene hydroperoxide were pooled. Purified GSTT2-2 was examined on 12% SDS/PAGE by the method of Laemmli (Laemmli, 1970) The gels were stained using a Silver Staining Plus kit (Biorad, CA. USA), following the manufacturer's protocol. Table 5.1 presents a summary of the purification steps.

5.4.4 Enzyme Characterisation

The isoelectric point of GSTT2-2 was estimated by isoelectric focusing on *Phast* gels (Amrad-Pharmacia, Australia). The pH optimum was determined with cumene hydroperoxide as a substrate using a previously described range of buffers (Beutler *et al.*,

1968). N-terminal amino acid sequencing was performed on an Applied Biosystems 477A automated protein sequencer.

5.5 RESULTS

5.5.1 Construction of the GSTT2 Expression Vector

The expression of GSTT2 cDNA was initially attempted using the expression vector, pKK233-2 (Amrad-Pharmacia, Australia). However, the level of protein expression was extremely low. Subsequently, an alternative expression system, utilising the ubiquitin protein fusion system was adopted. The expression of proteins as fusions with ubiquitin has been shown to enhance the levels of protein expression in a bacterial system (Baker *et al.*, 1994). Figure 5.2 outlines the construction of the ubiquitin-GSTT2 fusion expression vector. The expression vector pRB269 contains a yeast ubiquitin cDNA downstream of a *tac* promoter. A multi-cloning site is available immediately after the ubiquitin cDNA for the insertion of cDNAs to be expressed as ubiquitin fusions. The coding region of GSTT2 was amplified from the pHT4 clone using a forward primer which contained a *Sac*II site and a reverse primer containing a *Hin*dIII site. These restriction sites enable the insertion of the GSTT2 cDNA downstream of the ubiquitin gene without any additional residues between the carboxy terminal glycine of ubiquitin and the amino terminal methionine of GSTT2-2 (Figure 5.1).

Initially, a ubiquitin-GSTT2 fusion expression vector that excluded the initial methionine of GSTT2 (using primer-pair HTExA/HTExB) was constructed. However, the level of protein expression was extremely low and the initial methionine of GSTT2 was subsequently encoded in the pRBKL3 expression vector (using primer-pair HT2ExA/HTExB). The ubiquitin-fused GSTT2 was cleaved by the co-expressed Ubp1 at the ubiquitin-GSTT2 junction, yielding a mature GSTT2-2 recombinant protein. N-terminal amino acid sequencing of the purified enzyme did not identify any ubiquitin sequence suggesting that the ubiquitin moiety of the fusion protein had been completely removed by the action of Ubp1. However, the amino acid sequence data yielded two

Table 5.1 : Summary of the purification of recombinant human GSTT2-2

Fraction	Volume (ml)	Total Protein (mg)	Activity		Yield (%)
			Specific (µmol/min/mg protein)	Total (µmol/min)	
Crude extract	125.0	1812.50	0.035	63.37	100.0
DEAE-cellulose	77.0	97.79	0.272	26.57	41.9
Sephacryl S-200	26.0	7.49	1.866	13.97	22.0
Orange A affinity matrix	20.0	0.76	2.175	1.65	2.6
Mono Q f.p.l.c.	2.0	0.068	6.885	0.47	0.7

Note : Specific activity of GSTT2-2 was determined using cumene hydroperoxide as substrate.

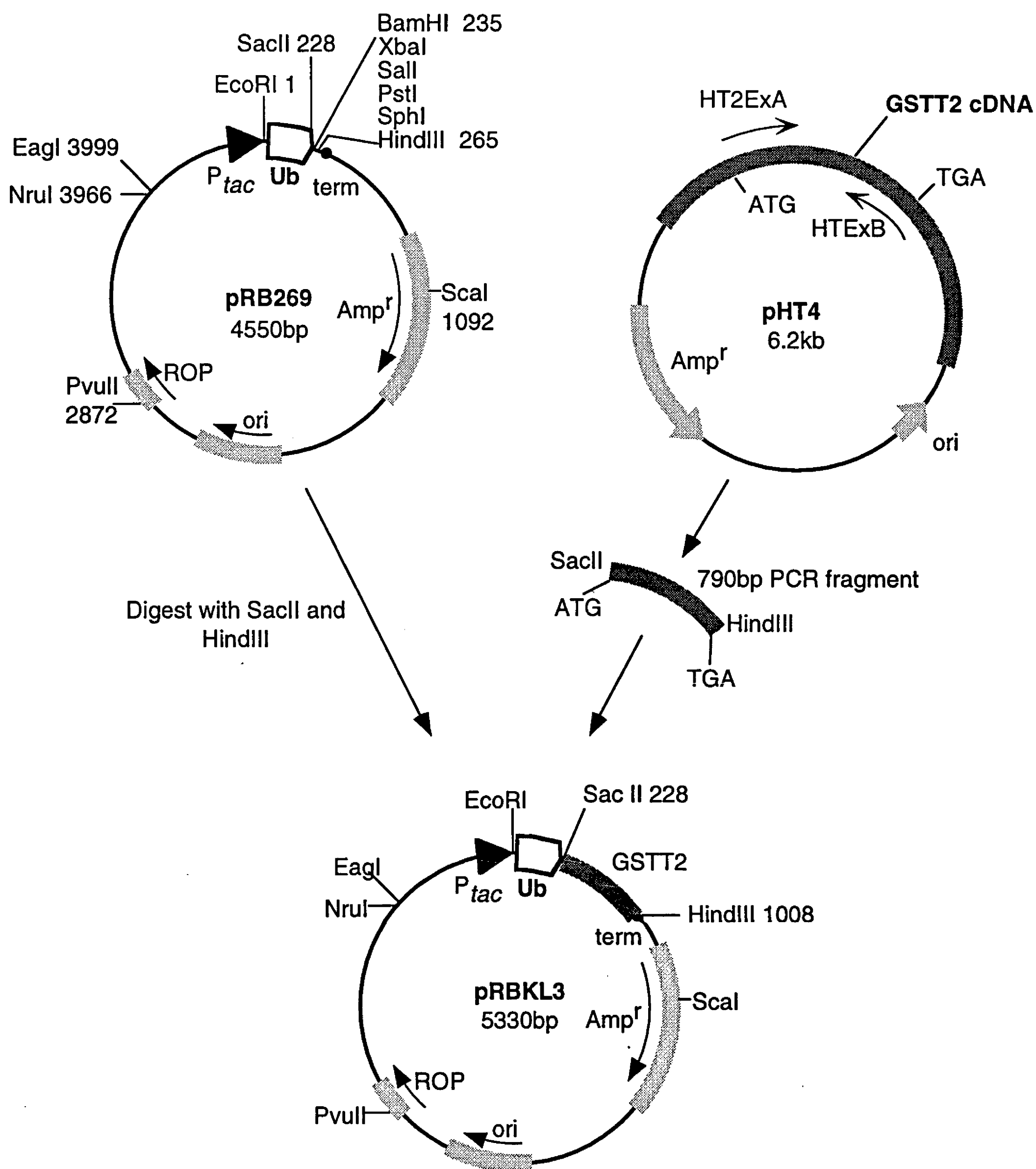


Figure 5.2 : Construction of the GSTT2 expression vector
The coding region of GSTT2 cDNA was amplified from the plasmid pHT4, containing the GSTT2 cDNA (see **Chapter 3**). The forward primer (HT2ExA) contained a *Sac*II site and the reverse primer (HTExB) contained a *Hin*dIII site for directional cloning of the amplified product into the pRB269 expression vector. The recombinant protein was produced as a ubiquitin-GSTT2 fusion protein and subsequently cleaved by the co-expressed ubiquitin protease, Ubp1.

residues of about equal concentration at each position suggesting two proteins were being sequenced. Further analysis of the data indicated that approximately 50% of the preparation had an amino terminal methionine residue followed by the expected GSTT2 sequence (see **Chapter 3**), while in the remaining 50%, the amino terminal methionine had been removed and the GSTT2 sequence started with the second encoded residue. In the experience of this laboratory with other recombinant proteins, it is not uncommon for a variable proportion of amino terminal methionine residues to be removed during expression in *E. coli* (Baker *et al.*, 1994).

The recombinant GSTT2-2 was expressed at a level of 1.6mg/L of culture. Although this rate of expression was able to produce sufficient protein for the present studies, the level of protein expression appears to be low when compared with 60mg/L for GSTP1-1 in the same expression vector (Baker *et al.*, 1994).

5.5.2 Purification of Recombinant GSTT2-2

Recombinant GSTT2-2 was purified to apparent homogeneity by a series of chromatographic steps involving anion exchange on DEAE cellulose, gel filtration on Sephacryl S-200, dye-ligand chromatography on Orange A Matrex gel, and high resolution anion exchange chromatography on Mono Q f.p.l.c. In all the purification steps, protein concentration was monitored by absorbance at 280nm and GSTT2-2 activity was monitored by the measurement of glutathione peroxidase activity with cumene hydroperoxide. Crude bacterial lysate was diluted to 3 vol. and after centrifugation was loaded on to an anion exchange DEAE cellulose. The column was washed with 4 bed volumes of Buffer A before elution with an NaCl gradient from 0-150mM (Figure 5.3). Fractions containing activity towards cumene hydroperoxide were eluted between 50-66mM NaCl.

Sephacryl S-200 was used to separate protein by size and in this case it also served as a convenient step to desalt and exchange buffers (from Buffer A to Buffer B). The separation profile from Sephacryl S-200 yielded 4 major protein peaks. Fractions

containing activity towards cumene hydroperoxide fell only within peak number 2 (Figure 5.4). Samples were pooled and loaded to Orange A Matrex gel (Figure 5.5). The Orange A retained approximately 15% of the protein which exhibited activity towards cumene hydroperoxide. However, about 30% of the protein which was active towards cumene hydroperoxide flowed through the column, presumably as a result of overloading. Therefore, fractions numbered from 13 to 32, containing the 30% flow through protein were rechromatographed after the column had been re-equilibrated. A gradient of KCl from 0-1M was used to develop the column. Fractions found to be active with cumene hydroperoxide were eluted between 0.26 and 0.52M KCl. The final purification step utilised high resolution anion exchange chromatography on a Mono Q f.p.l.c. column. The fractions which contained activity towards cumene hydroperoxide were eluted from the Mono Q column as a single peak between 4 and 12mM NaCl gradient (Figure 5.6). The purity of the protein recovered from Mono Q was analysed on 12% SDS/PAGE. Silver staining of the gel showed there were no contaminating proteins (Figure 5.7). The purified protein had a molecular mass of about 27kD as compared with the standard markers, which was in accordance with the calculated molecular mass of 27,489Da. A summary of the purification steps and the yield of purified protein is given in Table 5.1.

The pH optimum of GSTT2-2 as determined with cumene hydroperoxide was found to be in the range of pH 7.6 to 8.3 (Figure 5.8). Isoelectric focusing in polyacrylamide gels indicated that GSTT2-2 had a isoelectric point between 5.2 to 5.3 (Figure 5.9). In this experiment, the purified recombinant protein did not migrate as a discrete band and was somewhat diffuse suggesting that there was some charge heterogeneity in the purified protein.

5.5.3 Substrate Specificity of GSTT2-2

Table 5.2 outlines the specific activity of GSTT2-2 with a variety of substrates. GSTT2-2 showed peroxidase activity towards cumene hydroperoxide and *tert*-butyl hydroperoxide. However, there was no detectable activity towards hydrogen peroxide. It is notable that GSTT2-2 was active towards unsaturated aldehyde compounds such as the alka-2,4-dienals and alk-2-enals. Further more, the activity with these substrates increased with the length of the carbonyl carbon chain. In line with the earlier report of Hussey and Hayes (1992), the enzyme was also found to be active towards 1-menapthyl sulphate. The specific activity of the recombinant GSTT2-2 with both 1-menapthyl sulphate and cumene hydroperoxide was comparable with the native enzyme purified from human liver. GSTT2-2 has no detectable activity towards dichloromethane (DCM) unlike the other human Theta class isoenzyme GSTT1-1 (Pemble *et al.*, 1994). Finally, low but detectable levels of activity were also demonstrated with ethacrynic acid and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole as substrates.

