ISOLATION AND CHARACTERIZATION OF TWO MURINE PI-CLASS GLUTATHIONE S-TRANSFERASES

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Declaration of originality

I declare that the work presented within this thesis, unless stated otherwise is my own.

Theo Bammler
February 1996

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Abstract

A 15-kilobase (kb) DNA fragment has been isolated from a mouse genomic library in λEMBL3 that contains two actively transcribed pi-class glutathione S-transferase (GST) genes termed Gst p-1 and Gst p-2. Each of the two genes is approximately 3 kb in size and composed of seven exons interrupted by six introns. In addition to a TATA box and a sequence motif matching the phorbol-ester-responsive element, the promoters of Gst p-1 and Gst p-2 exhibit one and two G+C boxes (5'-GGGCGG-3') respectively. The hepatic expression of both pi genes was found to be significantly higher in males compared to females, and Gst p-1 mRNA levels were more abundant in both sexes than Gst p-2. The cDNAs corresponding to these genes were isolated from total liver RNA employing RT-PCR. The proteins encoded by the two murine pi-class genes, GSTP1-1 and GSTP2-2, which share 97% identity and differ in six amino acids only were expressed in *Escherichia coli*. Surprisingly, the catalytic activities of these recombinant enzymes toward a panel of electrophilic substrates were remarkably different, such that GSTP2-2 displayed 1-3 orders of magnitude lower activity than GSTP1-1. Site directed mutagenesis and kinetic analysis revealed that the amino acid differences at positions 10 (Val/Ser), 11 (Arg/Pro), and 104 (Val/Gly) were responsible for the dramatically reduced catalytic activity of GSTP2-2, affecting both binding of the electrophilic substrate and glutathione to the enzyme.

List of publications

The following publications have arisen from this thesis:

Bammler, T.K., Smith, C.A.D., and Wolf, C.R. (1994) Isolation and characterization of two mouse pi-class glutathione S-transferase genes. *Biochem. J.* **298**, 385-390

Bammler, T.K., Driessen, H., Finnstrom, N., and Wolf, C.R. (1995) Amino acid differences at positions 10, 11, and 104 explain the profound catalytic differences between two murine pi-class glutathione S-transferases. *Biochemistry* **34**, 9000-9008

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Abbreviations

A	adenine
A	ampere
AFB_1	aflatoxin B1
AFBO	aflatoxin-8,9-epoxide
AMV	avian myeloblastosis virus
AMPS	ammonium persulphate
ARE	antioxidant response element
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
ddATP	dideoxyadenosine triphosphate
BHA	butylated hydroxyanisole
Bis	N,N'-methylene-bis-acrylamide
bp	base pair
BSA	bovine serum albumin
C	cytosine
^oC	degree Celsius
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
Ci	curie
CIP	calf intestinal phosphatase
CTP	cytidine triphosphate
dCTP	deoxycytidine triphosphate
ddCTP	dideoxycytidine triphosphate
D	dalton
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
ssDNA	single strand deoxyribonucleic acid
dsDNA	double strand deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
G	guanine

g	gram
GPEI/II	GST-P enhancer I/II
GRE	glucocorticoid response element
GSH	reduced glutathione
GSSG	oxidized glutathione or glutathione disulfide
GST	glutathione S-transferase
GTP	guanosin triphosphate
dGTP	deoxyguanosin triphosphate
ddGTP	dideoxyguanosin triphosphate
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HNF	hepatocyte nuclear factor
Ig	immunoglobulin
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
kD	kilodalton
l	litre
LB	Luria-Bertani medium
LMP-agarose	low melting point agarose
M	molar concentration
3-MC	3-methylcholanthrene
MDR	multidrug resistance
min	minute
MOPS	3-[N-morpholino] propane sulphonic acid
m.o.i.	multiplicity of infection
mRNA	messenger RNA
MW	molecular weight
NADP	nicotinamide adenine dinuclotide phosphate
NADPH	nicotinamide adenine dinuclotide phosphate (reduced)
ND	not determined
OD	optical density
OLB	oligo labeling buffer
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
pfu	plaque forming unit
pH	measure of H^+ concentration in a solution
PVP	polyvinylpyrollidone
RFLV	restriction fragment length variant
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
T	thymine
TBS	tris-buffered saline
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylenediamine
TRE	12-O-tetradecanoate 13-acetate response element
Tris	N-tris[hydroxymethyl]aminomethane
TTP	thymidine triphosphate
dTTP	deoxythymidine triphosphate
ddTTP	dideoxythymidine triphosphate
TWEEN 20	polyoxyeyhylene-sorbitan monolaurate
U	enzyme unit
UV	ultraviolet
UTR	untranslated region
V	volt
vol	volume
W	watt
wt	weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XRE	xenobiotic response element

The standard one and three letter abbreviations for amino acids are used as follows:

A	Ala	Alanine
B	Asx	Asparagine or aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine or glutamic acid

Decimals

k	kilo-	($X\ 10^{3}$)
m	milli-	($X\ 10^{-3}$)
μ	micro-	($X\ 10^{-6}$)
n	nano-	($X\ 10^{-9}$)
p	pico	($X\ 10^{-12}$)

Chapter 1: Introduction

1.1 General introduction to drug metabolism

Living organisms are constantly exposed to a multitude of man made or naturally occurring chemicals, which cannot be utilized in normal biological processes to provide energy or precursors for the synthesis of cellular macromolecules. These compounds are referred to as xenobiotics. Most xenobiotics are lipophilic and can therefore penetrate or accumulate in cellular membranes. As a consequence, the chemically polluted cell would be damaged and ultimately die. However, protective mechanisms have evolved which enable higher organisms to excrete potentially harmful chemicals. Before a lipophilic xenobiotic can be excreted, it has to be converted to a more polar compound. This conversion is usually achieved by a process, which can be divided into two phases (Figure 1.1-1). In phase I, the often relatively inert hydrophobic compound is activated by the introduction of a reactive functional group on the molecule, which can subsequently serve as a target for conjugation reactions occurring in phase II. Phase I and phase II reactions are catalyzed by the so called drug metabolizing enzymes.

The majority of phase I reactions is carried out by members of the cytochrome P-450 superfamily (Wolf, C.R, 1986; Gonzalez, F.J., 1988). These haem containing proteins are located in the membrane of the endoplasmic reticulum and mediate reactions which require a transfer of electrons. In most cases, the flavoprotein NADPH-cytochrome P-450 reductase catalyzes electron transfer from reduced NADPH to cytochrome

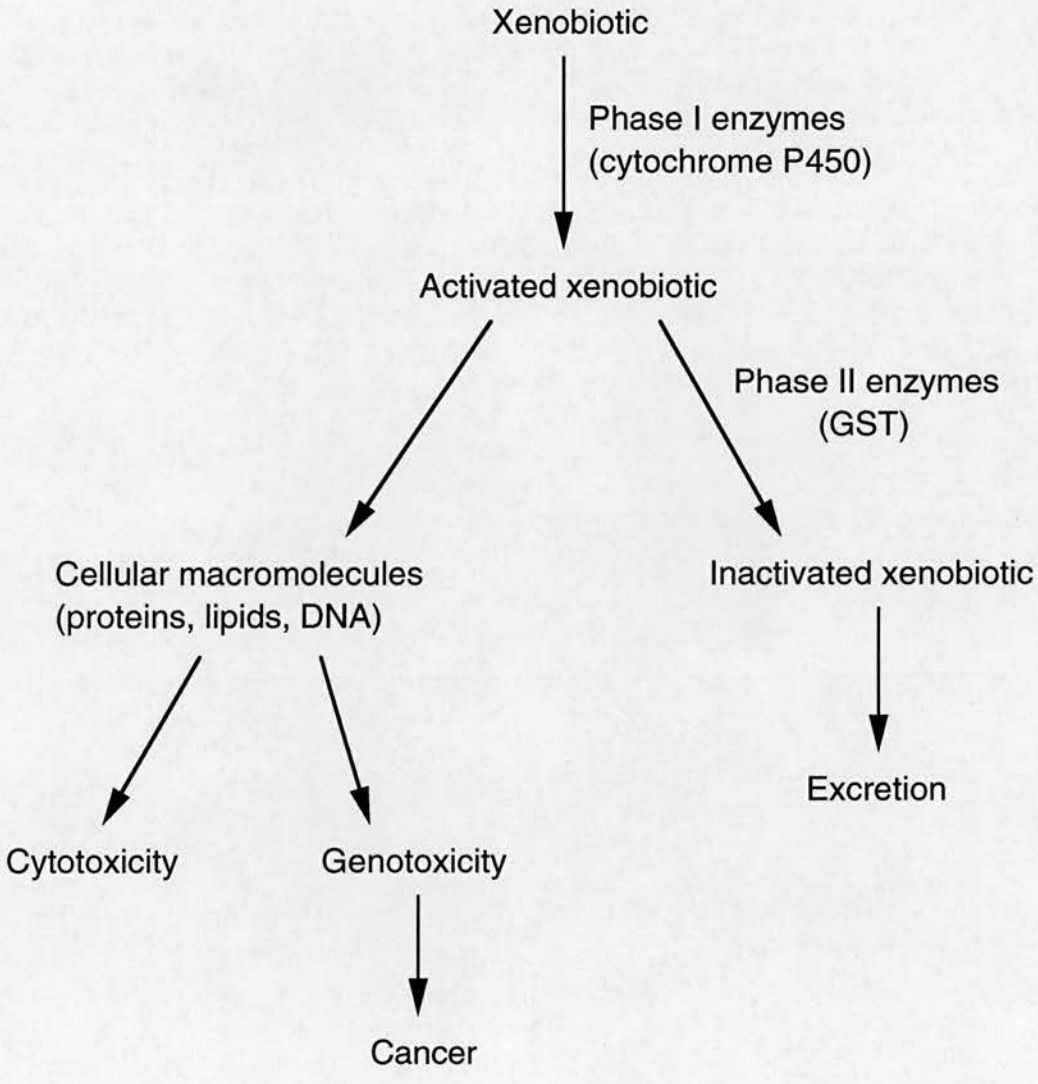


Figure 1.1-1 Overview of drug metabolism. See text for details.

P-450. However, in some cases electrons can also be donated via cytochrome b-5 to cytochrome P-450. In both cases, the reduced form of the P-450 enzyme activates molecular oxygen. All members of the cytochrome P-450 family catalyze essentially the same reaction, namely the insertion of a single molecule of oxygen in the substrate (Equation 1.1-1).

$$R\text{-}H + O_2 + NADPH + H^+ \xrightarrow[\text{P-450}]{\text{cytochrome}} ROH + NADP^+ + H_2O$$

Equation 1.1-1

At this stage, a large number of activated electrophilic xenobiotics have the potential to interact with cellular macromolecules such as proteins, lipids, and DNA, resulting in cytotoxicity and mutagenesis. Alternatively, phase II conjugating enzymes, including UDP-glucuronosyltransferases, sulphotransferases and glutathione S-transferases, can inactivate reactive electrophilic intermediates, thus preventing attack on cellular macromolecules. In addition, the conjugation of electrophilic xenobiotics with polar molecules (e.g. glucuronic acid, sulphate, glutathione) increases their hydrophilicity and, thus, facilitates their excretion. Therefore, phase II xenobiotic metabolism represents an important detoxification mechanism.

As the work presented in this thesis is concerned with the characterization of glutathione S-transferases (GSTs; E.C.2.5.1.18), an overview of this family of phase II drug metabolizing enzymes is provided in the following.

1.2 Historical perspective

Originally, in 1961 Booth and co-workers described an enzymatic activity present in rat liver cytosol that catalyzed the conjugation of glutathione (GSH) with 1,2-dichloro-4-nitrobenzene (DCNB). In the following years of this decade, many different glutathione S-transferase substrates were identified (Boyland and Chasseaud, 1969), and evidence for the existence of several distinct isoenzymes started to emerge (Fijellstetd *et al.,* 1973; Habig *et al.,* 1974b). Early attempts to classify enzymes displaying glutathione S-transferase activity were based on the chemical structure of the electrophilic substrates and resulted in names such as aryl-, alkyl-, alkene-, and epoxide- transferases (Boyland and Chasseaud, 1969; Johnson, 1966; Chasseaud,1973). Investigators from Jakoby's laboratory were the first to report the successful purification of GSTs from rat liver (Fjellstedt *et al.,* 1973; Habig *et al.,* 1974b). Subsequently, characterization of homogeneous enzyme preparations confirmed the existence of several catalytically distinct GSTs (Habig *et al.,* 1974b). In addition to catalyzing the conjugation of reduced glutathione with numerous eletrophilic compounds, GSTs were shown to possess ligandin (Habig *et al.,* 1974a; Kaplowitz *et al.,* 1973) , selenium independent peroxidase (Prohaska and Ganther, 1977), and ketosteroid isomerase activity (Benson *et al.,* 1977).

SDS-PAGE analysis (Bass *et al.,* 1977) and reversible denaturation experiments (Hayes *et al.,* 1981) of individual rat GSTs revealed the existence of homo- and hetero-dimeric isoenzymes, and provided the basis for dividing GSTs into structurally distinct groups. More recently, the isolation of different cDNA clones provided the ultimate proof that GST-subunits with distinct electrophoretic behaviour were indeed the products of

distinct genes (Lai *et al.,* 1984, 1986; Pickett *et al.,* 1984; Telakowski-Hopkins *et al.,* 1985).

Catalytic, immunochemical, and N-terminal sequence analyses indicated that certain GST isoenzymes isolated from different species (e.g. mouse, rat, human) were more similar than distinct GSTs from the same species. This observation led Mannervik and co-workers (1985b) to propose a species independent classification system, according to which mammalian cytosolic GSTs were grouped into three classes termed alpha, mu and pi. In more recent years, an additional class of cytosolic GSTs has been identified and designated theta (Meyer *et al.,* 1991).

Since the initial pioneering work by Jakoby and co-workers (Fjellstedt *et al.,* 1973; Habig *et al.,* 1974b), a large number of GSTs have been isolated and purified from bacteria, yeast, nematodes, insects, fish, birds, mammals, and plants (Buetler and Eaton, 1992). The ubiquitous occurrence suggests, that GSTs play an important role in protecting organisms from the potentially devastating effects caused by chemical insult.

In 1991, Reinemer and colleagues were the first to report the three dimensional structure of a pi-class GST isolated from pig lung. Since then, the structures of alpha (Sinning *et al.,* 1993), mu (Ji *et al.,* 1992), and further pi-class GSTs (Reinemer *et al.,* 1991, 1992; Garcia-Saez *et al.,* 1994) have been resolved.

1.3 Classification and nomenclature of GSTs

As indicated earlier, based on nucleotide and amino acid sequence similarities mammalian cytosolic GSTs can be grouped into four subfamilies named alpha, mu, pi, and theta (Mannervik *et al.,* 1985b, 1992; Meyer *et al.,* 1991). Members of the same subfamily share more than 50% sequence identity, whereas members of distinct subfamilies possess less than 30% identity. In addition to the cytosolic GSTs, a membrane-associated form has been isolated (Morgenstern and DePierre, 1983; Morgenstern *et al.,* 1985). Amino acid sequence comparison revealed that the microsomal GST shares no homology with the cytosolic isoenzymes, suggesting both groups of enzymes have arisen through independent evolutionary pathways.

Historically, the nomenclature of GSTs has been problematic, as different investigators have developed their own independent designations. The lack of a commonly used nomenclature system often resulted in multiple names for the same GST enzyme, confusing the matter. In order to resolve this problem, Mannervik and colleagues (1992) proposed a unifying nomenclature system for human GSTs (Table 1.3-1). These investigators suggested to use upper case Roman letters to indicate the class (A: alpha; M: mu; P: pi; T: theta) and Arabic numerals separated by a hyphen to specify the subunit composition. Arabic numerals are assigned to the subunits, on the basis of the chronological order of discovery. Allelic variants are represented by lower case Roman letters (e.g. GSTM1a-1a). The corresponding gene loci are also named according to this system with the modification that only one Arabic letter is used. As the microsomal form is not a member of the cytosolic GSTs, no change in the nomenclature

Table 1.3-1 Human GST nomenclature as proposed by Mannervik et al. (1992). Information presented was compiled from Mannervik et al. (1992), and Beckett and Hayes (1993).

Class	Nomenclature based on Mannervik et al. (1992)	Previously used nomenclature
alpha	GSTA1-1	ε, B1B1, GST2-type1, Ha-subunit 1
alpha	GSTA2-2	γ, B2B2, GST2-type2, Ha-subunit 2
alpha	GSTA1-2	δ, GST2-type1-2
mu	GSTM1a-1a	μ, GST1-type2, Hb-subunit4
mu	GSTM1b-1b	ψ, GST1-type 1
mu	GSTM1a-1b	GST2-type1-2
mu	GSTM1a-2	N1N2
mu	GSTM1b-2	N1N2
mu	GSTM2-2	GST4
mu	GSTM2-3	N2N3
mu	GSTM3-3	GST5
pi	GSTP1-1	π, GST3
theta	GSTT1-1	GSTθ
theta	GSTT2-2	– – –
microsomal	GSTMi1-1	Microsomal GST

of this enzyme has been proposed. In addition, Mannervik and colleagues (1992) suggested to extend this system for naming GSTs from other mammalian species by using an appropriate lower case letter prefix to indicate the origin (e.g. h: human; r: rat; m: mouse).

However, so far this nomenclature system has not been used for GSTs isolated from species other than human (e.g. rat or mouse). The two nomenclature systems commonly used in the literature for naming rat/mouse GSTs are as follows. The one most widely used is based on the suggestion by Bass *et al.* (1977) to name the different subunits Ya, Yb, Yc, e.t.c. based on their mobility on SDS-PAGE (Table 1.3-2), whereas according to the other system Arabic numerals are assigned to subunits based on the chronological order of their characterization (Jakoby *et al.,* 1984).

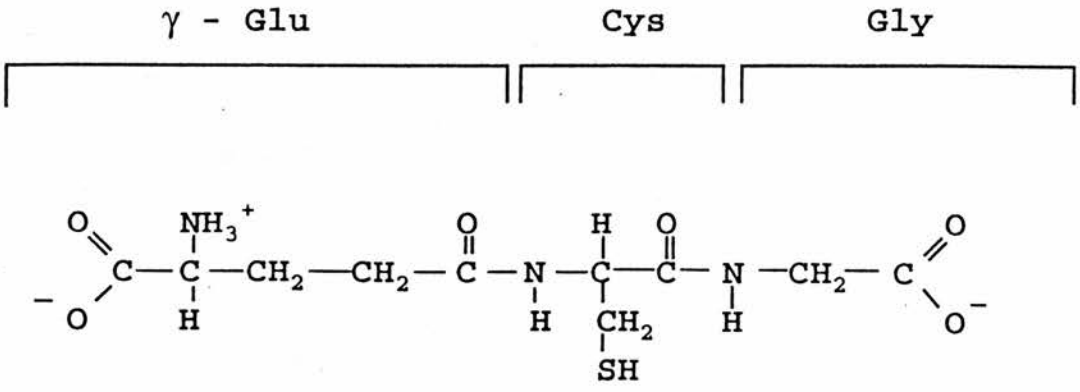
Table 1.3-2 Nomenclature of GST subunits isolated from rat and mouse. The information presented was taken from Buetler and Eaton (1992), Mannervik and Danielson (1988), and Bass et al. (1977).

Class	Subunit designations according to Bass et al. (1977)	Alternative designation
alpha	rat Ya	1
alpha	rat Yc1	2
alpha	rat Yc2	10
alpha	rat Yk	8
alpha	mouse Ya1	- - -
alpha	mose Ya2	- - -
alpha	mouse Yc	GT10.6
alpha	mouse Yk	GST5.7
mu	rat Yb1	3
mu	rat Yb2	4
mu	rat Yb3 or Yn1	6
mu	rat Yn2	9
mu	rat Yo	11
mu	mouse Yb1	GT8.7
mu	mouse Yb2	- - -
mu	mouse Yb3	Ft
mu	mouse Yb4	GT9.3
mu	mouse Yb5	- - -
pi	rat Yf or Yp	7
pi	mouse Yf	GT9.0
theta	rat Yrs	12
theta	rat Y?	5
theta	rat Y?	13

1.4 GSTs are multifunctional proteins

1.4.1 Glutathione conjugation reactions

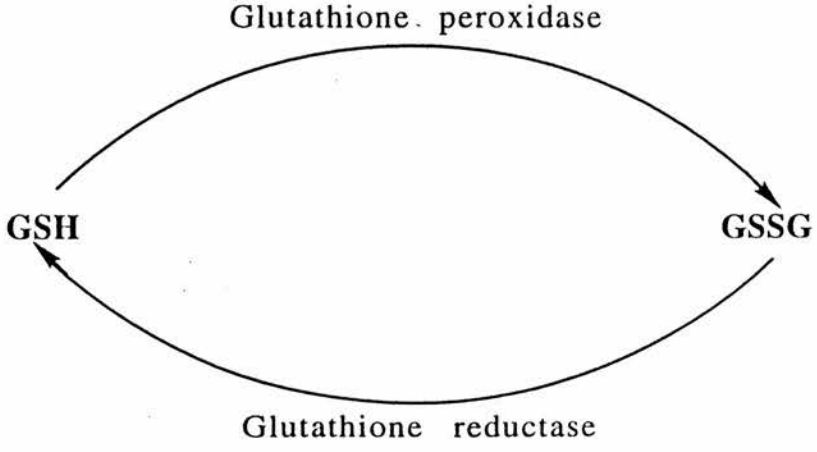
The tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is the major intracellular non-protein thiol that is found in all aerobic life forms. Glutathione concentrations are as high as 15 mM in the lens, 5 to 10 mM in the liver, and 1 to 2 mM in many other mammalian tissues (Kosower and Kosower, 1978). The glutathione molecule exists either in the reduced (GSH) or in the oxidized form (glutathione disulfide or GSSG). GSSG is generated from GSH by the action of the selenium dependent glutahione peroxidase, which degrades hydrogen peroxide and lipid peroxides. In turn, GSSG can be reduced to GSH by the enzyme GSSG-reductase, using NADPH as the reducing agent (Figure 1.4.1-1) (Arias and Jakoby, 1976).



a: Reduced glutathione (GSH)

γ - Glu - Cys - Gly
S
S
γ - Glu - Cys - Gly

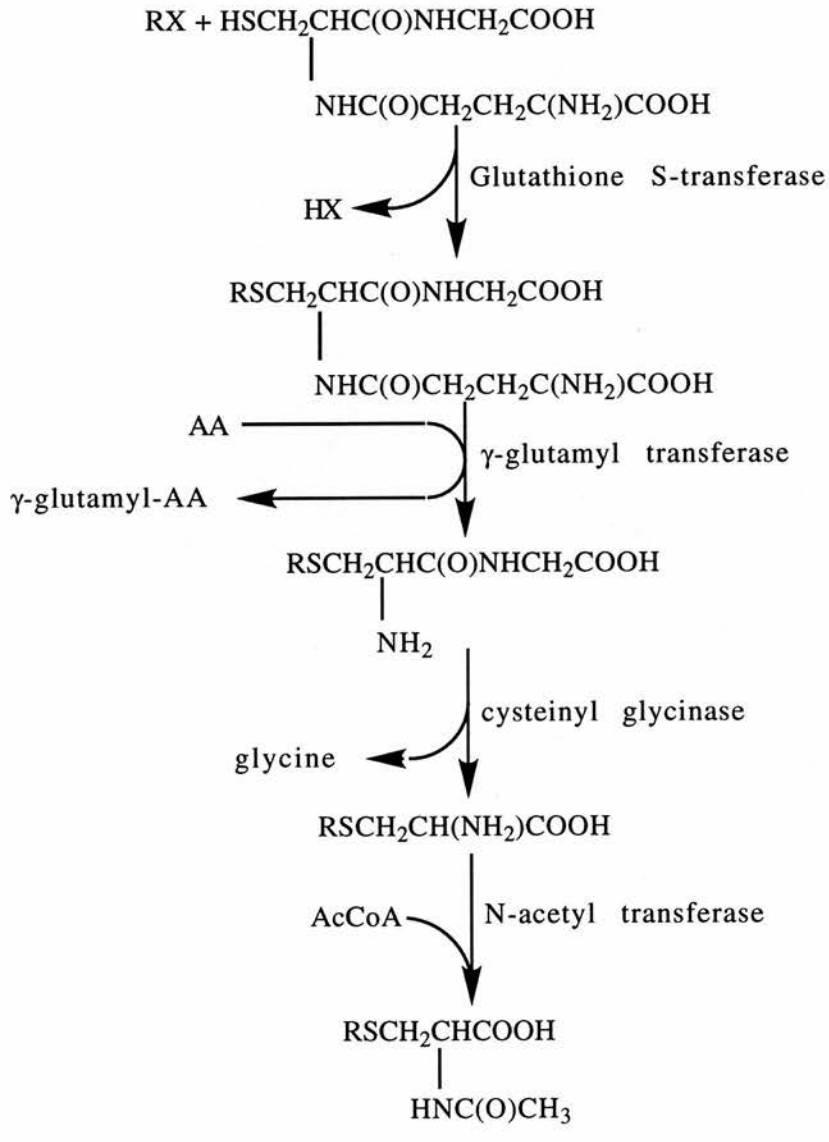
b: Oxidized glutathione (GSSG)



c: Relationship between reduced GSH and oxidised GSSG

Figure 1.4.1-1 Glutathione Glutathione is a tripeptide composed of glutamic acid, cysteine, and glycine. Shown are the reduced (a) and oxidized forms (b) of glutathione, and their inter-relationship (c).

At physiological pH, GSH exists as a nucleophilic thiolate anion (GS^-), which can react with a large number of electrophilic compounds, and by doing so detoxifying them. However, it has to be noted that the detoxification of toxic electrophiles by the spontaneous reaction with GSH is not very efficient and depends largely on the thiol concentration and the pH. In contrast, GSTs catalyze the conjugation of GSH with a large number of toxic electrophiles extremely efficiently. Thus it becomes clear, that GSTs are particularly important for the detoxification of numerous electrophilic foreign compounds. Furthermore, the conjugation of xenobiotics with GSH catalyzed by GSTs represents the first step in the mercapturic acid pathway (Habig *et al.,* 1974b), which is an important route for the biotransformation and excretion of foreign compounds. Figure 1.4.1-2 summarizes the reactions occurring during the formation of mercapturic acids, which are water soluble and can be readily excreted from the body.



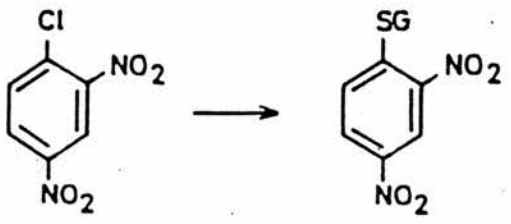
Mercapturic acid

Figure 1.4.1-2 Formation of mercapturic acid. Glutathione S-transferases catalyse the conjugation of an electrophilic compound (RX) with glutathione (GSH). The GSH-conjugate may be directly excreted into bile, or can undergo further metabolism. The renal enzyme g-glutamyl transferase catalyses the transfer of the g-glutamyl moiety to neutral amino acids (AA) or peptides. Subsequently, glycine is released by hydrolysis of the cysteinyl-glycine peptide bond catalysed by cysteinyl glycinase. N-acetyl transferase catalyses the N-actetylation of the cysteinyl derivative resulting in the mercapturic acid which can be excreted either in bile or in the urine (adapted from Hodgson, 1987).

1.4.1.1 Substrate specificity of GSTs

Individual GSTs belonging to distinct classes display overlapping substrate specificities. For example, 1-chloro-2,4-dinitrobenzene (CDNB) is generally considered a universal GST substrate (Figure 1.4.1.1-1). However, despite a certain degree of overlap in substrate specificities, each GST has a characteristic profile of substrates. Certain substrates are considered diagnostic for each GST class. High activity toward organic hydroperoxides (e.g. cumene hydroperoxide) is indicative of alpha-class GSTs, whereas epoxides (e.g. trans-stilbene oxide) and trans-4-phenyl-3-butene-2-one are characteristic substrates for the mu-class (Mannervik and Danielson, 1988). Ethacrynic acid has been shown to be a typical substrate for the pi-class (Mannervik and Danielson, 1988), whereas 1-menaphthyl sulphate and 1,2-epoxy-3-(p-nitrophenox)propane are metabolized by members of the theta class (Hiratsuka *et al.,* 1990; Meyer *et al.,* 1991). In addition, hexachlorobuta-1,3-diene can be used as a marker substrate for microsomal GSTs (McLellan *et al.,* 1989).

(1)



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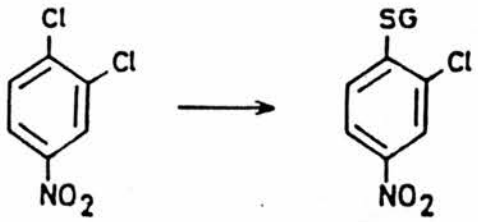

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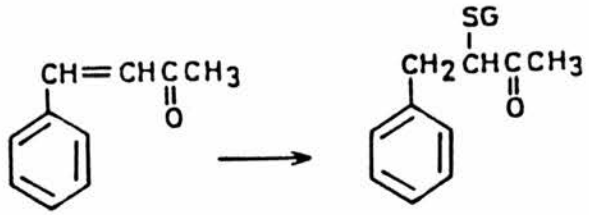
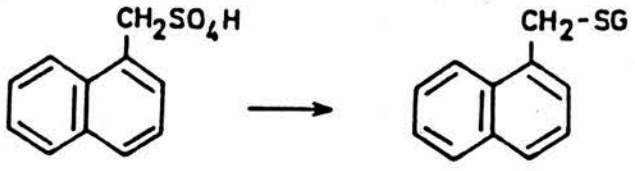


Figure 1.4.1.1-1 (to be continued) Metabolism of electrophilic substrates by GSTs. The conjugation of the follwing compounds with GSH is catalyzed by GSTs: (1) 1-chloro-2,4-dinitrobenzene, (2) 1,2-dichloro-4-nitrobenzene, (3) *trans*-4-phenyl-3-buten-2-one, (4) menaphthyl sulphate, (5) ethacrynic acid, and (6) 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (adapted from Beckett and Hayes, 1993).

(4)



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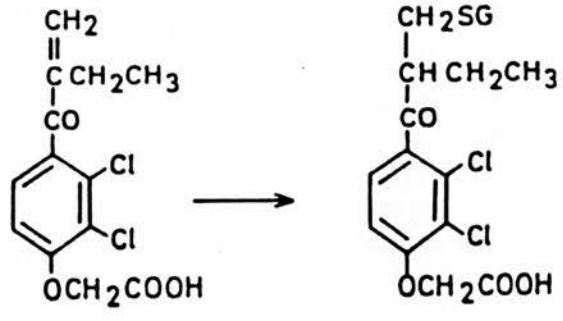


(6)

Figure 1.4.1.1-1 (continued)

1.4.1.2 Examples of carcinogens detoxified by GSTs

1.4.1.2.1 Aflatoxin B1

Aflatoxin B1 (AFB_1) is a potent naturally occurring hepato-carcinogen produced by the mould *Aspergillus flavus* which grows on cereal crops and nuts in areas of high humidity. AFB_1 is activated by cytochrome P450 to give rise to the highly reactive 8,9-epoxide (AFBO) which is the ultimate carcinogenic metabolite. It has been demonstrated that AFBO binds to guanine residues in DNA at the N-7 position (Martin and Garner, 1977; Essigman *et al.,* 1977). Several studies have provided evidence that the conjugation of AFBO with GSH (Figure 1.4.1.2.1-1) represents the major detoxification mechanism in rodents (Buetler and Eaton, 1992; Hayes *et al.,* 1991a, 1992; Ramsdell and Eaton, 1990). Certain species are highly susceptible to the toxic effects of AFB_1, whereas others are resistant. It has been shown that the degree of resistance to AFB_1 exhibited by a certain species is correlated with the expression of a GST that has high catalytic activity toward AFBO. For example, the mouse, which is highly resistant to both cytotoxic and genotoxic effects of AFB_1, expresses constitutively an alpha class GST termed Yc that is highly active toward AFBO (Buetler and Eaton, 1992; Hayes *et al.,* 1992; Ramsdell and Eaton, 1990). In contrast, the rat, which is susceptible to the adverse effects of AFB_1, fails to express a GST constitutively that exhibits such a property (Hayes *et al.,* 1991a).

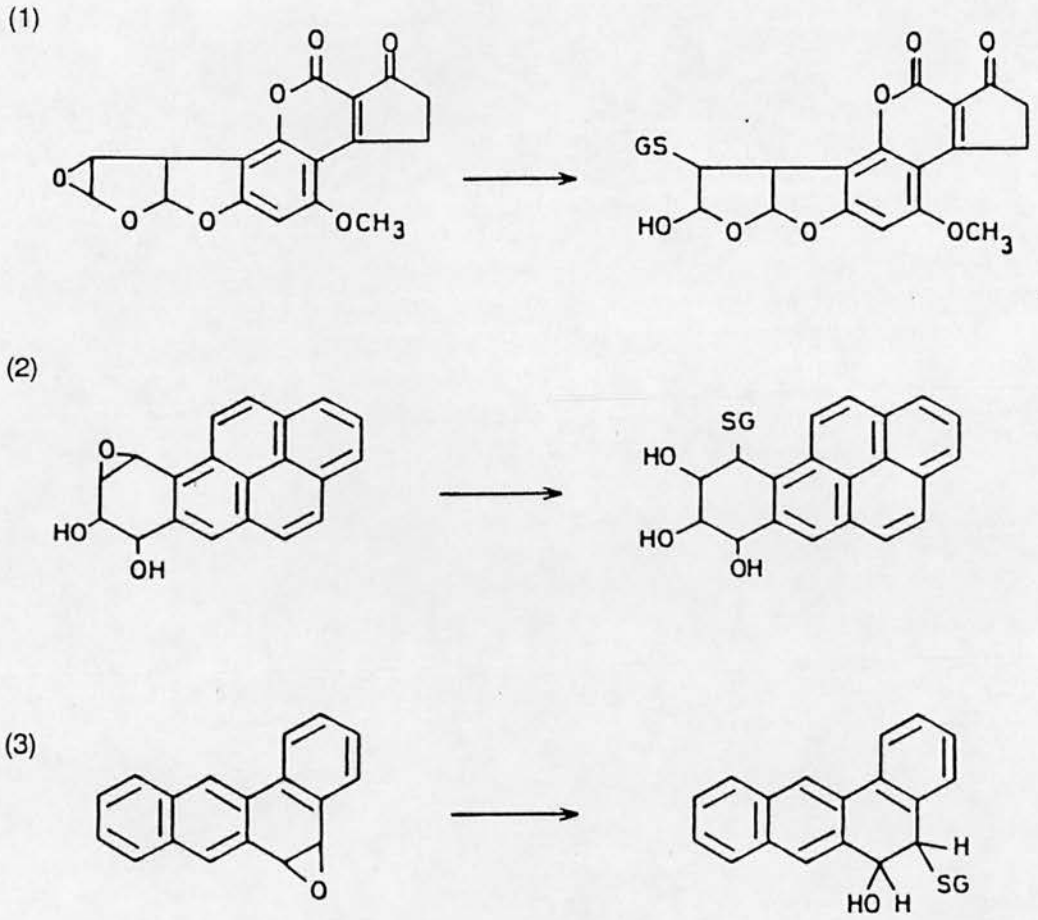


Figure 1.4.1.2.1-1 Examples of carcinogens detoxified by GSTs. The carcinogens (1) aflatoxin B_1-8,9-epoxide, (2) anti-benzo[*a*] pyrene 7,8-diol-9,10-oxide, and (3) benz[*a*]anthracene-5,6-epoxide are detoxified by GSTs (adapted from Beckett and Hayes, 1993).

1.4.1.2.2 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) represent a group of carcinogens (e.g. benz[a]anthracene and benzo[a]pyrene) that occur commonly in a number of environmental products such as coal tar, tobacco smoke, petroleum, and air pollutants generated by the combustion of fossil fuels (Cooke and Dennis, 1988). It is estimated that in the United States approximately 900 tons of benzo[a]pyrene alone, are emitted into the air each year. Reactive metabolites of PAHs include epoxides, phenols, and quinones. Several of the reactive intermediates of benzo[a]pyrene have been demonstrated to be conjugated with GSH. Several GSTs have been shown to catalyze the conjugation of the K-region epoxide benzo[a]pyrene-4,5-oxide with GSH (Nemoto *et al.,* 1975; Warholm *et al.,* 1981). Although this and other K-region epoxides are genotoxic in bacterial mutagenicity tests, no DNA adducts have been detected in animals. Consequently, these K-region epoxides have not been implicated in carcinogenesis (Coles and Ketterer, 1990), presumably because they are efficiently detoxified by GSTs and epoxide hydrolase. In contrast, Bay-region diol epoxides such as benzo[a]pyrene-7,8-diol-9,10-oxide (Figure 1.4.1.2.1-1) are responsible for the carcinogenic effects of PAH (Jerina and Lehr, 1977). These metabolites are poor substrates for epoxide hydrolase. However, they are metabolized and detoxified by GSTs (Jernstrom *et al.,* 1985; Robertson *et al.,* 1986).