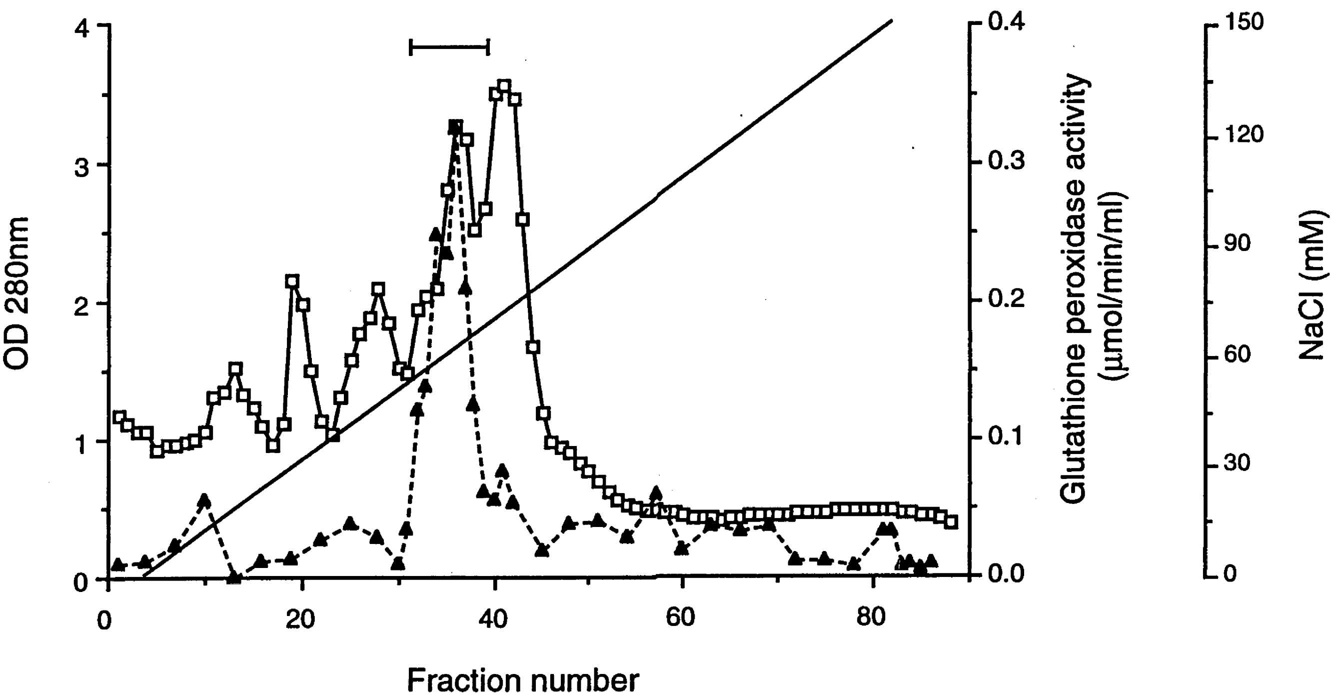


Figure 5.3 : Purification of recombinant GSTT2-2 by DEAE cellulose chromatography
Cell lysate was diluted to 3 vol. and centrifuged at 26,000g for 20 minutes. The supernatant was applied to a DEAE-cellulose column (29 x 2.5cm) equilibrated with Buffer A. A NaCl gradient from 0-150mM was used to develop the column. Fractions of 12ml were collected and GSTT2-2 activity towards cumene hydroperoxide (▲) and absorbance at A280 (□) were monitored. The horizontal bar represents fractions pooled for the subsequent purification step.

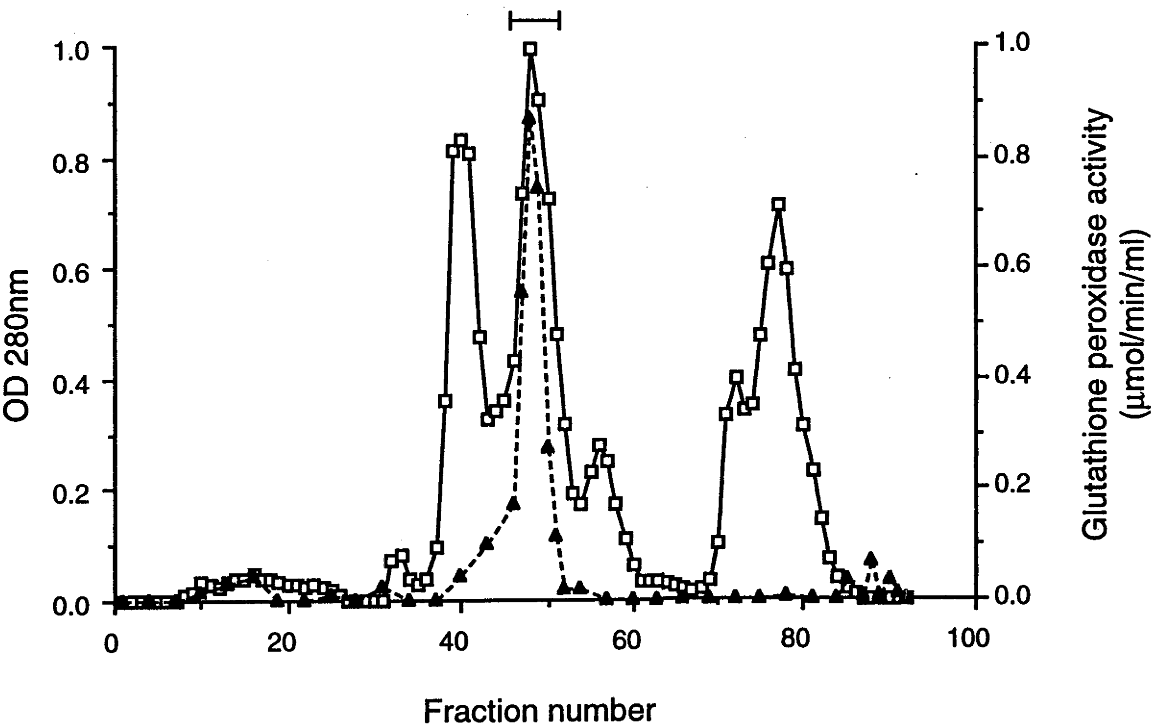


Figure 5.4 : Gel filtration chromatography of recombinant GSTT2-2 on Sephacryl S-200
Pooled fractions from the DEAE cellulose column were concentrated and applied immediately to a Sephacryl S-200 gel filtration column (60 x 3cm). The column was eluted with Buffer B at a flow rate of 1.0ml/min. Samples were collected as 2ml fractions and GSTT2-2 activity was determined using cumene hydroperoxide (▲). Protein concentration was monitored at A280 (□). Active fractions in protein peak number 2, indicated by the horizontal bar, were pooled for further purification.

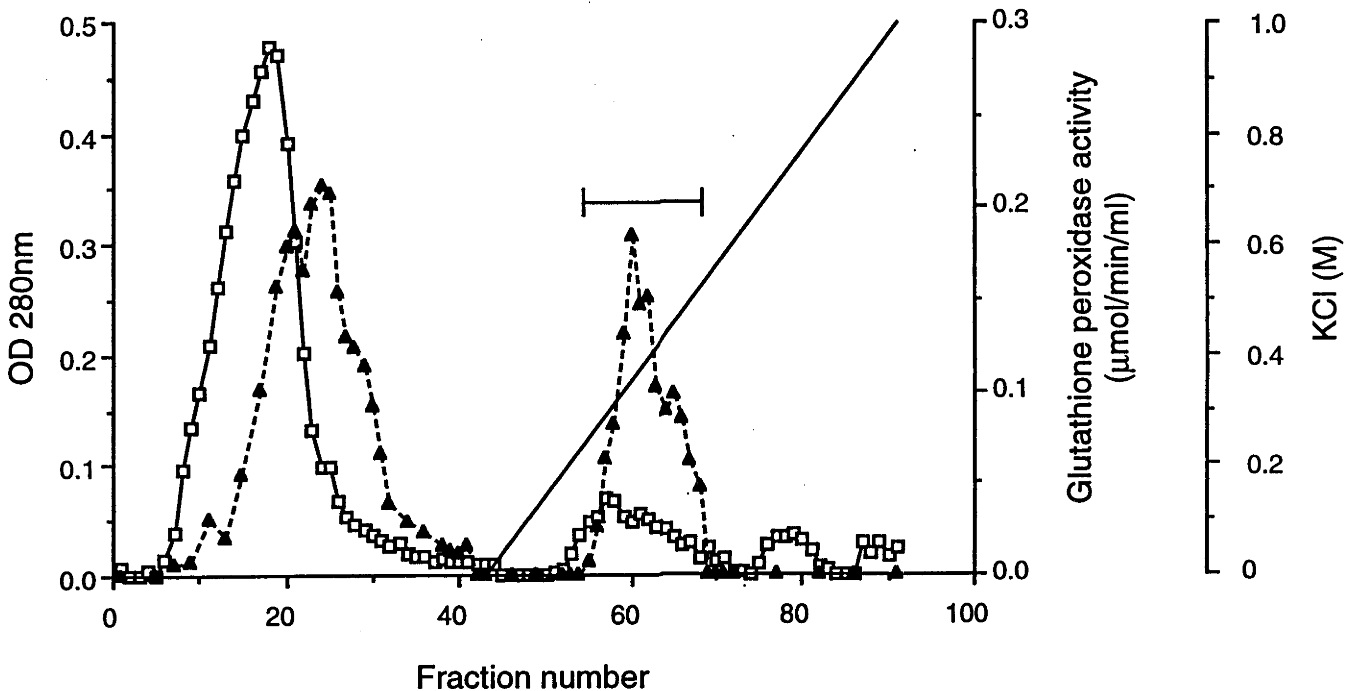


Figure 5.5 : Orange A Matrex gel chromatography
Active fractions from gel filtration chromatography were subjected to dye-ligand chromatography using Orange A Matrex gel. The column was pre-equilibrated in Buffer B and developed with a 0-1M KCl gradient formed in the same buffer. Fractions of 2.5ml were collected and GSTT2-2 activity was assayed with cumene hydroperoxide (▲) and protein concentration monitored at A280 (□). Fractions with activity towards cumene hydroperoxide were pooled as indicated by the horizontal bar.

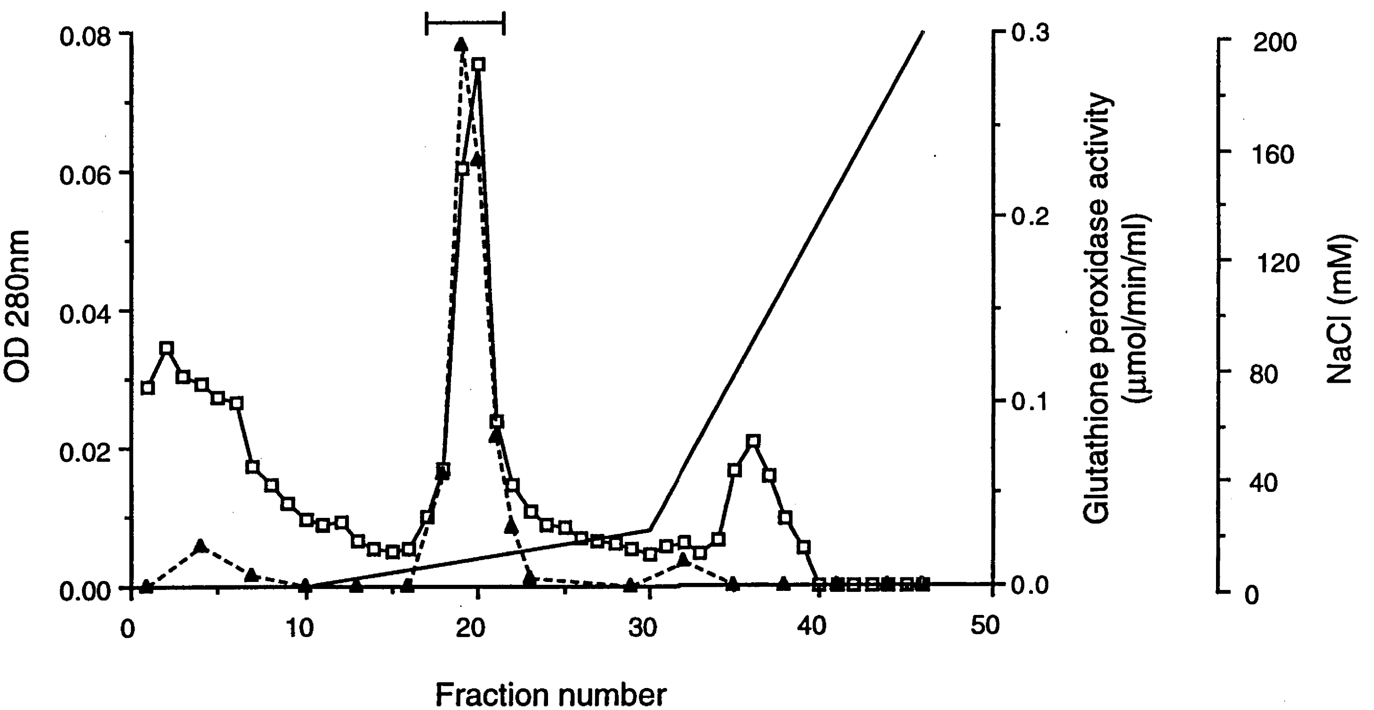
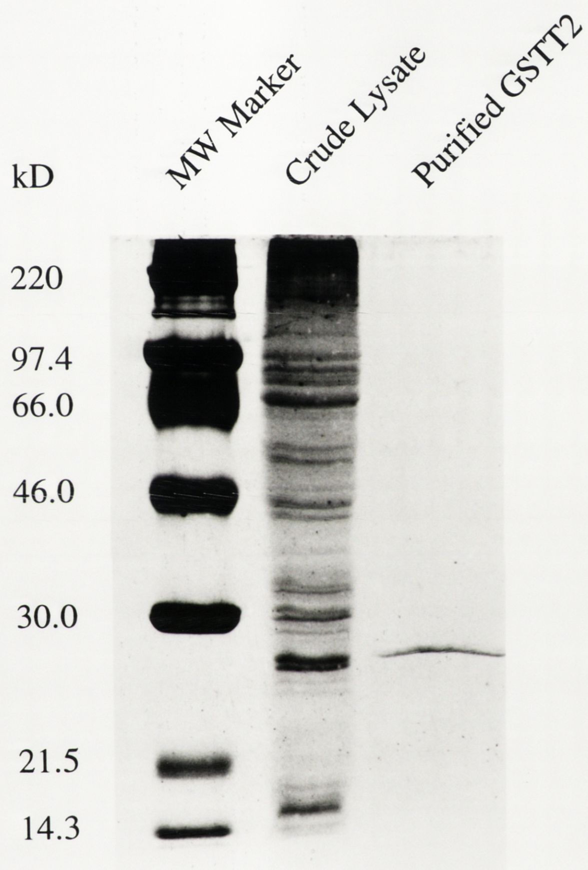


Figure 5.6 : Purification of recombinant GSTT2-2 by Mono Q f.p.l.c. anion exchange chromatography
Pooled fractions from Orange A Matrex gel chromatography were desalted, buffer exchanged from Buffer B to Buffer A during concentration and loaded onto a Mono Q HR 5/5 f.p.l.c.. The column was pre-equilibrated with Buffer A and two NaCl gradients of 0-20mM and 20-200mM were used to develop the column at a flow rate of 0.5ml/min. Fractions of 1.0ml were collected and GSTT2-2 activity towards cumene hydroperoxide (▲) and absorbance at A280 (□) were monitored. Fractions that contained activity towards cumene hydroperoxide were pooled as indicated by the horizontal bar.

Figure 5.7 : SDS/PAGE analysis of recombinant GSTT2-2.

Recombinant GSTT2-2 protein was analysed on a 12% SDS/PAGE gel. GSTT2-2 was purified to apparent homogeneity. The molecular mass of GSTT2-2 in SDS/PAGE as compared with the standard markers is about 27kDa.


kD
MW Marker
Crude Lysate
Purified GSTT2
220
97.4
66.0
46.0
30.0
21.5
14.3

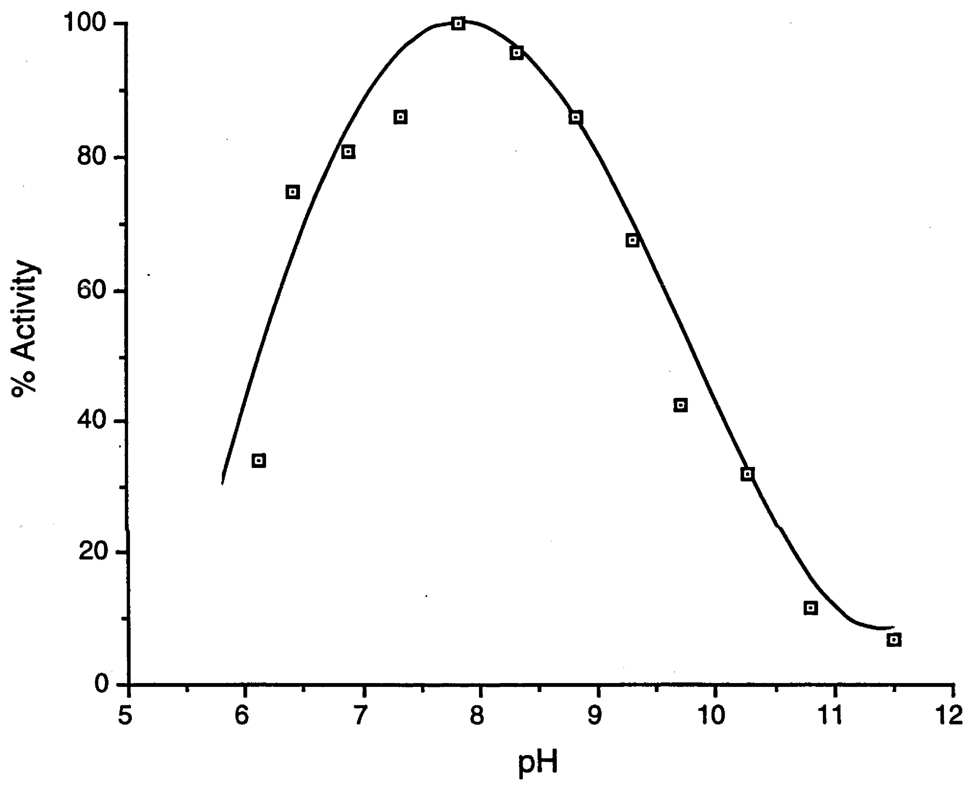


Figure 5.8 : pH profile of GSTT2-2 with cumene hydroperoxide as substrate

Figure 5.9 : Isoelectric focusing of recombinant GSTT2-2

Lane 1 : Recombinant GSTT2-2

Lane 2 : Standard proteins of known isoelectric points

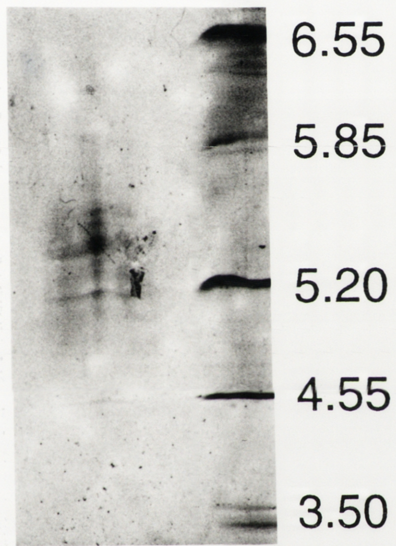

1
2
pI
6.55
5.85
5.20
4.55
3.50

Table 5.2 : Activity of recombinant human GSTT2-2 with various substrates

Substrate	**Specific activity (μmol/min/mg of protein)**
Cumene hydroperoxide	6.885 ± 0.482
tert-Butyl hydroperoxide	0.406 ± 0.042
Hydrogen peroxide	ND
1- menaphthyl sulphate	0.317± 0.028
Ethacrynic acid	0.290 ± 0.020
7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	0.057 ± 0.004
Dichloromethane	ND
1,2-Dichloro-nitrobenzene	ND
1-Chloro-2-4-dinitrobenzene	ND
1,2-Epoxy-3-(4-nitro phenoxy)-propane	ND
4-Phenyl-but-3-en-2-one	ND
2-Cyano-1,3-dimethyl-1-nitrosoguanidine	ND
Hexa-2,4-dienal	ND
trans,trans-Hepta-2,4-dienal	ND
trans,trans-Nona-2,4-dienal	$(50 \pm 3) \times 10^{-3}$
trans,trans-Deca-2,4-dienal	$(113 \pm 15) \times 10^{-3}$
trans-Hex-2-enal	ND
trans-Oct-2-enal	$(68.2 \pm 4.8) \times 10^{-3}$
trans-Non-2-enal	$(119.6 \pm 9.6) \times 10^{-3}$

The mean values ± S.D. were calculated from 4 samples.
ND : Not detectable.

5.6 DISCUSSION

GSTT2-2 was previously purified from human liver (Hussey and Hayes, 1992). A cDNA clone thought to encode GSTT2 was subsequently cloned from a λgt 11 human liver cDNA library (see **Chapter 3**) and the amino acid sequence derived from the cDNA clone was found to be identical to the N-terminal amino acid sequence provided by Hussey and Hayes (1992). The GSTT2-2 purified from human liver by Hussey and Hayes (1992) was active towards both cumene hydroperoxide and 1-menapthyl sulphate and the recombinant enzyme produced in the present study showed similar specific activities with these substrates, confirming that the cDNA described in **Chapter 3**, encodes the isoenzyme described by Hussey and Hayes (1992).

5.6.1 Expression and Purification of Recombinant GSTT2-2

Different expression plasmids that have previously achieved high levels of GST expression were used to express recombinant GSTT2. The highest expression level was obtained in pRBKL3. However, in comparison with the expression levels reported previously with other GSTs (Board and Pierce, 1987; Ross and Board, 1993; Baker *et al.*, 1994) the expression of GSTT2 in pRBKL3 was relatively low. In the earlier cases, the expressed GSTs were rapidly purified in high yield by glutathione affinity chromatography. The apparent low level of GSTT2-2 expression appears to reflect the low recovery of the enzyme during the extensive purification steps required for Theta class GSTs (see Table 5.1 and Hussey and Hayes, 1992). In the present case, the final yield of purified protein does not appear to be a good measure of protein expression in bacterial cells.

5.6.2 Role of GSTT2-2 in Detoxifying Products from Lipid Peroxidation

In the present study we tested the catalytic activity of GSTT2-2 with a number of compounds that have been shown to be substrates for other GSTs. Of particular interest is the activity of GSTT2-2 with secondary lipid peroxidation products such as the alka-2,4-dienals and alk-2-enals. Aldehydic products have been identified in rat liver microsomes as a result of ADP-Fe^{2+} stimulated lipid peroxidation (Esterbauer *et al.*,

1982). These products are known to inhibit several microsomal and plasma-membrane enzymes as well as inhibiting DNA and protein synthesis (Esterbauer *et al.*, 1982). The activity of GSTT2-2 with these lipid peroxidation products together with its glutathione peroxidase activity points to an important role of this enzyme in the protection of cells against the toxic products of oxygen and lipid peroxidation. The specific activity of GSTT2-2 doubled when the carbonyl carbon chain of alka-2,4-dienals and alk-2-enals increased from 8 to 9 or 9 to 10 carbons. Appreciable increases in specific activity with chain length of alka-2,4-dienals and alk-2-enals were also documented for a GST isolated from tapeworm *Moniezia expansa* (Brophy *et al.*, 1989) and the human Mu class GSTs (Ross and Board, 1993). This increased activity may reflect the increased hydrophobicity of the longer carbon chain compounds rendering an easier access to the hydrophobic active site.

The structure of a Theta class GST from *Lucilia cuprina* revealed that the insect GST has a deeper active site compared to other mammalian GSTs (Wilce *et al.*, 1995). Further more, amino acid sequence alignment has suggested that the extended α-helix 5 in the mammalian Theta class isoenzymes would result in an even deeper active site in these enzymes (Wilce *et al.*, 1995). Because of the apparent increased affinity of GSTT2-2 for substrates with increased carbon chain length, and the inability of GSTT2-2 to bind to conventional glutathione/agarose affinity matrices, an attempt was made to purify GSTT2-2 using a GSH-agarose matrix with a very long spacer arm, equivalent to 20 carbon atoms. Unfortunately, this attempt was no more successful than conventional glutathione affinity matrices (Tan and Board, unpublished data).

5.6.3 Role of GSTT2-2 in Detoxifying Reactive Sulphate Esters

The conjugating activity of GSTT2-2 with 1-menapthyl sulphate suggests the possibility of its action against a wide range of reactive sulphate esters such as, 7,12-dihydroxymethylbenz[*a*]anthracene (DHBA) and 5-hydroxymethylchrysene (HCR) as in the case of its rat and mouse orthologues (Hiratsuka *et al.*, 1990; Hiratsuka *et al.*, 1995). Conjugation of these compounds may be an important mode of protection against

hepatocarcinogenesis. The reaction mechanism of GSTT2-2 with sulphate esters will be discussed in greater detail in **Chapter 7**.

5.7 CONCLUSION

Very few of the compounds tested with GSTT2-2 gave significant levels of activity and the natural substrates for this enzyme has yet to be identified. However, the activities observed with 1-menapthyl sulphate, cumene hydroperoxide, alka-2,4-dienals and the alk-2-enals suggest classes of compounds that may include important natural substrates. Activity was also detected with ethacrynic acid as a substrate. The level of activity obtained appears to be intermediate between that of the human Alpha and Pi class GST isoenzymes (Mannervik and Widersten, 1995).

GSTT2-2 did not exhibit dichloromethane (DCM) conjugating activity with GSH. DCM conjugating activity was also lacking in the mouse GST Yrs-Yrs (Hiratsuka *et al.*, 1995). In contrast, the alkyl halides appear to be good substrates for GSTT1-1 and the rat 5-5 Theta class isoenzymes (Pemble *et al.*, 1994, Thier *et al.*, 1993). A genetically determined deficiency of GSTT1-1 occurs in approximately 16% of European Caucasians (Pemble *et al.*, 1994, Warwick *et al.*, 1994, Chenevix-Trench *et al.*, 1995). The absence of any activity of GSTT2-2 with alkyl halides may make the deficiency of GSTT1-1 of greater physiological significance following exposure to this class of compounds.

The role of GSTT2-2 in cellular metabolism is still largely unknown. Its distinct characteristics when compared with GSTT1-1 or members of the other GST classes may indicate that it has a novel functional role.

CHAPTER 6 : TISSUE DISTRIBUTION OF GSTT2-2

6.1 INTRODUCTION

The GST isoenzymes are ubiquitous and distributed in many tissues. Though multiple GST isoenzymes can exist in the same tissue, certain GSTs are expressed more abundantly than others in a particular tissues. For example, the Alpha class GSTs are the predominant GST expressed in human kidney and liver (Wheatley *et al.*, 1994; Meikle *et al.*, 1992). The Mu class GSTM2-2 is found most abundantly in skeletal muscle (Suzuki *et al.*, 1987; Vorachek *et al.*, 1991) while GSTM3-3 is a brain and testis specific GST (Suzuki *et al.*, 1987; Campbell *et al.*, 1990). The tissue distribution of human GSTs has been reviewed in **Chapter 1** of this thesis.

6.2 OBJECTIVES

While human GSTT2-2 has been purified from liver (Hussey and Hayes, 1992) and the cDNA cloned from a human liver library (see **Chapter 3**), nothing is known about its expression in extrahepatic tissues. The objective of this chapter is to gain a greater understanding of the tissues in which GSTT2-2 is expressed by the use of Northern blot and Western blot analysis of a variety of human tissues.

6.3 MATERIALS

Freund's Adjuvant is a product of Gibco BRL, Life Technologies Inc., NY, USA. New Zealand White Rabbit breed at the Animal Services Division of the Australian National University, Australia, was used to raise polyclonal antibodies against GSTT2-2. Goat anti-rabbit IgG was obtained from Sigma Chemical Co. (USA).