1.4.1.3 GSH conjugation resulting in increased toxicity

As outlined above, the vast majority of conjugation reactions catalyzed by GSTs result in detoxification. However, in a small number of cases the conjugation of electrophilic compounds with GSH increases their toxicity. For example, the conjugation of ethylene dibromide with GSH leads to 2-bromoethylglutathione (Figure 1.4.1.3-1) which reacts with DNA and has been shown to be a carcinogen in the rat (Ozawa and Guengerich, 1983). Trichloroethene and hexachloro-1,3-butadiene are also activated following the conjugation with GSH (Figure 1.4.1.3-1) catalyzed by microsomal GST (Anders *et al.,* 1988; Vamvakas and Anders, 1991).

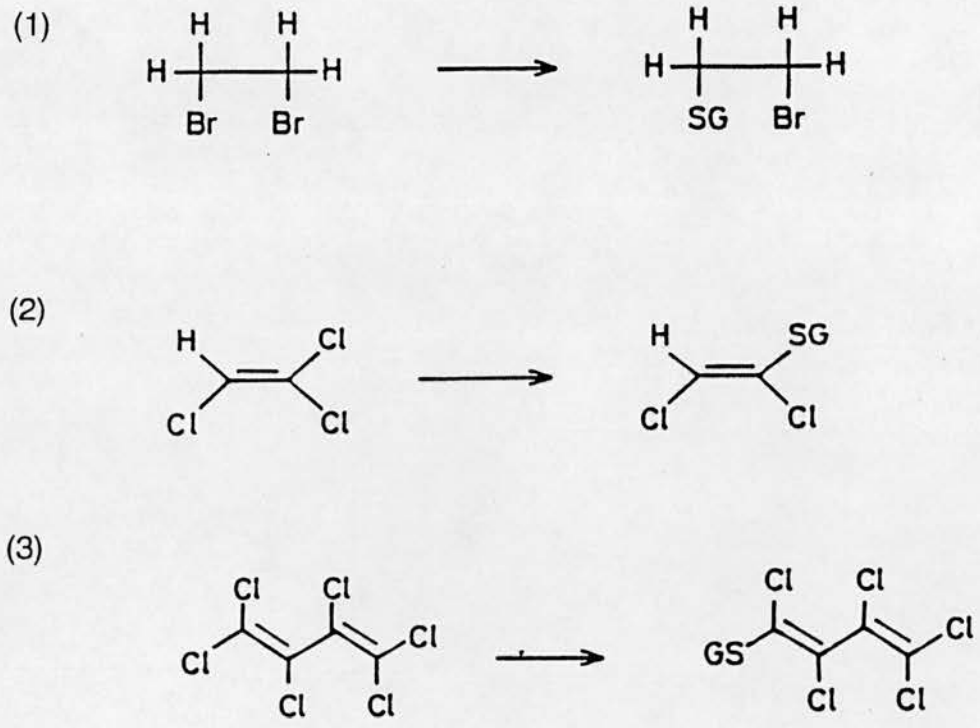


Figure 1.4.1.3-1 GSH conjugations catalyzed by GSTs leading to increased toxicity. The conjugation of (1) ethylene dibromide, (2) trichloroethene, and (3) hexachloro-1,3-butadiene with GSH increases their toxicity (adapted from Beckett and Hayes, 1993).

1.4.2 Endogenous GST substrates

During aerobic metabolism, reactive oxygen species (e.g. superoxide anion, hydrogen peroxide, hydroxyl radicals) are constantly generated. As a consequence, free radicals can react with polyunsaturated fatty acids of biomembranes, resulting in lipid peroxidation. The selenium independent peroxidase activity of GSTs helps to reduce peroxidized lipids (e.g. hydroperoxides of linoleic acid and arachidonic acid) (Ketterer *et al.,* 1987). Furthermore, toxic byproducts of lipid peroxidation such as malondialdehyde, 2-alkenal, 4-hydroxyalkenal, and cholesterol-5,6-epoxide are also detoxified by GSTs (Danielson *et al.,* 1987) (Figure 1.4.2-1). In addition, it has been demonstrated that peroxidized DNA and thymine hydro-peroxide also serve as substrates for GSTs (Tan *et al.,* 1986, 1988). The above examples illustrate the important function of GSTs in protecting cells from oxidative damage. In addition to their detoxifying functions, GSTs are also involved in the biosynthesis of biologically active molecules such as leukotrienes (Bach *et al.,* 1984; Nicholson *et al.* 1992, 1993) and prostaglandins (Figure 1.4.2-1) (Cagen *et al.,* 1975; Chaudhari *et al.,* 1978), which exhibit hormonal properties. For example, leukotrienes are potent inflammatory mediators. As mentioned earlier, GSTs also catalyze the isomerization of physiologically important molecules such as Δ^5-3-ketosteroids (Benson *et al.,* 1977) (Figure 1.4.2-1).

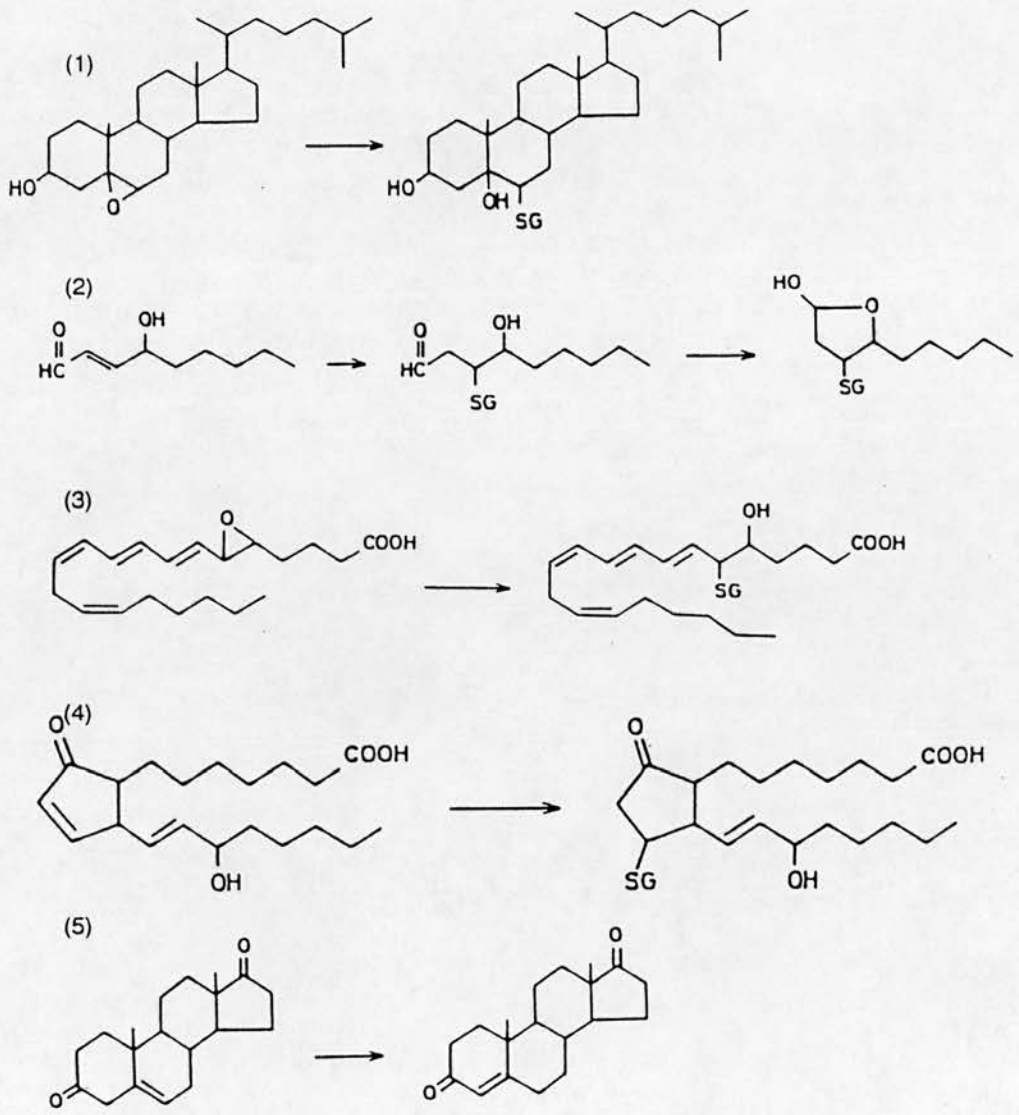


Figure 1.4.2-1 Endogenous GST substrates. (1) cholesterol-5,6-oxide, (2) 4-hydroxynon-2-enal, (3) leukotriene A_4, (4) prostaglandin A_1, and (5) Δ^5-androstene-3,17-dione (adapted from Beckett and Hayes, 1993).

1.4.3 GSTs, binding proteins

Besides their catalytic activities discussed so far, many GSTs are also able to bind covalently and non-covalently various hydrophobic non-substrate ligands such as steroids, bilirubin, and bile acids (Abramovitz *et al.,* 1988; Levi *et al.,* 1969; Hayes *et al.,* 1988; Listowski et al, 1988). Based on this ability, it has been suggested that these proteins may play a role in the storage and/or the intracellular transport of hydrophobic compounds. For example, it has been proposed that GSTs are involved in the intracellular transport of haem from mitochondria to appropriate apoenzymes (Husby *et al.,* 1981)

Furthermore, certain reactive xenobiotic metabolites, including those arising from 3-methylcholanthrene (Ketterer, 1972) and the azo-dye carcinogen (Ketterer *et al.,* 1967), have been shown to bind covalently to rat GSTs (YaYa and YaYc), which were originally named ligandin or Y protein. Although a physiological role has not been firmly established, it is reasonable to speculate that the covalent binding of reactive eletctrophiles to GSTs may represent an alternative detoxification mechanism, which could prevent the attack of other cellular macromolecules by these ligands.

1.5 Kinetic aspects of GSTs

The first analysis concerned with the kinetic mechanism of GSTs was carried out by Pabst and co-workers (1974). These investigators reported a complex biphasic kinetic mechanism for the rat mu class homodimer Yb1 (also called A, or subunit 3). At high concentrations of GSH, an ordered sequential mechanism seemed to predominate, such that GSH binds first, whereas at low concentrations a ping-pong pathway, in which the electrophilic substrate binds first, was found to fit the experimental data best. Subsequently, however, Jakobson *et al.* (1977) performed a detailed analysis of all the available data, including the data set generated by Pabst an co-workers (1974). Interestingly, these researchers demonstrated that a steady state random mechanism best explained the complex initial rate pattern observed for the rat Yb1Yb1 enzyme. Danielson and Mannervik (1988) reported also a random-order sequential mechanism for another rat mu class homodimer Yb2 (also called 4).

Schramm and collaborators (1984) studied the kinetic behaviour of the rat alpha class homodimer Ya (also called 1), and suggested a rapid equilibrium random mechanism. However, it has to be noted that these workers did not rule out a steady state random mechanism.

More recently, Ivanetich and Goold (1989) proposed a rapid equilibrium random mechanism for the human GST pi enzyme. Another member of the pi family isolated from mouse liver (YfYf) was examined by Phillips and Mantle (1991), and also found to obey a rapid equilibrium random mechanism.

In addition, GSTs isolated from insects have been shown to obey a steady state random mechanism (Clark *et al.,* 1984, 1986).

1.6 Structural aspects of cytosolic GSTs

Cytosolic GSTs exist as homo- and heterodimeric proteins. Only subunits encoded by distinct genes belonging to the same subfamily can hybridize and form heterodimers (Hayes *et al.,* 1981; Kitahara and Sato, 1981). Detailed kinetic studies of homo- and heterodimeric rat GSTs indicated that the activities measured using several substrates at different concentrations were additive (Danielson and Mannervik, 1985). In addition, inhibition studies of homo- and heterodimeric enzymes showed that the kinetic properties of a heterodimer could be accurately predicted from the corresponding homodimers (Tahir and Mannervik, 1986). These results provided good evidence that each GST subunit is kinetically independent. Consequently, each subunit must contain an active site. Mannervik (1985a) suggested a model, based on which the active site consists of two adjacent subsites; a GSH binding region (G-site) and a hydrophobic region (H-site) that can accommodate the electrophilic substrates.

Recently, Reinemer and co-workers (1991) were the first to report the three dimensional structure of a pi-class GST isolated from pig lung. Analysis of the porcine pi GST crystals complexed with glutathione sulfonate revealed that each subunit is folded into two distinct domains. The smaller N-terminal domain I (residues 1-74) exhibits an α/β motif folding with the sequence $\beta\alpha\beta\alpha\beta\beta\alpha$ describing the secondary structure elements (Table 1.6-1). In the tertiary structure, the central four-stranded β-sheet is flanked on one side by two α-helices and on the other side by a bent irregular helix structure. An oligopeptide, represented by residues 75-80, links domain I with domain II. The C-terminal domain II (residues 81-207) is composed of five α-helices (Table 1.6-1). Residues lining the G-site were identified and include Tyr-7, Gly-12, Arg-13, Trp-38, Lys-42, Gln-49,

Pro-51, Gln-62, Ser-63, and Glu-95. Tyr-7, a residue that is conserved in mammalian GSTs, was shown to interact with the sulfonate group of the inhibitor. In contrast to the G-site, the H-site could not be mapped accurately from the porcine pi crystals. Subsequently, Reinemer *et al.* (1992) also resolved the structure of the human pi-class protein, which closely resembled that of the porcine enzyme. Crystals obtained from the human pi GST allowed the identification of residues participating in the lining of the H-site. These residues include Tyr-7, Phe-8, Pro-9, Val-10, Val-35, Tyr-106, and Gly-203.

More recently, the three dimensional structures of a rat mu (Ji *et al.,* 1992), a human alpha class GST (Sinning *et al.,* 1993), and a mouse pi-class GST (Garcia-Saez *et al.,* 1994) have also been reported. Although members of different GST classes share little identity at the primary sequence level, they exhibit a high degree of structural similarity, reflecting overlapping enzymatic activities (Dirr *et al.,* 1994).

Table 1.6-1 Secondary structure elements of porcine and human pi-class GSTs. Adapted from Reinemer et al. (1993). Domains I and II are connected by a short segment consisting of residues 75-80. The amino acid numbering used is according to the position of residues with respect to the porcine sequence. The primary sequence of the human subunit is two amino acids longer than the homologous porcine peptide.

	porcine GSTP1-1	human GSTP1-1
Domain I	β1 (3-7)	β1 (3-7)
	αA (15-23)	αA (15-23)
	β2 (29-32)	β2 (29-32)
	αB (38-41)	αB1* (35-39)
	3_{10} (42-44)	αB2* (40-44)
	β3 (52-55)	β3 (52-55)
	β4 (58-61)	β4 (58-61)
	αC (63-74)	αC (63-74)
Domain II	αD (81-107)	αD (81-107)
	αE (109-132)	αE (109-133)
	3_{10} (135-137)	3_{10} (135-137)
	αF (148-163)	αF (148-163)
	3_{10} (167-170)	3_{10} (168-170)
	αG (172-182)	αG (172-183)
	αH (185-192)	αH (185-191)
	αI (194-197)	αI (194-197)

* The helices αB1 and αB2 are separated by the insertion sequence Glu39aGly39b

1.7 Regulation of GSTs

The regulation of cytosolic GSTs is subject to a complex set of endogenous and exogenous parameters. These include developmental (Hatayama *et al.,* 1986) and tissue specific factors (Rushmore *et al.,* 1990), as well as a number of structurally diverse xenobiotics such as planar aromatic compounds(e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methyl-cholanthrene), antioxidants (e.g. butylated hydroxyanisole, ethoxyquin) and barbiturates (e.g. phenobarbital) which are known to induce these enzymes (Daniel, 1993). Northern blot analyses and nuclear run on experiments demonstrated that induction occurs at the transcriptional level (Ding and Pickett,. 1985).

1.7.1 Transcriptional regulation of alpha class GSTs

Pickett and co-workers investigated the mechanisms underlying the transcriptional activation of the rat Ya gene by xenobiotics (Rushmore *et al.,* 1990). These investigators identified five distinct *cis*-acting elements in the 5'-flanking region of the rat Ya gene (Figure 1.7.1-1). Two of these contain core DNA sequences that are recognized by the liver specific transcription factors HNF1 and HNF4 (hepatocyte nuclear factors 1 and 4), and are required for maximal basal level expression. In addition, a glucocorticoid-response element (GRE), a xenobiotic-response element (XRE), and an antioxidant-response element (ARE) were identified (Rushmore *et al.,* 1990, 1991; Paulson *et al.,* 1990). The XRE core sequence (5'-GCGTG-3') is also found in the 5'-regulatory region of the cytochrome P450 IAI gene, and is known to interact with the Ah receptor complex. Following high affinity binding of planar aromatic hydrocarbons, the liganded Ah receptor

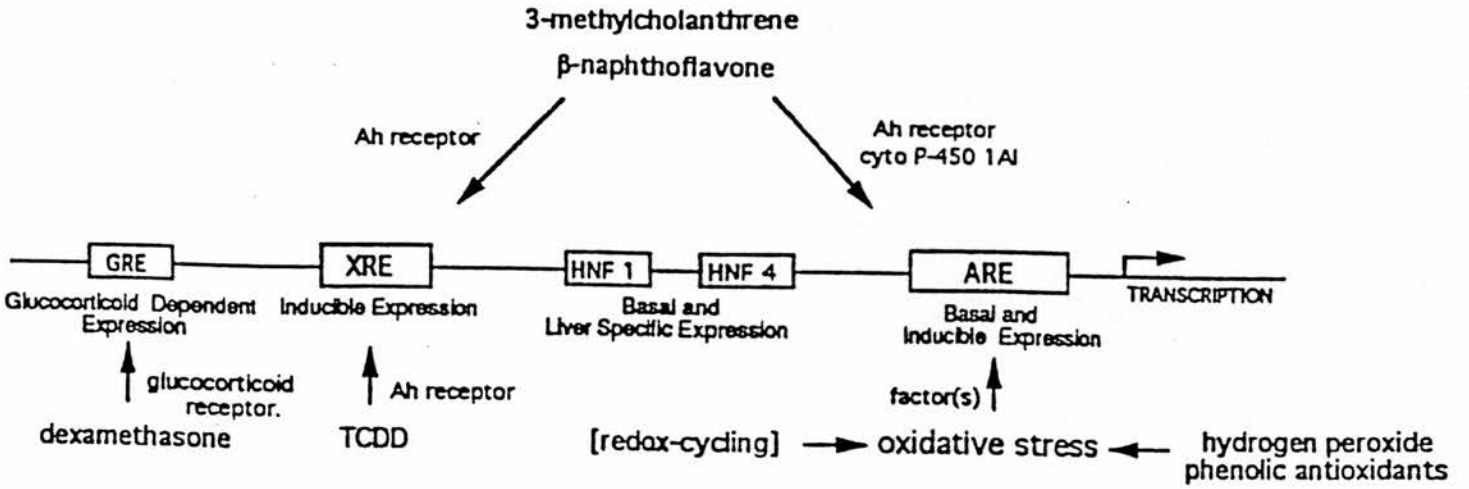


Figure 1.7.1-1 Transcriptional regulation of the rat GST Ya gene. See text for details (adapted from Rushmore and Pickett, 1993).

complex translocates from the cytosol to the nucleus, where it binds to the XRE sequence and activates transcription (Swanson and Bradfield, 1993). Pickett and colleagues demonstrated that indeed the Ah receptor was required for transcriptional activation of the Ya gene by planar aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Rushmore *et al.,* 1990). Certain planar aromatic compounds, such as 3-methyl-cholanthrene or β-naphtoflavone cannot activate transcription via the ARE in cells deficient in either Ah receptor or functional P450IAI, suggesting these compounds need to be metabolized prior to induction through the ARE (Rushmore and Pickett, 1990).

Furthermore, it was demonstrated that transcriptional activation of the rat Ya gene by phenolic antioxidants (e.g. *tert*-butylhydro-quinone, 3,5-di-*tert*-butylcatechol) was mediated through the ARE by an Ah receptor independent mechanism (Rushmore and Pickett, 1990).

Interestingly, Friling and co-workers (1990; 1992) analyzed the 5'-flanking region of the murine Ya gene, and identified a regulatory element, termed the electrophile-response element (EpRE), located between nucleotides -754 and -714, which exhibits a high degree of sequence homology with the ARE found in the rat Ya gene. The EpRE was shown to be responsive to both, planar aromatic hydrocarbons (β-naphtoflavone, 3-methylcholanthrene, TCDD) and electrophiles (e.g. *trans*-4-phenyl-3-buten-2-one) (Friling *et al.,* 1990).

1.7.2 Transcriptional regulation of pi-class GSTs

Muramatsu and colleagues have investigated the transcriptional regulation of the rat pi-class GST gene, by fusing various 5'-flanking regulatory regions to the promoterless bacterial chloramphenicol acetyl transferase (CAT) gene and transfecting these constructs into a rat hepatoma cell line (Sakai *et al.,* 1988; Okuda *et al.,* 1989). Using this approach, these investigators identified two enhancing elements, termed GPEI and GPEII (GST-P enhancer I and II), located approximately 2.0 kilo base pairs (kb) and 2.5 kb upstream from the transcriptional start site. GPEI comprises two imperfect phorbol 12-O-tetradecanoate 13-acetate-response elements (TRE), which together act as an enhancer, whereas two SV40 enhancer corelike sequences and a polyoma corelike sequence were found in GPEII. Furthermore, 400 bp upstream of the cap site a silencing element was identified, that consisted of several cooperatively functioning *cis*-acting elements. One of the proteins interacting with the silencer was identified as the transcription factor LAP/IL-DBP (Imagawa *et al.,* 1991). Muramatsu's laboratory provided evidence that in addition to the transcription factors *Jun* and *Fos*, other as yet unidentified *trans*-acting elements interact with GPEII (Diccianni *et al.,* 1992).

1.7.3 Regulation of mu and theta class GSTs

The regulation of mu and theta class GSTs is poorly understood, and studies designed to elucidate the molecular mechanisms governing the expression of these GST classes are eagerly awaited.

1.8 Biomedical applications of GSTs

1.8.1 GSTs and drug resistance

Farber and colleagues (Farber, 1984; Farber and Cameron, 1980) reported that foci and hyperplastic nodules induced in rat liver by different carcinogens were resistant to a number of structurally distinct chemicals; hence the term multidrug resistance (MDR). In addition, a number of researchers observed that malignant cells derived from human tissues often exhibit the MDR phenotype (Moscow and Cowan, 1988; Wolf *et al.,* 1987; Hayes and Wolf, 1990). Several different mechanisms may be involved in contributing to the MDR phenotype. These include transport mechanisms resulting in increased drug efflux (P-glycoprotein), repair mechanisms (topoisomerases), decreased activation of the prodrug (phase I enzymes), and increased activity of detoxification reactions (phase II).

Several lines of evidence have implicated elevated expression of GSTs in the drug resistant phenotype (Black and Wolf, 1991). Firstly, certain cell lines resistant to adriamycin (Batist *et al.,* 1986), cis-platinum and adriamycin (Wolf *et al.,* 1987), and nitrogen mustard (Buller *et al.,* 1987; Robson *et al.,* 1987) exhibit significantly elevated levels of GSTs, including human and rat pi-class GSTs. Secondly, glutathione depleting agents (Black *et al.,* 1990.; Somfai-Relle *et al.,* 1984) and GST inhibitors (Tew *et al.,* 1988) overcome drug resistance and re-sensitize cells to drugs. Thirdly, transfection of cDNAs encoding GSTs into eukaryotic cells confer certain degrees of resistance to alkylating agents (Black *et al.,* 1990; Manoharan *et al.,* 1987; Puchalski and Fahl, 1990). However, it has to be pointed out that further studies are required to define more clearly the role GSTs play in the drug resistant phenotype.

1.8.2 GST polymorphism and its effect on cancer susceptibility

An individual's susceptibility to chemical insult will be determined by the balance between phase I and phase II metabolism. Therefore, it is reasonable to hypothesize that the failure to express a phase II detoxification enzyme will result in increased sensitivity to chemicals which are substrates for the enzyme in question. Such a scenario is the case for the human mu class GSTM1. Approximately 50% of the Caucasian population is lacking the gene encoding GSTM1 (Seidegard *et al.,* 1988; Board *et al.,* 1990; Strange *et al.,* 1984). This enzyme has been shown to possess high conjugating activity toward mutagenic epoxides, such as styrene-7,8-oxide and benzo[a]pyrene-4,5-oxide. Therefore, it has been proposed that individuals carrying the null allele are likely to be more sensitive to the toxic effects of GSTM1 substrates.

Seidegard and colleagues (1986, 1990) used *trans*-stilbene oxide to measure GSTM1 activity in a group of lung cancer patients and a control group represented by healthy smokers. These researchers reported that individuals exhibiting the GSTM1 null-phenotype were at significantly higher risk to develop lung cancer compared to the control group. Strange and co-workers (1991) used a starch gel based zymogram assay to determine the frequency of the GSTM1 null allele in a group of patients suffering from adenocarcinoma of the colon or stomach. In agreement with the reports by Seidegard *et al.* (1986, 1990), these investigators also found an increased frequency of the GSTM1 null allele in patients with adenocarcinoma.

However, studies reported by other laboratories contradict these findings. Using an immunochemically based assay, Peters and co-workers (1990) found no difference in the frequency of the GSTM1 null allele between a group of patients with breast or colon carcinomas and the

control group. In addition, using a genotyping assay, Zhong *et al.* (1991) found no increased frequency of the mu class GST null phenotype in a lung cancer group compared with a healthy group of smokers that served as a control. In view of the contradicting findings, more studies are required to clarify the significance of the human mu class GST polymorphism with respect to cancer susceptibility.

1.8.3 GSTs as tumour markers

Sato and co-workers (1984, 1985) identified the pi-class GST as a marker for preneoplastic lesions occurring at early stages of chemically induced hepatocarcinogenesis in the rat. This enzyme is essentially undetectable in normal rat hepatocytes, whereas it is expressed at high levels in preneoplastic foci and nodule bearing livers. Importantly, the pi-class enzyme is not only a marker for chemically induced hepatic preneoplasia, but also for spontaneously occurring preneoplastic foci and nodules (Sawaki *et al.,* 1990). The fact that rat pi GST is not significantly induced by chemicals such as phenobarbital and 3-methylcholanthrene suggests that its hepatic expression is intrinsically linked to the neoplastic transformation process. Alternatively, its expression could also reflect a stress response. In any case, the pi-class enzyme is one of the most reliable markers for the detection of preneoplastic lesions during rat hepatocarcinogenesis (Sato, 1989).

It has to be pointed out that the regulation of pi-class GSTs in different species varies considerably. As a consequence, this family cannot be utilized as a tumour marker in all species. For example, pi-class GST is constitutively expressed in mouse liver, with males exhibiting approximately

10 fold higher levels than females (Hatayama *et al.,* 1986; McLellan and Hayes, 1987). In addition, murine pi GST is inducible by the antioxidant butylated hydroxyanisole (Hayes *et al.,* 1991a; McLellan *et al.,* 1991) and interferon (Adams *et al.,* 1987). These properties make the pi GST unsuitable as a reliable tumour marker in the mouse.

There are several reports demonstrating a significant increase in the expression of GSTP1-1 in a number of human tumours including oral, colon, stomach and lung (Sato, 1989; Howie *et al.,* 1990 a, b; Black and Wolf, 1991; Volm *et al.,* 1991; Hirata *et al.,* 1992). In contrast, Hayes and co-workers (1991c) provided evidence that GSTP1-1 expression is not increased in hepatocellular carcinomas.

In addition to the pi-class, alpha class GSTs have also the potential to be useful tumour markers. The rat alpha class GST designated Yc2, which is not expressed in hepatocytes of normal rat liver, has been found to be expressed in preneoplastic nodules and hepatomas, induced by aflatoxin B_1 (Hayes et al, 1991a). Studies investigating the expression of alpha class GSTs in human tumour samples failed to provide evidence supporting the use of this class of GSTS as tumour markers. However, much remains to be learned about the expression patterns of alpha class GSTs in human tumours.

1.9 Murine GSTs

As this thesis is concerned with the characterization of murine GSTs, an overview of the different GST classes isolated from the mouse is provided in the following.

Murine cytosolic and microsomal GSTs have been studied in several laboratories, and the physical properties of the enzymes isolated by independent groups of researchers differ to varying degrees. Possible reasons for these disparate findings include strain and sex differences. However, it is noteworthy that McLellan and Hayes (1987) found no qualitative differences concerning the physical properties of cytosolic GSTs isolated from three distinct strains of inbred mice (DBA/2, C3H/He, C57BL6). Therefore, it is likely that methodological differences account for the observed inconsistencies. The fact that different laboratories used their own nomenclature systems adds to the difficulty comparing results reported by individual investigators. In order to avoid confusion and to present a clear picture, I have, whenever possible, restricted the following overview of murine GSTs to reports which have identified subunits based on partial or full lenght primary sequences.

1.9.1 Murine alpha-class GSTs

To date, the complete primary sequences of four murine alpha class subunits termed Ya1 (Daniel *et al.,* 1987; McLellan *et al.,* 1991), Ya2 (Pearson *et al.,* 1988), Yc (Buetler and Eaton, 1992; Hayes *et al.,* 1992), and Yk (Zimniak *et al.,* 1992; McLellan *et al.,* 1992) have been reported. The enzymes Ya1 and Ya2 share 95% sequence identity and are the two

most closely related murine alpha class GSTs, whereas Yc and Yk form two separate subgroups within this class.

In contrast to the Ya and Yk type GSTs, the Yc enzyme is the major murine alpha class GST expressed in the liver of untreated animals. However, treatment of mice with the anti carcinogenic antioxidant butylated hydroxyanisole (BHA) induces the expression of the Ya and Yk type subunits in the liver and kidney up to 50 fold, whereas it has little effect on the expression of the Yc protein in either of these organs (McLellan *et al.,* 1989, 1992; Pearson *et al.,* 1988). Interestingly, a pronounced sex difference in the expression of the Ya and Yk type GSTs in kidney, and the Yc and Yk subunits in lung have been reported, with females expressing significantly higher levels compared to males (McLellan *et al.,* 1992).

Organic hydroperoxides (e.g. cumene hydroperoxide) are useful substrates for the identification of alpha class GSTs, as this class of enzymes possesses selenium independent peroxidase activity (Mannervik and Danielson, 1988; McLellan and Hayes, 1987, 1989).

Interestingly, the murine Yc enzyme, which is unlike its rat counterpart Yc2 constitutively expressed in the liver, has high catalytic activity toward activated aflatoxin B_1 (AFB_1) (Hayes *et al.,* 1992). Unlike rats, mice are highly resistant to the hepato-carcinogenic effects of AFB_1. This is believed to be due to the efficient detoxification of AFB_1-8,9-epoxide by the murine Yc enzyme.

1.9.2 Murine mu-class GSTs

Like the alpha class, the mu class is also represented by multiple GST subunits in the mouse. At least five distinct mu class subunits, termed

Yb1 (Pearson *et al.,* 1983; Townsend *et al.,* 1989), Yb2 (Townsend *et al.,* 1989), Yb3 (Lee, 1982), Yb4 (Pearson *et al.,* 1988), and Yb5 (Hayes *et al.,* 1991b) have been identified. The full length primary sequences of the subunits Yb1, Yb2, and Yb4, and a partial sequence of the Yb5 subunit, have been determined, whereas no sequence information of the Yb3 subunit is, to my knowledge, currently available.

Yb1 is the major mu class subunit constitutively expressed in the liver and intestinal mucosa, but is also found at lower levels in kidney, heart, lung and brain (Townsend, *et al.* 1989). In addition to Yb1, Yb4 has also been detected as a minor hepatic mu class subunit (Pearson *et al.,* 1988).

Although the Yb2 and Yb5 type subunits have not been detected in the liver of mice fed on normal diets, their hepatic expression is, like those of Yb1 and Yb4, significantly induced by treatment of animals with the antioxidant BHA (Townsend *et al.,* 1989; Hayes *et al.,* 1991b). The Yb3 subunit has been purified as the major form from testis, but with the exception of brain, it has not been detected in any other tissues (Lee, 1982).

1,2-Dichloro-4-nitrobenzene (DCNB) and *trans*-4-phenyl-3-buten-2-one serve as marker substrates for most mu-class enzymes (Mannervik and Danielson, 1988; Hayes *et al.* 1991b).

1.9.3 Murine pi-class GST

Unlike alpha and mu-class enzymes, the pi-class has been shown to be represented by one subunit, encoded by a single gene, in human (Morrow *et al.,* 1989; Board *et al.,* 1993) and rat (Okuda *et al.,* 1987) .

Although the characterization of the murine pi locus had not been reported at the beginning of my Ph.D. project, it was reasonable to assume that the murine pi-class is also encoded by a single gene. The knowledge of murine pi-class GST, as at the start of this project, was as follows.

As mentioned earlier, in contrast to rat (Sato *et al.,* 1984a,b) and human (Hayes *et al.,* 1991), the murine pi-class enzyme is expressed constitutively in liver, with male mice expressing approximately 10 fold higher levels than females. Hatayama and co-workers (1986) demonstrated that testosterone is the endogenous agent which is at least partially responsible for the pronounced sex difference observed in the expression of the YfYf homodimer. In addition, Adams *et al.* (1987) reported the induction of the pi enzyme in female nude mice by interferone (α/β), suggesting that this GST is inducible by stress. Furthermore, the antioxidant BHA has been shown to induce YfYf in liver and intestine of mice (McLellan *et al.,* 1992).

Ethacrynic acid has been established as a diagnostic substrate for pi-class GSTs isolated from a number of species, including mouse, rat, and human (Mannervik and Danielson, 1988).

1.9.4 Theta class GSTs

To my knowledge, no reports describing the purification or the cloning of murine theta class GSTs, had been published at the time of the writing of this thesis. Therefore, I will describe theta class GSTs isolated from other species, to point out properties which are characteristic for this class of enzymes.

Unlike other cytosolic GSTs, mammalian theta class GSTs are not retained on GSH affinity matrices (Meyer *et al.,* 1984, 1991), and with few exceptions (e.g. dog Ydf, rat mitochondrial GST 13-13) (Igarashi *et al.,* 1991; Harris *et al.,* 1991) they do not exhibit any appreciable activity toward CDNB, a classic substrate for virtually all cytosolic GSTs belonging to the alpha-, mu-, and pi-classes (Mannervik and Danielson, 1988). Substrates metabolized by theta class GSTs include ethacrynic acid (rat 13-13, rat YrsYrs) (Harris *et al.,* 1991), cumene hydroperoxide (rat 5-5) (Meyer *et al.,* 1991), dichloromethane (rat 5-5) (Meyer *et al.,* 1991), and arylmethyl sulfates, such as 1-menaphthyl sulfate (rat YrsYrs) (Hiratsuka *et al.,* 1990).

Based on the observation that the primary sequences of theta class GSTs isolated from rat, the fruitfly *Drosophila* , maize (*Zea mays*), and *Methylobacterium*, share a surprisingly high degree of identity, Pemble and Taylor (1992) proposed that this class of GSTs, is representative of the ancient progenitor gene. This proposal is further supported by the finding that unlike theta class GSTs, members of the alpha-, mu-, and pi-families have not been identified in plants. A more detailed primary sequence analysis of cytosolic GSTs lead these investigators to suggest an evolutionary tree of cytosolic GSTs, according to which the alpha-, mu-, and pi-classes evolved by gene duplication from a progenitor gene belonging to the theta class.

1.9.5 Murine microsomal GST

Andersson and co-workers (1988) reported the purification of a microsomal GST from mouse liver. Like it is the case for the rat orthologue (Morgenstern *et al.,* 1979), the activity of the murine enzyme could be

enhanced significantly by N-ethylmaleimide treatment, resulting in an approximately 7-fold increase of specific activity toward CDNB. The murine enzyme also exhibited peroxidase activity toward cumene hydroperoxide, suggesting a protective role against lipid peroxidation (Andersson *et al.,* 1988).

As discussed earlier, in contrast to cytosolic GSTs, the quaternary structure of the microsomal enzyme is homo-trimeric (Andersson *et al.,* 1993)

1.10 Aims of thesis

The aim of this thesis was to provide a thorough characterization of both the murine pi-class GST locus and its gene products. The experimental approaches chosen included gene cloning, sequencing, and heterologous expression of the corresponding cDNAs. In addition, site-directed mutagenesis was employed to explore the relationship between structure and function of the gene products in question. The results presented in this thesis form the basis for gene targeting experiments which are currently being carried out.

Chapter 2: Materials and Methods

2.1 Chemicals and reagents

Unless otherwise indicated, all chemicals and reagents were purchased from either Sigma Chemical Co., Poole, Dorset, U.K. or BDH, Glasgow, U.K., and were of analytical grade or better. All non-commercial materials are acknowledged.

2.2 Bacterial strains, growth media and antibiotics

2.2.1 Bacterial strains used

E.coli:	BL21(DE3)	F^-, *hsd*S, *gal* (λcI*ts* 857, *ind*1, *Sam*7, *nin*5, *lac* UV5-T7 gene), pLysS This strain was used for heterologous expression experiments.
	BMH71-18 mutS	*thi*, *sup*E, Δ(*lac-pro*AB), [*mut*S::Tn10], [F', *pro*AB, *laq*I^{q}ZΔM15] This strain was used for the generation of mutants by site directed mutagenesis.
	JM109	*end*A1, *rec*A1, gyr A96, *thi*, *hsd*R17, *rel*A1, *sup*E44, Δ (*lac-pro*AB), [F'; *tra*D36, *pro*AB, *lac*I^{q}ZΔM15] This strain was used for plasmid preparation.