6.4 METHODS

6.4.1 Preparation of GSTT2-2 Antiserum

Purified GSTT2-2 from the Mono Q f.p.l.c. purification step (see **Chapter 5**) was used to raise polyclonal antibodies in a rabbit. 7μg of purified GSTT2-2 was mixed with Freund's Complete Adjuvant and injected subcutaneously for an initial dose. The rabbit was given another 2 booster doses of 7μg GSTT2-2 in Freund's Incomplete Adjuvant at weekly intervals. At the end of the 4th and 5th weeks, the rabbit was bled by intravenous puncture of the marginal ear veins of the ear and blood was collected into 10ml sterile glass centrifuge tubes. Approximately 20ml of blood was collected and left to clot at room temperature. Upon clotting, the content of the tubes were carefully freed from the glass wall by a sealed pasteur pipette. The clot was left to retract to about half of its original volume for 2-3 hours at room temperature. Blood serum was aspirated carefully from the clotted material into a sterile glass tube and the remainder of the clotted material was centrifuged at 1500g for 10min at room temperature. The supernatant (serum) was pooled with the previously collected serum and sodium azide was added to a concentration of 0.02% to prevent microbial growth. The antisera was aliquoted into 1.5ml screw cap tubes and stored frozen at -20°C.

6.4.2 Western Blot Analysis

Human tissue samples were obtained at autopsy from individuals who had died from accidental causes. Approximately 2g of tissue from brain, placenta, spleen, lung, liver and kidney were homogenised in a IKA-Ultra-Turrax T25 homogeniser in phosphate buffered saline containing 10mM β-mercaptoethanol. The homogenised samples were centrifuged at 12,000g for 10 minutes and protein concentration of the supernatant was quantitated using the method of Bradford (1976). Approximately 2mg of total protein from the supernatant was resolved on 12% SDS/PAGE and electroblotted onto nitrocellulose membrane as described by Towbin *et al.* (1979). The membrane was probed with rabbit polyclonal antibodies raised against GSTT2-2 and goat anti-rabbit IgG alkaline phosphatase as previously described by Board *et al.* (1984).

6.4.3 Northern Blot Analysis

A Multiple Tissue Northern (MTN) Blot was purchased from CLONTECH Laboratories, Inc., Palo Alto, CA (USA). Each lane of the blot contains approximately 2μg of purified Poly-(A) RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The probe used in the hybridisation was an 860bp *Eco*RI fragment derived from pHT1 (see **Chapter 3**). The *Eco*RI fragment of pHT1 was labelled with [α-^{32}P]dATP by random priming and used as a hybridisation probe. Hybridisation, washing and exposure conditions of the blot were carried out according to the supplier's protocol.

6.5 RESULTS

6.5.1 Tissue Distribution of GSTT2-2

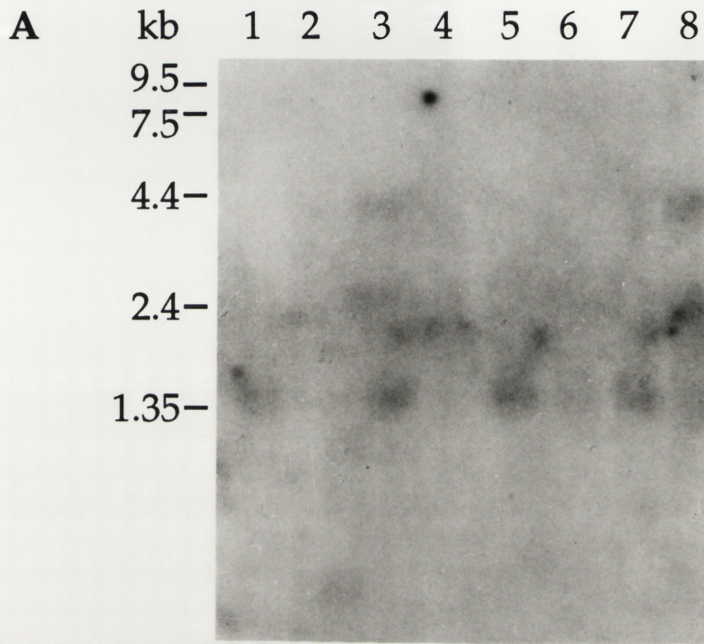
Northern blot analysis of mRNA, obtained from various tissues identified a 1.35kb hybridising band. This experiment suggested that GSTT2-2 was expressed at varying levels in liver, placenta, kidney, pancreas, lung, heart, and skeletal muscle (Figure 6.1, panel A). Although this filter was hybridised on several occasions, only weak signals were ever obtained. This may reflect a naturally low level of GSTT2 message in the tissue samples, or degradation of the fractionated mRNA. Western blots using rabbit antiserum raised against recombinant GSTT2-2 also showed weak staining suggesting that GSTT2-2 is not expressed abundantly in any of the tissues studied. The Western blots confirmed that placenta expressed GSTT2-2 at a level comparable to that in liver (Figure 6.1, panel B). There appeared to be little or no expression of GSTT2-2 in brain as observed from both the Northern and Western Blot results. This observation is rather surprising, in view that the brain is an organ with a high turnover in oxygen metabolism. Perhaps other peroxidases with higher catalytic efficiency may play a more important role in scavenging the products of lipid peroxidation in the human brain.

Figure 6.1 : (A) Northern blot and (B) Western blot analysis

(A) Northern blot of Poly-(A) mRNA from various tissues was probed with the GSTT2 cDNA cloned in pHT1. Tissues from which mRNA was extracted include:- Lane 1: heart; Lane 2: brain; Lane 3: placenta; Lane 4: lung; Lane 5: liver; Lane 6: skeletal muscle; Lane 7: kidney; Lane 8: pancreas. Molecular weight markers are indicated on the side.

(B) Western blot of cytosolic extracts from various tissues probed with antiserum to recombinant GSTT2-2. Cytosolic extracts were from, Lane 1: purified recombinant GSTT2-2; Lane 2: brain; Lane 3: placenta; Lane 4: spleen; Lane 5: lung; Lane 6: liver; Lane 7: kidney. Protein size markers are indicated on the side.

The GSTT2 in placenta appears to be a different size to that in other tissues. This was a consistent finding in three experiments. This result is probably an artefact generated by the presence in placenra of another abundant protein of similar but slightly larger molecular size This protein may displace the GSTT2 in the gel forcing it out of alignment.


A
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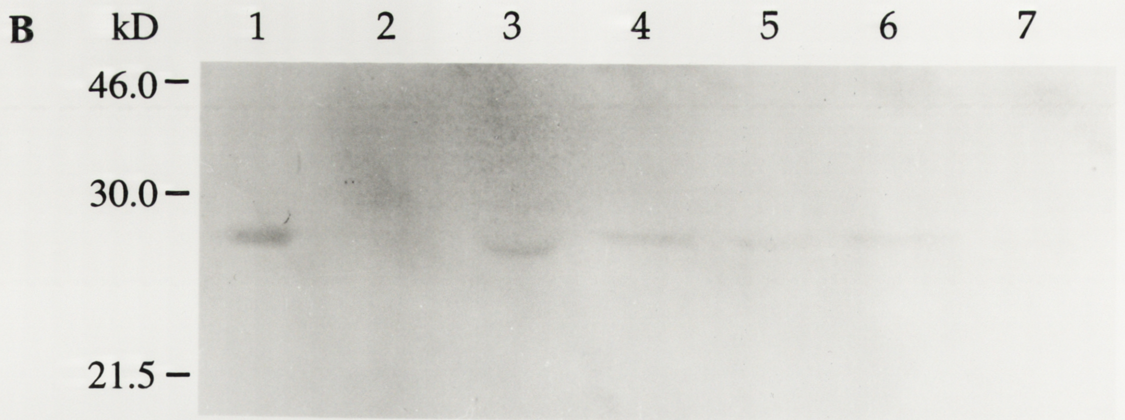
9.5
7.5
4.4
2.4
1.35


B
kD
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2
3
4
5
6
7
46.0
30.0
21.5

6.6 DISCUSSION AND CONCLUSION

Despite the poor signal levels on the Northern and Western blots, GSTT2-2 is evidently expressed in many tissues. It appears to be predominantly expressed in placenta and liver, although detectable amounts of protein or mRNA were also observed in kidney, pancreas, lung, spleen, heart, and skeletal muscle but not in brain. Previous studies by Guthenberg *et al.* (1979) have suggested that GSTP1-1 is the major GST expressed in placenta. That conclusion was based on the activity of the Alpha, Mu and Pi class enzymes with CDNB and their ability to bind to a *S*-hexyl glutathione affinity matrix. The present study indicates that GSTT2-2 is an additional GST expressed in placenta that was previously over looked. Equivalent studies of the distribution of GSTT1 have not yet been reported. However, the rat GST Yrs-Yrs (Hiratsuka *et al.*, 1990) and mouse GST mYrs-mYrs (Hiratsuka *et al.*, 1995) that are orthologous to hGSTT2 are widely distributed in many tissues.

The broad tissue distribution of GSTT2-2 suggests that its role is probably not highly specialised as would be indicated by restricted expression in a specific tissue. Therefore, its role in protection against organic hydroperoxides or secondary products of lipid peroxidation seems highly probable.

Differences in expression of GSTT2 may occur during development however this possibility was not addressed during this study because of a lack of time and the lack of the appropriate samples.

CHAPTER 7: SITE-DIRECTED MUTAGENESIS AND FUNCTIONAL STUDIES OF GSTT2-2

7.1 INTRODUCTION

Mammalian cytosolic glutathione transferases (GSTs) have been grouped into four distinct classes, termed Alpha, Mu, Pi and Theta in a recent consensus nomenclature for the human isoenzymes (Mannervik *et al.*, 1992). Amino acid sequence similarity within the Alpha, Mu and Pi class tends to be around 70% or greater while inter-class sequence similarity is usually less than 30%. The most conserved region lies within the N-terminal half of the protein where the common glutathione binding domain is located, while the C-terminal half shows greater diversity in each isoenzyme. The diversity in the C-terminal sequence is likely to account for the varying specificities for the hydrophobic substrates.

The structure and function of GSTs have been studied by various approaches. Examples of the many studies in this area include chemical modification (Awasthi *et al.*, 1987, Meyer *et al.*, 1993; Xia *et al.*, 1993); site directed mutagenesis (Stenberg *et al.*, 1991, Liu *et al.*, 1992, Kolm *et al.*, 1992, Bjornestedt *et al.*, 1995); construction of deletion mutants and chimeric enzymes (Board and Mannervik, 1991, Zhang *et al.*, 1992; Bjornestedt *et al.*, 1992); photoaffinity labelling (Hoesch and Boyer, 1989, Barycki and Colman, 1993; Cooke *et al.*, 1994); X-ray diffraction studies (Reinemer *et al.*, 1991, Reinemer *et al.*, 1992, Ji *et al.*, 1992, Sinning *et al.*, 1993, Wilce *et al.*, 1995) and NMR spectroscopy (Penington and Rule, 1992). In general, these studies have suggested that the catalytic mechanism of GSTs depends on the ability of the isoenzymes to lower the pKa of the thiol group of glutathione (GSH) resulting in the formation of a glutathione thiolate anion (GS^-) which acts as a nucleophile to attack the electrophilic centres of xenobiotic or endogenous substrates (Chen *et al.*, 1988, Graminski *et al.*, 1989). Studies of the three dimensional structure of the Alpha, Mu and Pi class isoenzymes supported by site directed mutagenesis have attributed this activation role predominantly to an

N-terminal domain tyrosine residue which is conserved through out all cytosolic GST classes. In lowering the pKa of the sulfhydryl group of enzyme-bound glutathione, the hydroxyl group of this tyrosine may function as a general base in abstracting the Sγ hydrogen (Karshikoff *et al.*, 1993) and subsequently stabilising the thiolate anion (GS^-) by hydrogen bonding (Tyr—OH ····· ^-SG) (Liu *et al.*, 1992). Site directed mutagenesis of this tyrosine residue to phenylalanine decreased the CDNB conjugating activity of the mutant enzyme to less than 2% compared with the wild type enzymes of the Alpha, Mu and Pi classes (Stenberg *et al.*, 1991, Liu *et al.*, 1992, Kolm *et al.*, 1992).

The molecular cloning and expression of a cDNA encoding the human Theta class isoenzyme GSTT2-2 has been described in **Chapters 3** and **5**. The protein sequence as translated from the cDNA as well as N-terminal amino acid sequencing (see **Chapter 3** and Hussey and Hayes, 1992) revealed the existence of a phenylalanine at position 9 and the absence of a conserved tyrosine residue near the N-terminal domain. This observation suggested the possibility that GSTT2-2 may utilise another residue in the catalytic mechanism. Recent studies of a Theta-like GST from *Lucilia cuprina* (Wilce *et al.*, 1995, Board *et al.*, 1995) have identified a serine residue that is conserved throughout all the Theta class isoenzymes and appears to be within hydrogen bonding distance of the glutathionyl sulphur. These studies have also shown that the conserved tyrosine in the N-terminal domain (Tyr5) of the *L. cuprina* GST is positioned away from the glutathionyl sulphur and unlike its counterparts in the Alpha, Mu and Pi classes, is unlikely to participate in the catalytic mechanism. Site directed mutagenesis of Ser9 in the *L. cuprina* GST has confirmed its importance in catalysis (Board *et al.*, 1995).

7.2 OBJECTIVES

To evaluate the role played by Ser11 in the catalysis of GSTT2-2 through site-directed mutagenesis and the analysis of a GSTT2 homology model, based on the co-ordinates of the Theta-like GST from *Lucilia cuprina* .

7.3 MATERIALS

Sources of chemicals, reagents and restriction enzymes used in this study have been described in **Chapter 2.** Substrates used in the GST assays were obtained from the following sources : Cumene hydroperoxide and 1-(chloromethyl)napthalene from Aldrich Chemical Co., Wisconsin (USA); reduced glutathione, ethacrynic acid and L-cysteine from Sigma Chemical Co. (St. Louis, MO), glutathione reductase from Boehringer Mannheim (Australia), β-mercaptoethanol from BDH Chemicals Ltd (England), 1-menapthyl sulphate (MS) was synthesised by the method of Clapp and Young (1970). *S*-methylglutathione was synthesised by the reaction of reduced glutathione with methyl iodide at pH9 in an ethanolic solution.

7.4 METHODS

7.4.1 Enzyme Assays

Glutathione peroxidase activity was determined by the procedure described by Beutler (Beutler, 1975). The determination of GST activity with MS was essentially the same as described by Gillham (1971), using an extinction coefficient for *S*-(1-menapthyl)glutathione of $3.9mM^{-1}cm^{-1}$. Glutathione transferase assays using ethacrynic acid were previously described in detail by Mannervik and Widersten (1995). Reaction between L-cysteine or β-mercaptoethanol with MS in the presence of *S*-methylglutathione and GSTT2-2 was measured spectrophotometrically at 298nm in 0.1M Tris.HCl, pH8.3, containing 10mM *S*-methylglutathione, 20mM L-cysteine (or 30mM β-mercaptoethanol) and 0.25mM MS, at 37°C. The extinction coefficient for *S*-(1-menapthyl)-L-cysteine is $4.8mM^{-1}cm^{-1}$ (Gillham, 1971) while the extinction coefficient for *S*-(1-menapthyl)-β-mercaptoethanol ($2.1mM^{-1}cm^{-1}$ at 298mn), was determined by allowing 50nmol of MS to react to completion with an excess of β-mercaptoethanol in the presence of GSTT2-2. Sulphatase activity was determined by measuring sulphate production by a turbidimetric procedure after reaction with $BaCl_2$ in a gelatine solution (Dodgson, 1960).

7.4.2 Site-Directed Mutagenesis

A *BglII/Hind*III fragment of pRBKL3 (see **Chapter 3**) was subcloned into an M13 vector digested with *Bam*HI and *Hind*III to generate single stranded DNA template for site-directed mutagenesis. Oligonucleotides for site-directed mutagenesis of the N-terminal Ser11 were :-

S11A 5' GCTGGGCTGGGCCACCAGGTC 3';

S11T 5' GCTGGGCTGGGTCACCAGGTC 3';

S11Y 5' GCTGGGCTGGTACACCAGGTC 3'.

Mutated residues were underlined. Site-directed mutagenesis was carried out using a *Sculptor*™ *in-vitro* mutagenesis kit (Amersham, Australia). The resulting mutations were confirmed by DNA sequencing using Sequenase™ Version 2.0 DNA sequencing kit (USB, USA) after subcloning the *Sac*II-*Hind*III fragment into pRB269 (Baker *et al.*, 1994). The plasmids bearing the Ser11→Ala, Ser11→Thr and Ser11→Tyr mutations were given the name pRBS11A, pRBS11T and pRBS11Y respectively.

7.4.3 Construction of pQE Expression Vectors

A 354bp fragment was amplified from a GSTT2 cDNA cloned in pRBKL3 using a forward primer, QET2 (5' GCGGATCCGTACCCCGCGGTATGGGCCTAGAGCTG 3') and a reverse primer, HTB3 (5' ATACCAAAGGTGCCACGG 3'). Primer QET2 contained additional *Bam*HI and *Sac*II restriction sites while primer HTB3 was designed 3' to a *Sph*I site, within the GSTT2 cDNA sequence. PCR was performed on a capillary thermal cycler (Corbett Research, Australia) in a 20µl reaction mix containing 200ng of pRBKL3, 200µM dNTPs, 10pmol of each primer, 1.5u of *Taq* polymerase (Promega) and 1x *Taq* polymerase buffer (Promega). PCR conditions were as follows:- A hot start (94°C, 10sec); 20 cycles of (94°C, 10sec; 56°C, 30sec; 72°C, 60sec); 10 cycles of (94°C, 10sec; 56°C, 30sec; 72°C, 80sec); and an extra extension cycle of 72°C for 2 minutes. The amplified product was digested with *Bam*HI and *Sph*I and cloned into the same restriction sites in the QIAexpress System pQE30 (QIAGEN, GmbH, Germany). The resulting plasmid, pQE130 was digested with *Sac*II and *Hind*III and ligated with a *Sac*II, *Hind*III fragment from either pRBKL3, pRBS11A, pRBS11T or pRBS11Y to

generate the wild type and mutant pQE expression vectors. The pQE vector incorporates a tract of 6 histidine residues at the amino terminal end of the expressed protein to facilitate purification (Figure 7.1). The construction of the pQE(GSTT2) expression vectors is described in Figure 7.2 and the expression constructs (wile type and mutants) were termed pQEHT1, pQES11A, pQES11T, and pQES11Y.

7.4.4 Protein Expression and Purification

For the production of recombinant protein, the wild type or mutant expression plasmids were transformed into *Escherichia coli* host strain TG1 and were each grown in a 10L fermenter. Standard Luria Broth supplemented with 100μg/ml of ampicillin was used as the growth medium. IPTG was added to a final concentration of 0.1mM when the density of the cells reached an OD_{600} between 0.7-0.9. Bacterial cells were harvested by centrifugation and resuspended in Buffer A (50mM sodium phosphate, 300mM NaCl, 10% glycerol at pH 6.0). The cells were lysed by passage through a Ribi cell disruptor and stored frozen in 50ml aliquots at -20°C if not used immediately.

The wild type and mutant GSTT2 isoenzymes were purified by immobilised metal ion chromatography. A 50ml sample of cell lysate was diluted with Buffer A to 80ml and centrifuged at 26,000g for 20 minutes. The supernatant was mixed with 20ml of a 50% slurry of Ni-NTA resin (QIAGEN, GmbH, Germany), pre-equilibrated in Buffer A, and allowed to mix overnight on an rotating mixer at 4°C. The resin was washed twice in Buffer A by centrifugation then packed into a Pharmacia XK16 column. The resin was washed with a continuous flow of Buffer A at a rate of 1.0ml/min at 4°C until the OD_{280} of the flow through fraction was less than 0.01. The column was then mounted on to a Pharmacia f.p.l.c. system and developed with three linear imidazole gradients of 0-50mM, 50-100mM and 100-200mM, pH 8.2, formed over a period of 20 minutes each. Each gradient was interspersed by elution for 40 minutes with a constant imidazole concentration equivalent to the concentration achieved at the end of each gradient. Protein concentration was monitored by absorbance at 280nm. Fractions containing the recombinant protein were detected by dot blot on nitrocellulose and probed with rabbit

Figure 7.1 : N-Terminal sequence of pQE expression vector

The intial methionine of the fusion protein is in bold. The pQE expression vector has a 6 histine residue at the N-terminal. The *Bam*HI and *Sac*II sites were introduced to the pQE30 expression vector by the PCR primer QET2. The *Sac*II and *Hin*dIII sites served as convenient restriction sites for shuttling the coding region of the respective cDNAs to the pQE expression vector. The restriction sites are underlined and the coding sequence of the inserted cDNAs and its translated amino acid sequence are in italics.

```
5' ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC
    M   R   G   S   H   H   H   H   H   H

  BamHI           SacII                             HindIII
  GGA TCC GTA CCC CGC GGT ATG GGC CTA GAG CTG....AAGCTT 3'
   G   S   V   P   R   G   M   G   L   E   L
```

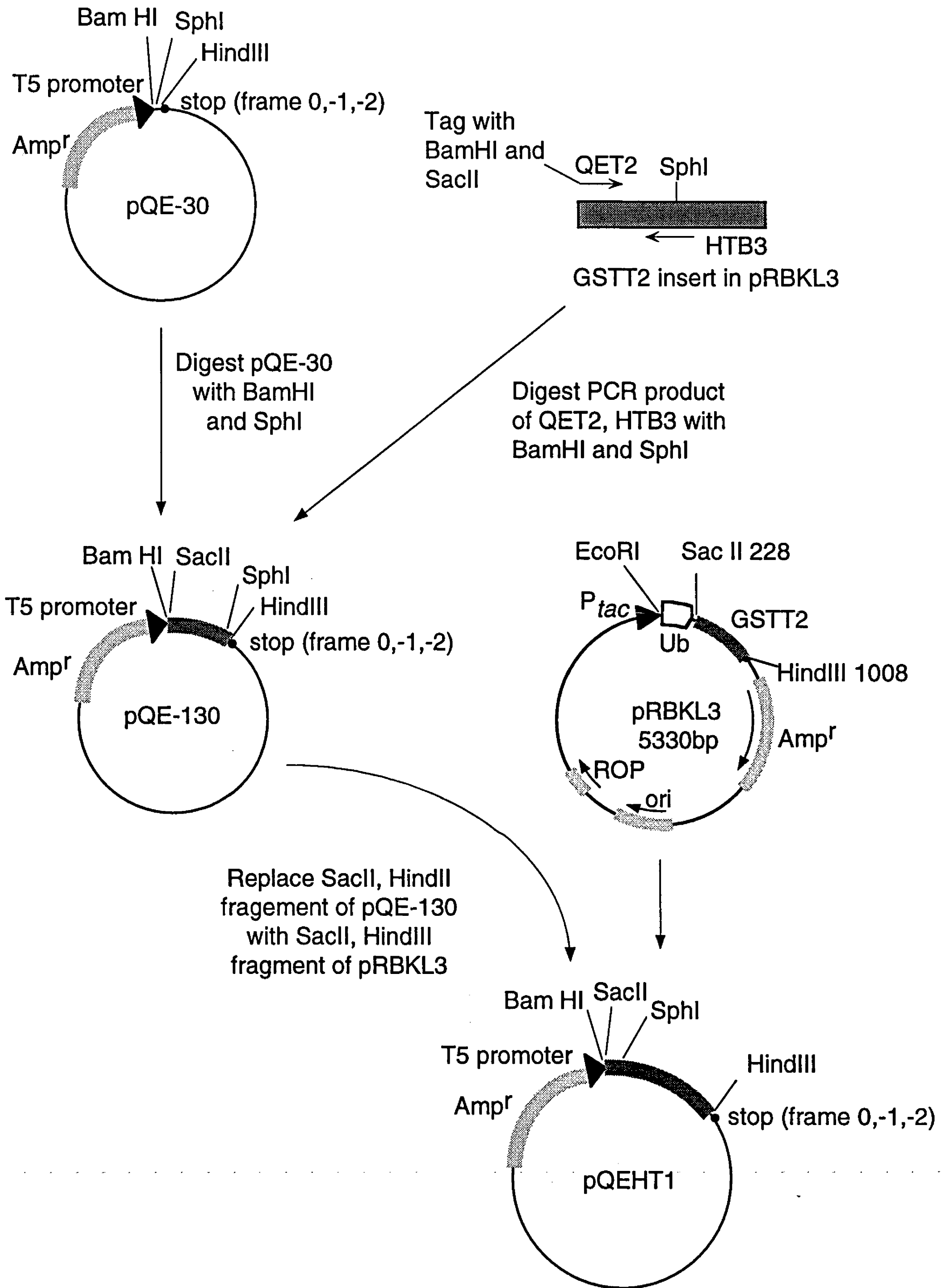

Figure 7.2 : Construction of the pQE expression vector
A forward primer QET2 with additional *Bam*HI and *Sac*II restriction sites and a reverse primer HTB3 were used to amplify a 5' coding region of pRBKL3. The amplified product (354bp) was digested with *Bam*HI and *Sph*I and cloned into the same restriciton sites in the QIAexpress System pQE30. The resulting plasmid pQE-130 was digested with *Sac*II and *Hin*dIII and ligated with the *Sac*II, *Hin*dIII fragment from either pRBKL3, pRBS11A, pRBS11T or pRBS11Y

polyclonal antibodies raised against recombinant GSTT2-2 (**Chapter 6**) and anti rabbit IgG alkaline phosphatase as previously described by Board (1984). Fractions that showed a positive signal with the dot blot were pooled and concentrated with Centricon-30 ultrafiltration apparatus (Amicon Inc, USA) and buffer change to 10mM Tris/1mM EDTA/0.5mM β-mercaptoethanol/pH8.25. To eliminate minor contaminating protein the concentrated samples were loaded on to a Mono Q HR 5/5 f.p.l.c. column equilibrated with 10mM Tris/1mM EDTA/0.5mM β-mercaptoethanol/pH8.25. Under these conditions, the His_6-tagged recombinant GSTT2-2 passed straight through the column while the contaminating proteins were retained.

7.4.5 Determination of Kinetic Parameters and pH Profile

Kinetic parameters for the wild type and mutant enzymes were obtained with cumene hydroperoxide and MS as the second substrates. The values for V_{max} and K_m were determined from the rate-saturation curves of GSH and the second substrates when the concentration of one was fixed while varying the other. Table 7.1 shows the different range of substrate concentrations used in determining the kinetic parameters. The value of K_{cat}/K_m was determined as $V/[S][E]_{tot}$ at low substrate concentrations when the rate saturation curve degenerates to first-order with respect to the concentration of the varied substrate (Mannervik *et al.*, 1985). The molecular mass of the enzyme used in these calculations was 54,978Da. Percentage of activity vs pH profiles for the wild type and mutant recombinant proteins were determined with cumene hydroperoxide or MS as the second substrate, using a previously described range of buffers (Beutler *et al.*, 1968). The non-enzymatic reaction rate at each pH was subtracted from the total recorded reaction rate and the results were expressed as a percentage of the maximum enzymatic rate.