NM522	*sup*E, *thi*, Δ(*lac-pro*AB), Δ*hsd*5, [F'; *pro*AB, *lac*I^{q}ZΔM15] This strain was used for the preparation of single strand DNA from phagemids.
NM538	F^{-}, *sup*F, *hsd*R, *trp*R, *lac*Y This strain was used to plate and propagate λEMBL3 bacteriophage.

2.2.2 Growth media

Each of the following recipes are for a total volume of one litre.

L-broth:	10 g Bacto-tryptone (Difco), 5 g Bacto-yeast extract (Difco), 5 g NaCl
Minimal agar:	200 ml 5 X M9 salts (64 g $Na_2HPO_4 \cdot 7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, 20 ml 20% glucose [w/v]), supplemented with maltose to 0.2% (w/v) and $MgSO_4$ to 10 mM
TYP broth:	16 g Bacto-tryptone, 16 g Bacto-yeast extract, 5 g NaCl, 2.5 g K_2HPO_4
H-top agar:	10 g Bacto-tryptone, 10 g Bacto-yeast extract, 6 g agarose (Sigma)

2.2.3 Antibiotics

Ampicillin: A stock solution of 50 mg/ml in distilled water is stored at -20°C and used at a working concentration of 50μg/ml.

Chloramphenicol: A stock solution of 34 mg/ml in ethanol is stored at -20°C and used at a working concentration of 34μg/ml.

Tetracycline: A stock solution of 5 mg/ml in ethanol is stored at -20°C and used at a working concentration of 20μg/ml.

Kanamycin: A stock solution of 10 mg/ml in distilled water is stored at -20°C and used at a working concentration of 20μg/ml.

2.3 Isolation and analysis of DNA

2.3.1 Vectors

2.3.1.1 Plasmid and phagemid vectors

Plasmid/phagemid vector	Use
pGEM 3Zf(+) (Promega)	General cloning
pAlter-1(Promega)	site directed mutagenesis, generation of single strand DNA
pET21a (Novagen)	Bacterial expression

2.3.1.2 Bacteriophage vectors

Vector	Use
λEMBL3	Screening of a mouse Balb/c genomic library in λEMBL3
M13KO7 (Promega)	Helperphage used to generate single strand DNA from phagemids

2.3.2 Preparation of plasmid DNA

2.3.2.1 Small scale preparation of plasmid DNA

The method by Birnboim and Doly (1979) was used to prepare plasmid DNA from an overnight culture (up to 2 ml) of bacteria. Bacteria were harvested by centrifugation at 12,000 g for 3 min and resuspended in 100 µl of Solution A (25 mM Tris/HCl, pH 8.0; 10 mM EDTA, pH 8.0; 50 mM glucose). The cells

were lysed by addition of 200 µl of Solution B (0.2 M NaOH; 1% SDS) and 150 µl of Solution C (3 M potassium acetate, pH 4.8), and incubated on ice for 5 minutes. The bacterial genomic DNA and cell debris were removed by centrifugation at 12,000 g for 5 minutes. The plasmid DNA was precipitated from the supernatant by the addition of 2 volumes of ethanol and centrifugation at 12,000 g for 10 minutes. The DNA pellet was washed with 1 ml of 70 % ethanol, air dried, and resuspended in 20 µl of distilled water.

2.3.2.2 Medium and large scale preparation of plasmid DNA

Plasmid DNA from 100-300 ml bacterial cultures was prepared using Quiagen DNA affinity columns (Quiagen Inc.). The protocol described under section 2.3.2.1 was scaled up accordingly and used up to the addition of Solution C. After the addition of Solution C the sample was centrifuged for 30 min at 20,000 g. An appropriately sized Quiagen column was equilibrated with buffer QBT (750 mM NaCl; 50 mM MOPS ((3-[N-morpholino]propane-sulfonic acid), pH 7.0; 15 % (v/v) ethanol; 0.15 % (v/v) Triton-X 100), and the supernatant containing the plasmid DNA was applied and allowed to enter the resin under gravity. The column was washed with 3 column volumes of buffer QC (1 M NaCl; 50 mM MOPS, pH 7.0; 15 % (v/v) ethanol). The plasmid DNA was eluted from the column with 1.5 column volumes of buffer QF (1.25 M NaCl; 50 mM Tris/HCl, pH 8.5; 15 % (v/v) ethanol). The plasmid DNA was precipitated by the addition of 0.7 volumes of isopropanol and centrifugation at 20,000 g for 30 min. The DNA was washed with 70 % ethanol, air dried and resuspended in 100 µl of distilled water per 100 ml of bacterial culture.

2.3.3 Isolation of genomic DNA from tissue

Genomic DNA was isolated from mouse liver following the procedure described in volume 2 of the laboratory manual by Sambrook *et al.,* (1989). Freshly excised liver tissue was weighed, snap frozen in liquid nitrogen and mechanically ground to a fine powder using pestle and mortar. The liquid nitrogen was allowed to evaporate and the tissue powder was added to 10 volumes of extraction buffer (10 mM Tris/HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 20 μg/ml pancreatic RNAse; 0.5 % SDS). The sample was incubated at 37°C for 1 hour, proteinase K was added to a final concentration of 100 μg/ml and the suspension of lysed cells was incubated at 50°C for 3 hours. An equal volume of phenol (pH 8.0) was added, the sample was mixed gently for 10 minutes, and the two phases were separated by centrifugation at 5,000 g for 15 minutes. The viscous aqueous phase was transferred to a fresh centrifuge tube using a wide-bore pipette and the phenol extraction step was repeated twice. After the third extraction, the aqueous phase was transferred to a fresh centrifuge tube, 0.2 volumes of 10 M ammonium acetate and 2 volumes of ethanol were added, and the sample was mixed thoroughly. The precipitated DNA was spooled using a pasteur pipette whose end was shaped into a U, washed with 70 % ethanol and air dried. The DNA was resuspended in an appropriate volume of TE buffer, (10 mM Tris/HCl, pH 8.0; 1 mM EDTA, pH 8.0).

2.3.4 Quantitation of DNA and RNA samples

DNA and RNA concentrations were measured spectophotometrically at 260 nm and the concentrations were calculated using the following formulae:

1 absorbance unit at 260 nm = 50 μg/ml for double strand DNA

1 absorbance unit at 260 nm = 40 μg/ml for single strand DNA or RNA

An estimate of sample purity is provided by the ratio OD_{260}/OD_{280}. Pure DNA and RNA samples have a value of 1.8 and 2.0 respectively.

2.3.5 Restriction endonucleases

Restriction endonucleases were purchased from Boehringer Mannheim, New England Biolabs, Bethesda Research Laboratories (BRL) or Amersham International (Amersham). Restriction endonucleases were used following the manufacturers recommendations.

2.3.6 Gel electrophoresis of DNA

2.3.6.1 Agarose gel electrophoresis

DNA samples were electrophoresed in 0.8-1% (w/v) agarose gels prepared from normal grade or low melting point agarose and TAE buffer (0.04 M Tris-acetate; 1mM EDTA, pH 8.0). 0.1 volumes of gel-loading dye (0.25% [w/v] bromophenol blue; 0.25% [w/v] xylene cyanol FF; 15% [w/v] ficoll; in distilled water) was added to the samples. After electrophoresis, gels were stained in a solution of ethidium bromide (0.5 µg/ml) and DNA was visualized using a UV transilluminator. In order to estimate the sizes of DNA fragments the following DNA-size markers were used: 1 kb ladder (BRL), and RF ΦX174/ *Hae* III (BRL).

2.3.6.2 Non-denaturing polyacrylamide gel electrophoresis (PAGE)

Products of DNA sequencing reactions were analyzed using PAGE gels (6% [w/v] acrylamide; 0.3% *N,N'*-methylene-bis-acrylamide; 1% Ammonium persulphate; 0.05% *N,N,N',N'*-tetra-methyl ethylendiamine [TEMED])

prepared in TBE buffer (0.09 M Tris/Borate; 0.002 M EDTA, pH 8.0). Electrophoresis was carried out using a BioRad Protean 2 vertical electrophoresis kit.

2.3.7 Southern blotting

DNA samples were subjected to agarose gel elctrophoresis and visualized with ethidium bromide. The gel was washed in distilled water, soaked for 45 minutes in denaturing solution (1.5 M NaCl; 0.5 M NaOH) followed by a 45 minute period in neutralizing solution (1.5 M NaCl; 0.5 M Tris/HCl, ph 7.5). Gels containing large DNA fragments (> 10 kb) were soaked in 0.1 M HCl for 10 minutes to facilitate the transfer of high molecular weight DNA. DNA fragments were transferred to Hybond-N filters (Amersham) in 10 X SSC (1.5 M NaCl; 0.15 M sodium citrate) by capillary action as described by Southern (1975). The DNA fragments were cross-linked to the nylon based membrane by ultra-violet irradiation (254 nm) for 30 seconds using a Stratalinker 2400 (Stratagene).

2.4 Screening and isolation of bacteriophage λEMBL3 clones

2.4.1 Preparation of the *E.coli* host strain NM538 and titer determination of the λEMBL3 library

The *E. coli* cells of the strain NM538 were grown overnight in L-broth supplemented with 0.2% maltose and 10 mM $MgSO_4$. The cells were harvested by centrifugation at 5,000 g for 5 minutes and resuspended in 10 mM $MgSO_4$ to give a final OD_{600} of approximately 2. Maltose induces the

maltose operon which includes the *lam*B gene encoding the λ receptor on the surface of the *E. coli* cells. Serial dilutions (10 fold) of the λ bacteriophage stock were made in SM-buffer (5.8 g NaCl; 2 g $MgSO_4$; 50 ml 1 M Tris/HCl, pH 7.5/ litre) to a final volume of 100 µl. These were mixed with 150 µl of host cells and incubated at 37°C for 20 minutes to allow the bacteriophage to adsorb to the *E.coli* host cells. This mixture was mixed with 3 ml molten H-top agar (45°C), poured onto L-agar plates and allowed to set. Plates were incubated overnight at 37°C. The plaque forming unit (pfu) concentration (pfu/ml) of the recombinant bacteriophage stock was determined from the number of plaques present on the lawn of *E. coli* .

2.4.2 Screening of a recombinant bacteriophage library in λEMBL3

The recombinant bacteriophages were plated on 20 cm^2 plates to give a near confluent plaque density. Approximately 2 X 10^6 plaques were screened as described by Benton and Davis (1977). Hybond-N (Amersham) filters were used to perform plaque lifts in duplicate (1st lift for 30 seconds, 2nd lift for 60 seconds) from each of the plates. Subsequently, these filters were placed (bound DNA side up) on 3MM Whatman paper soaked in denaturing solution (0.5 M NaOH; 1.5 M NaCl) for 1 minute, neutralizing solution (1.5 mM NaCl; 0.5 M Tris/HCl, pH 7.4) for 2 minutes and allowed to air dry. The DNA was crosslinked as described in section 2.3.7. A 2.2 kb DNA fragment (MPCR2.2, see Chapter 3) was radiocatively labeled with [α-^{32}P]dCTP (3000Ci/mmol) by the method of Feinberg and Vogelstein (1983) and used as a probe. The area of the plate containing a plaque hybridizing to the probe on both filters was excised and placed into SM-buffer overnight. The resulting recombinant-bacteriophage stock was used to repeat the whole procedure until single clonal bacteriophage were isolated, which hybridized to the probe.

2.4.3 Isolation of recombinant-bacteriophage DNA from λEMBL3

Approximately 1 X 10^{10} cells (OD_{600} of 1 = 8 X 10^8 cells/ml) from a fresh overnight culture of NM538 was mixed with approximately 5 X 10^7 clonal recombinant bacteriophage in a final volume of 1ml and allowed to adsorb to the host cells for 20 minutes at 37°C. The mixture was added to 100 ml of L-broth (containing 10 mM MgSO4; 0.2% [w/v] maltose) and grown overnight at 37°C in an orbital shaker. Next morning the lysis of the remaining intact host cells was completed by adding 200 µl of chloroform and shaking was continued for another 10 minutes. The bacterial debris was removed by centrifugation at 5,000 g for 10 minutes. The supernatant was centrifuged at 100,000 g for 90 minutes to pellet the recombinant-bacteriophage, and the pellet was resuspended in 1.5 ml of SM-buffer. 10 µl each of DNAse (10 µg/ml) and RNAse A (10 µg/ml) were added to digest the bacterial DNA and RNA for 1 hour at 37°C; the bacteriophage DNA is protected from digestion as it is contained within the phage coat proteins. Subsequently, proteinase K was added to a final concentration of 100 µg/ml and the sample incubated at 65°C for 2 hours. Proteins were removed from the sample by 3 X phenol/ chloroform extractions. 0.1 volumes of 3 M sodium acetate, ph 4.8, and 2.5 volumes of ethanol (-20°C) were added to the aqueous phase taken from the final phenol/chloroform extraction and the recombinant-phage DNA was precipitated at -70°C for 30 minutes. The DNA concentration was estimated as described in section 2.3.4.

2.4.4 Isolation of single strand DNA from phagemid vectors

A 2 ml overnight culture of host cells containing phagemid DNA was prepared using TYP broth supplemented with the appropriate antibiotic, and grown at

37°C. The following morning 5 ml of TYP broth supplemented with the appropriate antibiotic was inoculated with 100 µl of the overnight culture and shaken vigorously at 37°C for 30 minutes. The culture was infected with helper phage M13K07 at a multiplicity of infection (m.o.i.) of approximately 10 (that is 10 helper phage particles per cell) and continued to be shaken vigorously at 37°C for 8-16 hours. The bacteria were pelleted by centrifugation at 5,000 g for 10 minutes and discarded. RNase A and DNase were added to the supernatant to a final concentration of 10 µg/ml) and left at room temperature for 30 minutes. 0.25 volumes of PEG solution (20% [w/v] polyethylene glycol [PEG 8,000, SIGMA]; 3.5 M NH_4OAc) was added and the sample was incubated on ice for 30 minutes. Subsequently, the phage particles were harvested by centrifugation at 12,000 g for 30 minutes and the pellet was resuspended in 100 µl of buffer containing 300 mM NaCl, 100 mM Tris/HCl, pH 8.0; 1 mM EDTA. The sample was phenol/chloroform extracted twice and ethanol precipitated at -70°C for 30 minutes by adding 0.2 volumes of 7.5 M NH_4OAc and 2.5 volumes of ethanol. Proteins produced by the M13K07 helper phage recognize the f1 origin initiation site in the phagemid and nick it at this site leading to the production of a single strand copy of the phagemid DNA downstream of this site until the termination signal in the f1 origin is reached. The ssDNA molecules are circularized by other f1 encoded proteins leading to the formation of functional f1 origins. The ssDNA is packaged in coat proteins and secreted from the cell.

2.5 DNA Sequencing and computer aided analysis of DNA

2.5.1 Sequencing of double strand DNA templates

Recombinant plasmid/phagemid DNA was prepared using Quiagen DNA affinity columns as described in section 2.3.2.2. Approximately 5 μg of double stranded DNA in a total volume of 18 μl of distilled water were denatured for 10 minutes at room temperature following the addition of 2 μl of 2 M NaOH (freshly prepared). Subsequently, 8 μl of 5 M NH_4OAc, ph 7.5, were added, the sample was mixed, 100 μl of ethanol were added and the DNA was precipitated at -70°C for 30 minutes. The DNA was collected by centrifugation at 12,000 g for 10 minutes. The DNA pellet was resuspended in 7 μl of distilled water, 1 μl (10 ng) of primer and 2 μl of sequencing reaction buffer (USB; 200 mM Tris/HCL, ph 7.5; 100 mM $MgCl_2$; 250 mM NaCl). The sample was heated to 65°C for 2 minutes and allowed to cool to room temperature over an approximately 30 minute period during which time the primer anneals to its complementary sequence in the template. The annealed primer was extended for 5 minutes at room temperature following the addition of 1 μl of 0.1 M dithiothreitol, 2 μl of 1 X Labeling mix (1.5 mM each of dCTP, dGTP, dTTP), 0.5 μl of [α-^{35}S]dATP (1000Ci/mmol, 10mCi/ml) and 2 μl of Sequenase version 2 diluted 1:8 in enzyme dilution buffer (10mM Tris/HCl, pH7.5; 5 mM dithiothreitol; 0.5 mg/ml BSA). 3.5 μl aliquots of the extension reaction were terminated by adding them to 4 separate tubes each containing 2.5 μl of 8 mM of one ddNTP. The termination reaction was incubated for 5 minutes at 37°C and stopped by the addition of 4μl of loading buffer (95% formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). The samples were heated to 90°C for 2 minutes and resolved on a non-denaturing PAGE gel as described in section 2.3.6.2.

After electrophoresis the gel was fixed for 10 minutes in 10% acetic acid (v/v), 10% methanol (v/v), transferred to Whatman 3MM paper, dried under vacuum at 80°C and exposed to X-ray film (section 2.13).

2.5.2 Analysis of DNA sequencing data using a computer

Sequencing data was analyzed using the GCG package (Devereux *et al.*, 1984), the GeneJockey package (Biosoft) and the MacPattern package (Macintosh).

2.6 Generation of cDNA mutants by site-directed mutagenesis

cDNA mutants were generated using the 'Altered Sites™ i*n vitro* Mutagenesis System' (Promega) following the manufacturer's recommendations. The cDNA was cloned into the pAlter phagemid vector and ssDNA was isolated using the *E.coli* host strain NM522 as described in section 2.4.4. The mutagenesis annealing reaction was carried out as follows:

recombinant pAlter ssDNA	0.05 pmol
phosphorylated ampicillin repair oligonucleotide	0.25 pmol
phosphorylated mutagenic oligonucleotide	1.25 pmol
10 X annealing buffer (200mM Tris/HCl, pH 7.5; 100 mM MgCl; 500 mM NaCl)	2 µl
with distilled H_2O to a final volume of	20 µl

Following the annealing reaction (which was performed as described in section 2.5.1.), the sample was put on ice and 3 µl of 10 X synthesis buffer (100 mM Tris/HCl, pH 7.5; 5mM dNTPs; 10 mM ATP; 20 mM DTT), 1 µl of T_4 DNA polymerase (10 U/ml), 1 µl of T_4 DNA ligase (2U/ml) and 5 µl of distilled water were added. The synthesis and the ligation of the mutant strand were carried out at 37°C for 90 minutes. The entire synthesis reaction was used to transform competent *E.coli* BMH71-18 mutS as described in section 2.10.2. This strain was used, because it is a repair minus strain and, therefore, suppresses in *vivo* mismatch repair. Following the transformation, 4 ml of L-broth were added, the cells were allowed to recover for 1 hour shaking at 37°C before ampicillin at a final concentration of 125 µg/ml was added (only the cells containing a repaired ampicillin resistance gene are able to grow) and the cells were incubated overnight at 37°C. The following morning, plasmid DNA was prepared from these transformants as described in section 2.3.2.1. Approximately 5 ng of this DNA was used to transform competent *E.coli* JM109. The transformants were plated on L-agar plates containing 125 µg/ml ampicillin and incubated overnight at 37°C. The next morning plasmid DNA was prepared from single colonies of these transformants and analyzed for the desired mutation by sequencing. An overall success rate of 73% was achieved by using this mutagenesis system.

2.7 Isolation of total RNA

Total RNA was isolated from tissue samples for RT-PCR using the method by Chomczynski and Sacchi (1987). 0.5 g of tissue was added to 5 ml solution D (Stock solution: 250 g guanidinium thiocyanate [Sigma]; 293 ml distilled water; 17.6 ml of 0.75 M sodium citrate, pH 7.0; 26.4 ml of 10%

sarcosyl; immediately before use 0.36 ml of 2-mercaptoethanol was added per 50 ml to produce stock solution D). The tissue was homogenized in solution D using a Silverson Laboratory mixer homogenizer. The homogenate was centrifuged at 10,000 g for 10 minutes to remove insoluble tissue debris and sequentially 0.5 ml of 2 M sodium acetate, pH 4.0, 5 ml of water saturated phenol, and 1 ml of chloroform/isoamylalcohol (49:1) were added to the supernatant. The sample was mixed thoroughly after the addition of each reagent, placed on ice for 20 minutes and centrifuged at 10,000 g for 20 minutes at 4°C. The upper aqueous phase containing the RNA was removed, 5 ml isopropanol was added and the RNA was precipitated at -20°C for 1 hour. The RNA was collected by centrifugation at 10,000 g for 20 minutes at 4°C and resuspended in 1.5 ml of solution D. The RNA was re-precipitated at -20°C for 1 hour following the addition of an equal volume of isopropanol. Subsequently, the precipitate was re-suspended in 1 ml of RNAse free water, RNA concentration was determined and the sample stored at -70°C.

2.8 The Polymerase Chain Reaction (PCR)

2.8.1 PCR involving mouse genomic DNA or cDNA containing plasmid DNA as a template

For reactions involving genomic DNA the following reaction mixture was prepared :

template DNA	2 µg
dNTPs (2.5 mM each dNTP [Pharmacia])	8 µl
oligonucleotide primers (100 ng/ml)	1 µl of each
10 X reaction buffer	10 µl
Taq DNA dependent polymerase	4 units
distilled water to	100 µl

10 X reaction buffer: 10 mM Tris/HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% (w/v) gelatin

The reaction mixture was overlaid with 40 µl of mineral oil (Sigma) to prevent evaporation and subjected to the following temperature cycles using a PHC-2 thermal cycler (Techne): 2 minutes at 94°C, followed by 30 cycles of 96°C, 15 seconds; 60°C, 1 minute; 72°C, 2.5 minutes. The mouse pi cDNAs were amplified to create appropriate restriction sites at their 5'- and 3'-ends using the same protocol as above with the only modification that 5-10 ng of plasmid DNA was used as a template. Oligonucleotides used are listed in Chapters 3 and 4.

2.8.2 PCR involving RNA

2.8.2.1 Isolation of the mouse pi-class cDNAs

2 μg of total male mouse liver RNA was reverse transcribed in a total volume of 20 μl using 1 X PCR buffer (Promega), 2 mM of each dNTP, 1 unit of RNasin (Promega), 100 pmol oligo dT, and 24 units of avian-myeloblastosis-virus (AMV) reverse transcriptase (Boehringer). Following 10 minutes at room temperature, the samples were incubated for 60 minutes at 42°C. In order to denature the RNA-cDNA hybrids and to inactivate the reverse transcriptase, the reaction mixture was heated to 95°C for 5 minutes and then cooled on ice. 80 μl of 1 X PCR buffer containing 50 pmol each of upstream and downstream primer, and 2.5 units of Taq polymerase (Promega) were added. 30 cycles were performed as follows : 20 seconds denaturing at 96°C, annealing for 1 minute at 60°C, and primer extension for 1 minute at 72°C. The primer pairs used for the PCR reactions were 5'-GAGAGAGAATTCCCTCACTCTGAGTACCCCTCTGTCTA CG-3' and 5'-GAGAGATCTAGATTTATTAGTGCTGGGAAAAC-3 for Gstp-1, and 5'-GAGAGAGAATTCCCATCCCTGAGACACCTCTCTGACTATT-3' and 5'-GAGAGATCTAGATTTATTAGTGCTGGGAAAAC-3' for Gst p-2.

2.8.2.2 Estimation of hepatic mRNA levels corresponding to the mouse pi-class GST genes

RT-PCR was carried out as described in section 2.8.2.1 with the exceptions that 1μg of total RNA, and 100 units of moloney-murine-leukaemia virus (MMLV) reverse transcriptase (BRL) were used instead of 24 units of AMV reverse transcriptase (Boehringer) per reaction. In order to asses if the two pairs of oligonucleotides differ in their affinity toward their target DNA, the

yields of the following PCRs were compared. Known amounts (0.1ng) of the subcloned cDNAs, Gst p-1 or Gst p-2, were used separately together with the corresponding primer pairs and 10, 15, 20, 25, and 30 PCR cycles were performed. Equal aliquots of the two PCR products (Gst p-1 and Gst p-2 cDNAs), generated by the same number of cycles were electrophoresed on an agarose gel, visualized with ethidium bromide, and yields were compared. No difference was detected, indicating that the two pairs of oligonucleotides do not vary in their affinity to anneal to their target DNA. Various numbers of PCR cycles (10, 15, 20, 25, 30) were performed to determine conditions under which the template DNA represented the limiting factor in the amplification reaction, so that the generation of the PCR product is directly proportional to the template present. The assay was carried out in triplicate and each experiment gave identical results.

2.9 Subcloning of DNA fragments

2.9.1 Gel purification of DNA fragments

The DNA fragment to be subcloned was separated from other DNA material by electrophoresis in an agarose gel (0.6% - 2% , [w/v] depending on the size of the fragment of interest), visualized with ethidium bromide and excised from the gel using a scalpel. The DNA fragment was isolated from the agarose using GlassMax (BRL) glass bead spin columns according to the manufacturer's instructions.

2.9.2 Preparation of vector for the subcloning of fragments

The vector into which a DNA fragment was to be subcloned was digested with the appropriate restriction endonucleases and subjected to any further necessary modification (e.g. dephosphorylation). Following these manipulations the vector was gel purified as described in section 2.9.1.

2.9.3 Dephosphorylation of 5' terminal phosphates

In order to minimize the possibility of re-circularization of vectors, which have been linearised using a single restriction endonclease, during a ligation reaction, the 5' terminal phospate groups were removed using calf intestinal phosphatase (CIP; Promega). 2-5 µg of digested vector DNA was re-suspended in a total volume of 40 µl of 10 mM Tris/HCl, pH 8.0 and 5 µl of 10 X CIP buffer (0.5 mM Tris/HCl, pH 9.0; 10 mM $MgCl_2$; 1mM $ZnCl_2$; 10 mM spermidine) and 0.5 units of CIP were added and incubated at 37°C for 30 minutes after which another 0.5 units of CIP were added. After an additional 30 minutes at 37°C the vector DNA was isolated using the GlassMax glass bead columns (see section 2.9.1).

2.9.4 Ligation of the DNA fragment and the vector

The ligation of DNA fragments and vectors with cohesive ends was carried out in a total volume of 10 µl using 50-100 ng of vector DNA and a 1:1 and 1:3 molar excess of DNA fragment together with 1 ml of 10 X ligation buffer (660 mM Tris/HCl, pH 7.5; 50 mM $MgCl_2$; 10 mM DTE; 10 mM ATP) and 0.5 units of T4 DNA ligase (Boehringer Mannheim). The ligation reaction was performed at 15°C overnight.

2.10 Transformation of bacterial cells with plasmid/phagemid DNA

2.10.1 Preparation of competent bacteria

A modified version of the acid salt method for the preparation of competent bacteria was used as described in 'Promega Protocols and Applications Guide' (Promega Corporation, Madison, U.S.A.). 10 ml of L-broth was inoculated with a single colony of the appropriate strain of *E.coli* and grown overnight at 37°C. Using a 1 l flask, 100 ml of L-broth was inoculated with 1 ml of cells grown overnight and the culture was grown shaking at 37°C until the OD_{600} reached 0.45 - 0.55 (mid-log phase). The cells were chilled in ice water for 2 hours and collected by centrifugation at 2,500 g for 15 minutes at 4°C. The bacteria were resuspended in 5 ml of freshly prepared ice-cold trituration buffer (100 mM $CaCl_2$; 70 mM $MgCl_2$; 40 mM sodium acetate, pH 5.5) , diluted to 100 ml with the same solution and incubated on ice for 45 minutes. Subsequently, the cells were collected by centrifugation at 1,800 g for 10 minutes at 4°C, gently resuspended in 10 ml of ice-cold trituartion buffer, and 80% (v/v) sterile glycerol was added to a final concentration of 15% (v/v). The cells were either used immediately for transfomation or snap frozen on dry ice and stored at -70°C for up to 4 weeks before being used.

2.10.2 Transformation of competent bacteria

5-20 ng of DNA was mixed with 200 µl of competent cells and incubated on ice for 30 minutes. The mixture was subjected to a heat shock at 42°C for 60 seconds and cooled on ice for 1 minute, before 4 ml of L-broth was added and the cells were allowed to recover for 45 minutes shaking at 37°C. The cells were collected by centrifugation at 1,800 g for 5 minutes, plated out on

agar plates supplemented with the appropriate antibiotic, and incubated overnight at 37°C.

2.11 Screening of bacterial colonies

2.11.1 α-Complementation screening of bacterial colonies

The pGEM 3Zf(+) and pAlter vectors carry a short segment of DNA that contains the regulatory sequence and the first N-terminal 146 amino acids of the *E.coli lac Z* gene encoding the β-galactosidase enzyme. On transformation of the parent plasmid into certain host strains, which produce the C-terminus of the β-galactosidase enzyme encoded on the F' episome, the two fragments can interact and produce an active protein (Ullmann *et al.*,, 1967). This process is called α-complementation. Bacteria transformed with vector lacking an insert form blue colonies in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) on de-repression of the *lacZ* gene fragments by addition of IPTG (isopropylthio-β-D-galactoside). However, insertion of the recombinant fragment into the cloning site of the vector disrupts the *lacZ* N-terminal fragment and colonies containing recombinant plasmids are white. Bacteria transformed with vectors allowing for blue/white screening, based on the α-complementation phenomenon, were grown on plates containing X-gal (40μg/ml) and IPTG (0.5 mM).

2.11.2 Screening of bacterial transformants using colony hybridization

A modification of the method by Grunstein and Hogness (1975) was used as an alternative to screen for recombinant bacterial colonies. Colonies were picked using sterile toothpicks and streaked out in duplicate onto Hybond-N

gridded membranes (Amersham) placed on appropriate agar plates. The plates were incubated at 37°C overnight. One plate was stored at 4°C (master plate), whereas the membrane from the second plate was removed and placed colony side up on Whatman 3MM paper soaked in 10% SDS (w/v) for 3 minutes. The membrane was transferred to another sheet of paper and left soaking in denaturing solution (1.5 M NaCl; 0.5 M NaOH) followed by neutralizing solution (0.5M Tris/HCL, pH 7.5; 1.5 M NaCl) for 5 minutes each time. The membrane was air dried and baked at 80°C for 2 hours. The filter was washed in 50mM Tris/HCl, pH 8.0, 1.5 mM EDTA, and 1% SDS (w/v) in a shaking water bath at 42°C for 1 hour. Subsequently, the membrane was prehybridized (for at least 1 hour) without, and hybridized (overnight) with radiolabeled insert containing probe, washed and exposed to X-ray film. The recombinant colonies which hybridized with the probe were picked from the duplicate colony of the master plate and grown for the preparation of plasmid DNA.

2.12 DNA and RNA hybridization techniques

2.12.1 Radiolabeling DNA probes

2.12.1.1 Random prime labeling of double-strand DNA probes

Double-strand DNA was labeled by the random priming method of Feinberg and Vogelstein (1983). The DNA fragment was heat denatured in the presence of a molar excess of random hexanucleotide primers and radioactive nucleotides were added into the DNA probe followed by primer extension using the large fragment of *E.coli* DNA polymerase I ("Klenow" fragment). 20-30 ng of DNA was denatured at 100°C for 3 minutes, chilled immediately on ice, the following components were added, and the labeling reaction was carried out for 2 hours at 37°C:

OLB	3 µl
BSA (10mg/ml)	2 µl
[α-^{32}P]dCTP (3,000 Ci/mmol; 10 Ci/ml)	3 µl
Klenow fragment	2 units
distilled water to	50 µl

OLB consists of a mixture of the solutions A, B, and C in a ratio of 2:5:3

A:	2 M Tris/HCl, pH 8.0	625 µl
	1 M $MgCl_2$	125 µl
	distilled water	82 µl
	2-mercaptoethanol	18 µl
	10 mM dATP, dGTP, dTTP	50 µl of each
B:	2 M Hepes, pH 6.6	

C: Hexadeoxyribonucleotide in 3 mM Tris/HCl, pH 8.0, 0.2 mM EDTA to a concentration of 9000 units/ml (Pharmacia)

2.12.1.2 Radiolabeling oligonucleotide probes

Synthetic oligonucleotides are synthesized without a phosphate group at the 5' end and can be labeled by the transfer of [γ-^{32}P] ATP to the 5' end using the bacterial T_4 polynucleotide kinase (PNK; Boehringer) in the following reaction. The labeling reaction was carried out at 37°C for 1 hour:

Oligonucleotide (10 pmoles/ml)	1 µl
10 X PNK buffer	2 µl
[γ-^{32}P] ATP (5.000 Ci/mmole; 10 Ci/ml)	5 µl
PNK (10 units/ml)	1 µl
distilled water	1 µl
total volume	10 µl

10 X PNK buffer: 0.5 M Tris/HCl, pH 7.6; 0.1 M $MgCl_2$; 50 mM dithiothreitol; 1 mM spermidine; 1 mM EDTA, pH 8.0

2.12.1.3 Checking the efficiency of radiolabeling both oligonucleotide and double-strand DNA probes

1 ml of the labeled probe mixture was spotted onto Whatman DE-81 filter paper. The filter is positively charged and binds oligonucleotide and double strand-DNA stronger than non-incorporated radiolabeled nucleotides. Therefore, non-incorporated radiolabeled nucleotides can be separated from incorporated radiolabeled nucleotides chromatographically using 0.3 M

ammonium formate, pH 8.0 as the mobile phase. The resultant chromatogram was exposed to X-ray film and the relative levels of incorporated to non-incorporated radiolabeled nucleotides was assessed.

2.12.2 Hybridization of radiolabeled double-strand DNA probes to membrane-bound DNA

All hybridization procedures were carried out in rotating glass tubes using a Hybridiser HB-1 (Techne). Membrane bound DNA was incubated for at least 2 hours at 65°C in prehybridization solution (5 X SSPE; 0.1% sodium pyrophosphate [w/v]; 0.5% SDS [w/v]; 100 µg/ml heat denatured herring sperm DNA; 2 X Denhardt's solution) after which this solution was exchanged for the hybridization solution (as for prehybridization solution omitting the herring sperm DNA; preheated to 65°C). Subsequently, the radiolabeled probe was denatured at 100°C for 3 minutes, added to the rotating glass tube containing the membrane bound DNA and hybridized overnight at 65°C. The following morning the filters were washed for 60-90 minutes at 65°C changing the washing solution (0.1-2 X SSC; 0.1% SDS [w/v]; 0.1% sodium pyrophosphate [w/v]) every 10-20 minutes until the background radioactivity attached to the filter was minimized.

20 X SSPE, pH 8.0/litre:	175.3 g NaCl; 27.6g NaH_2PO_4; 7.4g EDTA
50 X Denhardt's solution/litre:	10 g each of BSA (Sigma), polyvinylpyrolidone, (Sigma), and Ficoll-400 (Sigma)
20 X SSC, pH 7.0/litre:	175.3 g NaCl; 88.2 g sodium citrate

2.12.3 Hybridization of radiolabeled oligonucleotide probes to membrane-bound DNA

Hybridization was carried out as described in section 2.12.2 with the following modifications:

(pre-)hybridization temperature was 40°C

prehybridization solution: 6 X SSPE; 0.5% SDS (w/v); 0.1% sodium pyrophosphate (w/v); 5 X Denhardt's; 100 µg/ml heat denatured herring sperm DNA

Filters were washed in prewarmed 6 X SSC, 0.1% sodium pyrophosphate (w/v) initially at 30°C for 20 minutes. Filters were examined with a monitor and, if they showed high levels of background radioactivity, they were washed in the same solution but with progressive 4°C increases in temperature until the background radioactivity was minimized.

2.12.4 Hybridization of radiolabeled double-strand DNA probes to membrane bound RNA

Membrane bound RNA was prehybridized for at least 2 hours at 42°C using a Hybridiser HB-1 (Techne). The probe was heat denatured at 100°C for 3 minutes, added to the hybridization mixture and hybridized overnight at 42°C.

Prehybridization/hybridization solution:

100% formamide	10 ml
50 X Denhardt's solution (see 2.12.2)	2 ml
10 % SDS (w/v)	1 ml
20 X SSC (see 2.11.2)	6 ml
50% Dextran sulphate	3 ml
100 µg/ml heat denatured herring sperm DNA	200 µl

All washes were carried out at 65°C as described in section 2.12.2.

2.13 Autoradiography

Autoradiography was performed at -70°C for filters containing the isotopes ^{14}C, ^{125}I and ^{32}P, and at room temperature for ^{35}S, using X-ray cassettes and Kodak X-AR film. The time of exposure was varied according to the intensity of the radioactive signal. Films were developed using a Gevamatic 60 (Agfa-Gevaert) automatic developer.

2.14 Heterologous expression, affinity purification, and analysis of recombinant glutathione S-transferase proteins

2.14.1 Heterologous expression of pi-class glutathione S-transferases in *E.coli*

Host cells [*E.coli*, BL21(DE3)] harbouring pET21a expression constructs were grown in L-broth supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) to an OD_{600} of approximately 0.6-0.7 at 37°C. The expression of the desired protein was induced (de-repressed) by the addition of IPTG (isopropyl-β-D-galactoside, Boehringer) to a final concentration of 1 mM. The culture was allowed to continue growing for 3 hours before the cells were harvested at 5,000 g for 5 minutes.

2.14.2 Purification of pi-class glutathione S-transferases

All purification procedures were carried out at 4°C. Following induction of the desired glutathione S-transferase protein, the bacterial cell pellet was lysed by freeze (60 minutes at -70°C) thawing and resuspended in buffer P1 (4 ml of P1 per 100 ml of starting culture). Subsequently, the sample was sonicated on ice twice for 20 seconds at full power (Soniprep 150 sonicator,

MSE) to complete lysis and to shear the bacterial DNA. The sample was centrifuged twice at 20,000 g to remove the bacterial debris and the supernatant was loaded onto a glutathione agarose (Sigma) affinity column (column dimensions: 1.5 cm X 10 cm) at a flow rate of approximately 30 ml/hour, which had previously been equilibrated with at least 10 column volumes of buffer P1. The column was washed with 20 column volumes of buffer P1 overnight. The next morning, the protein was eluted from the column with buffer P2. The OD_{280} of the 1.5 ml fractions was measured and fractions showing a value greater than 0.8 were pooled and dialysed against 4 X 2 litre of buffer P3 over a period of 48 hours. The purified protein sample was divided into 250 ml aliquots, frozen on dry ice and stored at -70°C. The homogeneity of the recombinant protein was checked by SDS-PAGE analysis and the protein concentration was determined.

P1-buffer: 50 mM Tris/HCl, pH 7.4; 200 mM NaCl; 0.5 mM DTT

P2-buffer: 200 mM Tris/HCl, pH 9.0; 50 mM glutathione (Sigma)

P3-buffer: 40 mM Na_2HPO_4, pH 7.4; 0.5 mM DTT; 1 mM EDTA

2.14.3 Enzymatic assays

Enzyme assays were performed either manually using a Shimadzu UV 160 spectrophotometer or by using a Cobas Fara centrifugal analyser. Glutathione S-transferase activities were determined at 30°C according to the method by Habig and Jakoby (1981). The assays were performed at least in triplicate. Table 2.14.3-1 shows the conditions under which assays for individual substrates were performed. The 1-chloro-2,4-dinitrobenzene (CDNB) assay was carried out using a Cobas Fara centrifugal analyser as described by Hayes and Clarkson (1982). This instrument allowed the simultaneous analysis of up to 29 samples. Selenium-independent glutathione peroxidase activity was measured using the coupled assay sytem described by Reddy *et al.,* (1981).

$$ROOH + 2GSH \text{ ----------> } H_2O + ROH + GSSG$$

$$GSSG + NADPH + H^+ \text{ ----------> } 2GSH + NADP^+$$

The spontaneous conjugation of the various substrates with GSH was subtracted from initial rates. Linear regression analysis was performed using the manufacturer's kinetic rate program for those assays carried out on the Cobas Fara. The extinction coefficients (Table 2.14.3-1) were used to calculate the enzymatic activities which were expressed as mmol/min/mg protein.

Table 2.14.3-1 Conditions for various GST assays (adapted from Habig and Jakoby, 1981).

Substrate	[Substrate] (mM)	[GSH] (mM)	pH	Wave length (nm)	$\Delta\varepsilon$ ($mM^{-1}\ cm^{-1}$)
1,2-Dichloro-4-nitrobenzene	1.0	5.0	7.5	345	8.5
1-Chloro-2,4-nitrobenzene	1.0	1.0	6.5	340	9.6
p-Nitrophenyl acetate	0.2	0.5	7.0	400	8.79
p-Nitrobenzyl chloride	1.0	5.0	6.5	310	1.9
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	0.5	5.0	6.5	360	0.5
trans-4-Phenyl-3-buten-2-one	0.05	0.25	6.5	290	24.8
Ethacrynic acid	0.2	0.25	6.5	270	5.0
Bromosulfophthalein	0.03	5.0	7.5	330	4.5
Δ^5-Androstene-3,17-dione	0.068	0.1	8.5	248	16.3

2.14.4 Kinetic analysis

Activity toward CDNB was assayed spectrophotometrically at 340 nm using a Cobas Fara centrifugal analyser. Assays were performed at 30°C. Initial rates were measured at 5 s intervals for a total period of 55 s, commencing 3 s after initial mixing. The reaction was initiated by the addition of the enzyme to 0.1 M sodium phosphate buffer, pH 6.5, containing the substrates CDNB and GSH. CDNB was dissolved in ethanol, and the final concentration of ethanol in the reaction mixture was constant at 3.5%. Non-enzymatic reaction rates were subtracted from enzymatic rates. The enzymatic rate was linear with time for up to 60 s after initiation. Each data point represents the mean of three measurements. Individual kinetic parameters for each enzyme represent the mean of three independent series of experiments, with the only exception being the values for GSTP2-2 which was calculated from two series of experiments. Kinetic analysis was based on the report by Phillips and Mantle (1991) describing the initial rate kinetics of murine pi-class GST YfYf (GSTP1-1) as a rapid-equilibrium random mechanism. Therefore, initial velocities were interpreted according to the equation:

$$Vo = \frac{V_{max}\,[A]\,[B]}{aK_AK_B + aK_B[A] + aK_A[B] + [A]\,[B]}$$

(A; GSH, B: CDNB). Double reciprocal plots of initial velocity data were linear for all enzymes. Individual kinetic parameters were determined as described by Ivanetich and Goold (1989). Regression analysis was performed using the Apple Mac program Cricket Graph, version 1.3.2. With CDNB as the varied substrate, GSH concentrations ranged from 0.05 mM to 2mM. With GSH as the varied substrate, CDNB concentrations ranged from 0.1 mM to 2

mM (the CDNB concentration cannot be increased above 2 mM for reasons of solubility).

2.14.5 Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS/PAGE)

Protein samples were prepared for SDS/PAGE following dilution to a suitable protein concentration in P3-buffer (see 2.14.2), and the addition of an equal volume of 2 X boiling mix (0.05 M Tris/HCl, pH 6.8; 2% [w/v] SDS; 5% [v/v] mercaptoethanol; 10% [v/v] glycerol; 0.05% bromophenol blue). The samples were heated to 100^{o}C for 5 minutes. Glutathione S-transferase protein samples (Mr of approximately 24,000 dalton) were electrophoresed in a 12% (w/v) polyacrylamide separating gel (11.7% acrylamide; 0.3% *N,N'*-methylene-bis-acrylamide) in 0.375 M Tris/HCl, pH 8.8, 0.15% (w/v) SDS. Ammonium persulphate (AMPS, 1% [w/v]) and N,N,N,N'-tetra-methyl ethylenediamine (TEMED) were added to a final concentration of 0.05% (v/v). A stacking gel (4.5% [w/v] acrylamide; 0.125 M Tris/HCl, pH 6.8; 0.125% SDS; 0.05% of each TEMED and AMPS) was cast onto the polymerized separating gel. The gels were prepared in a BioRad Protean 2 vertical electrophoresis kit and electrophoresed in electrode buffer (0.05M Tris/HCl, pH 8.3; 0.05 M glycine; 0.1% SDS) at a constant voltage of 100 V.

2.14.6 Western blot analysis

Western blots were performed as described by Towbin *et al.,* (1979) with the following modifications. Following SDS-PAGE the proteins were transferred electrophoretically to nitrocellulose for 1 hour at a constant current of 250 mA, using the transblot apparatus from Biorad, (transblot buffer: 25 mM Tris/HCl; 193 mM glycine, pH 8.3). Following transfer, the nitrocellulose was washed for two 10 min periods in TBST (50 mM Tris/HCl, pH 7.9; 0.15 M NaCl, 0.05% Tween 20) and blocked for at least 60 min in 3% (w/v) non-fat milk protein (Marvel, Cadbury) in TBST. Two 10 min washes in TBST were followed by a 60 min incubation with the first antibody (a polyclonal antibody raised against murine pi-class GST YfYf which was a kind gift of Dr. Lesley McLellan was used at a 1:2000 dilution). The nitrocellulose filter was washed three times for 10 min in TBST and incubated for 60 min with the second antibody (1:1000 dilution of anti-rabbit IgG) conjugated to horseradish peroxidase. A further three 10 min washes in TBST followed before the protein bands were visualized with peroxidase staining using 4-chloro-1-naphtol (Sigma) as substrate. The nitrocellulose filter was placed in a sandwich box containing 200 ml TBS (50 mM Tris/HCl pH 7.9, 0.5 M NaCl), 4-chloro-1-naphtol (120 mg in 40 ml methanol), and 80 µl of 30% hydrogen peroxide were added. Filters were incubated for 10 min and washed for 10 min in distilled water, before they were labeled with ^{125}I-protein A (Amersham). Filters were put in 50 ml of TBST, containing 0.19 MBq of ^{125}I-protein A for 45 min. Non-hybridized ^{125}I protein A was removed by washing filters four times for 15 min in TBST. Subsequently, filters were air dried,and subjected to autoradiography.

2.14.7 Determination of protein concentration

The method by Lowry *et al.,* (1951) was employed to estimate the protein concentration of samples. Bovine serum albumin (BSA) was used in the concentration range of 20-200 mg/ml to generate a standard curve. Samples were diluted such that they showed OD_{650} values which were within the linear range of the standard curve. A Shimadzu UV 160 spectrophotometer was used to measure the absorbance of the samples. The determination of the protein concentration was carried out in triplicate for each sample.

2.15 Animals

2.15.1 Animal maintenance

Animals were kept at the University of Edinburgh Faculty Animal Care Aerea. Animals were fed a standard laboratory chow diet *ad libitum* and acclimatized for at least 4 days to a 12 hour light/dark cycle prior to sacrifice.

2.15.2 Animal sacrifice and tissue removal

Mice were killed by cervical dislocation, organs were removed, rinsed in phosphate buffered saline (PBS/litre: 1.5 g Na_2HPO_4; 8.g NaCl;0.1 g MgCl; 1.5 g KH_2PO_4; 0.2 g KCl; 0.132 g $CaCl \cdot H_2O$) and snap frozen in liquid nitrogen.

Chapter 3: Isolation and characterization of two murine pi-class glutathione S-transferase genes

3.1 Introduction and aims

Sato and co-workers (1984, 1985) demonstrated that the expression of pi-class GST is markedly elevated in rat liver tumour development. Subsequent studies established the pi enzyme as one of the most reliable marker for the identification of both chemically-induced and spontaneously occurring hepatocarcinogenesis in the rat (Sato, 1989; Sawaki *et al.,*1990). In the early stages of the carcinogenic process, single cells and foci appear that display a phenotype very different from that of normal hepatocytes. It is generally thought that these foci represent precursors of liver tumours. The GST pi protein (YfYf) is virtually non-detectable in normal, healthy rat hepatocytes, whereas it is expressed at high levels in foci- or nodule bearing livers (Sato, 1989).

Importantly, several investigators have also reported a significant increase of the pi enzyme in a number of human tumours including oral, colon, stomach and lung (Sato, 1989; Howie *et al.,*1990; Black and Wolf, 1991; Volm *et al.,*1991; Hirata *et al.,*1992). However, unlike in rat, human GSTP1-1 levels are not elevated in hepatocellular carcinoma (Hayes *et al.,*1991). Furthermore, numerous studies have found significantly increased levels of the pi enzyme in cell lines made resistant to anticancer drugs and to chemical toxins in culture (Batist *et al.,*1986; Cowan *et al.,*1986; Wolf *et al.,*1990; Black and Wolf, 1991).

The mechanism(s) underlying the overexpression of pi-class GSTs in carcinogenesis and drug resistance are poorly understood. Therefore,

studying the regulation and function of this group of enzymes will improve our understanding of these fundamental processes.

As the mouse is genetically well characterized and recent advances in transgenic and gene targeting technology provide unique opportunities for investigating gene regulation and function, the characterization of the murine pi-class gene locus was chosen as the subject for this study. It has to be noted, unlike its rat and human homologues, the mouse GST pi gene is expressed constitutively in hepatocytes and is subject to regulation by both, endogenous agents, such as testosterone and interferone (Hatayama *et al.,*1986; Adams *et al.,*1987) and exogenous agents, such as the anti-carcinogen butylated hydroxyanisole (McLellan *et al.,*1989).

The aim of this part of my project was to study the murine pi-class gene locus at the molecular level employing cloning and sequencing techniques. As outlined in Chapter 1, in contrast to rat and human, the mouse pi gene had not been isolated at the beginning of this project. The analysis of the murine pi locus presented here is also the basis for gene targeting experiments which are being carried out currently.

3.2 Results and Discussion

3.2.1 Isolation of genomic clones

The amino acid sequences deduced from the mouse pi-class GST cDNA reported by Hatayama *et al.*(1990) and the rat pi-class gene, GST-P, (Okuda *et al.*,1987) share 92% identity. Based on the conservation of the exon/intron organization of homologues mammalian genes, the exon/intron boundaries of the mouse pi gene was predicted from the rat gene reported by Okuda *et al.*(1987). A pair of oligonucleotides (see Chapter 2) to sequences in exon 2 and 7 of the predicted mouse gene and mouse genomic DNA were then used to amplify part of the mouse pi-class GST gene emloying PCR (Saiki *et al.*,1989). Exon 1 was omitted as it only contains the A of the ATG initiation codon in both the rat and the human pi-class genes. As a result, I obtained two PCR products, approximately 0.7 kb and 2.2 kb (MPCR2.2) in size (Figure 3.2.1-1). Attempts to subclone these blunt-ended PCR fragments into the plasmid pGEM3Zf(+) were unsuccessful.

Gel purified MPCR2.2 was digested with a number of restriction endonucleases to identify those, which would not cleave this DNA fragment. Beside others, the restriction enzymes *Sph* I and *Xba* I were found not to cut MPCR2.2 as judged by electrophoretic analysis in a 1% agarose gel stained with ethidium bromide. In order to optimize the conditions for subcloning the PCR fragments, two new oligonucleotides were designed, such that a *Sph* I or *Xba* I restriction site was added to the 5' ends of the previously used oligonucleotides. The PCR reaction was repeated with the new oligonucleotides, and the two resulting products (0.7 kb, 2.2 kb) were ligated separately into the *Sph* I and *Xba* I restriction sites of the plasmid

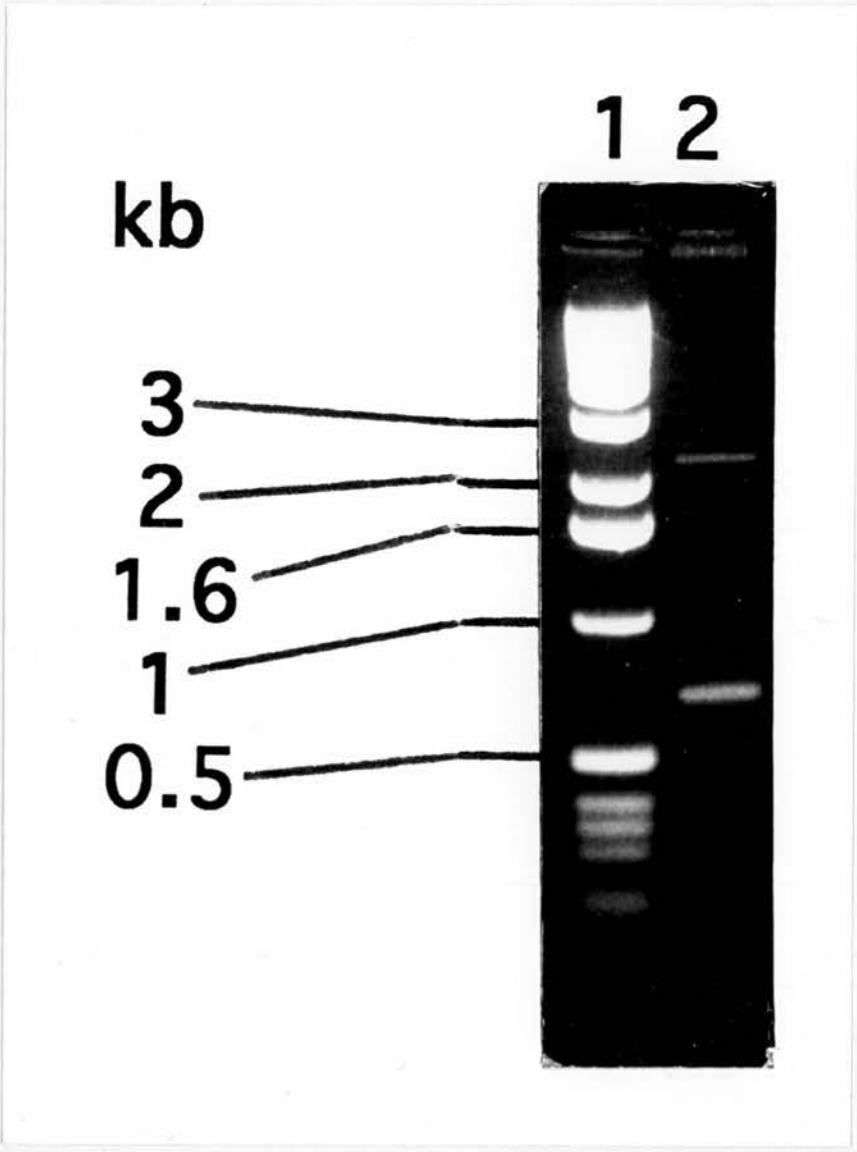


Figure 3.2.1-1 Two PCR products, 0.7 and 2.2 kb in length, share a high degree of identity with the murine pi-class GST genes Gst p-2 and Gst p-1 respectively. Mouse genomic DNA together with oligonucleotides matching sequences in exons 2 and 7 of the murine Gst p-1 gene produce two products in a PCR-amplification, approximately 0.7 and 2.2 kb in size. These DNA fragments were electrophoresed in a 1 % agarose gel, stained with ethidium bromide and visualized using a UV source; lane 1, molecular weight standards; lane 2, 0.7 and 2.2 kb PCR products.

pGEM3Zf(+). Initially, only the bigger fragment, MPCR2.2, was characterized by partial sequencing, because this fragment was approximately of the size predicted for the partial murine gene ranging from exons 2-7. The sequence over the coding region was identical to the murine pi-class cDNA described by Hatayama *et al.* (1990). This clone, MPCR2.2, was then used as a probe to screen a mouse Balb/c liver genomic library in λEMBL3. Four positive plaques were isolated, and restriction analysis revealed that three of these were identical. The two distinct clones were named MGSTP1 and MGSTP2. They contained inserts of approximately 12 kb, and 15 kb respectively. The restriction maps of these clones are shown in Figure 3.2.1-2. The maps also show the regions that hybridized to MPCR2.2.

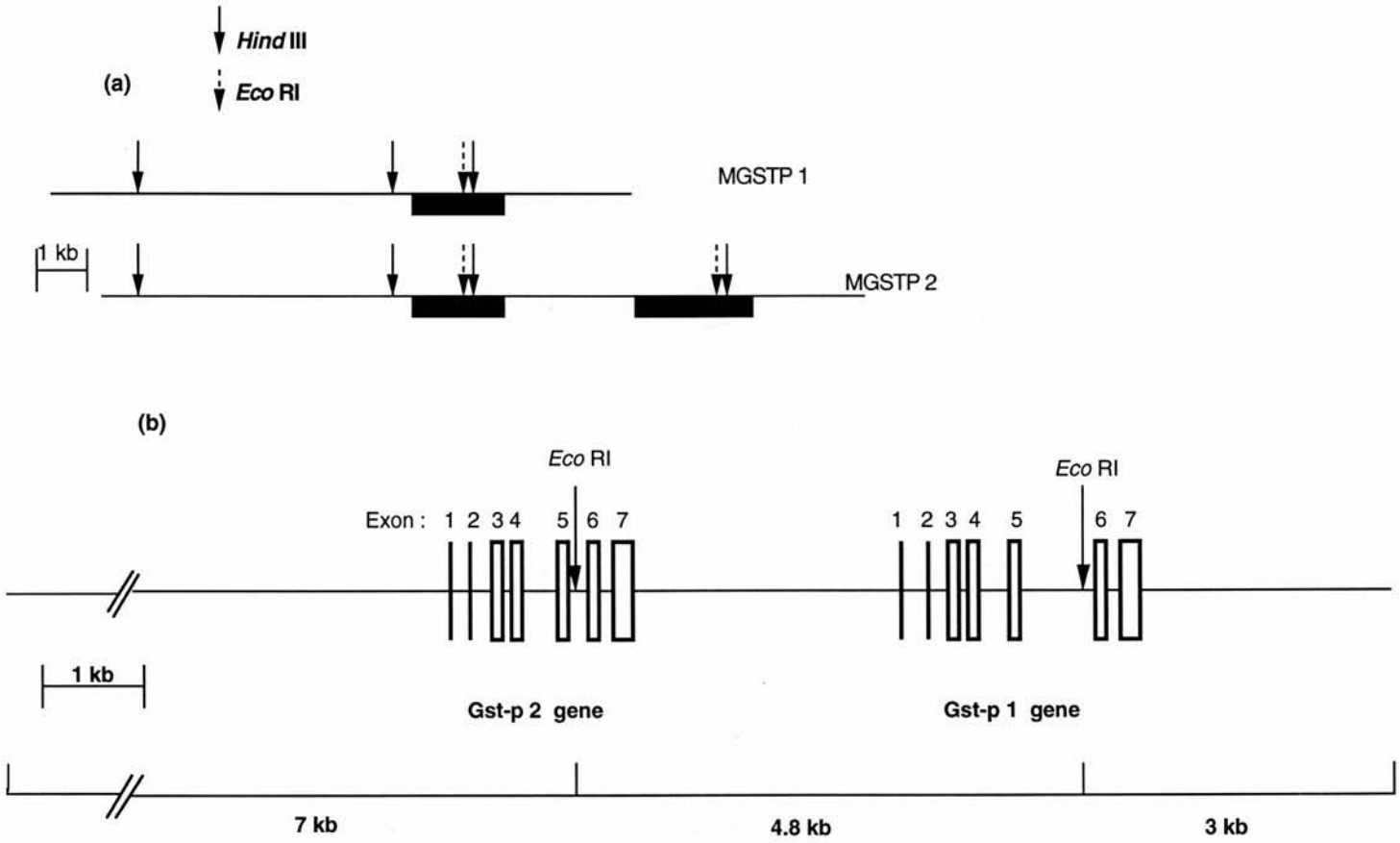

(a)
Hind III
Eco RI
MGSTP 1
1 kb
MGSTP 2
(b)
Eco RI
Eco RI
Exon : 1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 kb
Gst-p 2 gene
Gst-p 1 gene
7 kb
4.8 kb
3 kb

Figure 3.2.1-2 Organization of the mouse GST pi locus. (a) Restriction maps of the phage clones MGSTP1 and MGSTP2, containing one and two mouse pi-class GST genes respectively. Solid and dashed arrows represent *Hind* III and *Eco*RI sites respectively; solid boxes indicate regions complementary to the probe MPCR2.2. (b) Restriction map and intron/exon arrangement of the two mouse pi-class GST genes, Gst p-1, and Gst p-2, as contained in clone MGSTP2. Exons are shown as boxes numbered 1-7. Length of introns was determined by sequencing the genes.

3.2.2 Sequence analysis of clone MGSTP2

The approximately 15 kb insert of clone MGSTP2 was digested separately with the restriction endonucleases *Eco*RI and *Hind*III to generate smaller overlapping DNA fragments which were individually cloned into the plasmid pGEM 3Zf(+). Restriction mapping, hybridization analysis, and DNA sequencing showed that clone MGSTP2 contained two closely related genes, Gst p-1 and Gst p-2, which had not been described previously. They are adjacent and separated only by approximately 3 kb intervening DNA (Figures 3.2.1-2, 3.2.2-1), and therefore do not represent allelic variants. The coding regions of Gst p-1 and Gst p-2 share 100 % and 93 % identity with the sequence of the mouse pi-class cDNA reported by Hatayama et. al. (1990). The positions of splice junctions were deduced by comparison of the nucleotide sequence of the genes with that of the murine pi-class cDNA reported previously (Hatayama et. al 1990). Both genes were sequenced completely and shown to contain 7 exons interrupted by 6 introns. Gst p-1 is approximately 2.7 kb in length, while Gst p-2 is approximately 2.1 kb long due to a smaller sized intron 5 (Figure 3.2.1-2). The nucleotide sequence at all splice junctions is consistent with the canonical GT/AG rule of Breathnach and Chambon (1981), indicating that the exonic segments within each gene transcript can be spliced accurately to form functional mRNA. The polyadenylation signal, AATAAA, occurs in the seventh exon of the two genes, which is further evidence that both gene transcripts can be processed to mature mRNAs.

Each gene possesses an open reading frame of 630 nucleotides encoding a protein of 210 amino acids including the initiator methionine. The calculated molecular weights of the proteins encoded by Gst p-1 and Gst p-2 are 23.6 kDa and 23.5 kDa, respectively. As found in the rat and the human

```
Gstp-1   1     GGAGGCGGGA GCTGCAGAGC TCCGCTACCG GCTTCTCTCT CCAACGTGTT GAGTCAG--- ---------C ATCCGGGGCG GAGCGCGATG CCCCTTATAA
Gstp-2   1     .......... ....G....A ...AA..... ...AT..... .......... .......CAT CCGGGGCGG. .......... ......A... ..........

Gstp-1   88    GGCTGGCGGC CCGGTGGCCT ACGCGTGCGT CCTCACTCTG AGTACCCCTC TGTCTACGCA GCACTGAATC CGCA------ ----CCCAGC AGGCAgtgag
Gstp-2   101   .......T.T ..T.G..... GT.TT...T. ..ATC.CTGA GAC...T... ..A...TT.. .....TG... .C..CTTCTC TCTG.A.... ..C.......

Gstp-1   179   ~~210 bp~~ ctaacattct tctcttgtcc acccc agTGC CACCATACAC CATTGTCTAC TTCCCAGTTC GAGgtaagac caaatgactg caggaagggg
Gstp-2   201   ~~197 bp~~ .......... ....c....- -.......... .G..G..... .......... ......AG.. C.....g..g .t........ ..........

Gstp-1   479   aggtctgggg taggggttgc agcccaccat tcctatcctc catgaagaaa ttccagttca ttttttattc tccttctgg- ---------- ----------
Gstp-2   485   .......... .......... .......... .......tg. ....g..... ..g..a.... .ca..a..c. ....gg.t.c aaggctctac ttccctcacc

Gstp-1   557   --ttgcaagt ggtccttgta cttgaaattg ctttgaggct gggcctgaat gctgt-cccc aaatccttac cctgactcca tacagGGCGG TGTGAGGCCA
Gstp-2   586   atggc..gtc a......... ..c....... .......... ......tt.c a..a.c.... .......... .......... .......... ..........

Gstp-1   654   TGCGAATGCT GCTGGCTGAC CAGGGCCAGA GCTGGAAGGA GGAGGTGGTT ACCATAGATA CCTGGATGCA AGGCTTGCTC AAGCCCACTT GTgtgagtga
Gstp-2   686   ....C..... .......... .......... .......... .......... ......... .......... .......... .......... ..........

Gstp-1   754   cacccgtagt ggaggggggca gaggtagggc cttaggaggg ctgtgactgg gaggcagcag catcaccaag gttcttgttc ctccctccag CTGTATGGGC
Gstp-2   786   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

Gstp-1   854   AGCTCCCCAA GTTTGAGGAT GGAGACCTCA CCCTTTACCA ATCTAATGCC ATCTTGAGAC ACCTTGGCCG CTCTTTGGgt aagtcctgaa cccaggtggt
Gstp-2   886   ....T..T.. .......... .......... .......... .......... .......... .......... .......... .......... ..........

Gstp-1   954   ~~281 bp~~ aacccgtagG GCTTTATGGG AAAAACCAGA GGGAGGCCGC CCAGATGGAT ATGGTGAATG ATGGGGTGGA GGACCTTCGC GGCAAATATG
Gstp-2   986   ~~275 bp~~ ......c... .......... .......... .......... ....G..... .......... .......... .......... ..........

Gstp-1   1325  TCACCCTCAT CTACACCAAC TATgtgagcc ~~742 bp~~ tctccctggc agGAGAATGG TAAGAATGAC TACGTGAAGG CCCTGCCTGG GCATCTGAAG
Gstp-2   1351  G....A.G.. .....GA... .......... ~~149 bp~~ .......... .......... .......... .......... .......... ..........

Gstp-1   2157  CCTTTTGAGA CCCTGCTGTC CCAGAACCAG GGAGGCAAAG CTTTCATCGT GGGTGACCAG gtgagcatct ~~140 bp~~ ctgcccctgc agATCTCCTT
Gstp-2   1590  .......... .......... .......... .......... .......... .......... .......... ~~146 bp~~ .......... ..........

Gstp-1   2387  TGCCGATTAC AACTTGCTGG ACCTGCTGCT GATCCACCAA GTCCTGGCCC CTGGCTGCCT GGACAACTTC CCCCTGCTCT CTGCCTATGT GGCTCGCCTC
Gstp-2   1826  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

Gstp-1   2487  AGTGCCCGGC CCAAGATCAA GGCCTTTCTG TCCTCCCCGG AACATGTGAA CCGTCCCATC AATGGCAATG GCAAACAGTA GTGGACTGAA GAGACAAGAG
Gstp-2   1926  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

Gstp-1   2587  CTTCTTGTCC CCGTTTTCCC AGCACTAATA AAGTTTGTAA GACAGAAGAG GTGTCTTTGG
Gstp-2   2026  .......... .......... .......... .......... .......... ..........
```

Figure 3.2.2-1 Nucleotide sequences of the mouse pi-class GST genes, Gst p-1 and Gst p-2. The nucleotide sequence of gene Gst p-1 is shown with substitutions in gene Gst p-2 indicated below. Intronic sequences are presented in lower case letters. Dashes indicate gaps inserted in the sequences to optimize the similarity between the two genes. The TATA box and the polyadenylation signal are double, and the GC boxes, the TRE motif, the GT/AG splice sites, and the stop codon are single underlined.

homologues, the start codon, ATG, is split by the first intron in both mouse genes (Figure 3.2.2-1). The coding nucleotide sequences of the two genes differ in 14 positions (Figure 3.2.2-1); 5 of these substitutions are neutral and the remaining 9 result in the replacement of 6 amino acids (Figure 3.2.2-2). Two of these amino acid differences, Met89/Val and Leu106/Met, are conservative (Miyata *et al.,*1979). A detailed structure-function analysis of the the two murine pi proteins with respect to the six amino acid differences is presented in Chapter 4. Interestingly, N-terminal sequence comparison clearly shows that these peptides are different from the murine pi-class GST reported by Mannervik and colleagues (1985b) (Figure 3.2.2-2).

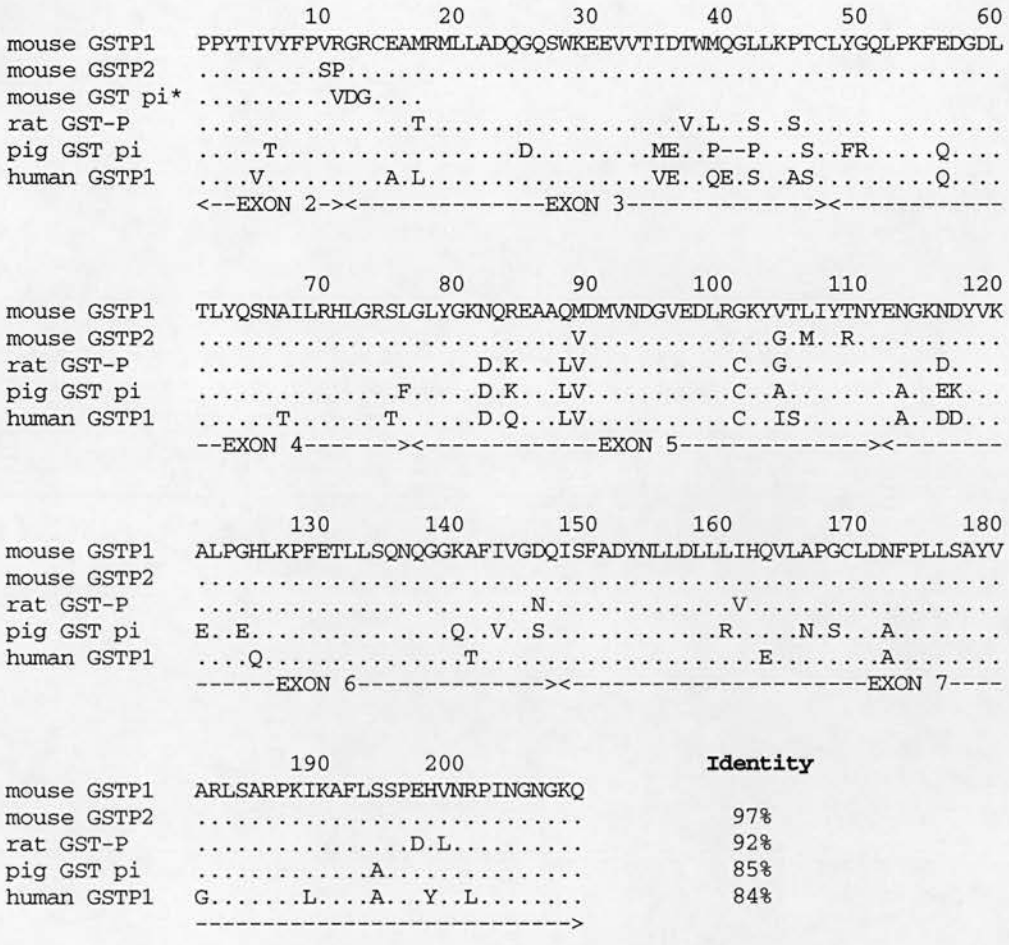


Figure 3.2.2-2 Amino acid sequence alignment of mammalian pi-class GSTs. The pi-class GST peptide sequences of mouse GST pi* (only partial sequence available), rat GST-P (YfYf), pig GST-pi, and human GSTP1 were taken from Mannervik et al. (1985b), Okuda et al. (1987), Dirr et al. (1991), and Kano et al. (1987) respectively. Dots represent amino acids identical to those in mouse GSTP1. Dashes indicate gaps inserted in the sequences to optimize the similarity between the sequences. The percentages of identities between mouse GSTP1 and other pi-class GSTs are shown in the last column. Exons corresponding to peptide sequences are indicated for the mouse, the rat, and the human sequences only, as the exon/intron junctions of the pig pi-class GST have not been determined.

3.2.3 Analysis of the promoter regions

A "TATAA" sequence and a possible "CCAAT" element (CCAAC) can be found in the 5' flanking regions of both genes (Figure 3.2.2-1). These elements, which are present in most eucaryotic promoters, are associated with the accurate initiation and promotion of transcription (Maniatis *et al.,*1987). Furthermore, a GC box, which matches precisely the consensus sequence 5'(G/T)GGGCGG(G/A)(G/A)(C/T)3' for the binding site of transcription factor SP1, forms part of the promoter of the two genes (Kadonaga *et al.,*1986). Gst p-2 has an additional GC box ,5'-GGGGCGG CAT-3', which differs from a perfect SP1 binding site in only one nucleotide, and is missing in Gst p-1. Immediately upstream of the GC boxes in both genes is the sequence 5'-TGAGTCAG-3' which corresponds to the consensus sequence found in promoters of genes that are responsive to phorbol esters (e.g. 12-O-tetradeconoylphorbol 13-acetate (TPA)). This motif has been called a TPA response element (TRE). In comparison, the human and the rat pi-class genes also possess two and one GC boxes, respectively, and a TRE (Cowell *et al.,*1988; Okuda et. al., 1987). Muramatsu and co-workers have suggested that a TRE is involved in the high level of transcription of GST-P observed during rat hepato-carcinogenesis (Diccianni et. al., 1992). In addition, the 5' untranslated regions of both genes exhibit various substitutions and deletions. The overall differences between the 5' non-coding regions of the two mouse genes suggest that they may be regulated differently.

3.2.4 Southern-blot analysis

The number of pi-class GST genes in the Balb/c mouse was estimated by Southern-blot analysis. Figure 3.2.4-1 shows that four *Eco* RI fragments of mouse DNA, approximately 2.5 kb, 4.8 kb, 6.8 kb, and 9 kb in size, hybridize to probe MPCR2.2. Based on the restriction map of clone MGSTP2 (Figure 3.2.1-2), this suggests that there are at least three pi-class GST related sequences in the genome of the Balb/c mouse strain. Clone MGSTP2 accommodates three of these hybridizing fragments, namely the approximately 4.8 kb, 6.8 kb, and 9 kb *Eco* RI fragments. However, the approximately 2.5 kb band is not present in any of the positive clones isolated. This fragment requires further characterisation to establish whether it is a pseudogene or a further functional GST pi gene. Pi-class GST pseudogenes have been found in rat and human (Okuda *et al.,*1987; Board, 1993).