7.4.6 Construction of a Homology Model of GSTT2-2

A homology model of GSTT2-2 was constructed based on the co-ordinates of the recently solved crystal structure of a Theta-like GST from the Australian Blowfly, *Lucilia cuprina*. The computational modelling of GSTT2-2 was carried out in collaboration with

Dr. Gareth Chelvanayagam of the Human Genetics Group, Division of Molecular Medicine, John Curtin School of Medical Research, ANU, Canberra, Australia. Detailed procedures in deriving the homology model have been submitted for publication (Chelvanayagam *et al.*, 1996).

7.5 RESULTS

7.5.1 Protein Expression in pQE Expression System

The pQE expression system offers a convenient means for the purification of recombinant enzymes. The extra 6 histidine residues tagged on the N-terminal of the recombinant protein enable it to be rapidly purified by immobilised metal ion affinity chromatography. The His_6-tagged GSTs were eluted from the column between 100-200mM imidazole, pH8.2. However, there were a few minor contaminating proteins in the region of 23kD and between 30-66kD. The minor contaminants were removed using a Mono Q f.p.l.c. column where the His_6-tagged proteins passed straight through while the other contaminants were retained. The existence of contaminating proteins that co-elute with recombinant His_6-tagged proteins were also reported by other groups (Hengen, 1995). This method of protein expression and purification was adopted because it was anticipated that some mutant proteins would be devoid of GST activity and could be difficult to purify by the multiple chromatography steps which have been used previously to purify recombinant GSTT2-2 (see **Chapter 5**). The degree of purity of the wild type and mutant proteins was evaluated by SDS/PAGE and is shown if Figure 7.3.

7.5.2 Kinetic Studies and pH Profiles

Wild type recombinant GSTT2-2 expressed in the pQE system was compared with the same recombinant protein expressed in a co-translationally cleaved ubiquitin fusion protein system previously described in **Chapter 5**. The GSTT2-2 expressed in the latter system has no additional N-terminal residues. However, both recombinant proteins showed essentially the same specific activity towards cumene hydroperoxide, ethacrynic acid and MS (Table 7.2). This shows that the extra residues on the

N-terminus of the recombinant GSTT2-2 do not interfere significantly with the activity and function of the enzyme. Furthermore, pH curves of GST activity with cumene hydroperoxide or MS showed a similar profile for each enzyme (Figure 7.4A, 7.4B). With this confirmation, it was possible to proceed to study the catalytic characteristics of the wild type and mutant proteins expressed in the pQE system.

The specific activities of each mutant enzyme with cumene hydroperoxide, ethacrynic acid or MS as a substrate are shown in Table 7.2. The S11Y mutant was inactive with all substrates. In comparison, while the S11A and S11T mutants were inactive with cumene hydroperoxide and ethacrynic acid, they were both active with MS as a substrate. In particular the S11A mutant had a higher specific activity than that of the wild type enzyme (1.5 times higher than the wild type). Several kinetic parameters for the wild type enzyme with cumene hydroperoxide as the second substrate are shown in Table 7.3 and the same values for the wild type enzyme and the active Ser11 mutants with MS as a substrate are shown in Table 7.4. Mutating Ser11 to Ala and Thr did not seem to impair the affinity of the enzyme for GSH as shown by the apparent K_m values of the Ser11 mutants, with MS as the second substrate.

The GSH peroxidase activity of pQE-expressed GSTT2-2 varies with pH and adopts the same bell-shaped profile as the recombinant GSTT2-2 expressed in the ubiquitin fusion system (see **Chapter 5,** Figure 5.8), with an optimum pH range between 7.6-8.5 (Figure 7.4A). The pH curve of GSH conjugating activity with MS did not show a similar bell-shaped profile for recombinant GSTT2-2 expressed in either expression system (Figure 7.4B). In this case, the enzyme activity increased as a function of the pH. Similar pH profiles with MS were also obtained with the S11A and S11T mutants. The non-enzymatic rates of reaction that were subtracted from the data in Figure 7.4A and 7.4B are shown in Figure 7.4C. Clearly, unlike most GST substrates, MS does not show increased non-enzymatic reaction rates at high pH.

The non-enzymatic rate of cumene hydroperoxide as substrate did appear to follow a bell-shaped curve with increasing pH. This may have been due to inhibition of the linking enzyme , (glutathione reductase) at high pH. In retrospect it would have been better to demonstrate the nonenzymatic reaction with ethacrynic acid which does not need a separate linking enzyme.

7.5.3 Thiol Specificity of GSTT2-2

Because the reaction between MS and GSH does not involve Ser11 and since its increasing rate with increasing pH resembles a non-enzymatic chemical reaction, the MS-glutathione conjugation reaction was investigated in greater detail. The enzyme shows a high specificity for glutathione as a thiol substrate and compounds such as L-cysteine or β-mercaptoethanol are not normally substrates (Table 7.5). However, as shown in Table 7.5, the specificity for GSH as the reactive thiol can be relaxed if L-cysteine or β-mercaptoethanol are added in the presence of non-reactive *S*-methylglutathione.

7.5.4 GSTT2-2 as a Sulphatase

Sulphate production from MS was determined in the presence or absence of GSH or *S*-methylglutathione. The data in Figure 7.5 show the production of sulphate in the presence of GSH or *S*-methylglutathione plus β-mercaptoethanol. Sulphate was also released in the presence of *S*-methylglutathione alone, however, the rate was only a tenth of that in the presence of GSH. It is possible that in the absence a[illegible] nucleophile [illegible], SO_4^{2-} could reassociate with the carbonium ion of 1-methylnapthalene thus limiting the appearance of free SO_4^{2-}. Essentially no sulphate was released in the absence of GSH or *S*-methylglutathione.

Figure 7.3: SDS/PAGE analysis of wild type and mutant recombinant GSTT2-2 expressed in pQE system

Wild type and mutant recombinant GSTT2-2 proteins were analysed in a 12.5% SDS/PAGE gel on a Phamacia Phast Gel System. All recombinant proteins were purified to apparent homogeneity. The molecular mass of recombinant GSTT2-2 in SDS/PAGE as compared with the standard markers is about 28kD. Lane 1: S11Y mutant. Lane 2: S11T mutant. Lane 3: S11A mutant. Lane 4 : wild type GSTT2-2. Lane 5: molecular weight marker.

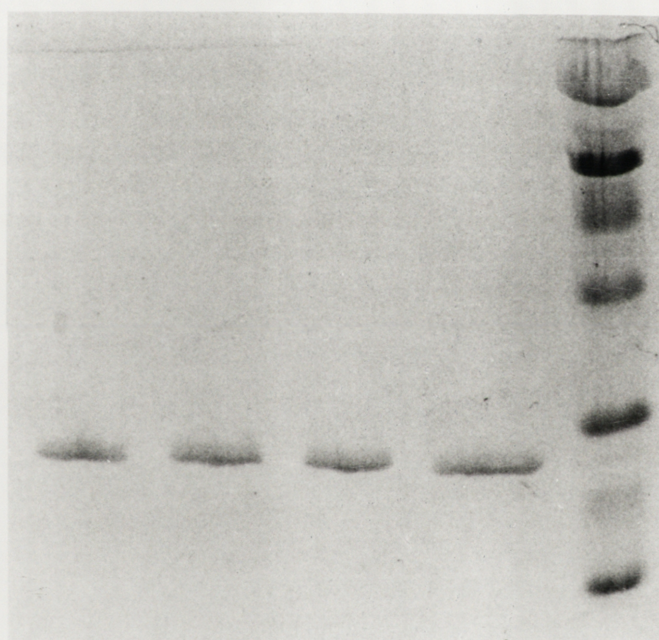
1
2
3
4
5
kD
220
97.4
66.0
46.0
30.0
21.5
14.3

Table 7.1 : Substrate concentrations used in determining the kinetic parameters.

	Second substrate concentration (mM)	GSH concentration (mM)
Cumene hydroperoxide	0.35	0.10 - 15
	0.07 - 0.56	2.0
Ethacrynic acid	0.04 - 3.0	0.25
	0.2	0.1 - 2.0
1-menapthyl sulphate	0.05	0.1 - 4.0
	0.003 - 0.04	5.0

Table 7.2 : Comparison of specific activity between wild type and Ser11 mutants

	Specific activity (μmol/min/mg protein)		
Enzyme	1-menapthyl sulphate	Cumene hydroperoxide	Ethacrynic acid
Wild type (pQE)[a]	0.237 ± 0.017	5.26 ± 0.041	0.159 ± 0.010
Wild type (Ubq)[b]	0.317 ± 0.028	6.89 ± 0.482	0.290 ± 0.020
S11A	0.352 ± 0.021	n.d.	n.d.
S11T	0.179 ± 0.010	n.d.	n.d.
S11Y	n.d.	n.d.	n.d.

The mean values ± s.d. were calculated from at least 3 replicates.
n.d. : non detectable.

a : Wild type recombinant GSTT2-2 expressed in the pQE system.
b : Wild type recombinant GSTT2-2 expressed in the ubiquitin fusion system.

Table 7.3 : Kinetic parameters for recombinant GSTT2-2 with cumene hydroperoxide as second substrate

Enzyme form	V_{max}^{GSH} (mmol/min/mg)	K_m^{GSH} (mM)	K_{cat}/K_m^{GSH} ($mM^{-1}.s^{-1}$)	$K_m^{Cu\text{-}OOH}$ (mM)	$K_{cat}/K_m^{Cu\text{-}OOH}$ ($mM^{-1}.s^{-1}$)
wild type	8.3 ± 0.35	4.3 ± 0.48	1.3 ± 0.01	1.2 ± 0.58	7.7 ± 0.16

Table 7.4 : Kinetic parameters for recombinant GSTT2-2 and its mutant forms with MS as second substrate

Enzyme form	V_{max}^{GSH} (mmol/min/mg)	K_m^{GSH} (mM)	K_{cat}/K_m^{GSH} ($mM^{-1}.s^{-1}$)	K_m^{MS} (mM)	K_{cat}/K_m^{MS} ($mM^{-1}.s^{-1}$)
wild type	0.27 ± 0.019	0.62 ± 0.138	0.24 ± 0.010	0.0048 ± 0.0026	28.92 ± 0.424
S11A	0.38 ± 0.014	0.37 ± 0.049	0.48 ± 0.011	0.0027 ± 0.0001	137.49 ± 7.301
S11T	0.22 ± 0.010	1.10 ± 0.070	0.13 ± 0.012	0.0067 ± 0.0013	19.93 ± 0.231

Results are expressed as mean ± standard error.

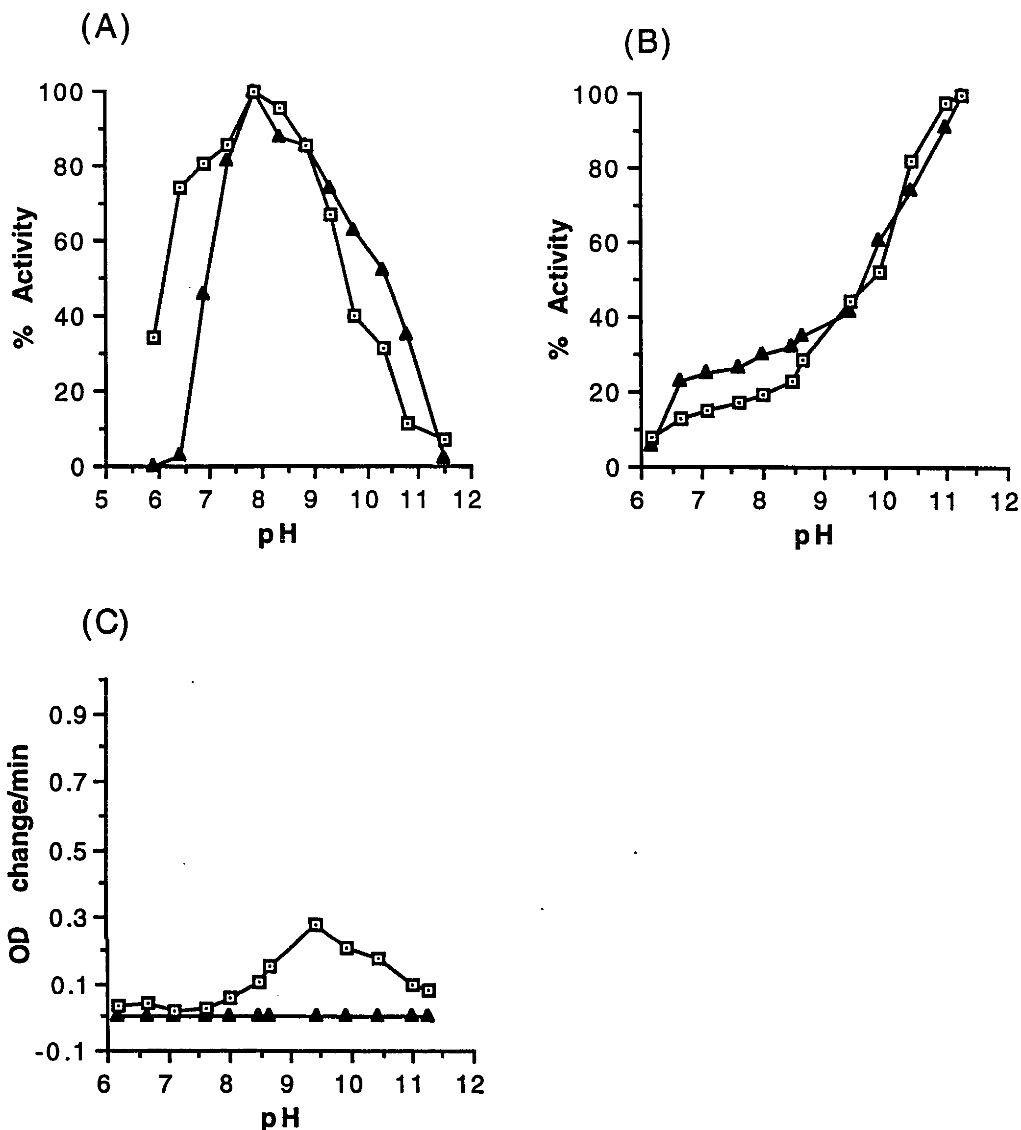

Figure 7.4 : pH profiles of recombinant GSTT2-2 activity with MS and cumene hydroperoxide as substrates and the effect of pH on non-enzymatic reaction rates

Comparison of pH profiles between wild type GSTT2-2 expressed in the pQE system and Ubiquitin-GSTT2 fusion system (**Chapter 5**) with (A) cumene hydroperoxide or (B) menapthyl sulphate as substrate. Activity of the recombinant enzymes are expressed as a percentage of the highest activity obtained. (▲): pQE % activity; (▫): Ubq % activity. (C): Non-enzymatic rates of GSH with either MS (▲) or cumene hydroperoxide (▫) as substrate.