Interestingly, Miles and co-workers (1991) detected two additional *Eco*RI fragments, approximately 3.8 kb and 10 kb in size, in the genomes of the C57BL/6 and DBA/2J mouse strains, which hybridized to a full length human GSTP1 cDNA probe. In addition, these researchers identified the approximately 2.5 kb fragment as a restriction fragment length variant (RFLV). This fragment was present in the C57BL/6 strain, whereas it was missing in the DBA/2J strain. There are several possible explanations for the differences in Southern blot analysis found between the C57BL/6, DBA/2J, and Balb/c mouse strains. Firstly, it cannot be ruled out that the approximately 3.8 kb and 10 kb fragments represent RFLVs between the mouse strains C57BL/6 and DBA/2J used by Miles *et al.*(1991) and the Balb/c strain used in this study. Secondly, these researchers used a full length human GSTP1 cDNA as a probe (Kano at al., 1987), whereas I used

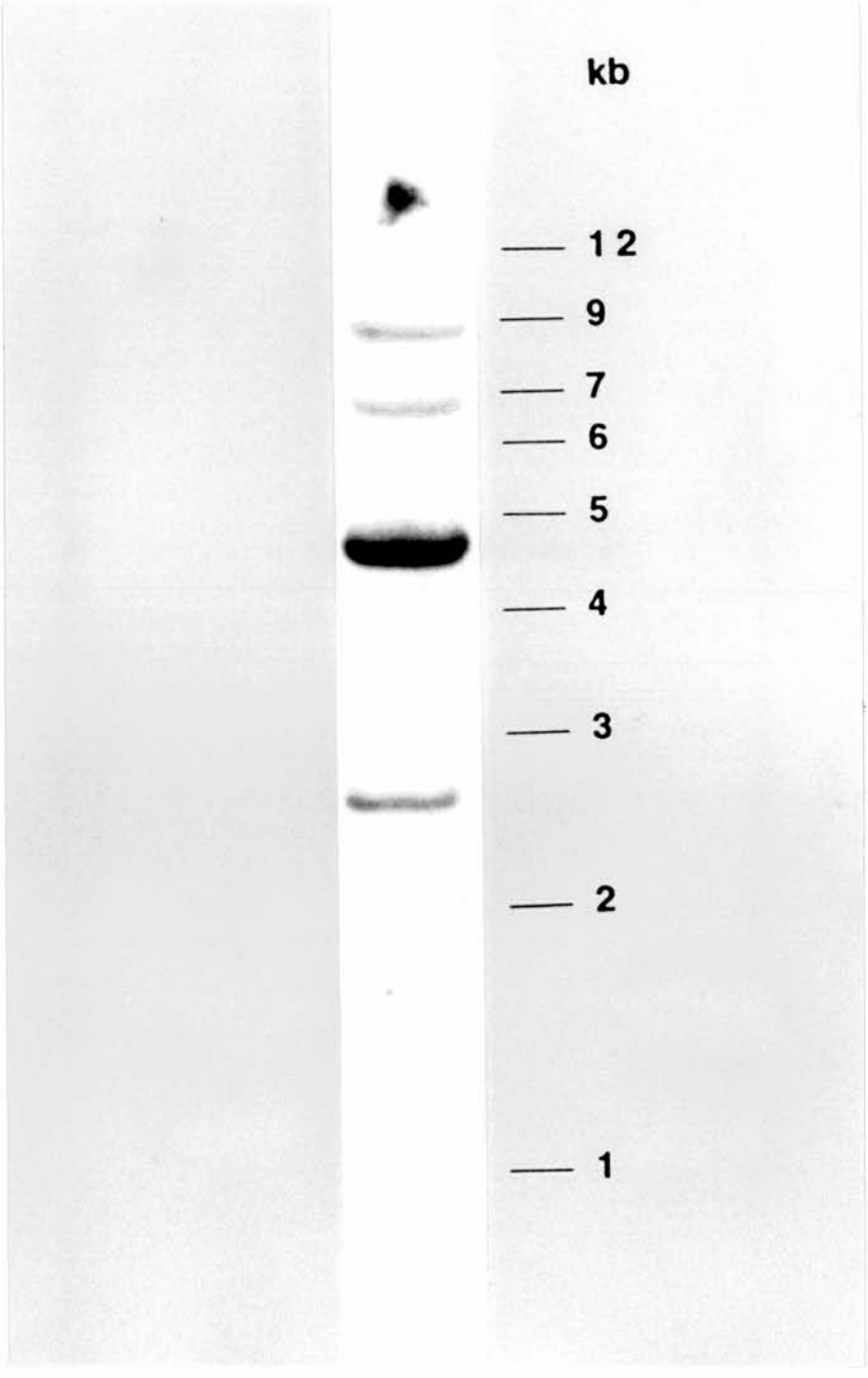


Figure 3.2.4-1 Southern-blot analysis of mouse liver DNA. Genomic DNA (20μg) was digested with *Eco*RI and separated on a 0.8% agarose gel. Following transfer to a Hybond-N filter (Amersham), hybridization was carried out with ^{32}P-labeled MPCR2.2 probe using the conditions described in Chapter 2.

MPCR2.2 as a probe, which represents part of the murine Gst p-1 gene spanning the region between exons 2 and 7 inclusively. Thirdly, Miles and colleagues washed their filters much less stringently (1 X SSC, 0.1% sodiumpyrophosphate, 0.1% SDS) than I did (0.1 X SSC, 0.1% sodium-pyrophosphate, 0.1% SDS). Finally, the blots were exposed to X-ray film up to 14 days by these investigators, whereas I limited the exposure time to 24 hours. Therefore, it is possible that the differences in the execution of the southern blot analyses may account for the varying results.

Miles and co-workers (1991) mapped the murine pi-class GST gene locus to the distal end of chromosome 1.

3.2.5 Characterization of the 0.7 kb PCR product

As described in section 3.2.1 of this chapter, two products, 0.7 kb and 2.2 kb in length, were obtained from a PCR reaction, when oligonucleotides to the coding regions of exon 2 and 7 of Gst p-1 were used together with mouse genomic DNA. As outlined earlier, the 2.2 kb fragment was identified by partial sequencing as part of the Gst p-1 gene. To characterize the nature of the smaller 0.7 kb fragment, one strand of its DNA was sequenced in its entirety (Figure 3.2.5-1). Interestingly, the sequence of the *Sph* I site containing oligonucleotide (representing the 5'- end of exon 2), used in the PCR reaction could not be found in this fragment. Further analysis revealed, that a *Sph* I restriction site was present in the 0.7 kb fragment close to its 5'-end. When this fragment was digested with *Sph* I and *Xba* I, prior to subcloning into the plasmid pGEM3Zf(+), the very 5'-end must have been lost. As this *Sph*I fragment was very small (estimated $\leq$ 50 bp), its loss

```
                [Sph I]
MPCR0.7     GCATGCTGCT GACTGACCAG GGCCAGAGCT GGAAGGAGGA GGTGGTTACC
p-2cDNA     .......... .G........ .......... .......... ..........

MPCR0.7     ATAGATACCT GGATGCAAGG CTTGCTCAAG CCCACTTGTC TGTATGGGCA
p-2cDNA     .......... .......... .......... .......... ..........

MPCR0.7     GCTTCCTAAG TTTGAGGATG GAGACCTCAC CCTTTACCAA TCTAATGCCA
p-2cDNA     .......... .......... .......... .......... ..........

MPCR0.7     TCTTGAGACA CCTTGGCCGC TCTTTGGGGC TTTATGGGAA AAACCAGAGG
p-2cDNA     .......... .......... .......... .......... ..........

MPCR0.7     GAGGCCGCCC AGGTGGATAT GGTGAATGAT GGGGTAGAGG ACCTTCGCGG
p-2cDNA     .......... .......... .......... .....G.... ..........

MPCR0.7     CAAATATGGC ACCATGATCT ACAGAAACTA TGAGAATGGT AAGAATGACT
p-2cDNA     .......... .......... .......... .......... ..........

MPCR0.7     ACGTGAAGGC CCTGCCTGGG CATCTGAAGC CTTTTGAGAC CCTGCTGTCC
p-2cDNA     .......... .......... .......... .......... ..........

MPCR0.7     CAGAACCAGG GAGGCAAAGC TTTCATCGTG GGTGACCAGg tgagcatctc
p-2cDNA     .......... .......... .......... .........- ----------
p-2Int.6                                               [. ..........

MPCR0.7     ttgcattgcc tttgccttgt ggataaggac aagagggagc catatgtaaa
p-2cDNA     ---------- ---------- ---------- ---------- ----------
p-2Int.6    .......... .......... .......... .......... ..........

MPCR0.7     gtggagcaga gctaaggggt ggaggtgggg g------ccc tcagctctct
p-2cDNA     ---------- ---------- ---------- ---------- ----------
p-2Int.6    .......... .......... .......... .tgaggg... ..........

MPCR0.7     ccccccccagc cccagcctcc tcccagcttc tgagacccac acctcctgcc
p-2cDNA     ---------- ---------- ---------- ---------- ----------
p-2Int.6    g......... .......... ........g. .......... ..........

MPCR0.7     cctgcagATC TCCTTTGCCG ATTACAACTT GCTGGACCTG CTGCTGATCC
p-2cDNA     -------... .......... .......... .......... ..........
p-2Int.6    .......]

MPCR0.7     ACCAAGTCCT GGCCCTTGGC TGCCTGGACA ACTTCCCCCT GCTCTCTGCT
p-2cDNA     .......... .....C.... .......... .......... ..........

MPCR0.7     ATGTGGCTCG CCTCAGTGCC CGGCCCAAGA TCAAGGCCTT TCTGTCCTCC
p-2cDNA     .......... .......... .......... .......... ..........

                                                        [STOP]
MPCR0.7     CCGGAACATG TGAACCGTCC CATCAATGGC AACGGCAAAC AGTAGTGGAC
p-2cDNA     .......... .......... .......... ...T....... ..........
                                                        [Xba I]
MPCR0.7     TGAAGAGACA AGAGCTTCTT GTCCCCGTTT TCCCAGCACT AATAAATCTA
p-2cDNA     .......... .......... .......... .......... ......

MPCR0.7     GA
```

Figure 3.2.5-1 Sequence alignment of the 0.7 kb PCR fragment with the murine Gst p-2 cDNA and intron 6 of the Gst p-2 gene. A comparison of the nucleotide sequences of the 0.7 kb PCR product (MPCR0.7), the Gst p-2 cDNA (p-2cDNA), and intron 6 of the Gst p-2 gene (p-2Int.6) is shown. The region of MPCR0.7 that is highly homologous to p-2Int.6 is presented in lower case. Potential splice signals (gt/ag) and the inverse sequence corresponding to the oligonucleotide (to exon 7 of Gst p-1) used in the PCR reaction that generated MPCR0.7 are underlined. Dots indicate nucleotides identical to those in MPCR0.7. Dashes represent gaps inserted in the sequences to optimize the similarity between them. The *Sph*I and *Xba*I restriction sites present at the 5'- and 3'-ends of MPCR0.7 are indicated in bold.

was not noticed by visual analysis of an ethidium bromide stained agarose gel.

The 0.7 kb PCR fragment shares a very high degree of identity with the Gst p-2 cDNA, with an additional sequence inserted between exons 6 and 7 that differs from that of intron 6 of the Gst p-2 gene in only 8 basepairs, namely a 6 bp deletion and 2 single bp substitutions (Figure 3.2.5-1). Interestingly, this fragment has an open reading frame (very similar to the Gst p-2 cDNA), which is interrupted by an intron-like sequence containing the GT and AG splice consensus sequences at its 5' and 3'-ends, respectively. Therefore, it is possible that the 0.7 kb PCR product is a partially processed RNA derived from the Gst p-2 gene, which has been reverse transcribed and integrated into the genome. However, whether the 0.7 kb fragment is a pseudogene or a further functional gene remains to be established.

3.2.6 Evidence for transcription of the mouse pi-class GST genes Gst p-1 and Gst p-2

In order to assess , if the two mouse pi-class GST genes are transcribed, I attempted to clone the corresponding cDNAs by RT-PCR. The nucleotide sequences of the Gst p-1 and Gst p-2 genes are identical in the 3' untranslated regions (Figure 3.2.2-1). However, differences in the 5' non-coding regions are sufficient to allow the design of two pairs of oligo-nucleotides which are specific for each gene (see 2.8.2.1 for sequence of oligonucleotides). These oligonucleotides were used together with total liver RNA isolated from male mice to perform RT-PCR. This procedure resulted in two DNA fragments of approximately 700 bp. The two fragments were cloned individually into the *EcoR*I and *Xba*I restriction sites of the plasmid

vector pGEM3Zf(+) and sequenced. As anticipated, these PCR fragments represented the two mouse pi-class cDNAs, with coding regions identical to those found in the genes, Gst p-1 and Gst p-2. The isolation of these cDNA clones clearly shows that both murine pi-class GST genes are transcribed. This observation is intriguing in view of the finding that only one active pi-class GST gene has been reported in both human and rat (Okuda *et al.,*1987; Cowell *et al.,*1988; Board, 1993). To the best of my knowledge, this was the first time, two actively transcribed murine pi-class GST genes have been reported.

3.2.7 Relative mRNA levels of Gst p-1 and Gst p-2 in male and female mouse liver

Using the same oligonucleotides which enabled me to amplify and isolate the Gst p-1 and Gst p-2 cDNAs, I developed a RT-PCR assay to estimate the expression levels of the two genes in mouse liver. Conditions under which RT-PCR was performed were identical for each reaction, and as described in Chapter 2. When a primer pair was used together with the non-compatible subcloned murine Gst pi cDNA as a template, no PCR product was generated, demonstrating that the primer pairs were indeed specific for the amplification of the corresponding cDNA. Using this procedure it became apparent that both of the murine pi-class GST genes are expressed at significantly higher levels in male compared to female liver (Figure 3.2.7-1). Moreover, mRNA encoding Gst p-1 is much more abundant (at least 20-fold) than that of Gst p-2 in both male and female mouse liver. Consequently, it is estimated that at least 95% of total hepatic pi-class enzyme present in mouse liver is represented by GSTP1-1.

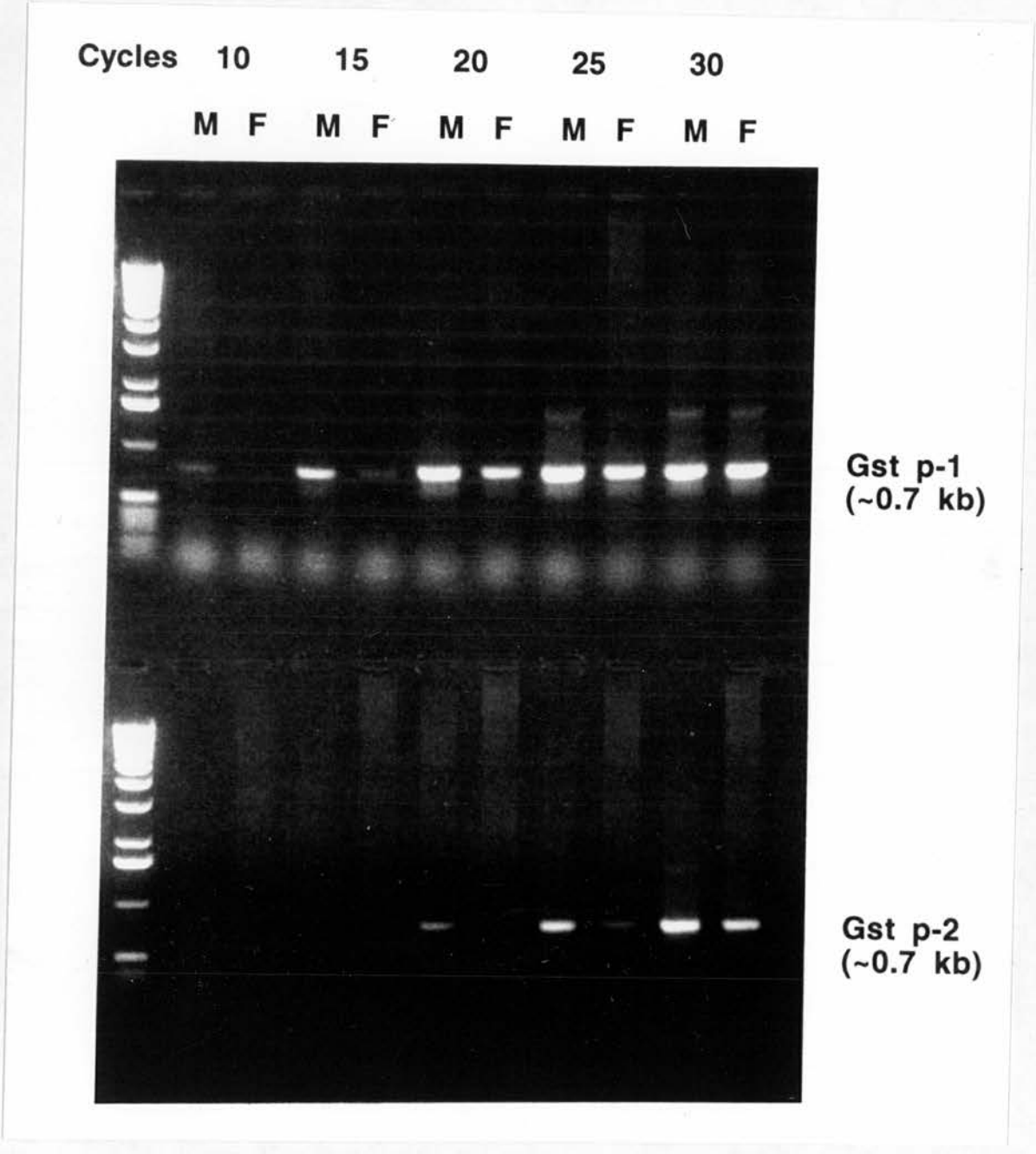


Figure 3.2.7-1 Relative levels of Gst p-1 and Gst p-2 mRNAs in male and female mouse liver. RT-PCR (10, 15, 20, 25, 30 cycles) was carried out using total mouse liver RNA to assess the relative expression of Gst p-1 and Gst p-2 in male (M) and female (F) mice as described in Chapter 2. 20 µl of the PCR product was subjected to electrophoresis in an 0.8% agarose gel and visualized with ethidium bromide.

3.3 Summary

Unlike other species characterized to date, the mouse contains at least two functional GST pi genes. These genes appear to have certain common regulatory characteristics to previously reported pi-class GST genes, in that both of their promoters contain each a TPA responsive element. However, the 5'-regulatory regions of the two genes differ significantly suggesting that both genes are expressed differentially. Indeed, the hepatic expression of both genes was found to be much higher in males compared to females, and Gst p-1 mRNA is more abundant than Gst p-2 in both sexes. As expected, the overall structure of pi-class genes in mouse, rat and human is highly conserved. The proteins encoded by the two murine pi genes are highly homologous and differ in 6 amino acids only. A detailed analysis of the proteins encoded by these genes is presented in Chapter 4.

Chapter 4: Heterologous expression, purification, and structure-function analysis of two recombinant murine pi-class GSTs

4.1 Introduction and aims

As outlined in Chapter 3, unlike other species characterized to date, the mouse contains at least two actively transcribed pi-class GST genes, Gst p-1 and Gst p-2. Alignment of the deduced amino acid sequences revealed that the proteins encoded by these genes share 97 % identity and differ in 6 amino acids only. Two of these amino acid differences, namely Met89/Val and Leu106/Met, are conservative (Miyata *et al.*,1979). Structural data obtained from porcine and human pi-class GSTs (Reinemer *et. al.,* 1991, 1992) suggest that the non-conservative changes Arg11/Pro, Val104/Gly, and Thr109/Arg do not directly participate in the lining of the active site. In contrast, the amino acid Val10 participates in the formation of the region of the active site to which the electrophilic substrate binds (Reinemer *et al.,*1992). In addition, Val10 is conserved in all mammalian pi-class GSTs isolated to date, with the exception of murine GSTP2 (Figure 4.1-1), in which it is substituted by a Ser. Therefore, it was reasonable to speculate that the substitution of Val10 by Ser was likely to alter the activity of the enzyme. However, although only the amino acid at position 10 is directly involved in the formation of the active site, it cannot be ruled out that the other 5 changes have also an influence on the catalytic activity.

It was the aim of this chapter to find out whether the proteins encoded by these genes display similar or distinct catalytic activities, and to study the effects each of the 6 amino acid differences has on the catalytic

performances of these enzymes. As I had cloned the two murine pi-class GST cDNAs previously (Chapter 3), I decided to generate the correseponding recombinant proteins by means of heterologous expression in *E. coli.* In addition, site directed mutagenesis was used to determine the effects of the amino acid differences on the catalytic activity.

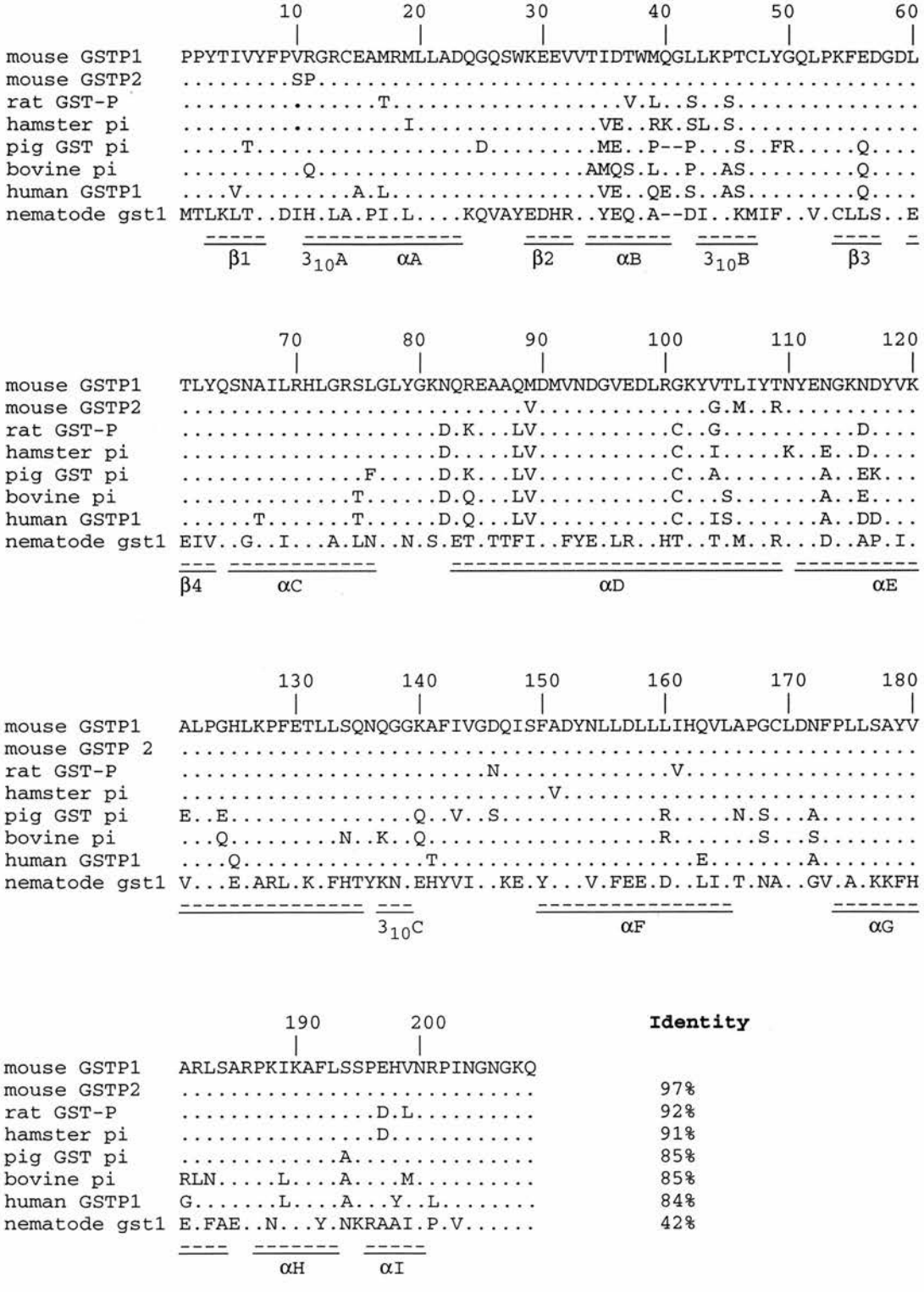
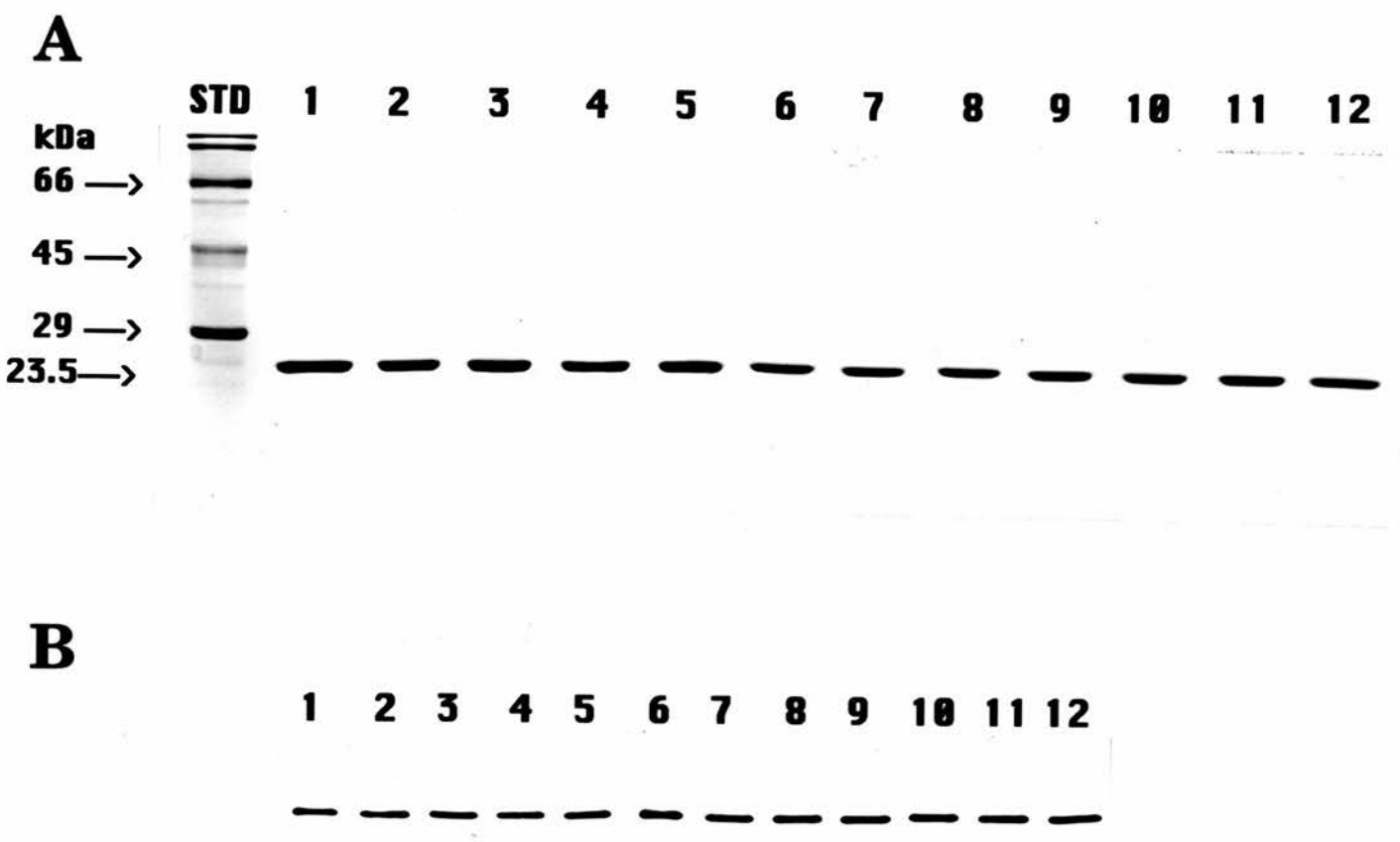

10 20 30 40 50 60
mouse GSTP1 PPYTIVYFPVRGRCEAMRMLLADQGQSWKEEVVTIDTWMQGLLKPTCLYGQLPKFEDGDL
mouse GSTP2 .....SP.....
rat GST-P .....T.....V.L..S..S.....
hamster pi .....I.....VE..RK.SL.S.....
pig GST pi .....T.....D.....ME..P--P...S..FR.....Q....
bovine pi .....Q.....AMQS.L..P..AS.....Q....
human GSTP1 ....V.....A.L.....VE..QE.S..AS.....Q....
nematode gst1 MTLKLT..DIH.LA.PI.L....KQVAYEDHR..YEQ.A--DI..KMIF..V.CLLS..E
β1 3₁₀A αA β2 αB 3₁₀B β3
70 80 90 100 110 120
mouse GSTP1 TLYQSNAILRHLGRSLGLYGKNQREAAQMDMVNDGVEDLRGKYVTLIYTNYENGKNDYVK
mouse GSTP2 .....V.....G.M..R.....
rat GST-P .....D.K...LV.....C..G.....D....
hamster pi .....D.....LV.....C..I.....K..E..D....
pig GST pi .....F.....D.K...LV.....C..A.....A..EK...
bovine pi .....T.....D.Q...LV.....C...S.....A..E....
human GSTP1 .....T.....T.....D.Q...LV.....C..IS.....A..DD...
nematode gst1 EIV..G..I...A.LN..N.S.ET.TTFI..FYE.LR..HT..T.M..R...D..AP.I.
β4 αC αD αE
130 140 150 160 170 180
mouse GSTP1 ALPGHLKPFETLLSQNQGGKAFIVGDQISFADYNLLDLLLIHQVLAPGCLDNFPLLSAYV
mouse GSTP 2 .....
rat GST-P .....N.....V.....
hamster pi .....V.....
pig GST pi E..E.....Q..V..S.....R.....N.S...A.....
bovine pi ...Q.....N..K..Q.....R.....S...S.....
human GSTP1 ....Q.....T.....E.....A.....
nematode gst1 V...E.ARL.K.FHTYKN.EHYVI..KE.Y...V.FEE.D..LI.T.NA..GV.A.KKFH
3₁₀C αF αG
Identity
190 200
mouse GSTP1 ARLSARPKIKAFLSSPEHVNRPINGNGKQ
mouse GSTP2 ..... 97%
rat GST-P .....D.L..... 92%
hamster pi .....D..... 91%
pig GST pi .....A..... 85%
bovine pi RLN.....L....A....M..... 85%
human GSTP1 G.....L....A...Y..L..... 84%
nematode gst1 E.FAE..N...Y.NKRAAI.P.V..... 42%
αH αI

Figure 4.1-1 Alignment of amino acid sequences of pi-class GSTs. The pi-class GST peptide sequences of rat, hamster, pig, cattle, human, and the nematode *C. elegans* were taken from Suguoka et al. (1985), Swedmark and Jenssen (1994), Dirr et al. (1991), Hernando et al. (1991), Kano et al. (1987), and Weston et al. (1989), respectively. Dots represent amino acids identical with those in mouse GSTP1. Dashes indicate gaps inserted in the sequences to optimize the alignment. Double lines (=====) indicate secondary structure elements. The percentage identities between the mouse GSTP1 and the other pi-class sequences are given in the last column.

4.2 Results

4.2.1 Catalytic activities of two recombinant murine pi-class GSTs, GSTP1-1 and GSTP2-2, heterologously expressed in *E. coli*

The cDNAs were amplified (see Chapter 2) by the polymerase chain reaction (PCR) to create an *Nde*I site immediately 5' to the ATG start codon and a *Bam*HI site approximately 50 bp 3' to the TAG stop codon. This was achieved using the oligonucleotides 5'-GAGAGAGACATATGCCACCATAC ACCATTGTC-3' and 5'-GAG AGAGAGGATCCTTTATTAGTGCTGGGAAA AC-3' for Gst p-1 and 5'-GAGAGAGACATATGCCGCCGTACACCATTG TC-3' and 5'-GAGAGAGAGGATCCTTTATT AGTGCT GGGAAAAC-3' for Gst p-2 (restriction sites are underlined). The fidelity of the cDNAs was confirmed by sequencing, and they were cloned into the *Nde*I and *Bam*HI sites of the procaryotic expression vector pET21a. The proteins encoded by these cDNAs were expressed in *E. coli* as described in Chapter 2. Immunoblotting analysis of the soluble fractions of individual *E. coli* cultures harbouring the two enzymes separately showed very similar expression levels of the recombinant proteins (results not shown). The recombinant enzymes were purified on a glutathione agarose affinity column to apparent homogeneity as judged by SDS/PAGE-analysis (Figure 4.2.1-1). One litre of *E. coli* cultures yielded approximately 60 mg of pure GSTP1-1 protein, whereas only approximately 15 mg of GSTP2-2 were obtained, suggesting the latter bound with lower affinity to the glutathione agarose affinity column. The apparent molecular weights of the subunits of GSTP-1-1 and GSTP2-2 (approximately 23.5 kDa) were indistinguishable as estimated by SDS/PAGE (Figure 4.2.1-1).


A
STD
1
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kDa
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45
29
23.5
B
1
2
3
4
5
6
7
8
9
10
11
12

Figure 4.2.1-1 SDS/PAGE and immunoblotting of purified recombinant wildtype and mutant murine pi-class glutathione S-transferase proteins. Purified recombinant wildtype (WT) and mutant murine pi-class GSTs were analysed by SDS/PAGE in 12% polyacrylamide resolving gels. The gel was loaded as follows: STD, molecular weight standards; lanes 1 and 12, mouse Yf marker isolated from mouse liver, provided by Dr. Lesley I. McLellan; lane 2, WT GSTP1; lane 3, WT GSTP2; lane 4, P-1-V10S-R11P; lane 5, P-2-S10V-P11R; lane 6, P-1-V10S; lane 7, P-1-R11P; lane 8, P-1-M89V; lane 9, P-1-V104G; lane 10, P-1-L106M; lane 11, P-1-T109R. Panel A shows the polyacrylamide gel stained with Coomassie Blue R250 (1 μg of protein was loaded per lane); panel B shows the immunoblot developed with a polyclonal antibody raised against purified pi-class GST (YfYf) isolated from mouse liver (100 ng of protein was loaded per lane).

In order to assess, whether the two enzymes have similar or distinct catalytic functions, a panel of established GST substrates was used to determine a profile of specific activities for GSTP1-1 and GSTP2-2. Table 4.2.1-1 shows that GSTP1-1 is much more catalytically active toward all of these substrates than GSTP2-2. Of the 10 substrates tested, GSTP2-2 exhibited approximately 700-, 150-, 25-, and 9-fold less activity toward the substrates 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid, *p*-nitrobenzyl chloride, and cumene hydroperoxide respectively, compared to GSTP1-1. Furthermore, GSTP2-2 displayed no detectable activity for 1,2-dichloro-4-nitrobenzene (DCNB), bromosulfophtalein, *trans*-4-phenyl-3-buten-2-one, 1,2-epoxy-3-(*p*-nitrophenoxy)-propane, *p*-nitrophenyl acetate, and Δ^5-androstene-3,17-dione.

Table 4.2.1-1 Specific activities (μmol/min/mg) of the two wildtype murine pi-class GSTs, GSTP1-1 and GSTP2-2.

All analyses were performed at least in triplicate (± SD); N.D., not detectable.

Substrate	GSTP1-1	GSTP2-2
1-Chloro-2,4-dinitrobenzene	84 ± 4	0.12 ± 0.01
1,2-Dichloro-4-nitrobenzene	0.10 ± 0.01	N.D.
Bromosulfophthalein	N.D.	N.D.
Ethacrynic acid	6.1 ± 0.5	0.04 ± 0.008
trans-4-Phenyl-3-buten-2 one	0.016 ± 0.0008	N.D.
1,2-Epoxy-3-(p-nitrophenoxy)propane	0.45 ± 0.017	N.D.
Cumene hydroperoxide	0.09 ± 0.01	0.01 ± 0.001
p-Nitrophenyl acetate	0.21 ± 0.012	N.D.
p-Nitrobenzyl chloride	1.24 ± 0.085	0.05 ± 0.01
Δ^5-Androstene-3,17-dione	0.09 ±0.004	N.D.

4.2.2 The generation of chimeric GSTP1-1/GSTP2-2 proteins, and their catalytic activities toward CDNB

The finding that the catalytic activities of GSTP1-1 and GSTP2-2 were so profoundly different (Table 4.2.1-1) was initially surprising, as both proteins differ in only six amino acids, and two of these differences are conservative (Met89/Val and Leu106/Met). This result raised the question, which of the six residues varying between GSTP1-1 and GSTP2-2 were responsible for the large differences in catalytic activities. As mentioned earlier, Reinemer and co-workers (1991, 1992) had previously solved the three-dimensional structures of the porcine and human pi-class GST homologues. This data suggested that the only amino acid difference between GSTP1-1 and GSTP2-2 directly involved in the lining of the active site was the residue at position 10 (Val/Ser). Therefore, it was reasonable to hypothesize that the differences at positions 10, and, due to its close proximity, possibly also residue 11, were likely candidates to account, at least partially, for the large differences in catalytic activities displayed by GSTP1-1 and GSTP2-2. Interestingly, the six amino acid differences occur in two clusters, one of which is located near the N-terminus (Val10/Ser, Arg11/Pro), whereas the other is found closer towards the C-terminus (Met89/Val, Val104/Gly, Leu106/Met, Thr109/Arg).