Table 7.5 : Activity of MS with L-cysteine or β-mercaptoethanol as alternative thiol substrate

Reaction mixture	Enzymatic activity (μmol/min/mg protein)
MS + *S*-methylglutathione + β-ME + GSTT2-2	0.3446 ± 0.0249
MS + *S*-methylglutathione + L-Cys + GSTT2-2	0.1670 ± 0.0011
MS + *S*-methylglutathione + GSTT2-2	n.d.
MS + *S*-methylglutathione + β-ME	n.d.
MS + *S*-methylglutathione + L-Cys	n.d.
MS + β-ME + GSTT2-2	n.d.
MS + L-Cys + GSTT2-2	n.d.
MS + GSH	n.d.

The reaction was determined at pH8.3, 37°C, in a 1.0ml cuvette. The final concentration of the respective components are as follows: 0.25mM MS, 0.1M Tris.HCl, 10mM *S*-methylglutathione and 20mM L-cysteine (or 30mM β-mercaptoethanol). Reaction rate was monitored by absorbance at 298nm.

n.d. : not detectable

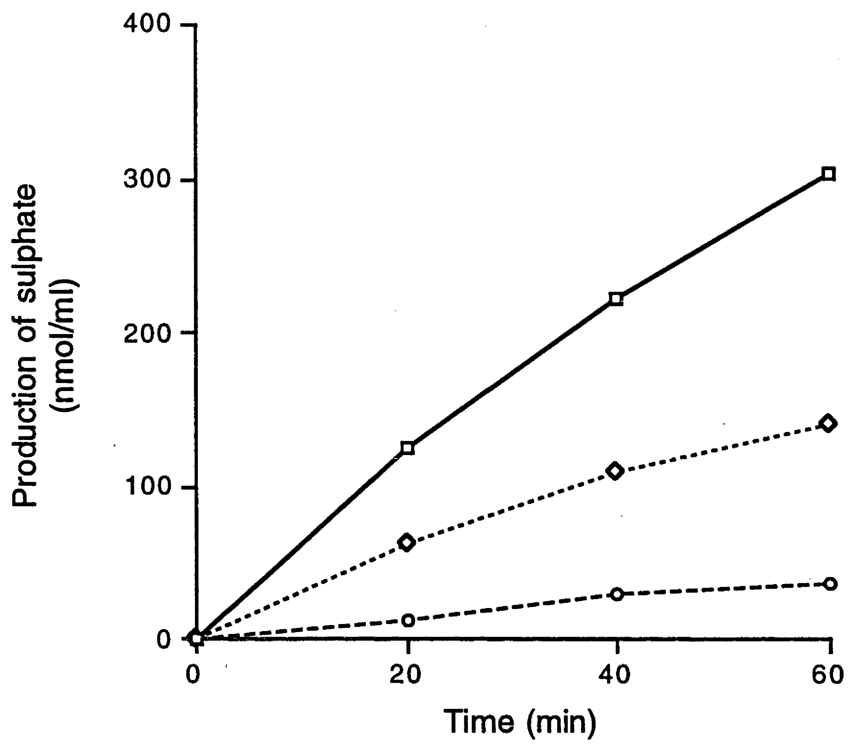

Figure 7.5 : Production of sulphate from MS by GSTT2-2 in the presence of GSH (◻), S-methylglutathione plus β-mercaptoethanol (◇) and S-methylglutathione (○) .

7.6 DISCUSSION

7.6.1 Purification of Recombinant GSTT2-2 with Ni^{2+} Matrix

The mammalian Theta class GSTs are distinct from the Alpha, Mu and Pi classes because they do not utilise the model substrate CDNB and do not bind to glutathione or *S*-hexylglutathione affinity matrices. Therefore, the purification of the Theta class GSTs has been more laborious, taking at least 4 or 5 chromatographic steps to purify the enzymes to apparent homogeneity (Hiratsuka *et al.*, 1990, Hussey and Hayes, 1992, and **Chapter 5**). As a multi-step purification scheme would largely preclude the purification of mutant enzymes with severely diminished activity an alternative expression system was adopted for this study. The QIAexpress system adds 6 histidine residues to the N-terminal of the recombinant protein which facilitates its purification on an immobilised Ni matrix. Comparison of the wild type His-tagged enzyme with the enzyme produced without an N-terminal extension (see **Chapter 5**) did not indicate any substantial differences in enzymatic function. Using the pQE system, wild type and mutant GSTT2-2 isoenzymes were purified to apparent homogeneity in two chromatographic steps.

7.6.2 The Role of Ser11 in Catalysis

Recently, the crystal structure of a Theta-like GST from *Lucilia cuprina* was solved and a serine residue at position 9 from the N-terminal domain was found to be within hydrogen bonding distance of the glutathionyl sulphur atom (Wilce *et al.*, 1995). Site directed mutagenesis of this residue to alanine or threonine lead to a marked decrease in GSH conjugating activity with CDNB, confirming the importance of Ser9 in catalysis (Board *et al.*, 1995). Based on the co-ordinates for the *Lucilia cuprina* GST structure and an alignment of all the Theta class amino acid sequences, a homology model of GSTT2-2 had been constructed (Chelvanayagam *et al.*, 1996). As shown in Figure 7.6, the hydroxyl of Ser11 appears to be within hydrogen bonding distance of the enzyme-bound glutathionyl sulphur. Replacement of Ser11 by site-directed mutagenesis to Ala, Thr or Tyr showed that this serine residue is essential for the GSH peroxidase activity of

GSTT2-2, as all three mutant enzymes lost activity completely with cumene hydroperoxide as the second substrate. A similar loss of activity was also observed for all Ser11 mutants when ethacrynic acid was used as the second substrate (Table 7.2). Therefore, it appears that Ser11 plays an important role in the catalysis with cumene hydroperoxide and ethacrynic acid, presumably by activating the enzyme-bound GSH to form a reactive glutathione thiolate anion (GS^-), in a similar mechanism to that of the N-terminal domain tyrosine found in the Alpha, Mu and Pi class GSTs.

An interesting contrary observation was noted when MS was used as the second substrate. The Ser11→Ala mutant showed a marked increase in specific activity, as well as a higher affinity for GSH and MS. When the side chain hydroxyl group of residue 11 was preserved by replacing Ser11 with Thr or Tyr, a decrease in specific activity was observed that correlated with the size of the substituting residue. The Ser11→Thr mutant retained 75% of the MS conjugating activity compared to the wild type, while replacement of Ser11 with a bulky residue like tyrosine abolished the conjugating activity of GSH with MS completely. These data suggest that the hydroxyl side chain of Ser11 is of little consequence in reaction with MS and that the size of the side chain on residue 11 is particularly significant. A favourable conformation of tyrosine substituted in position 11 in the homology model suggests that this mutation would occupy part of the GSH binding site. This may well explain its inactivity with cumene hydroperoxide, ethacrynic acid and MS. The K_{cat} value for MS in the Ser→Ala mutant was 3 fold higher (0.3712 s^{-1}) than the wild type, whereas the K_{cat} value for the Ser→Thr mutant is comparable to the wild type (~0.1362s^{-1}). This suggests that the high $K_{cat}/K_m{}^{MS}$ value for the S11A mutant is due to a faster product release as well as a higher affinity for MS as the second substrate. It is possible that the elimination of the side chain hydroxyl group at residue 11 facilitates the entry of substrates as well as the release of products.

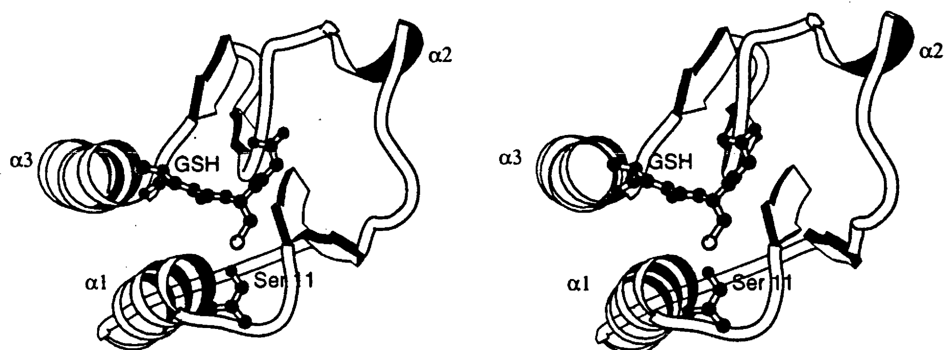

Figure 7.6: A schematic stereo illustration of the N-terminal domain of recombinant GSTT2 highlighting the potential interaction between the GSH thiol and side chain of Ser11. The figure was generated by the program MOLSCRIPT (Kraulis, 1991) from the co-ordinates of a homology model of GSTT2 (Chelvanayagam *et al.*, 1996) based on the structure of a *L. cuprina* GST (Wilce *et al.*, 1996).

7.6.3 Reaction Mechanism with MS

The conjugation reaction between GSH and MS may employ a different reaction mechanism and clearly involves different residue(s) in the active site. In most cases, substrates of GSTs are electrophiles that will react non-enzymatically with the glutathione thiolate anion (GS^-) at high pH. This is clearly not the case with MS, as even at high pH when GSH should be fully deprotonised, MS does not react non-enzymatically with GSH (Figure 7.4C). However, the conjugation rate between GSH and MS which increases as a function of pH in the presence of GSTT2-2 (Figure 7.4B), appears to be like a chemical reaction between a thiolate anion and a reactive electrophile. These observations suggest that the reaction between GSH and MS is not purely enzymatic. In this reaction, the enzyme may modify the MS to a form that is reactive with GS^-, which is increasingly present at elevated pH approaching the pKa (~9) of glutathione in aqueous solution (Jung *et al.*, 1972).

Gillham (1973) reported the purification of a rat liver GST which has GSH conjugating activity with MS. This rat GST is likely to be the rat GST Yrs-Yrs isoenzyme (Hiratsuka *et al.*, 1990), an orthologue of human GSTT2-2, and the only rat liver GST known to utilise MS as a substrate. Gillham suggested the reaction between GSH and MS followed an Ordered Bi Bi mechanism in which GSH is first added to the enzyme before MS. The order of product release is SO_4^{2-} followed by *S*-(1-menaphthyl glutathione. If the reaction of GSTT2-2 also follows an Ordered Bi Bi mechanism, then GSH has to be bound with the enzyme before MS. It is proposed in this study, that GSH must be added first to the enzyme to induce a conformational change. This is followed by the entry of the second substrate, MS. The SO_4^{2-} group of MS is then removed by the enzyme in a mechanism similar to the action of a sulphatase. Removal of SO_4^{2-} releases the newly formed 1-menapthyl carbonium ion which reacts with the thiolate anion of GSH to form *S*-(1- menaphthyl glutathione. The increase in the reaction rate between MS and GSH at elevated pH can therefore be explained by the reaction between the carbonium ion and the GS^- species which predominates at high pH. The reaction between the carbonium ion and the GS^- may take place in free solution since

Ser11 which stabilises the bound GSH as a thiolate anion appears not to take part in the reaction.

7.6.4 Relaxed Thiol Specificity of GSTT2-2

If the proposed GSH-induced conformational change is true, then a GSH analogue should be able to fulfil a similar role and the 1-menapthyl carbonium ion (electrophile) released following the removal of SO_4^{2-}, should be capable of reacting with an alternative thiol substrate (nucleophile). To test this hypothesis, GSH was replaced with its analogue, *S*-methylglutathione, while L-cysteine or β-mercaptoethanol were used as the alternative thiol substrates. As shown in Table 7.5, there was no detectable activity in the absence of either *S*-methylglutathione, L-cysteine, β-mercaptoethanol or GSTT2-2. However, when *S*-methylglutathione was added in combination with either L-cysteine or β-mercaptoethanol in the presence of MS and GSTT2-2, the formation of MS-thiol conjugates could be detected spectrophotometrically. Principato *et al.* (1988) reported the relaxed thiol substrate specificity of the rat GST4-4 which utilised β-mercaptoethanol as an alternative thiol substrate for reaction with 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of *S*-methylglutathione. They proposed a glutathione-induced conformation change in the enzyme, rendering a relaxed thiol specificity for any sterically acceptable thiol substrate(s). However, it is not clear in their studies whether β-mercaptoethanol and the carbonium ion of 2,4-dinitrobenzene were enzyme-bound during this reaction, or if the carbonium ion is released to react in free solution. It is unlikely that alternative thiol substrates could occupy the common glutathione binding site since glutathione needs to reside at this site to induce the proposed conformation change. Therefore, the relaxed thiol specificity of GSTT2-2 could be explained as the reaction between the carbonium ion and the alternative thiol substrates in free solution.

7.6.5 GSTT2-2 as a Glutathione Dependent Sulphatase

The removal of the sulphate (SO_4^{2-}) moiety of MS precedes the formation of the 1- menaphthyl carbonium ion. As shown in Figure 7.5, GSTT2-2 catalysed the release of SO_4^{2-} from MS in the presence of GSH or *S*-methylglutathione. The reaction between

GSH and MS can be regarded as a typical substitution reaction catalysed by GST, with the concomitant release of SO_4^{2-} and the *S*-(1-menapthyl)glutathione conjugate. However, when *S*-methylglutathione was used as a glutathione analogue, GSTT2-2 also catalysed the removal of the sulphate moiety of MS. While the standard reaction between GSH and MS catalysed by GSTT2-2 results in the production of *S*-1-menapthyl)glutathione conjugate, the reaction between *S*-methylglutathione and MS does not result in the formation of any glutathione conjugate. In this case, the unconjugated products released from the enzyme are the carbonium ion and SO_4^{2-}. Indeed SO_4^{2-} release was assayed by direct measurement of SO_4^{2-} production and the carbonium ion release was measured by an indirect assay through the conjugation with alternative thiol substrates. SO_4^{2-} production was not detected in the absence of GSH or *S*-methylglutathione, suggesting that the sulphatase activity of GSTT2-2 was a glutathione (or *S*-methylglutathione) dependent reaction. This observation was in agreement with the GSH- (or *S*-methylglutathione-) induced conformational change, conferring the sulphatase activity of GSTT2-2.

In order to further investigate the function of GSTT2-2 as a glutathione transferase and/or as a sulphatase, 1-(chloromethyl)napthalene was used as an alternative substrate for MS. 1-(chloromethyl)napthalene resembles MS in structure but only differs in the methyl side chain group, substituting Cl^- for the SO_4^{2-} group. As a GST, GSTT2-2 should catalyse the removal of the chloride group on the methyl side chain and give rise to *S*-(1-menapthyl)glutathione, in a same fashion as the reaction between MS and GSH. However, there was no detectable activity with this substrate, suggesting the removal of the sulphate group on the methyl side chain of MS was a specific reaction. Similarly, Gillham (1971) demonstrated that a rat liver GST which utilised a range of aralkyl sulphate esters as substrates including benzyl sulphate and 1-menapthyl sulphate, did not utilise non-sulphate ester substrates such as benzyl orthophosphate, 1-menapthyl acetate or 1-menapthyl alcohol.

In view of the results obtained in the present study, it is proposed that the route for inactivation of aralkyl sulphate esters by GSTT2-2 as well as the rat and mouse orthologues GST Yrs-Yrs (Hiratsuka *et al.*, 1990; Hiratsuka *et al.*, 1995) involves binding of glutathione, desulphation, formation of a carbonium ion, conjugation with GSH or a thiol substrate and the subsequent breakdown of the conjugated product in the mercapturic acid pathway (Clapp and Young, 1970). Although it has been shown *in vitro* that there is a relaxed specificity for the nucleophilic substrate in the presence of glutathione, it is highly likely that GSH acts as the nucleophile *in vivo* as it is likely to be the most abundant nucleophile in the intracellular environment.

Studies of the Alpha and Mu class GSTs have shown that residues other than the conserved tyrosine can contribute significantly to catalysis (Bjornestedt *et al.*, 1995, Johnson *et al.*, 1993). In the case of GSTT2-2, residue(s) other than Ser11 must be responsible for its apparent sulphatase activity with MS. Although the homology model of GSTT2-2 predicts the position of GSH, the position or orientation of MS in the active site cannot be predicted with certainty. This was partly due to the lack of certainty in predicting the C-terminal secondary structure of GSTT2-2 in which the C-terminal domain of GSTT2-2 extends beyond that of the Theta-like GST of *L. cuprina*. This unfortunately precludes the reliable identification of residues that could contribute to the sulphatase activity.

7.6.6 Glutathione-Induced Conformational Change of GSTT2-2

The data suggest the reaction between MS and GSH is preceded by a conformation change or change in the electrostatic environment of the active site induced by the binding of GSH or *S*-methylglutathione. An example of a similar substrate induced modification is provided by the relatively flexible α2 helix of the Pi class GSTs which appears to undergo a conformational change upon the binding of GSH in the G-site (Wilce and Parker, 1994, Lo Bello *et al.*, 1993). Furthermore, the three dimensional structural analysis of human GSTA1-1 has suggested that the α9 helix which is otherwise disordered in the apo-form, forms a lid over the H-site upon binding of the

hydrophobic substrate (Sinning *et al.*, 1993). The GSH- (or *S*-methylglutathione-) dependent sulphatase activity of GSTT2-2 and the relaxed thiol specificity of GSTT2 in the presence of *S*-methylglutathione support the GSH-induced conformational change hypothesis and the role of GSTT2-2 as a glutathione dependent sulphatase.

7.7 CONCLUSION

Amino acid sequence comparison of the Theta class isoenzymes has identified a conserved serine residue in the N-terminal domain (Wilce *et al.*, 1995). This conserved serine has been implicated in the activation of the enzyme-bound glutathione (Board *et al.*, 1995). Mutating the equivalent serine (residue 11) of GSTT2-2 to Ala, Thr or Tyr abolished the catalytic properties of GSTT2-2 with cumene hydroperoxide and ethacrynic acid. However, with 1-menapthyl sulphate (MS) as the second substrate, the specific activity of the S11A mutant was elevated 1.5 times, the S11T mutant retained 75% of the wild type specific activity and the S11Y mutant was inactive. The role of Ser11 in catalysis seems to vary with different second substrates. In the substitution reaction with MS, GSTT2-2 activity appears to depend on the size of the Ser11 replacement rather than the presence of a side chain hydroxyl. In addition, the reaction rate appears to be a function of pH, and there is no non-enzymatic reaction even at high pH.

As a reaction between MS and an alternative thiol such as L-cysteine or β-mercaptoethanol can take place in the presence of *S*-methylglutathione and GSTT2-2, it is proposed that the catalytic activity of GSTT2-2 with MS is preceded by a conformational or charge modification to the enzyme upon the binding of glutathione or *S*-methylglutathione. This is followed by the binding of MS and the subsequent removal of the sulphate group, giving rise to the carbonium ion of 1-methylnapthalene as the electrophile to react with the nucleophilic species. The reaction mechanism of GSTT2-2 with MS appears to represent a novel function of GSTT2-2 as a glutathione dependent sulphatase.

CHAPTER 8 : CONCLUDING REMARKS AND FUTURE DIRECTIONS

Much of the knowledge on the mammalian Theta class GSTs comes from the work carried out in the laboratories of Ketterer (Meyer *et al.*, 1991; Pemble and Taylor, 1992; Thier *et al.*, 1993; Pemble *et al.*, 1994; Wiencke *et al.*, 1995), Watabe (Hiratsuka *et al.*, 1990; Ogura *et al.*, 1991; Ogura *et al.*, 1994; Hiratsuka *et al.*, 1994; Hiratsuka *et al.*, 1995) and Board (Tan *et al.*, 1995; Tan and Board, 1996; Board *et al.*, 1995; Board *et al.*, 1994; Wilce *et al.*, 1994; Wilce *et al.*, 1995; Whittington *et al.*, 1996). The research conducted in this thesis, has contributed to a greater understanding of the human Theta class isoenzyme GSTT2-2. It described the molecular cloning of the cDNA encoding the human liver GSTT2*-2* which was first purified by Hussey and Hayes (1992). This cDNA with an open reading frame of 732bp, encodes a polypeptide of 244 amino acids, represents the longest mammalian GST protein characterised to date. The isolation of 3 partial cDNA clones of GSTT2-2 showed evidence of alternative splicing in GSTT2 mRNA transcripts. As alternative splicing had also been observed in GSTM4, the discovery of similar events in GSTT2 suggest that alternative splicing may play a role in the regulation of the GST supergene family. It is proposed from the results of this study that, alternative splicing may represent a post-transcriptional regulatory mechanism for *GSTT2*. Further investigations need to be carried out to confirm this possibility. Studies on gene regulation at the post-transcriptional level should also be extended to the other GST classes.

GSTT2 has been mapped to chromosome 22 by somatic cell hybrid analysis and sublocalised to q11.2 by *in situ* hybridisation. There was no evidence for reverse transcribed pseudogenes of *GSTT2* dispersed through out the genome. Southern blot analysis of human genomic DNA revealed that the gene of *GSTT2* is less that 4kb in length. In a current ongoing project in this laboratory, the *GSTT2* gene has been isolated using the GSTT2 cDNA as a probe and the characterisation of the human *GSTT2* gene is currently in progress.

The heterologous expression of the GSTT2 cDNA has allowed a more extensive characterisation of its protein. The specific activities of recombinant GSTT2-2 with cumene hydroperoxide and 1-menapthyl sulphate (MS) are comparable to those of natural GSTT2-2 purified from human liver (Hussey and Hayes, 1992). The most active substrate tested with GSTT2-2 was cumene hydroperoxide. GSTT2-2 was also found to be active towards the alka-2,4-dienals and alk-2-enals. In view of the peroxidase activity of GSTT2-2 and its activity towards aldehydic compounds, which are secondary products of lipid peroxidation, the role of GSTT2-2 in protecting cells against the products of lipid peroxidation seems probable. Like the other members of subfamily 2 of the Theta class, GSTT2-2 exhibits activity towards MS. An area for future studies is to investigate the activity of GSTT2-2 with reactive sulphate esters arising from carcinogenic arylmethanols such as the 7-hydroxymethyl benz[a]anthracene, 7-hydroxymethyl-12-methyl-BA (7-HMBA), 12-hydroxymethyl-7-methyl-BA (12-HMBA), 7,12-dihydroxymethyl-BA (DHBA) and 5-hydroxymethylchrysene (5-HCR) and to establish a relationship between the role of GSTT2-2 and the carcinogenicity of the reactive sulphate esters. Lipid hydroperoxide substrates such as arachidonic acid 15-hydroperoxide, linoleic acid 13-hydroperoxide and linolenic acid 13-hydroperoxide may represent a category of endogenous substrates of GSTT2-2 which can be evaluated in future studies.

The expression of GSTT2-2 is not restricted to a specific tissue but was found to be expressed in liver, placenta, kidney, pancreas, lung, spleen, heart and skeletal muscle. Liver and placenta were the tissues with the most abundant expression. On the contrary, there was no detectable expression in brain. The broad tissue expression of GSTT2-2 is consistent with its general protective role in scavenging hydroperoxides and aldehydic products arising from oxygen metabolism. Whether GSTT2-2 is subject to sex-dependent or developmental-dependent expression is unknown at this stage. The cell types in which GSTT2-2 is expressed, in a particular tissue, are also unclear. With the

availability of polyclonal antibodies raised against GSTT2-2, these studies shall be undertaken in future investigations.

Site-directed mutagenesis of a serine (residue 11) at the N-terminal domain of GSTT2-2 revealed that, this residue which is conserved through out the Theta class GSTs, is not necessarily essential in every catalytic reaction. Mutating Ser11 to Ala, Thr or Tyr produced a mutant enzyme with severely impaired activity towards cumene hydroperoxide and ethacrynic acid. However with MS as a substrate, the Ser11→Ala mutant showed marked increase of specific activity (1.5 times higher than the wild type), Ser11→Thr mutant retained 75% of the wild type activity and the Ser→Tyr mutant was inactived. The varying activities of the mutants which seemed to be dependent on the size of the substituting residues, led to further investigations of the mechanism of the reaction between GSH and MS catalysed by GSTT2-2. In a series of experiments, it was shown that the enzymatic conjugation rate between GSH and MS accelerated with increasing pH, while no non-enzymatic reaction between GSH and MS was observed even at high pH. Also, GSTT2-2 was shown to exhibit relaxed thiol specificity and the release of sulphate (SO_4^{2-}) from MS was detected in the presence of GSH (or *S*-methylglutathione) and GSTT2-2. As discussed in detail in **Chapter 7**, this evidence pointed to the ability of GSTT2-2 to catalyse the release of the sulphate moiety of MS in a mechanism similar to that of a sulphatase. However, the sulphatase activity of GSTT2-2 is glutathione-dependent. Presumably, the role of GSH (or *S*-methylglutathione) in this reaction is to induce an enzyme conformational change. In summary, the functional study of GSTT2-2 has led to the discovery of a novel function of GSTT2-2 as a glutathione-dependent sulphatase. In retrospect, GSTs have been found to exhibit many other catalytic functions besides their conventional GSH conjugating role. These functions which have been reviewed in **Chapter 1** include, reduction, isomerisation, thiolysis and denitrosation. The findings in this current study suggest that there may be other novel roles within the supergene family of GSTs which have yet to be discovered and the glutathione dependent sulphatase activity of GSTT2-2 is just one of them.

The determination of the crystal structure of GSTT2-2 is a high priority for future study. The construction of a His_6-tagged GSTT2-2 expression vector (pQEHT1), has shown that the recombinant protein can be produced and purified by two simple chromatographic steps in sufficient quantity for crystallographic study. *S*-(1-menapthyl)glutathione and 1-(chloromethyl)napthalene may be a useful substrate-product analogues for co-crystallisation studies to investigate the residues involved in substrate binding and the sulphatase reaction of GSTT2-2.

In conclusion, the studies carried out in this thesis have contributed to the overall understanding of the GST supergene family and in particular to the knowledge of the human Theta class GSTs, of which little was known at the commencement of this study. These studies have provided the knowledge and means by which many additional studies of GSTT2-2 can be undertaken in the future.

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