In order to test this hypothesis, two chimeric Gst p-1/Gst p-2 cDNAs were generated such that the 5'-end of Gst p-1 (carrying the coding regions for the amino acids at positions 10 and 11) and the 3'-end of Gst p-2 (carrying the coding regions for the amino acids at positions 89, 104, 106, and 109) were interchanged and *vice versa* (Figure 4.2.2-1). The two corresponding recombinant chimeric proteins (P1-V10S-R11P and P2-S10V-P11R) were expressed in *E. coli* at very similar levels as judged by Western

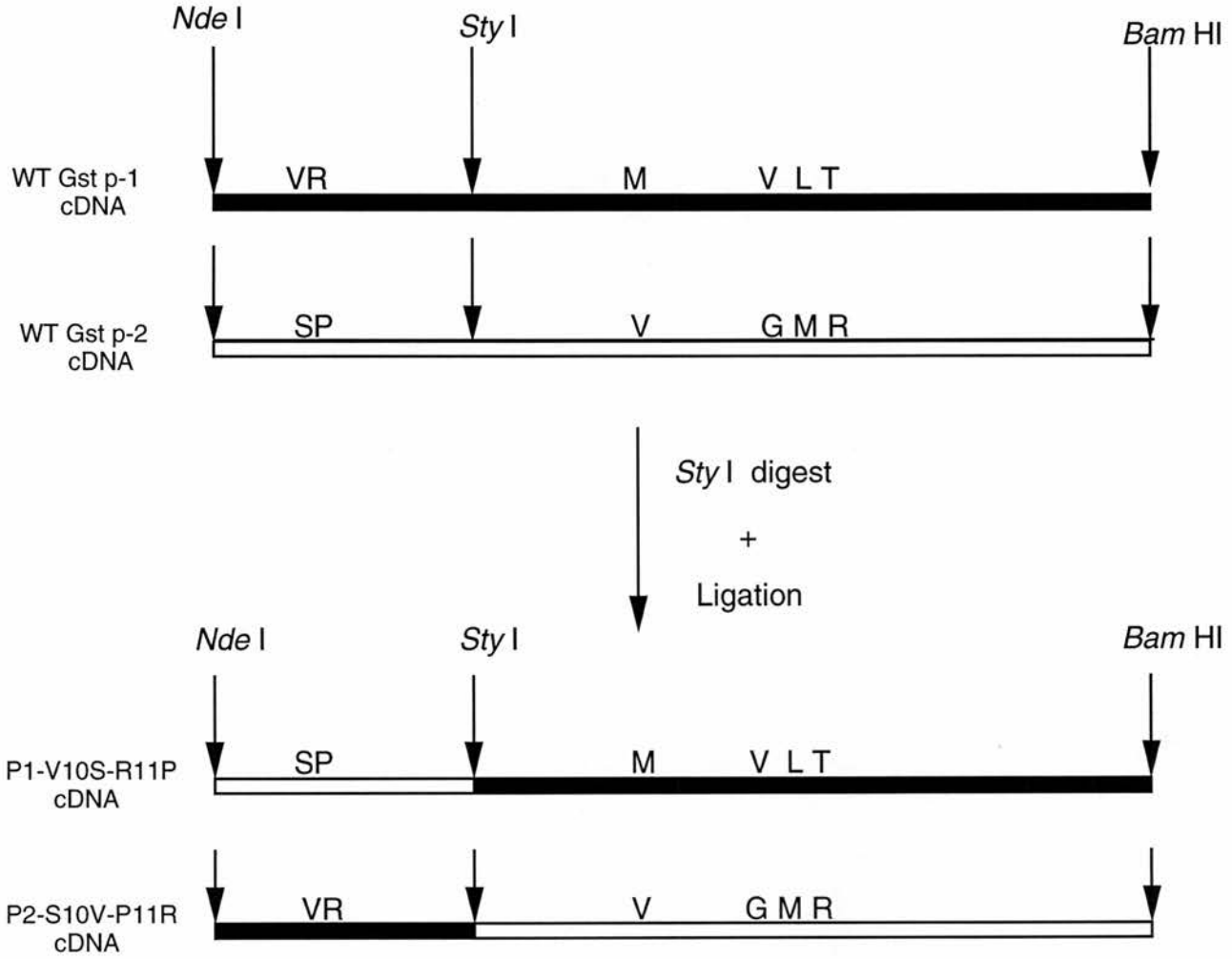

Nde I
Sty I
Bam HI
WT Gst p-1 cDNA
VR
M
V L T
WT Gst p-2 cDNA
SP
V
G M R
Sty I digest
+
Ligation
Nde I
Sty I
Bam HI
P1-V10S-R11P cDNA
SP
M
V L T
P2-S10V-P11R cDNA
VR
V
G M R

Figure 4.2.2-1 Schematic diagram showing the generation of the chimeric cDNAs P-1-V10S-R11P and P-2-S10V-P11R.
The *Nde* I/*Sty* I fragment of the wildtype (WT) cDNA Gst p-1 was ligated with the *Sty*I /*Bam* HI fragment of the wildtype cDNA Gst p-2 and *vice versa*. The 6 amino acid differences encoded by the two wildtype cDNAs are indicated by single letter abbreviations.

blotting analysis of the soluble fractions of individual *E. coli* cultures (results not shown). Approximately 60 mg and 15 mg of affinity purified P2-S10V-P11R and P1-V10S-R11P were obtained, respectively, from 1 litre of bacterial culture, suggesting the latter hybrid protein bound with lower affinity to the glutathione-agarose affinity column.

The specific activities of these chimeric enzymes, which can also be interpreted as mutants of GSTP1-1 and GSTP2-2 carrying a double mutation at positions 10 and 11, were determined using the substrate CDNB. The replacement of Val10 and Arg11 in GSTP1-1 with Ser and Pro, respectively, resulted in a 99 % loss of specific activity for the substrate CDNB as compared with the wildtype GSTP1-1. In contrast, substituting Ser10 and Pro11 in GSTP2-2 for Val and Arg, respectively, increased CDNB activity 217-fold compared to the wildtype GSTP2-2 (Table 4.2.2-1). These results provided strong evidence that at least one of the amino acids at positions 10 and 11 was indeed important in determining the catalytic activities of the murine pi-class GSTs. However, the catalytic activity of the chimeric enzyme P2-S10V-P11R exhibited only 31 % of that displayed by the wildtype GSTP1-1 (Table 4.2.2-1), suggesting that at least one of the C-terminal differences at positons 89, 104, 106, and 109 was also contributing to the large catalytic differences between the two murine pi enzymes.

Table 4.2.2-1 Specific activities of the wildtype (WT) and chimeric murine pi-class glutathione S-transferases toward CDNB.

Type of GST	CDNB activity (μmol/min/mg) ± SD	Percentage of activity compared to WT GSTP1-1
WT GSTP1-1	84.0 ± 4	100 %
WT GSTP2-2	0.12 ± 0.01	0.1 %
P1-V10S-R11P	0.76 ± 0.17	0.9 %
P2-S10V-P11R	26.04 ± 3.37	31 %

4.2.3 Design and expression of mutant murine pi-class GSTs

In order to assess the contribution of each of the six amino acid differences to the loss of enzymatic activity of GSTP2-2, six mutants of GSTP1-1 were created, in each of which one single amino acid was replaced as present in GSTP2-2 (Table 4.2.3-1). I decided to introduce the single mutations in GSTP1-1, because in contrast to GSTP2-2, GSTP1-1 exhibited much higher catalytic activities toward all the substrates tested. Therefore, even subtle changes in catalytic activity, caused by a single mutation, were expected to be much more readily detectable in GSTP1-1 compared to GSTP2-2 mutants.

To characterize these mutant enzymes further, they were expressed and purified (Figure 4.2.1-1) as described earlier. All single mutant proteins were expressed at very similar levels as judged by immunochemical analysis of the soluble fractions of individual *E. coli* cultures (results not shown). Approximately 60 mg of pure protein was obtained from a 1 litre culture for each of the single-mutant proteins with the exception of the mutant P1-R11P. This mutant was likely to bind with lower affinity to the glutathione agarose column resulting in an approximately 4 fold lower yield of purified protein. All mutant proteins displayed the same mobility and were stable as judged by SDS-PAGE analysis (Figure 4.2.1-1).

Table 4.2.3-1. Oligonucleotide primers used for site-directed mutagenesis

Mutant	Amino acid changed in GSTP1-1	Oligonucleotide sequence [a]	Sense
P1- V10S	Val-10 ----> Ser	5'-TCACACCGCCCTCGACTTGGGAAGTAGACAAT- 3'	Inverse
P1- R11P	Arg-11 ----> Pro	5'-CACACCGCCCTGGAACTGGGAAG- 3'	Inverse
P1- M89V	Met-89 ----> Val	5'-CACCATATCCACCTGGGCGGCCT- 3'	Inverse
P1- V104G	Val-104 ---->Gly	5'-GATGAGGGTGCCATATTTGCCGC-3'	Inverse
P1- L106M	Leu-106 ---->Met	5'-ATAGTTGGTGTAGATCATGGTGACATATTTGCC-3'	Inverse
P1-T109R	Thr-109 ---->Arg	5'-ATTCTCATAGTTTCTGTAGATGAGGG-3'	Inverse

a: Mismatches between the mutagenic oligonucleotide and the Gst p-1 cDNA are underlined.

4.2.4 Kinetic parameters of wildtype and mutant enzymes

The kinetic parameters of purified wild-type, double mutant (chimeric proteins P1-V10S-R11P, P2-S10V-P11R), and single mutant enzymes for the conjugating reaction between CDNB and GSH were determined as described in Chapter 2 and are shown in Table 4.2.4-1. CDNB was chosen as the electrophilic substrate for the kinetic analysis, because its specific activities for GSTP1-1 and GSTP2-2 showed by far the biggest difference (700-fold) of all the 10 substrates tested, making it a sensitive probe for the detection of even subtle differences in catalytic activities caused by the mutated residues. Due to the very low activity, the wild-type enzyme GSTP2-2 could not be fully characterised.

Kinetic analysis revealed that the wildtype enzyme GSTP1-1 and the mutants P1-M89V, P1-L106M, and P1-T109R share similar kinetic parameters. In contrast, the mutants P1-V10S, P1-R11P, and P1-V104G exhibited significantly lower catalytic efficiencies (k_{cat}/K_m) than GSTP1-1. The kcat values for P1-V10S, P1-R11P, and P1-V104G were 80%, 97%, and 55% decreased, respectively, as compared to the wild-type GSTP1-1. The change in K_m values for CDNB for the mutants P1-V10S and P1-R11P was approximately 2-fold, whereas the mutant P1-V104G showed a three-fold higher value. While an approximately 2-fold change in K_m for GSH was observed for P1-V10S and P1-V104G, a dramatic increase of approximately 24-fold was noticed for P1-R11P.

The chimeric enzyme P1-V10S-R11P displayed kinetic parameters which were very similar to those observed for the single mutant P1-R11P. The wildtype enzyme GSTP2-2 displayed a k_{cat} value of approximately 0.5% of GSTP1-1. When both Ser10 and Pro11 were replaced by Val and Arg (P2-S10V-P11R) in GSTP2-2, the k_{cat} value of GSTP2-2 increased

Table 4.2.4-1. Kinetic constants for wild-type and mutant enzymes in the reaction involving CDNB as the elctrophilic substrate. Values represent the means (± SD) of three independent series of measurements as described in Chapter 2. * Mean calculated from two series of measurements only.

Enzyme	V_{max} (μmol/min/mg)	k_{cat} (s^{-1})	GSH		CDNB	
			K_m^{GSH} (mM)	k_{cat}/K_m^{GSH} (mM^{-1} s^{-1})	K_m^{CDNB} (mM)	K_{cat}/K_m^{CDNB} (mM^{-1} s^{-1})
Wild-type						
GSTP1-1	141.1 ± 6.3	55.5 ± 2.5	0.12 ± 0.01	446 ± 33	0.75 ± 0.11	75 ± 6
GSTP2-2	0.8 ± 0.4*	0.3 ± 0.1*				
Double mutants						
P1-V10S-R11P	1.7 ± 0.4	0.7 ± 0.2	3.27 ± 0.52	0.2 ± 0.007	2.22 ± 0.58	0.3 ± 0.04
P2- S10V-P11R	73.9 ± 9.5	29.2 ± 3.7	0.38 ± 0.09	81 ± 10	2.84 ± 0.25	10 ± 0.8
Single mutants						
P1-V10S	28.2 ± 4.0	11.1 ± 1.6	0.07 ± 0.02	163 ± 10	0.43 ± 0.09	24 ± 3
P1-R11P	4.7 ± 1.6	1.8 ± 0.6	2.86 ± 0.23	0.46 ± 0.04	1.52 ± 0.42	1.6 ± 0.3
P1-M89V	172.6 ± 8.5	67.8 ± 3.4	0.21 ± 0.05	371 ± 63	0.55 ± 0.18	134 ± 23
P1-V104G	62.9 ± 3.8	24.7 ± 1.5	0.28 ± 0.08	100 ± 18	2.24 ± 0.29	11 ± 0.6
P1-L106M	120.6 ± 7.7	47.5 ± 3.0	0.15 ± 0.03	321 ± 29	0.60 ± 0.03	79 ± 0.4
P1-T109R	116.7 ± 8.4	46.0 ± 3.3	0.14 ± 0.05	293 ± 23	0.70 ± 0.06	65 ± 1

approximately 97-fold and reached approximately 53% of the value of GSTP1-1.

The results of the kinetic analysis of the two single mutants P1-V10S and P1-R11P, and the hybrid enzymes P1-V10S-R11P and P2-S10V-P11R strongly suggest that the two amino acid differences at positions 10 and 11 are of major importance for the catalytic activity of the two wildtype enzymes. In addition, the replacement of Val104 by Gly has also a significant effect on the catalytic performance of the wildtype enzyme GSTP1-1, by lowering the k_{cat} value approximately 55% and increasing the K_m for GSH and CDNB approximately 2-, and 3- fold respectively.

4.2.5 A three dimensional model of mouse GSTP2-2

By the time I had generated and analyzed the wildtype and mutant murine pi-class enzymes, a paper by Garcia-Saez *et al.* (1994) appeared in the literature describing the three dimensional structure of the major pi-class GST (GSTP1-1) isolated from mouse liver. Dr. Huub Driessen (I.C.R.F., Dept. of Crystallography, Birkbeck College, London) used the molecular coordinates published by Garcia-Saez and co-workers (1994) and constructed and analyzed a three-dimensional computer model of the GSTP2-2 protein to find a structurally based explanation for my results. Alignment of GSTP1-1 and GSTP2-2 was unambiguous as the sequences have an equal number of amino acids with only six differences in 209 residues. These could be modeled by Dr. Driessen with little difficulty.

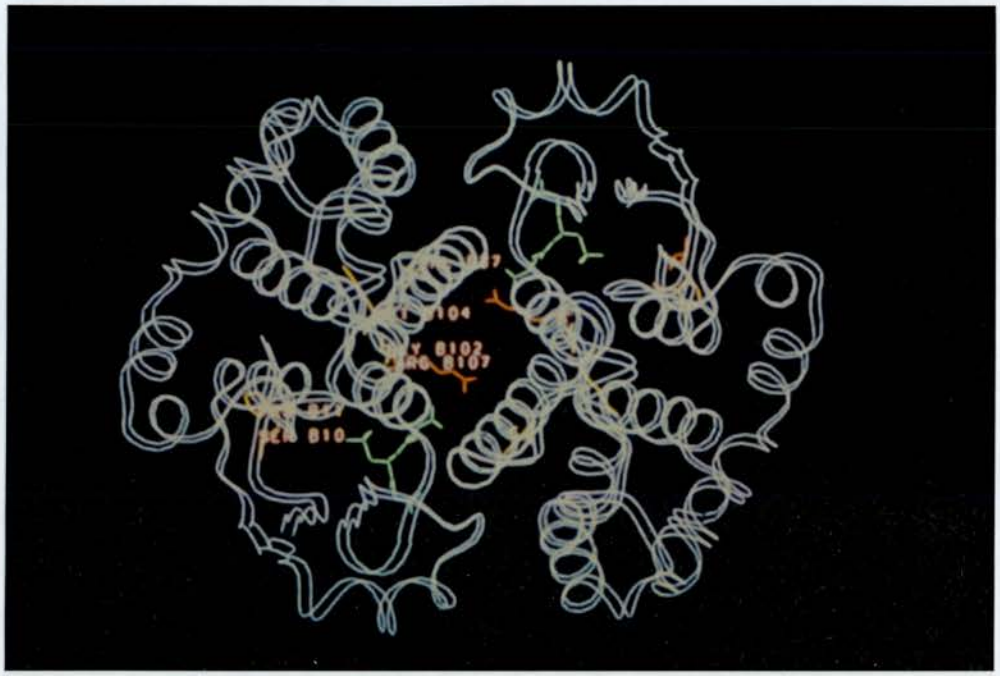
4.3 Discussion

In contrast to other species characterized to date, the mouse genome contains two actively transcribed pi-class GST genes. The recombinant proteins encoded by these genes were found to display profoundly different enzymatic activities. The specific activity profile of the enzyme named GSTP1-1 (Table 4.2.1-1) was strikingly similar to that reported previously for the pi-class GST (YfYf, MII) isolated from mouse liver (Warholm *et al.,* 1986; McLellan & Hayes, 1987). In addition, the kinetic analysis of GSTP1-1 gave very similar results to those reported by Phillips and Mantle (1991) for the major hepatic murine pi-class GST. Furthermore, as outlined in Chapter 3, using reverse PCR the hepatic mRNA level corresponding to the Gst p-1 gene was estimated at least 20-fold higher than that for Gst p-2 in both sexes. Together these findings clearly identify GSTP1-1 as the major pi-class GST expressed in both male and female mouse liver, whereas the GSTP2-2 enzyme is present at very low levels ($\leq$ 5 %) in this organ. The specific activities of GSTP2-2 for the electrophilic substrates CDNB, ethacrynic acid, p-nitrobenzyl chloride and cumene hydroperoxide were 700-, 150-, 25- and 9-fold lower, respectively, compared to GSTP1-1. Moreover, GSTP2-2 was not catalytically active toward any of the other substrates tested (DCNB, bromosulfophtalein, trans-4-phenyl-3-buten-2-one, 1,2-epoxy-3-(p-nitrophenoxy)-propane, p-nitrophenyl acetate, and Δ^5-androstene-3,17-dione; Table 4.2.1-1). This marked difference in catalytic activity appears at first astonishing, in view of the fact that the two proteins share 97% sequence identity (Figure 4.1-1). As the two enzymes differ in only six amino acids, it was possible to examine, which of these are responsible for the greatly reduced catalytic activity of GSTP2-2.

Recently, crystal structures of mammalian GSTs, belonging to the

alpha-(Sinning *et al.,*1993), mu-(Ji *et al.,*1992), and pi- class (Reinemer *et al.,*1991, 1992; Garcia-Saez *et al.,*1994), have been solved and the molecular architecture of the active site determined. Each subunit of the dimeric enzyme has an active site which is composed of two distinct functional regions: a hydrophilic G-site which binds the physiological substrate GSH, and an adjacent H-site which provides a hydrophobic environment for the binding of structurally diverse electrophilic substrates (Danielson & Mannervik, 1985, Reinemer *et al.,*1991, 1992). The outer wall of the active site cave is made up by helix αB and helix 3_{10}B connecting it to strand β3, the inner wall by the C-terminal part of helix αD and the C-terminus, and the bottom by the segment between strand β1 and αA. The differences between GSTP1-1 and GSTP2-2 are concentrated in just two secondary structure elements located at the bottom and the inner wall of the active site (Figure 4.1-1). Residues 10 and 11 are positioned on the segment between strand β1 and αA comprising a γ-turn followed by a 3_{10} helix, while residues 87, 104, 106, 109 are situated on helix αD, the latter three in the C-terminal part.

Based on the three dimensional structure of the murine GSTP1-1 crystal complexed with S-(p-nitrobenzyl)glutathione (Garcia-Saez *et al.,* 1994), the residues in the H-site are all hydrophobic and highly conserved: Phe8, Val10, Ile35, Tyr108, the Cα of Gly205, while Pro9 and Pro202 are in the vicinity (Garcia-Saez *et al.,*1994). In monomer A of murine GSTP1-1, the hydroxyl group of Tyr108 points almost perpendicular to the plane of the benzyl ring and is within van der Waals contact range of the Cε, Cζ2 and Cγ2 atoms of the S-(p-nitrobenzyl) group, in subunit B also of Cζ1 (Figure 4.3-1). Val10 interacts with the edge of the aromatic ring of the S-(p-nitrobenzyl) group.



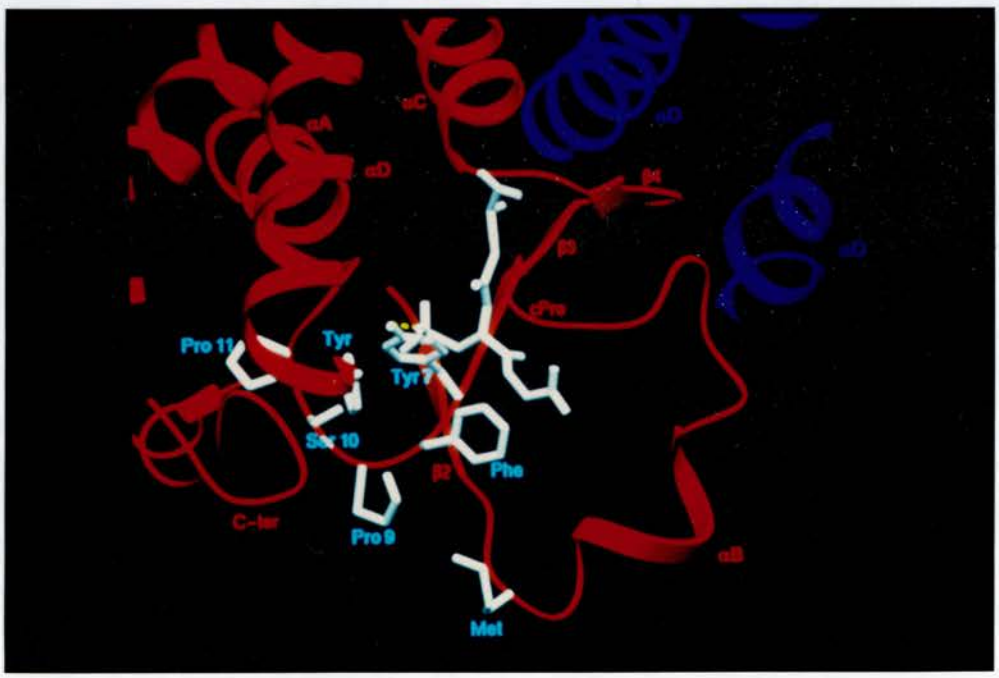

Pro 11
Tyr
Tyr 7
Ser 10
Phe
C-ter
Pro 9
Met

Figure 4.3-1 Three dimensional structure of GSTP2-2 as predicted by the computer model generated by Dr. H. Driessen. Top: Ribbon diagram of the mouse GSTP-2-2 model based on the structure of mouse GSTP1-1 (Garcia-Saez et al., 1994). The side chains of the six amino acids which differ between GSTP1 and GSTP2 subunits are in orange. GSH is shown in pale green. Bottom: Active site of the GSTP2-2 model based on the structure of mouse GSTP1-1 (Garcia-Saez et al., 1994). The secondary structure elements are shown in red and blue ribbons. The GSH-substrate conjugate and the side chains of relevant residues are shown in white and are labeled in pale blue.

When Val10 was replaced by Ser in GSTP1-1, the catalytic efficiencies (k_{cat}/K_m $^{GSH/CDNB}$) decreased approximately 3-fold. This was due to an approximately 5-fold decreased k_{cat} value and somewhat lower K_m values for both CDNB and GSH. As described above, Val10 participates directly in the lining of the H-site (Reinemer *et al.,*1992, Garcia-Saez *et al.,*1994). Moreover, this residue is conserved in all mammalian pi-class GSTs isolated so far, the only exception being GSTP2-2 (Figure 4.1-1). In addition, in the pi GST isolated from the nematode *C. elegans* (Weston *et al.,*1989) Val10 is replaced conservatively by Ile (Figure 4.1-1). Furthermore, in the mu-class GST the comparable residue is implicated in control of stereoselectivity (Ji *et al.,*1994). In the light of these observations, therefore, it is not surprising that a replacement of the hydrophobic residue Val10 by the more hydrophilic Ser residue results in a loss of enzymatic activity. Based on the above arguments, one would expect that the affinity of the mutant P1-V10S for CDNB would be decreased significantly as compared to GSTP1-1. However, judging by the K_m for CDNB this does not seem to be the case. The computer generated model did not offer a clear explanation for the unexpected subtle differences in the K_m for CDNB between the wildtype GSTP1-1 and the mutant P1-V10S.

It is noteworthy that theta class GSTs do possibly have a Ser in this same position, and dog theta (YdfYdf) has the combination Ser10-Pro11. Both GSTP2-2 and most theta GSTs have in common that they do not have appreciable activity with many substrates metabolized by other GSTs, the exception being the dog YdfYdf enzyme.

The replacement of Arg11 by Pro in GSTP1-1 resulted in a 97% loss of activity and an approximately 24-fold and 2-fold increase in the K_m for GSH and CDNB, respectively. Importantly, Stenberg *et al.* (1991) and Wang

et al. (1993) reported a similar result, when they replaced Arg12 (the equivalent to Arg11 in pi-class GSTs) in a rat (YaYa) and a human (GSTA1-1) alpha class GST by an uncharged amino acid. Arg11 is a conserved residue in pi-, mu-, and alpha-class GSTs. However, it does not directly participate in the binding of GSH and electrophilic substrates in the active site, as the dramatic decrease in k_{cat} and increase in the K_m value for GSH suggest. The charge of this amino acid appears to be important for maintaining the correct conformation of the active site. Arg11, present in the N-terminal domain, holds the helix αI in the C-terminal domain close with two hydrogenbonds (11NH2 - O198 and 11Nε – O201), and the C-terminal tail by another two (11N - O202 and 11N - O203). Furthermore, the carbonyl oxygen of Arg11 is hydrogen bonding the amide nitrogens of Cys14 and Glu15. Arg11, therefore, stabilizes the segment between strand β1 and αA, and its position at the bottom of the active site with respect to the inner wall and the 3_{10}A helix 11-14. Arg11 has phi -74 and psi -61 angles which on substitution by Pro with its fixed phi angle of -60 can be accommodated surprisingly well, when the C-terminal area 200-204 is moved away slightly. However, 3 out of the 6 hydrogen bonds are now lost, leading to weakened interaction with the inner wall.

The enzymes GSTP2-2, P1-R11P and P1-V10S-R11P containing a Pro at position 11, instead of an Arg, were noticed to bind with significantly lower affinity to the GSH-agarose affinity column. This observation is consistent with the high K_m of these two mutants with respect to GSH. The only residue close to residues 10 and 11 implicated in stabilizing the GSH molecule, is Arg13 which is near the terminal carboxyl group of the gamma-glutamyl group of GSH. Therefore, it is possible that there is a greater disturbance of the conformation than suggested by the simple model, in

which no N-terminal residues beyond Arg11 are affected. Residue Ser10 in GSTP2-2 is positioned between 2 relatively rigid prolines, and the γ-turn 9-11 may actually not be present. In addition, it cannot be excluded that the Pro has a cis-conformation.

Electrostatic calculations by Karshikoff and colleagues (1993) suggest that the Tyr7 OH-group is deprotonated by the influence of the protein charge constellation and the peptide dipoles, especially residues Gly12, Arg13, and Cys14. A drastic difference in the conformation of the 3_{10} helix 11-14, or a shift of residue Tyr7 would have an influence on the catalytic activation.

The simultaneous replacement of Ser10 by Val and Pro11 by Arg in GSTP2-2 (P2-S10V-P11R) results in an approximately 100-fold increase of k_{cat}. In contrast, an approximately 80-fold decrease in the k_{cat} of GSTP1-1 is observed, when Val10 and Arg11 are replaced by Ser and Pro (P1-V10s-R11P). These results provide strong evidence that the two N-terminal amino acid differences (Val/Ser, Arg/Pro) between GSTP1-1 and GSTP2-2 have a profound effect on the catalytic activities of these enzymes. With both mutations present, there may be realignment of bottom and innner wall of the active site, because of the shift of hydrogen bonding from helix αI towards the C-terminus, possibly affecting the positions of residues 202 and 205, which are close to the active site. This might have an effect on dissociation, when the GSH conjugate leaves the active site.

The single substitution of Met89 by Val has very little effect on the catalytic activiy of GSTP1-1. This is not surprising as this change is of a conservative nature. In addition, with the exception of mouse GSTP1-1, all mammalian pi-class GSTs isolated so far have a Val residue present at position 89 (Figure 4.1-1). In the case of the *C. elegans* enzyme, this residue is conservatively replaced by Ile. Val 89 in the pig pi enzyme and Met89 in

mouse GSTP1-1 are virtually inaccessible to solvent. Although Met is slightly larger than Val, a comparison of both murine GSTP1-1 and pig GST pi structures does not indicate a difference in core packing. This difference, although situated in helix αD, is some distance from the active site, and is therefore not expected to have a large effect on substrate binding on its own. This explains that a change from a Met to a Val in GSTP1-1 leads to similar kinetic parameters.

The differences between mouse GSTP1-1 and GSTP2-2 do not include any of the residues involved in the contact areas within the GST-dimer, therefore GSTP2-2 is expected to be a dimer. However, Val89 in mouse GSTP2-2 is situated next to some residues involved in the dimer interface, Ala87, Leu88 (marginally), Asp90 and Met91. Comparison of the mouse liver GSTP1-1 and pig lung GST pi structures does not indicate any significant differences in the dimer interface around residue 89. The only residue implicated in inter-subunit stabilisation of bound GSH, Asp98, is not affected.

Residues 104, 106, and 109 are in the C-terminal part of helix αD, close to residue Tyr108 which lines the H-site. The single substitution of Leu106 by Met in GSTP1-1 has very little effect on the catalytic activiy. Again, this is not surprising as this change is also of a conservative nature. As for position 106, the only other pi-class GST which has a Met instead of a Leu present is the *C. elegans* enzyme. The replacement of Leu106 by a Met requires only small rearrangements to allow positioning with correct van der Waals distances in the core of the inner wall of the active site, which is unlikely to cause changes in substrate binding on its own.

The non-conservative replacement of Thr109 by Arg has also little impact on the catalytic activitiy of GSTP1-1. Thr109 is conserved in all pi-

class GSTs, with the exceptions of GSTP2-2 and the *C. elegans* enzyme, which both have an Arg present at position 109. In the model, Arg 109 sticks out of the C-terminal part of helix αD into the large cavity between the two monomers making up the active GST dimer. It is accessible to the solvent. It is therefore pointing away from the H-site, and not directly interacting with substrates in the H-site. Because of its position, it is unlikely to interfere with the substrate entering or the adduct leaving the active site. Although this difference is situated in helix αD, it is not expected to have a significant effect on substrate binding.

In contrast, the non-conservative replacement of Val104 by Gly results in an approximately 55% decrease in the k_{cat} value of GSTP1-1, and a 2-, and 3-fold increase in the K_m for GSH and CDNB respectively. This finding is initially somewhat surprising, as Val104 is not conserved in the members of the pi-class. Human P1-1, which has an Ile at position 104, has a specific activity for CDNB similar to mouse GSTP1-1 (Mannervik and Danielson, 1988). Compared with human P1-1, the human isoform of P1-1 which has a Val at position 104 has approximately half the specific activity for CDNB, a similar K_m for GSH and a 4-fold increase of the K_m for CDNB (Zimniak *et al.*,1994), data which are in very good agreement with my results. The rat pi enzyme also has a Gly present at position 104, and has significantly lower specific activity for CDNB than mouse GSTP1-1, but not as low as mouse GSTP2-2. Based on a comparison of human P1-1 and its Val104 isoform, Zimniak and colleagues (1994) proposed that the residue at position 104 helps in defining the geometry of the H-site, with bulkier residues resulting in a smaller more restrictive H-site. In addition, the residue at this position may also influence activity by interacting with Tyr108 directly involved in substrate binding. Tyr108 is located within 4 Å of Ile (or Val) at position 104 in human

P1-1, suggesting that apart from size also hydrophobicity may play a role in influencing the function of Tyr108. Since for both human P1-1 and mouse GSTP1-1 the K_m for CDNB increases as the size of residue 104 decreases, a similar interpretation appears to be applicable. The human P1-1 and the mouse GSTP1-1 structures have in common that the aliphatic side chain of Val104 is within van der Waals range of the ring of Tyr108, possibly keeping it in a relatively fixed position with respect to the lipohilic substrate. In mouse GSTP2-2 and rat P1-1 Val104 is replaced by the much smaller Gly, this sabilisation will not be present at all, and Tyr108 will be more free to move. This may affect both its position and ability to aid in controlling stereoselectivity, as well as its steric hindrance of dissociation of the product. CDNB may therefore be held less firmly, explaining the 3-fold increase in K_m for CDNB of the mutant P1-V104G.

Another possibility that needs to be considered is, that due to its size, Gly can have main chain conformations which other residues cannot accommodate. The replacement may cause a change in direction in the helix αD, changing the position of Tyr108 in the active site and the size of the active site itself. In the mouse GSTP2-2, this might bring Tyr108 closer to Ser10. Since no residues implicated in binding GSH are directly affected, when replacing Val104 by Gly in GSTP1-1, a similar K_m for GSH is expected. Ile104 in human P1-1 is at roughly van der Waals distance to the carbonyl group of the glutamic acid in GSH, possibly aiding in the catalytic reaction by stabilising the position of GSH in the G-site. Val104 in the human P1-1 isoform is at a similar distance, therefore not causing a significant difference in the K_m for GSH. In contrast, Val104 in mouse GSTP1-1 is already at a distance of 6.5 Å, making such stabilisation less effective. There is no possibility of such an interaction with the much smaller Gly, possibly

explaining the small increase in K_m for GSH when replacing Val104 by Gly in GSTP1-1.

The hybrid enzyme P2-S10V-P11R, which can be interpreted as a mutated GSTP1-1 carrying the four mutations Met89Val, Val104Gly, Leu106Met, and Thr109Arg simultaneously, displays kinetic parameters remarkably similar to those found for the mutant P1-V104G. This finding strongly suggests that the four amino acids present in GSTP2-2 at positions 89, 104, 106, and 109, do not affect the catalytic activity in a combined way.

4.4 Summary

In conclusion, primary sequence-, catalytic activity-, and kinetic analyses clearly identify GSTP1-1 as the major pi-class GST expressed in both male and female mouse liver. In contrast to GSTP1-1, GSTP2-2 displays very low conjugating activity of various electrophilic substrates with glutathione, very low selenium independent peroxidase activity, and no isomerase activity toward Δ^5-androstene-3, 17-dione. Three out of the six amino acid diffrences occurring between the two enzymes are responsible for the loss of activity of GSTP2-2; these are Ser10, Pro11, and Gly104. The two N-terminal residues at positions 10 (Val/Ser) and 11 (Arg/Pro) are very important in determining CDNB activity of the two murine pi-class GSTs. In addition, the amino acid at position 104 (Val/Gly) plays also a significant role in CDNB metabolism of these enzymes.

Chapter 5: Thoughts for future work

5.1 Function of the two murine pi-class GSTs

Differences in promoter sequences, levels of hepatic expression, and catalytic activities of the two murine pi-class GSTs suggest that each gene has a distinct physiological function. However, what these functions are remains to be established.

In contrast to mouse GSTP1-1, GSTP2-2 has very little GSH conjugating activity toward a range of electrophilic substrates. There are a number of reasons which could explain this lack of catalytic activity. Future work should consider the following points:

(1) Despite the fact that a relatively high K_m for GSH is predicted for GSTP2-2 (Chapter 4), it is possible that this GST possesses a very narrow range of substrate specificity which has not been identified yet. Future work should include probing GSTP2-2 with numerous other substrates (e.g. benzo-[a]-pyrene metabolites, acrolein, menaphthyl sulphate, 4-hydroxynon-2-enal, e.t.c.) to test this hypothesis. Interestingly, GSTP2-2 and theta class GSTs have in common a relatively high K_m for GSH (approximately 3-5 mM) (Meyer, 1993).

(2) Although unlikely, it cannot be ruled out that GSTP2-2 requires post-translational modification to gain full catalytic activity. If this is the case, it would have been missed in the studies presented in this thesis, as the recombinant pi-class GSTs were generated in a procaryotic expression system lacking such post-translational activities. Based on this line of thought, it appears intriguing that the amino acid present at postion 10 in GSTP2-2 is a Ser which is a well established substrate for a

number of kinases. Furthermore, the sequence motive SP is recognized by at least two serine-kinases termed mitogen-activated protein kinase (MAPK) and mitotic protein kinase p34cdc2 (Peter et al, 1992; Maller et al., 1989). In the light of these observations, it is tempting to speculate that GSTP2-2 could be activated by phosphorylation of Ser10.

Interestingly, Taniguchi and Pyerin (1989) reported that certain rat GST subunits (Ya and Yc1) are indeed phosphorylated by the calcium-phospholipid-dependent kinase *in vitro*. However, the physiological significance of these phosphorylations with respect to GST-activity has not been firmly established by these investigators. In addition, Pyerin and Taniguchi (1989) demonstrated that the Ser residue at position 128 in the major phenobarbital-inducible rat cytochrome P-450 is phosphorylated by cAMP-dependent protein kinase both *in vitro* and *in vivo*. Importantly, these investigators showed that aforementioned phosphorylation is accompanied by a significant decrease in enzyme activity.

The three-dimensional computer model designed by Dr. Driessen may be suitable to give clues as to the structural effects of the phosphorylation of Ser10 in GSTP2-2. Furthermore, recombinant GSTP2-2 could be used to see whether it is a substrate for the MAPK or p34cdc2 kinases. Depending on the outcome, further experiments could be designed to examine, if phosphorylation of Ser10 would activate GSTP2-2.

(3) Another possibility is that GSTP2-2 has indeed very limited GSH-conjugating activity and acts instead as a "cellular sponge" which sequesters electrophilic compounds, or binds and transports so far unidentified endogenous compounds. As outlined in Chapter 1, it has

indeed been shown that GSTs bind covalently and non-covalently a number of compounds.

(4) It is well established that distinct GST subunits belonging to the same class can form heterodimeric enzymes. Judged by the results of the structure-function analysis presented in Chapter 4, it is likely that murine GSTP1-2 heterodimers can be formed in tissues expressing both subunits. It can be envisaged that these heterodimers possess unique properties.

(5) Investigating the tissue specific expression pattern of the two pi genes will provide further clues as to the biological role of these enzymes. Oligonucleotides specific for each of the two pi mRNAs (see Chapter 3) can be used as probes in Northern blotting analysis.

(6) Finally, the characterization of the murine pi-class locus presented in Chapter 3 is the basis for gene targeting experiments which are currently in progress. These experiments will generate mice lacking one or both functional pi-class genes. Such genetically manipulated animals will provide a powerful model for studying pi-class GSTs with respect to their function(s), in the metabolism of both endogenous and foreign compounds.

5.2 Regulation of murine pi GSTs

Both of the murine pi genes isolated contain sufficient 5'-untranslated regions (UTR) to carry out studies that will identifiy both cis- and trans-acting elements governing the regulation of these genes. Such studies would involve fusing various portions of the 5'-flanking regulatory regions to a promoterless reporter gene (e.g. the bacterial

chloramphenicol acetyl transferase gene) and transfecting these constructs into a suitable cell line. Measuring chloramphenicol acetyl transferase activity provides an indirect way of assessing transcriptional activity of the constructs.

5.3 Is there another functional pi gene present in the mouse genome?

Additional work is required to clarify whether the 0.7 kb PCR-product described in Chapter 3 is a pseudogene or a further functional gene.

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Isolation and characterization of two mouse Pi-class glutathione S-transferase genes

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Pi-class glutathione S-transferases (GSTs) play an important role in the detoxification of chemical toxins and mutagens and are implicated in neoplastic development and drug resistance. In all species characterized to date, only one functional Pi-class GST gene has been described. In this report we have identified two actively transcribed murine Pi-class GST genes, Gst p-1 and Gst p-2. The coding regions of Gst p-1 and the mouse Pi-class GST cDNA (GST-II) reported by Hatayama, Satoh and Satoh (1990) (Nucleic Acids Res. **18**, 4606) are identical, whereas Gst p-2 encodes a protein that has not been described previously. The two genes are approximately 3 kb long and contain seven exons interrupted by six introns. In addition to a TATA box and a sequence motif matching the phorbol-ester-responsive element, the promoters of Gst p-1 and Gst p-2 exhibit one and two G+C boxes (GGGCGG) respectively. The cDNAs of the two genes were isolated from total liver RNA using reverse PCR. The peptide sequences deduced from the cDNAs share 97% identity and differ in six amino acids. Both genes are transcribed at significantly higher levels in male mouse liver than in female, and Gst p-1 mRNA is more abundant in both sexes than Gst p-2.

INTRODUCTION

The cytosolic glutathione S-transferases (GSTs) form a family of dimeric enzymes which exhibit a number of catalytic activities, including the conjugation of glutathione to lipophilic electrophiles (Coles and Ketterer, 1990). On the basis of nucleotide and amino acid sequence similarities, mammalian cytosolic GSTs can be grouped into four subfamilies designated Alpha, Mu, Pi and Theta (Mannervik et al., 1985, 1992; Meyer et al., 1991). The Pi subfamily has attracted considerable attention following the observation by Sato and co-workers that the Pi-class GST enzyme is markedly elevated in rat liver tumour development (Sato et al., 1984a,b).

In the early stages of rat liver carcinogenesis, single cells and foci appear that exhibit a different phenotype from that of normal hepatocytes; these foci are believed to represent precursors of tumours. One of the most reliable markers for the identification of such (pre-)neoplastic foci is the Pi-class enzyme, GST-P (Sato, 1989). This protein is essentially absent from normal hepatocytes, whereas it is abundant in foci- or nodule-bearing livers. GST-P is expressed not only in foci or nodules induced by chemicals but also in spontaneously occurring hepatic (pre-)neoplastic lesions, without administration of any exogenous carcinogens (Sawaki et al., 1990).

In the human, there are several reports demonstrating a significant increase in the expression of the Pi-class enzyme, GST-π (GSTP1–1), in a number of tumours including oral, colon, stomach and lung (Sato, 1989; Howie et al., 1990; Black and Wolf, 1991; Volm et al., 1991; Hirata et al., 1992). However, GST-π levels are not increased in hepatocellular carcinomas (Hayes et al., 1991). In addition, Pi-class GST is expressed at elevated levels in cell lines made resistant to anticancer drugs and to chemical toxins in culture (Batist et al., 1986; Cowan et al., 1986; Wolf et al., 1990; Black and Wolf, 1991). Therefore, studying the function and regulation of Pi-class GSTs will lead to a better understanding of the mechanisms underlying carcinogenesis and drug resistance.

Studies in the mouse are of particular importance, as this species is genetically well characterized, and advances in transgenic and gene-targeting technology provide powerful tools for investigating gene regulation and function. Furthermore, the mouse GST Pi gene, unlike its rat counterpart is expressed constitutively in hepatocytes and is also subject to regulation by both hormones (Hatayama et al., 1986) and exogenous agents (McLellan and Hayes, 1989). In order to examine these GSTs, we have isolated and characterized two actively transcribed mouse Pi-class GST genes, as well as their corresponding cDNAs. We have also investigated relative hepatic mRNA levels of the two genes in male and female mice.

MATERIALS AND METHODS

Chemicals and reagents

Unless otherwise indicated, all chemicals were purchased from either Sigma Chemical Co., Poole, Dorset, U.K. or BDH, Glasgow, U.K., and were of analytical grade or better.

Screening of the mouse genomic library

An adult Balb/c mouse genomic library in λ EMBL3 purchased from Clontech (Palo Alto, CA, U.S.A.) was screened using standard protocols (Benton and Davis, 1977). A 2.2 kb DNA fragment (MPCR2.2), generated by PCR, was radioactively labelled with [α-^{32}P]dCTP (3000 Ci/mmol) by the method of Rigby et al. (1977), and used as a probe. MPCR2.2 was obtained using the oligonucleotides 5′-TGCCACCATACACCATTGTC-3′

Abbreviations used: GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate ('TPA'); PRE, PMA-responsive element; AMV, avian myeloblastosis virus.

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The mouse Pi-class GST nucleotide sequences, as well as the corresponding amino acid sequences, will appear in the GenBank/EMBL/DDBJ under the accession nos. X76143 and X76144.

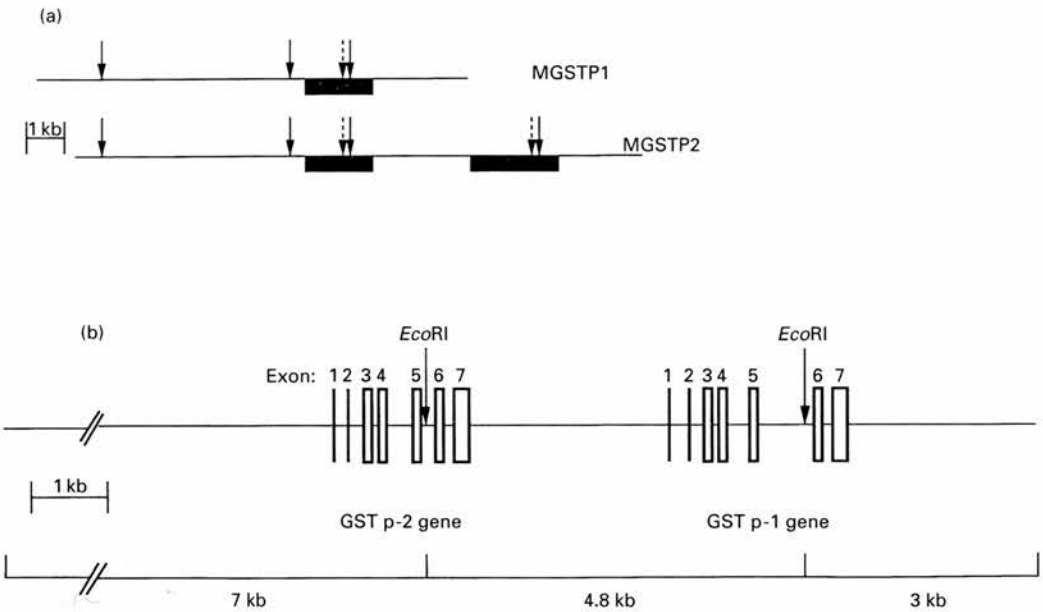

Figure 1 Organization of the murine GST Pi locus

(**a**) Restriction maps of the phage clones MGSTP1 and MGSTP2, containing one and two mouse Pi-class GST genes respectively. Solid and dashed arrows represent *Hin*dIII and *Eco*RI sites respectively; solid boxes indicate regions complementary to the probe MPCR2.2. (b) Restriction map and intron/exon arrangement of the two mouse Pi-class GST genes, Gst p-1, and Gst p-2, as contained in clone MGSTP2. Exons are shown as boxes numbered 1–7. Intron sizes were determined by DNA sequencing.

and 5′-TTTATTAGTGCTGGGAAAAC-3′ which hybridize to the 5′ end of exon 2 and to the 3′ end of exon 7 of the mouse gene, Gst p-1. The following temperatures/reaction times were used on a PHC-2 thermal cycler (Techne, Cambridge, U.K.): 2 min at 94 °C, followed by 30 cycles of 96 °C, 15 s; 60 °C, 1 min; 72 °C, 2.5 min. Hybridization was carried out in buffer containing 1 M NaCl, 50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 5× Denhardt's solution [1 × Denhardt's = 0.02 % BSA, 0.02 % polyvinylpyrrolidone, 0.02 % Ficoll], 0.1 % SDS, 50 μg/ml denatured herring sperm DNA and ^{32}P-labelled DNA probe MPCR2.2 at approx. 1×10^6 c.p.m./ml, for 16 h at 65 °C. The Hybond-N membranes (Amersham International plc, Amersham, Bucks., U.K.) were washed twice in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate)/0.1 % SDS at room temperature and then twice in 0.2 × SSC/0.1 % SDS at 65 °C, for 30 min on each occasion, before being exposed to Kodak XAR-5 autoradiograph film at −70 °C. Positive clones were plaque purified, and DNA was prepared by the method of Grossberger (1987). Inserts were excised from the clones by the restriction endonuclease *Sal*I, and separately digested with *Eco*RI and *Hin*dIII. The DNA fragments were individually cloned into the plasmid pGEM3Zf(+) (Promega, Madison, WI, U.S.A.).

Southern blot analysis

Balb/c mouse liver DNA was prepared by standard methods (Sambrook et al., 1989), and 20 μg was digested to completion with the restriction enzyme *Eco*RI, and subjected to electrophoresis in a 0.8 % agarose gel. After alkali treatment and neutralization, the DNA was transferred to a Hybond-N membrane (Amersham International) according to the manufacturer's instructions. Hybridization was carried out as described for the plaque hybridization. The membrane was washed twice in 2 × SSC/0.1 % SDS at room temperature and then twice in 0.1 × SSC/0.1 % SDS at 65 °C, for 30 min each time, before being exposed to autoradiographic film as described above.

Isolation of total RNA

Total RNA was isolated from the liver of adult male and female Balb/c mice fed *ad libitum* on standard laboratory diet by the method of Chomczynski and Sacchi (1987).

Reverse PCR

Isolation of the mouse Pi-class cDNAs

Total male mouse liver RNA (2 μg) was reverse-transcribed in a total volume of 20 μl using 1 × PCR buffer (Promega), 2 mM of each dNTP, 1 unit of RNasin (Promega), 100 pmol of oligo(dT) and 24 units of avian-myeloblastosis-virus reverse transcriptase (Boehringer, Mannheim, Germany). After 10 min at room temperature, the samples were incubated for 60 min at 42 °C. In order to denature the RNA–cDNA hybrids and to inactivate the reverse transcriptase, the reaction mixture was heated to 95 °C for 5 min and then cooled on ice. Then 80 μl of 1 × PCR buffer containing 50 pmol each of upstream and downstream primer and 2.5 units of *Taq* polymerase (Promega) were added. Then 35 cycles were performed as follows: 20 s denaturing at 96 °C, annealing for 1 min at 60 °C, and primer extension for 1 min at 72 °C. The primer pairs used for the PCR reactions were 5′-CCTCACTCTGAGTACCCCTCTGTCTACG-3′ and 5′-TTTATTAGTGCTGGGAAAAC-3′ for Gst p-1, and 5′-CCATCCCTGAGACACCTCTCTGACTATT-3′ and 5′-TTTATTAGTGCTGGGAAAAC-3′ for Gst p-2.

mRNA levels of the mouse Pi-class GST genes

Reverse PCR was carried out as described above with the exceptions that 1 μg of total RNA and 100 units of Moloney

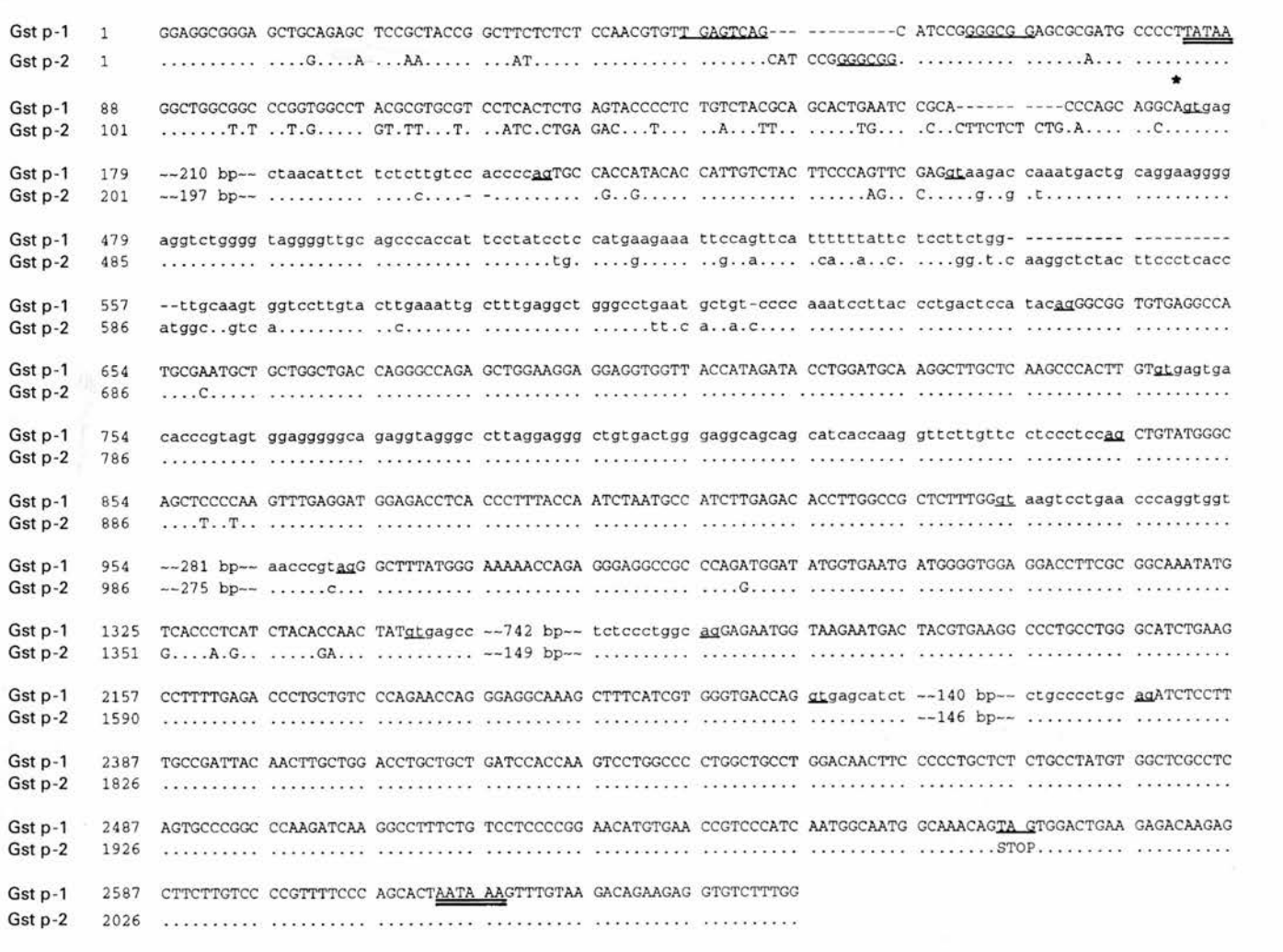

Figure 2 Nucleotide sequences of the mouse Pi-class GST genes, Gst p-1 and Gst p-2

The nucleotide sequence of gene Gst p-1 is shown with substitutions in gene Gst p-2 indicated below. Intronic sequences are presented in lower case. Dashes indicate gaps inserted in the sequences to optimize the similarity between the two genes. The TATA box and the polyadenylation signal are double, and the GC boxes, the phorbol-ester-responsive-element motif, the GT/AG splice sites and the stop codon are single underlined. An asterisk indicates the translational start site.

murine leukaemia virus reverse transcriptase (Gibco/BRL, Paisley, U.K.) were used instead of 24 units of AMV reverse transcriptase (Boehringer) per reaction.

In order to assess whether the two pairs of oligonucleotides differ in their affinity toward their target DNA, we compared the yields of the following PCRs. Known amounts (0.1 ng) of the subcloned cDNAs, Gst p-1 or Gst p-2, were used separately together with the corresponding primer pairs, and 10, 15, 20, 25 and 30 PCR cycles were performed. Equal amounts of the two PCR products (Gst p-1 and Gst p-2 cDNAs), generated by the same number of cycles, were electrophoresed on an agarose gel, visualized with ethidium bromide and yields were compared. No difference was detected (results not shown), indicating that the two pairs of oligonucleotides do not vary in their ability to anneal to their target DNA.

Various numbers of PCR cycles (10, 15, 20, 25, 30) were performed to determine conditions under which the template DNA represents the limiting factor in the amplification reaction, so that the generation of the PCR product is directly proportional to the template present. In the experiment shown in Figure 4, this was the case for cycle numbers 10, 15 and 20, 25 for Gst p-1 and Gst p-2. The assay was carried out in triplicate and each experiment gave identical results.

DNA sequencing

Three *Eco*RI (3 kb, 4.8 kb and 7 kb) and two overlapping *Hin*dIII (1.4 kb and 4.8 kb) restriction fragments of clone MGSTP2, which each hybridized to the MPCR2.2 probe, were cloned into the plasmid pGEM3Zf(+). Sequence data were determined by the dideoxy chain termination method of Sanger et al. (1977). All of the sequence data presented were determined from both strands of DNA.

RESULTS AND DISCUSSION

Isolation of genomic clones

The amino acid sequences deduced from the mouse Pi-class GST cDNA reported by Hatayama et al. (1990) and the rat Pi-class

Table 1 Sequence identities of the mouse Gst p-1 gene with mouse Gst p-2, rat GST-P and human GST-π genes

The values given in parentheses represent identity comparison with the mouse Gst p-1 sequence. The sequences for rat GST-P and human GST-π (GST P1–1) were taken from Okuda et al. (1987) and Marrow et al. (1989) repectively.

	Number of nucleotides (bp)			
	Gst p-1	Gst p-2	GST-P	GST-π
Exon 1*	1	1 (100%)	1 (100%)	1 (100%)
Intron 1	242	226 (80%)	209 (69%)	288 (38%)
Exon 2	36	36 (86%)	36 (94%)	36 (86%)
Intron 2	188	212 (75%)	185 (60%)	293 (36%)
Exon 3	107	107 (99%)	107 (92%)	107 (78%)
Intron 3	98	98 (100%)	101 (83%)	114 (48%)
Exon 4	88	88 (98%)	88 (94%)	88 (81%)
Intron 4	312	306 (94%)	329 (75%)	365 (40%)
Exon 5	104	104 (93%)	104 (88%)	104 (85%)
Intron 5	761	168 (14%)†	716 (75%)	862 (37%)
Exon 6	108	108 (100%)	108 (96%)	108 (87%)
Intron 6	162	168 (96%)	156 (80%)	177 (54%)
Exon 7*	186	186 (100%)	186 (95%)	186 (85%)

* Only the number of coding nucleotides of exons are given.
† The low identity is due to the different intron sizes of Gst p-1 and Gst p-2

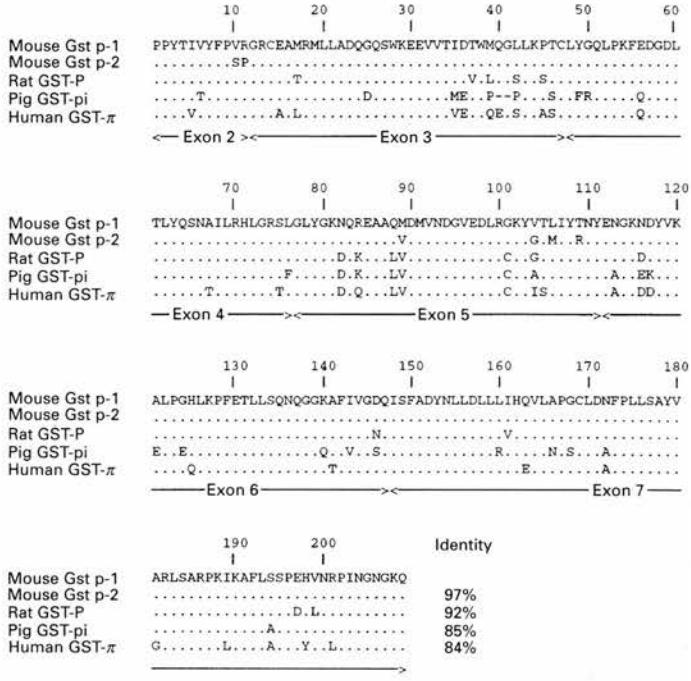

Figure 3 Alignment of amino acid sequences of mammalian Pi-class GSTs

The Pi-class GST peptide sequences of rat GST-P, pig GST-pi and human GST-π were taken from Okuda et al. (1987), Dirr et al. (1991) and Kano et al. (1987). Dots represent amino acids identical with those in mouse Gst p-1. Dashes indicate gaps inserted in the sequences to optimize the similarity between the sequences. The percentage identities between mouse Gst p-1 and other Pi-class GSTs are shown in the last column. Exons corresponding to peptide sequences are indicated for the mouse, the rat and the human sequences only, as the exon/intron junctions of the pig Pi-class GST have not been determined.

gene, GST-P (Okuda et al., 1987), share 92% identity. On the basis of the conserved intron/exon organization of homologous mammalian genes, we predicted the intron/exon boundaries of the mouse Pi-class gene from the rat gene sequence reported by Okuda et al. (1987). A pair of oligonucleotides to sequences in exon 2 and 7 were then used to amplify part of the mouse Pi-class GST gene using PCR (Saiki and Gelfand, 1989). Exon 1 was omitted as it only contains the A of the ATG initiation codon. An approx 2.2 kb PCR product (MPCR2.2) was obtained which was cloned into the plasmid pGEM3Zf(+) and partially sequenced. The sequence over the coding region was identical with the murine Pi-class cDNA described by Hatayama et al. (1990). This clone, MPCR2.2, was then used as a probe to screen a mouse Balb/c liver genomic library in λEMBL3. Four positive clones were isolated, three of which were identical and represented MGSTP2. The two distinct clones, MGSTP1 and MGSTP2, contained inserts of approx. 12 kb and approx. 15 kb respectively. The restriction maps of these clones are shown in Figure 1(a). The maps also show the region that hybridized to MPCR2.2.

Sequence analysis of clone MGSTP2

The approximately 15 kb insert of clone MGSTP2 was digested separately with the endonucleases *Eco* RI and *Hin*dIII to generate smaller overlapping DNA fragments which were individually cloned into the plasmid pGEM3Zf(+). Restriction mapping, hybridization analysis and DNA sequencing showed that clone MGSTP2 contains two closely related genes, Gst p-1 and Gst p-2, which have not been described previously (Figures 1b and 2). They are adjacent and only separated by about 3 kb of intervening DNA. The coding regions of Gst p-1 and Gst p-2 share 100% and 98% identity with the sequence of the mouse Pi-class cDNA reported by Hatayama et al. (1990). The positions of splice junctions were deduced by comparison of the nucleotide sequence of the genes with that of the murine Pi-class cDNA reported previously (Hatayama et al., 1990). Both genes were sequenced completely and shown to contain seven exons interrupted by six introns. Gst p-1 is about 2.7 kb in length, whereas Gst p-2 is about 2.1 kb long because of a smaller-sized intron 5 (Table 1).

The nucleotide sequence at all splice junctions is consistent with the canonical GT/AG rule of Breathnach and Chambon (1981), indicating that the exonic segments within each gene transcript can be spliced accurately to form functional mRNA. The polyadenylation signal, AATAAA, occurs in the seventh exon of the two genes, which is further evidence that both gene transcripts can be processed to mature mRNAs.

Each gene possesses an open reading frame of 630 nucleotides encoding a protein of 210 amino acids including the initiator methionine. The calculated molecular masses of the proteins encoded by Gst p-1 and Gst p-2 are 23.6 kDa and 23.5 kDa respectively. As found in the rat and human homologues, the start codon, ATG, is split by the first intron in both mouse genes (Figure 2). The coding nucleotide sequences of the two genes differ in 14 positions (Figure 2); five of these substitutions are neutral and the remaining nine result in the replacement of six amino acids (Figure 3). The substitutions at positions 10 and 11 allow the protein encoded by Gst p-1 to be identified as the major Pi form purified from mouse liver (Phillips and Mantle, 1993).

Two of the amino acid differences, namely Met^{89}/Val, and Leu^{106}/Met, are conservative (Miyata et al., 1979). The crystal structure of porcine Pi-class GST (Reinemer et al., 1991), which shares 85% and 83% identity with Gst p-1 and Gst p-2 respectively, suggests that the non-conservative changes, Arg^{11}/Pro, Val^{104}/Gly and Thr^{109}/Arg are distant from the active site. Val^{104} is not conserved in mammalian Pi-class GSTs isolated so far (rat, pig and human; Figure 3), which indicates that the amino acid at position 104 is not critical for enzyme activity. However, the Ser for Val difference at the tenth amino acid may be of significance, as Val^{10} is conserved in all Pi-class GSTs

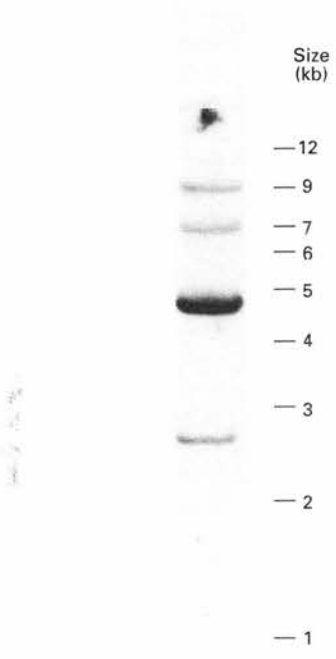

Figure 4 Complexity of the murine GST pi-gene cluster

Genomic DNA (20 μg) was digested with *Eco*RI and separated on a 0.8% agarose gel. After transfer to a Hybond-N filter (Amersham), hybridization was carried out with a ^{32}P-labelled MPCR2.2 probe using the conditions described in the Materials and methods section. The approx. 4.8 kb *Eco*RI fragment gives the strongest hybridization signal, because it contains parts of both Pi genes (see Figure 1), whereas the approx. 2.5 kb, approx. 4.8 kb, and approx. 9 kb fragments contain only a part of one of the pi genes.

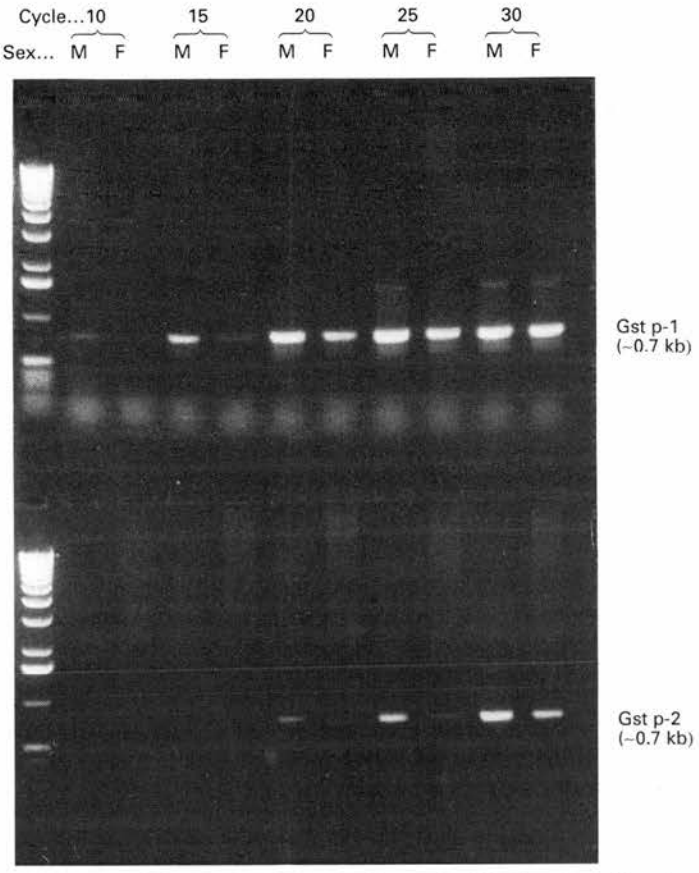

Figure 5 Relative levels of Gst p-1 and Gst p-2 mRNA in male and female mouse liver

Reverse PCR (10, 15, 20, 25, 30 cycles) was carried out using total mouse liver RNA to assess the relative expression of Gst p-1 and Gst p-2 in male (M) and female (F) mice as described in the Materials and methods section: 20 μl of the PCR product was subjected to electrophoresis in a 0.8% agarose gel and visualized with ethidium bromide. The first, unlabelled, lane contained size markers.

isolated to date, with the exception of murine Gst p-2 (Figure 3). Reinemer et al. (1991) proposed three possible locations in the active site to which the electrophilic substrate binds (H-site). One of them implicates the participation of amino acid Val^{10}. The catalytic activities of Gst p-1 and Gst p-2 remain to be characterized.

Analysis of the promoter regions

A TATAA sequence and a possible CCAAT element (CCAAC) can be found in the 5′ flanking regions of both genes (Figure 2). These elements, which are present in most eukaryotic promoters, are associated with the accurate initiation and promotion of transcription (Maniatis et al., 1987). Furthermore, a GC box, which precisely matches the consensus sequence 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′ for the binding site of transcription factor SP1, forms part of the promoter of the two genes (Kadonaga et al., 1986). Gst p-2 has an additional GC box, 5′-GGGGCGGCAT-3′, which differs from a perfect SP1-binding site in only one nucleotide, and is missing in Gst p-1. Immediately upstream of the GC boxes in both genes is the sequence 5′-TGAGTCAG-3′ which corresponds to the consensus sequence found in promoters of genes that are responsive to phorbol esters [e.g. phorbol 12-myristate 13-acetate (PMA; 'TPA')]. This motif has been called a PMA-responsive element (PRE). In comparison, the human and the rat Pi-class genes also possess two and one GC boxes and a PRE (Cowell et al., 1988; Okuda et al., 1987). Muramatsu and co-workers have shown that a PRE is involved in the high level of transcription of GST-P observed during rat hepatocarcinogenesis (Diccianni et al., 1992). In addition, the 5′ untranslated regions of both genes exhibit various substitutions and deletions. The overall differences between the 5′ non-coding regions of the two mouse genes suggest that they are regulated differently. Comparison of the intron sequences of Gst p-1 with Gst p-2, rat GST-P and human GST-π shows that all the introns have diverged to a similar extent from each other indicating that a specific intron does not play an important role in the regulation of the genes (Table 1).

Southern-blot analysis

The number of Pi-class GST genes in the mouse genome was estimated by Southern-blot analysis. Figure 4 shows that four *Eco* RI fragments of mouse DNA, approx. 2.5 kb, approx. 4.8 kb, approx. 6.8 kb and approx. 9 kb in size, hybridize to probe MPCR2.2. On the basis of the restriction map of clone MGSTP2 (Figure 1b), this suggests that there are at least three Pi-class GST-related sequences in the mouse genome. Clone MGSTP2 accommodates three of these hybridizing fragments, approx. 4.8 kb, approx. 6.8 kb and approx. 9 kb in length. However, the approx. 2.5 kb band is not present in any of the positive clones isolated. This fragment requires further characterization to establish whether it is a pseudogene or a further functional Pi-class Gst gene. Pi-class GST pseudogenes have been found in rat and human (Okuda et al., 1987; Board, 1993).

Evidence for transcription of the mouse Pi-class GST genes Gst p-1 and Gst p-2

In order to assess whether the two mouse Pi-class GST genes are transcribed correctly, we attempted to clone the corresponding cDNAs by reverse PCR. The nucleotide sequences of the Gst p-1 and Gst p-2 gene are identical in the 3′ untranslated regions (Figure 2). However, differences in the 5′ non-coding regions are sufficient to allow the design of two pairs of oligonucleotides (5′-CCTCACTCTGAGTACCCCTCTGTCTACG-3′ and 5′-TTTATTAGTGCTGGGAAAAC-3′ for Gsp p-1, and 5′-CCATCCCTGAGACACCTCTCTGACTATT-3′ and 5′-TTTATTAGTGCTGGGAAAAC-3′ for Gst p-2) which are specific for each gene. These oligonucleotides were used together with total liver RNA from male mice to perform reverse PCR. This procedure resulted in two DNA fragments of approx. 700 bp. The two fragments were cloned individually into the plasmid vector pGEM3Zf(+) and sequenced. As anticipated, these PCR fragments represented the two mouse Pi-class cDNAs, with coding regions identical with those found in the genes, Gst p-1 and Gst p-2. The isolation of these cDNA clones clearly shows that both mouse Pi-class GST genes are transcribed. This observation is intriguing in view of the finding that only one active Pi-class GST gene has been reported in both human and rat (Okuda et al., 1987; Cowell et al., 1988; Board et al., 1993).

Relative mRNA levels of Gst p-1 and Gst p-2 in male and female mouse liver

Using the same oligonucleotides that enabled us to amplify and isolate the Gst p-1 and Gst p-2 cDNAs, we developed a reverse PCR assay to estimate the expression levels of the two genes in mouse liver. Conditions under which reverse PCR was performed were identical for each reaction and as described in the Materials and methods section. When a primer pair was used together with the non-compatible subcloned Gst p cDNA as a template, no PCR product was generated (results not shown), demonstrating that the primer pairs were specific for the amplification of the corresponding cDNA. Using this analysis procedure, we found that both of the murine Pi-class GST genes are expressed at significantly higher levels in male compared with female liver (Figure 5). Moreover, mRNA encoding Gst p-1 is much more abundant than that of Gst p-2 in both male and female mouse liver (Figure 5). It remains to be established whether the two homologous proteins encoded by Gst p-1 and Gst p-2 have similar substrate specificities. This work is currently in progress.

Conclusions

Unlike other species characterized to date, the mouse contains at least two functional Pi-class GST genes. These genes appear to have certain regulatory characteristics common to previously reported Pi-class GST genes, in that both of their promoters contain a PRE. The proteins encoded by these genes differ in six amino acids. On the basis of data obtained from the crystal structure of porcine Pi-class GST, the replacement of Ser^{10} by Val is most likely to cause a difference in the enzymic activity. Further work will establish the relative tissue distribution, substrate specificity and mode of regulation of these two proteins, and whether they have common or distinct functions.

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Amino Acid Differences at Positions 10, 11, and 104 Explain the Profound Catalytic Differences between Two Murine Pi-Class Glutathione *S*-Transferases

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ABSTRACT: The glutathione *S*-transferases play a pivotal role in the detoxification of toxic and carcinogenic electrophiles. We have previously reported the isolation of two actively transcribed murine pi-class glutathione *S*-transferase genes. In this study the two proteins encoded by these genes, Gst p-1 and Gst p-2, were expressed in *Escherichia coli* and found to exhibit profoundly different catalytic activities, the activity of Gst p-2 toward a panel of electrophilic substrates being 1−3 orders of magnitude lower than that of Gst p-1. In order to establish the basis for the difference between these highly homologous proteins, mutants were generated where specific amino acids had been exchanged. Kinetic analysis of the wild-type and mutant enzymes revealed that the amino acid differences occurring at positions 10 (Val/Ser), 11 (Arg/Pro), and 104 (Val/Gly) are responsible for the reduced enzymatic activity of Gst p-2. This analysis together with computer graphics modeling for Gst p-2 indicated that these changes affected both substrate and glutathione binding to the enzyme.

Cytosolic glutathione *S*-transferases (GSTs)[1] are a family of dimeric enzymes which play a pivotal role in the conjugation of reduced glutathione (GSH) to a wide range of lipophilic electrophiles, including carcinogens and other toxins (Coles & Ketterer, 1990). Furthermore, certain GSTs catalyze the reduction of organic peroxides and protect membranes and DNA from oxidative damage (Ketterer et al., 1987). These enzymes also play a role in the intracellular transport and storage of hydrophobic molecules like hematin, bilirubin, and bile salts (Listowsky et al., 1988). In addition to their role as detoxification enzymes, GSTs also catalyze the isomerization of 3-ketosteroids and are involved in the biosynthesis of leukotrienes (Benson et al., 1977).

On the basis of nucleotide and amino acid sequence similarities, mammalian cytosolic GSTs can be grouped into four classes termed alpha, mu, pi, and theta (Mannervik et al., 1985, 1992; Meyer et al., 1991). Members within a class can form homo- or heterodimers which possess one active site per subunit. Recent reports on the crystal structures of alpha, mu, and pi class GSTs have had a tremendous impact on our understanding of the molecular architecture and function of these enzymes (Reinemer et al., 1991, 1992; Ji et al., 1992; Sinning et al., 1993; Garcia-Saez et al., 1994). Following the observation of Sato and co-workers that the pi-class GST is markedly elevated in rat liver tumor development (Sato et al., 1984a,b), much attention has focused on this group of enzymes. Subsequent studies demonstrated that this protein is one of the most reliable markers for pre-neoplastic lesions during rat liver carcinogenesis (Sato, 1989). Furthermore, several reports showed a significant increase in the levels of pi-class GST in a number of human tumors including oral, colon, stomach, and lung (Sato, 1989; Howie et al., 1990; Black & Wolf, 1991; Volm et al., 1991). In addition, these enzymes are expressed at elevated levels in cell lines made resistant to anticancer drugs and chemical toxins (Batist et al., 1986; Cowan et al., 1986; Wolf et al., 1990; Black & Wolf, 1991).

Inspite of numerous studies, the precise role of pi-class GSTs in carcinogenesis and multidrug resistance remains unclear. In order to answer these questions, we have been studying pi-class genes in the mouse because of the ability to apply mouse genetics to this problem, particularly because of the ability to generate transgenic animals and to carry out gene targeting experiments.

Recently, we reported the cloning of two actively transcribed murine pi-class GST genes, as well as their cDNAs (Bammler et al., 1994). In the present study, we have characterized the proteins encoded by these genes in detail and related amino acid differences to the observed profound differences in catalytic activity.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of analytical grade or better and were purchased from either Sigma Chemical Co., Poole, Dorset, U.K., or BDH, Glasgow, U.K., unless otherwise indicated. Restriction endonucleases used in this study were obtained from Gibco BRL (Life Technologies Ltd., Paisley, Renfrewshire, Scotland, U.K.).

Generation of Mutant GST cDNAs. The two GST cDNAs, Gst p-1 and Gst p-2, were isolated as described previously (Bammler et al., 1994). The oligonucleotides used for site-directed mutagenesis of Gst p-1 are listed in Table 1. The Gst p-1 cDNA was cloned into the *Eco*RI and *Xba*I restriction sites of the pAlter vector (Promega, Madison, WI),

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[1] Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 2,4-dichloro-4-nitrobenzene; GST, glutathione *S*-transferase; GSH, glutathione.

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Table 1: Oligonucleotide Primers Used for Site-Directed Mutagenesis

mutant	amino acid changed in Gst p-1	oligonucleotide sequence[a]	sense
P1-V10S	Val10 → Ser	5′-TCACACCGCCCTCGACTTGGGAAGTAGACAAT-3′	inverse
P1-R11P	Arg11 → Pro	5′-CACACCGCCCTGGAACTGGGAAG-3′	inverse
P1-M89V	Met89 → Val	5′-CACCATATCCACCTGGGCGGCCT- 3′	inverse
P1-V104G	Val104 → Gly	5′-GATGAGGGTGCCATATTTGCCGC-3′	inverse
P1-L106M	Leu106 → Met	5′-ATAGTTGGTGTAGATCATGGTGACATATTTGCC-3′	inverse
P1-T109R	Thr109 → Arg	5′-ATTCTCATAGTTTCTGTAGATGAGGG-3′	inverse

[a] Mismatches between the mutagenic oligonucleotide and the cDNA of GST p-1 are underlined.

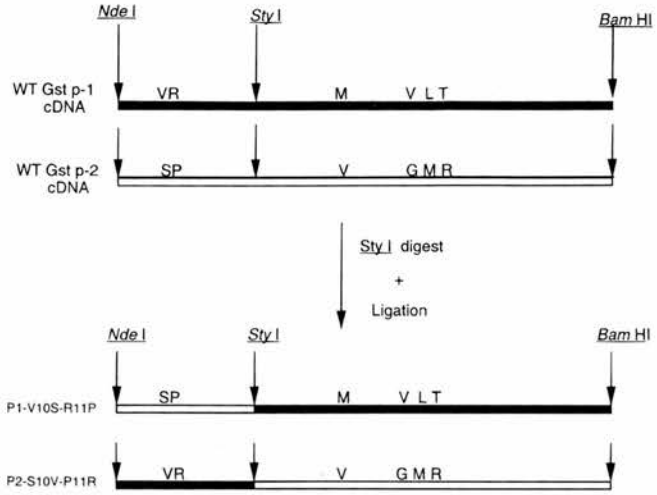

FIGURE 1: Schematic diagram showing the generation of the double mutant cDNAs P1-V10S-R11P and P2-S10V-P11R. The *Nde*I/*Sty*I fragment of the wild-type (WT) cDNA Gst p-1 was ligated with the *Sty*I/*Bam*HI fragment of the wild-type cDNA Gst p-2 and *vice versa*. The six amino acid differences between the two wild-type cDNAs are indicated by single-letter abbreviations.

and oligonucleotide-directed mutagenesis was carried out according to the manufacturer's recommendations. An overall success rate of 73% was achieved using the pAlter mutagenesis system.

Both murine pi-class cDNAs, Gst p-1 and Gst p-2, contain a unique *Sty*I restriction site 215 base pairs downstream of the ATG start codon. This enabled us to generate chimeric cDNAs, which contain the 5′ end of Gst p-1 and the 3′ end of Gst p-2, and *vice versa* (Figure 1). Briefly, the *Sty*I fragments of the two cDNAs were gel purified, and the appropriate fragments were ligated using T4 DNA ligase (Boehringer, Mannheim, Germany). The entire sequence of all mutant cDNAs was determined by the chain termination method of Sanger et al. (1977) before they were used for heterologous expression in *Escherichia coli*.

Expression and Purification of Murine Pi-Class GSTs. The pET expression system (AMS Biotechnology U.K. Ltd., Witney, Oxon, England) was used to express the wild-type and mutant GSTs. Expression was carried out according to the manufacturer's instructions. Briefly, all cDNAs were cloned into the *Nde*I and *Bam*HI restriction sites of the expression vector pET-21a, and the GSTs were transfected into the *E. coli* strain BL21(DE3). Expression was induced with a 2-h exposure to 1 mM isopropylthiogalactose (Boehringer), after which time the bacteria were harvested by centrifugation at 10000*g* for 15 min. Harvesting and all of the following steps were carried out at 4 °C. The cell pellet was resuspended in buffer A (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.5 mM dithiothreitol), and the bacterial cells were lysed by ultrasonication. Intact cells and cell debris were removed by two centrifugation steps at 50000*g* for 30 min each time. The resulting supernatant was loaded onto a glutathione−agarose affinity column, which had been equilibrated previously with buffer A. The bound protein was washed with approximately 15 column volumes of buffer A, and the recombinant GSTs were eluted with buffer B (200 mM Tris-HCl, pH 9.0, 50 mM glutathione). The absorbance at 280 nm of the collected fractions (1.5 mL each) was measured. Fractions showing an absorbance value >0.8 were pooled and dialyzed against four changes of 2 L of buffer C (40 mM sodium phosphate buffer, pH 7.4, 0.5 mM dithiothreitol, 0.5 mM EDTA). The purified proteins were frozen on dry ice and stored at −70 °C for up to 8 weeks, without a significant loss of activity. The purity of the proteins was analyzed by SDS/PAGE (Laemmli, 1970). When the same procedure was repeated using *E. coli* BL21-(DE3), which did not harbor the expression vector, no bacterial protein bound to the affinity column.

Enzyme Assays. All enzyme assays were carried out at 30 °C. The 1-chloro-2,4-dinitrobenzene (CDNB) assay was carried out using a Cobas Fara centrifugal analyzer as described by Hayes and Clarkson (1982). Assays with either 1,2-dichloro-4-nitrobenzene (DCNB, Aldrich Chemical Co. Ltd., Gillingham, Dorset, England, U.K.), ethacrynic acid, bromosulfophthalein, *trans*-4-phenyl-3-buten-2-one (Aldrich), 1,2-epoxy-3-(*p*-nitrophenoxy)propane, *p*-nitrobenzyl chloride, *p*-nitrophenyl acetate, or Δ^5-androstene-3,17-dione were performed as described by Habig et al. (1974, 1981). Selenium-independent glutathione peroxidase activity for organic hydroperoxide was determined by using the coupled assay system described by Reddy et al. (1981). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Kinetic Analysis. Activity toward CDNB was assayed spectrophotometrically at 340 nm using a Cobas Fara centrifugal analyzer. Assays were performed in triplicate at 30 °C. Initial rates were measured at 5 s intervals for a total period of 55 s, commencing 3 s after initial mixing. The reaction was initiated by the addition of the enzyme to 0.1 M sodium phosphate buffer, pH 6.5, containing the substrates CDNB and GSH. CDNB was dissolved in ethanol, and the final concentration of ethanol in the reaction mixture was constant at 3.5%. Nonenzymatic reaction rates were subtracted from enzymatic rates. The enzymatic rate was linear with time for up to 60 s after initiation. Each data point represents the mean of three measurements. Individual kinetic parameters represent the mean of three independent series of experiments, with the only exception being the values for Gst p-2 which are calculated from two experiments. Kinetic analysis was based on the report by Phillips and Mantle (1991) describing the initial rate kinetics of murine pi-class GST YfYf (Gst p-1) as a rapid-equilibrium random mechanism. Therefore, initial velocities can be

interpreted according to the equation:

$$V_0 = \frac{V_{max}AB}{\alpha K_A K_B + \alpha K_B A + \alpha K_A B + AB}$$

(A, GSH; B, CDNB). Double-reciprocal plots of initial velocitiy data were linear for all enzymes. Individual kinetic parameters were determined as described by Ivanetich and Gould (1989). Regression analysis was performed using the Apple Mac program Cricket Graph, version 1.3.2. With CDNB as the varied substrate, GSH concentrations ranged from 0.05 to 2 mM. With GSH as the varied substrate, CDNB concentrations ranged from 0.1 to 2 mM (the CDNB concentration cannot be increased above 2 mM for reasons of solubility).

Model Building. The sequence of mouse Gst p-2 was modeled into the 1.8 Å three-dimensional structure of Gst p-1 (Garcia-Saez et al., 1994) by computer graphics on an Evans and Sutherland PS390, using the FRODO program (Jones, 1978, as modified by P. Evans). Side chains which were not identical were placed in similar positions and then adjusted interactively to minimize unfavorable contacts and to optimize van der Waals and electrostatic interactions, keeping in mind the need to form a close-packed core. Where necessary, other residues were adjusted. The resulting coordinates were also compared with those of pig lung P-1 GST (Reinemer et al., 1991). Water accessibilities were calculated using the program DSSP of Kabsch and Sander (1983). Contacts were calculated with the program MODEL (Driessen, unpublished). Residues for Gst p-2 are numbered according to the mouse liver Gst p-1 structure.

RESULTS

Catalytic Activities of Two Recombinant Wild-Type Murine Pi-Class GSTs. We recently reported the cloning of a novel murine pi-class glutathione *S*-transferase cDNA termed Gst p-2 (Bammler et al., 1994). N-Terminal sequence comparison showed that Gst p-2 is different from the murine pi-class GST reported by Mannervik and colleagues (1985) and Hatayama et al. (1990). In order to obtain Gst p-1 (YfYf, MII) and Gst p-2 protein, their corresponding cDNAs were expressed in *E. coli*. Immunoblotting of the soluble fractions of individual *E. coli* cultures showed that both constructs expressed GST protein at very similar levels (results not shown). The recombinant enzymes were purified on a glutathione−agarose affinity column to apparent homogeneity as judged by SDS/PAGE analysis (Figure 2). One litre of *E. coli* culture yielded approximately 60 mg of pure Gst p-1 protein, whereas only approximately 15 mg of Gst p-2 was obtained. This suggested that Gst p-2 bound with a lower affinity to the glutathione−agarose column. The relative mobilities of the proteins in SDS/PAGE were indistinguishable, with an apparent molecular mass of 23.5 kDa (Figure 2). In order to establish whether the two enzymes have similar or distinct catalytic activities, we determined their activity toward a panel of established GST substrates (CDNB, DCNB, bromosulfophthalein, ethacrynic acid, *trans*-4-phenyl-3-butene-2 one, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, cumene hydroperoxide, *p*-nitrophenyl acetate, *p*-nitrobenzyl chloride, Δ^5-androstene-3,17-dione) (Table 2). Gst p-1 was much more active toward all of these substrates than Gst p-2.

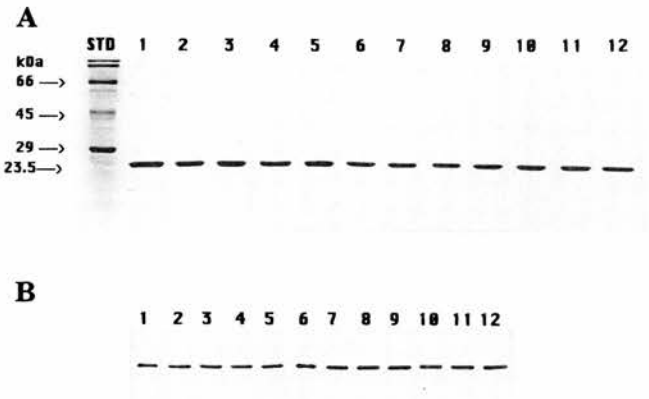

FIGURE 2: Purified recombinant wild-type and mutant murine pi-class glutathione *S*-transferase proteins. Purified recombinant wild-type (WT) and mutant murine pi-class GSTs were analyzed by SDS/PAGE in 12% polyacrylamide resolving gels. The gel was loaded as follows: STD, molecular weight standards; lanes 1 and 12, mouse Yf marker isolated from mouse liver; lane 2, WT Gst p-1; lane 3, WT Gst p-2; lane 4, P1-V10S-R11P; lane 5, P2-S10V-P11R; lane 6, P1-V10S; lane 7, P1-R11P; lane 8, P1-M89V; lane 9, P1-V104G; lane 10, P1-L106M; lane 11, P1-T109R. Panel A shows the polyacrylamide gel stained with Coomassie Blue R250 (1 μg of protein was loaded per lane); panel B shows the immunoblot developed with a polyclonal antibody raised against purified pi-class GST (YfYf) isolated from mouse liver (100 ng of protein was loaded per lane).

Table 2: Specific Activities (μmol min^{-1} mg^{-1}) of the Two Wild-Type Murine Pi-Class GSTs, Gst p-1 and Gst p-2[a]

substrate	Gst p-1	Gst p-2
1-chloro-2,4-dinitrobenzene	84 ± 4	0.12 ± 0.01
1,2-dichloro-4-nitrobenzene	0.10 ± 0.01	ND
bromosulfophthalein	ND	ND
ethacrynic acid	6.1 ± 0.5	0.04 ± 0.008
trans-4-phenyl-3-buten-2-one	0.016 ± 0.0008	ND
1,2-epoxy-3-(*p*-nitrophenoxy)propane	0.45 ± 0.017	ND
cumene hydroperoxide	0.09 ± 0.01	0.01 ± 0.001
p-nitrophenyl acetate	0.21 ± 0.012	ND
p-nitrobenzyl chloride	1.24 ± 0.085	0.05 ± 0.01
Δ^5-androstene-3,17-dione	0.09 ± 0.004	ND

[a] All analyses were performed at least in triplicate (±SD); ND, not detectable.

Expression of Mutant Murine Pi-Class GSTs. There are six amino acid differences between Gst p-1 and Gst p-2 in two clusters: one located in the N-terminal domain (amino acids 10 and 11) and the other in the C-terminal domain (amino acids 89, 104, 106, and 109). In order to assess the contribution of each of the six amino acid differences between Gst p-1 and Gst p-2 to the profound difference in enzymatic activity, six mutants of Gst p-1 were created, where one single amino acid was replaced with the equivalent amino acid in Gst p-2 (Table 1). In addition, two hybrid enzymes were generated, in which the N-termini, containing the amino acid differences Val/Ser10 and Arg/Pro11, were interchanged. These hybrid enzymes were designed to examine the possible combined effect of the amino acid differences. To characterize the mutant enzymes further, they were expressed and purified (Figure 2). All mutant proteins were expressed at very similar levels as judged by immunochemical analysis of the soluble fractions of individual *E. coli* cultures (results not shown). They all bound to the GSH affinity column, and approximately 60 mg of pure protein was obtained from a 1 L culture, with the exception of the mutants P1-R11P and P1-V10S-R11P.

Table 3: Kinetic Constants for Wild-Type and Mutant Enzymes in the Reaction Involving CDNB as the Electrophilic Substrate[a]

			GSH		CDNB	
enzyme	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_m^{GSH} (mM)	k_{cat}/K_m^{GSH} (mM^{-1} s^{-1})	K_m^{CDNB} (mM)	k_{cat}/K_m^{CDNB} (mM^{-1} s^{-1})
wild type						
Gst p-1	141.1 ± 6.3	55.5 ± 2.5	0.12 ± 0.01	446 ± 33	0.75 ± 0.11	75 ± 6
Gst p-2	0.8[b]	0.3[b]				
double mutants						
P1-V10S-R11P	1.7 ± 0.4	0.7 ± 0.2	3.27 ± 0.52	0.2 ± 0.007	2.22 ± 0.58	0.3 ± 0.04
P2- S10V-P11R	73.9 ± 9.5	29.2 ± 3.7	0.38 ± 0.09	81 ± 10	2.84 ± 0.25	10 ± 0.8
single mutants						
P1-V10S	28.2 ± 4.0	11.1 ± 1.6	0.07 ± 0.02	163 ± 10	0.43 ± 0.09	24 ± 3
P1-R11P	4.7 ± 1.6	1.8 ± 0.6	2.86 ± 0.23	0.46 ± 0.04	1.52 ± 0.42	1.6 ± 0.3
P1-M89V	172.6 ± 8.5	67.8 ± 3.4	0.21 ± 0.05	371 ± 63	0.55 ± 0.18	134 ± 23
P1-V104G	62.9 ± 3.8	24.7 ± 1.5	0.28 ± 0.08	100 ± 18	2.24 ± 0.29	11 ± 0.6
P1-L106M	120.6 ± 7.7	47.5 ± 3.0	0.15 ± 0.03	321 ± 29	0.60 ± 0.03	79 ± 0.4
P1-T109R	116.7 ± 8.4	46.0 ± 3.3	0.14 ± 0.05	293 ± 23	0.70 ± 0.06	65 ± 1

[a] Values represent the means (±SD) of three independent series of measurements as described in Experimental Procedures. [b] Mean calculated from two series of measurements only; therefore, no standard deviation is provided.

These two mutants bound with lower affinity to the glutathione−agarose column, resulting in an approximately 4-fold lower yield of purified proteins. All mutant proteins displayed the same mobility and were stable as judged by SDS/PAGE analysis.

Kinetic Analysis of the Wild-Type and Mutant Enzymes. The kinetic properties of the purified wild-type and mutant enzymes in the conjugation of CDNB with GSH are shown in Table 3. Due to the very low activity, the wild-type enzyme Gst p-2 could not be reliably characterized. Kinetic analysis revealed that the wild-type enzyme Gst p-1 and the mutants P1-M89V, P1-L106M, and P1-T109R exhibited similar kinetic properties. In contrast, the mutants P1-V10S, P1-R11P, and P1-V104G displayed significantly lower catalytic efficiencies (k_{cat}/K_m) than Gst p-1. The k_{cat} values for P1-V10S, P1-R11P, and P1-V104G were 80%, 97%, and 55% decreased, respectively, as compared to the wild-type Gst p-1. There was an approximately 2-fold increase in the K_m value for CDNB for the mutant P1-R11P, whereas the mutant P1-V104G showed a 3-fold higher value. While an approximately 2-fold change in K_m for GSH was observed for P1-V104G, a profound increase of approximately 24-fold was measured for P1-R11P. Interestingly, the P1-V10S mutant did not exhibit a significant change in K_m for either CDNB or GSH. The hybrid enzyme P1-V10S-R11P gave kinetic constants very similar to those observed for the single mutant, P1-R11P. The wild-type enzyme Gst p-2 had a k_{cat} value of approximately 0.5% of Gst p-1. When both Ser10 and Pro11 were replaced by Val and Arg (P2-S10V-P11R) in Gst p-2, the k_{cat} value of Gst p-2 increased approximately 97-fold and reached approximately 53% of the value of Gst p-1.

Kinetic constants for the two single mutants P1-V10S and P1-R11P and the hybrid enzymes P1-V10S-R11P and P2-S10V-P11R strongly suggest that the two amino acid differences between Gst p-1 and Gst p-2 at positions 10 and 11 are major determinants for the catalytic activity differences of the two wild-type enzymes. In addition, the replacement of Val104 by Gly has also a significant effect on the wild-type enzyme Gst p-1, lowering the k_{cat} value approximately 55% and increasing the K_m for GSH and CDNB approximately 2- and 3-fold, respectively.

Relationship between the Activity of the Mutants and the Three-Dimensional Model of Mouse Gst p-2. In order to rationalize the different activities of the mutant proteins, a model for Gst p-2 was constructed on the basis of the three-dimensional coordinates of the crystal structure of Gst p-1, complexed with *S*-(*p*-nitrobenzyl)glutathione (Garcia-Saez et al., 1994). Alignment of Gst p-1 and Gst p-2 was unambiguous as the sequences have an equal number of amino acids with only six differences in 209 residues (Figure 3). These data are discussed below.

DISCUSSION

Unlike other species characterized to date, the mouse contains two actively transcribed pi-class GST genes. The recombinant proteins encoded by these genes exhibit a profound difference in enzymatic activity. The catalytic activity of Gst p-1 was extremely similar to the GST pi form isolated previously from mouse liver (Warholm et al., 1986; McLellan et al., 1987). Importantly, the kinetic parameters for Gst p-1 were very similar to those reported for the murine pi-class GST isolated by Phillips and Mantle (1991). In contrast, the specific activities of Gst p-2 for the substrates CDNB, ethacrynic acid, *p*-nitrobenzyl chloride, and cumene hydroperoxide were 700-, 150-, 25-, and 9-fold lower, respectively. Furthermore, Gst p-2 was not catalytically active toward any of the other substrates tested. This marked difference in catalytic activity could be considered surprising, as the two proteins share 97% sequence identity. As the two enzymes differ in only six amino acids, it was possible to examine which of these are responsible for the differences observed in catalytic activity of Gst p-2.

Recently, crystal structures of mammalian GSTs, belonging to the alpha, mu, and pi class, have been solved, and the molecular architecture of the active site has been determined. Each subunit of the dimeric enzyme has an active site which is composed of two distinct functional regions: a hydrophilic G-site which binds the physiological substrate GSH and an adjacent H-site which provides a hydrophobic environment for the binding of structurally diverse electrophilic substrates (Mannervik et al., 1988; Reinemer et al., 1991, 1992). The outer wall of the active site cave is made up by helix αB and helix 3_{10}B connecting it to strand β3, the inner wall by the C-terminal part of helix αD, the C-terminus, and the middle of helix αD of the neighboring subunit, and the bottom by the segment between strand β1 and αA. The differences between Gst p-1 and Gst p-2 are concentrated in just two secondary structure elements located at the bottom and the inner wall of the active site (Figure 4a). Residues

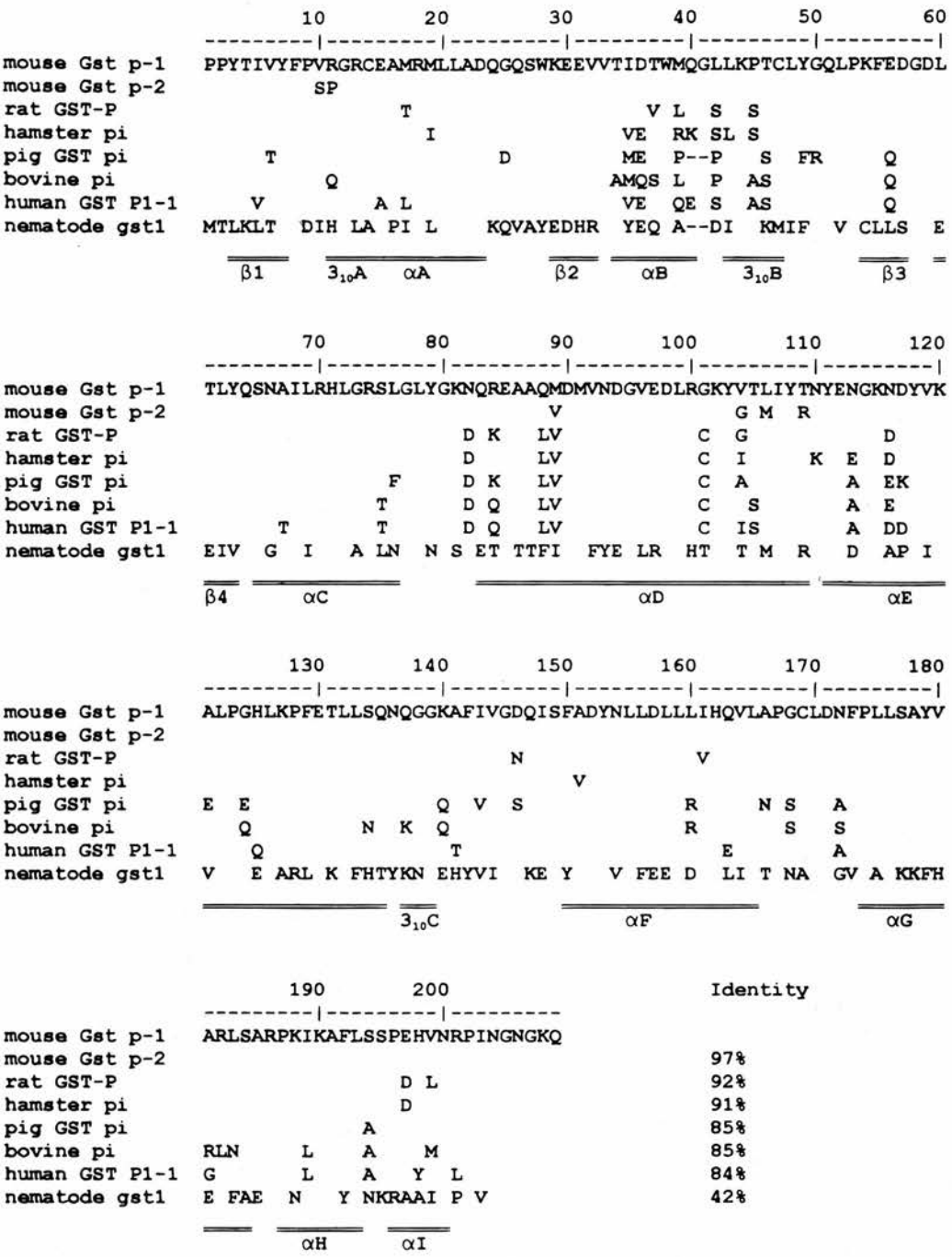

FIGURE 3: Sequence alignment of all known GST pi sequences. Sequence identities are given with respect to mouse Gst p-1. The secondary structure elements as determined for this protein (Garcia-Saez et al., 1994) are indicated with a double line. Sequences are from Bammler et al. (1994), Suguoka et al. (1985), Swedmark and Jensson (1994), Dirr et al. (1991), Hernando et al. (1991), Kano et al. (1987), and Weston et al. (1989).

10 and 11 are positioned on the segment between strand $\beta 1$ and αA comprising a γ-turn followed by a 3_{10} helix, while residues 89, 104, 106, and 109 are situated on helix αD, the latter three in the C-terminal part.

The residues in the H-site of Gst p-1 are all hydrophobic and are highly conserved: Phe8, Val10, Ile35, Tyr108, the Cα of Gly205, while Pro9 and Pro202 are in the vicinity (Reinemer et al., 1991; Garcia-Saez et al., 1994). In monomer A of the structure of murine Gst p-1, the hydroxyl group of Tyr108 points toward the plane of the aromatic ring of the *S-p*-nitrobenzyl group and is within van der Waals contact range of the Cε, Cζ2, and Cγ2 atoms, in subunit B also of Cζ1 (Figure 4b). Val10 interacts with the edge of the aromatic ring of the *S-p*-nitrobenzyl group.

When Val10 was replaced by Ser in Gst p-1, the catalytic efficiency ($k_{cat}/K_m^{GSH/CDNB}$) decreased approximately 3-fold. This was due to an approximately 5-fold decreased k_{cat} value and somewhat lower K_m values for both CDNB and GSH. The binding of GSH therefore does not appear to be affected significantly. As described above, Val10 participates directly in the lining of the H-site and is within van der Waals range of the ring of Tyr7. Moreover, this residue is conserved in all mammalian pi-class GSTs isolated so far, the only exception being Gst p-2 (Figure 3). In addition, in the pi-class GST isolated from the nematode *Caenorhabditis elegans* (Weston et al., 1989) Val10 is replaced conservatively by Ile (Figure 3). In the mu-class GST the comparable residue is implicated in control of stereoselectivity (Ji et al.,

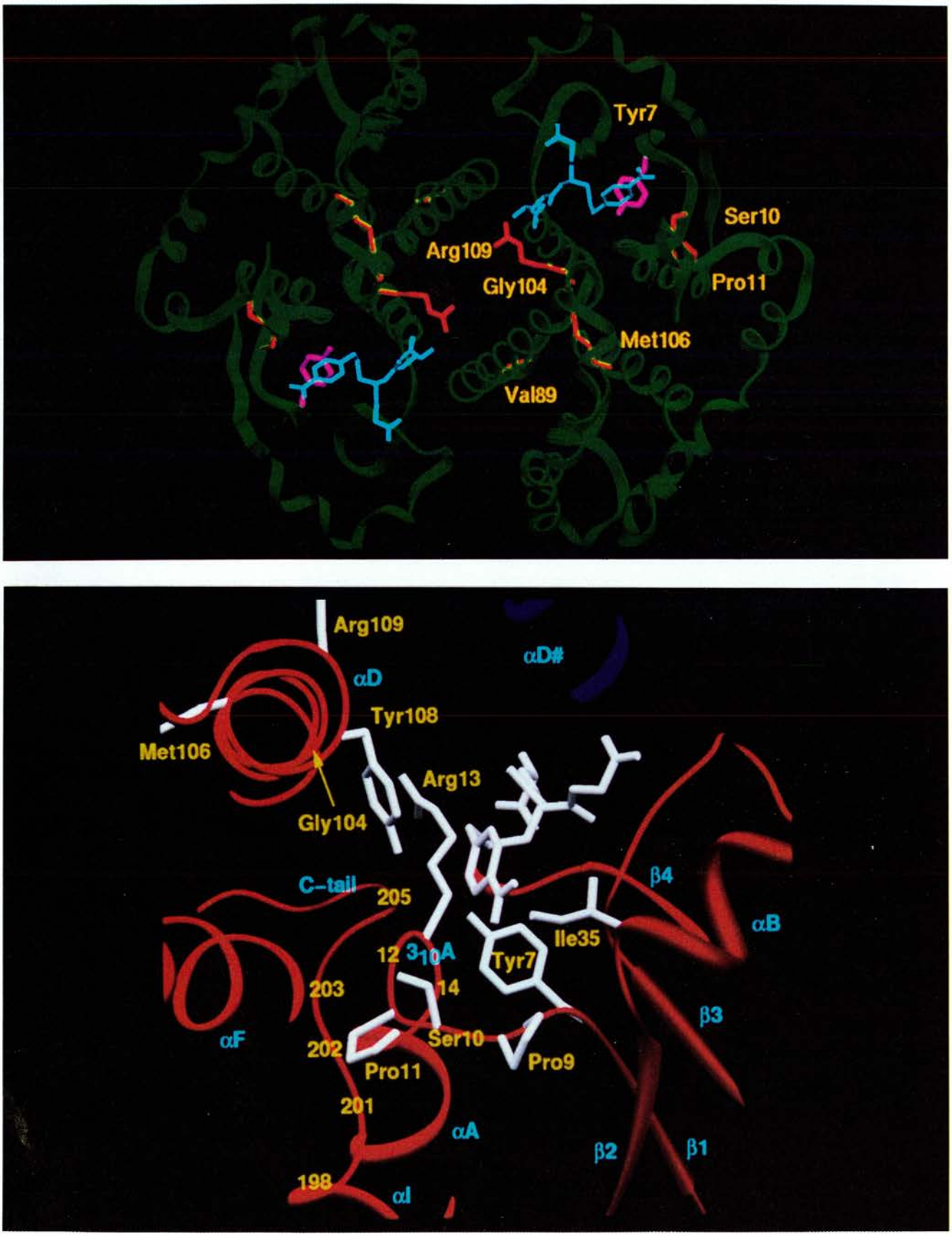

FIGURE 4: Predicted structures of Gst p-2. (a, top) Ribbon diagram of the mouse Gst p-2 model, based on the structure of mouse Gst p-1. The GSH−substrate conjugate is shown in blue, Tyr7 is in purple, and the side chains of the six amino acids which differ between Gst p-1 and p-2 are in red. (b, bottom) Active site of the Gst p-2 model, based on the structure of mouse Gst p-1. The secondary structure elements are shown as red and blue ribbons and are identified in pale blue. The GSH−substrate conjugate and the side chains of relevant residues are shown in white and are identified in yellow. Where necessary, the positions of other residues are indicated by a yellow number only.

1994). These observations therefore rationalize the reduction in enzyme activity when the hydrophobic residue Val10 is replaced by the more hydrophilic Ser residue. On the basis of the above arguments it would be expected that the affinity of the mutant P1-V10S for CDNB would be decreased significantly as compared to Gst p-1. However, judging by the K_m, this was not the case.

If the hydroxyl group of Ser 10 points toward the substrate, which would require some main-chain movement, Tyr108 and Ser10 could both be involved in electrophilic assistance in addition to GSH to an as yet unknown substrate in a similar way as has been shown for the equivalent Tyr115 in mu3-3 (Ji et al., 1994). Tyr108 is about 2.8 Å deeper in the active site of Gst p-1 than in the mu class, while no equivalent to mu Ser209 is hydrogen-bonded. In the model, the OH of Tyr108 and the Cβ of Ser10 are 5.85 Å apart (Figure 4b). In this respect it is noteworthy that theta-class GSTs do possibly have a Ser in this area, while dog theta (YdfYdf) has the combination Ser-Pro (Igarashi et al., 1991). Both Gst p-2 and most theta GSTs have in common that they do not have appreciable activity with many of the substrates metabolized by other GSTs, the exception being the dog theta enzyme.

On the other hand, the OH group of the Ser10 can hydrogen bond to the carbonyl atoms of residues Arg202 and Pro203. The carbonyl atom of the latter is also hydrogen bonding to the nitrogen atom of Gly12. This would tighten the binding of the C-terminal loop.

The replacement of Arg11 by Pro in Gst p-1 results in a 97% loss of activity and an approximately 24- and 2-fold increase in the K_m for GSH and CDNB, respectively. Importantly, Stenberg et al. (1991) and Wang et al. (1993) reported a similar result when they replaced Arg12 (the equivalent to Arg11 in pi-class GSTs) in a rat (YaYa) and a human (GST2, GST B1B1) alpha-class GST with an uncharged amino acid. Arg11 is a conserved residue in pi, mu, and alpha GSTs. However, it does not directly participate in the binding of GSH and electrophilic substrates in the active site, as the marked decrease in k_{cat} and increase in the K_m value for GSH suggests. The charge of this amino acid appears important for maintaining the correct conformation of the active site. Arg11, present in the N-terminal domain, holds the helix αI in the C-terminal domain close with two hydrogen bonds (11NH2-O198 and 11Nϵ-O201) and the C-terminal tail by another two (11N-O202 and 12N-O203). Furthermore, the carbonyl oxygen of Arg11 is hydrogen bonding the amide nitrogens of Cys14 and Glu15. Arg11 therefore stabilizes the segment between strand β1 and helix αA and its position at the bottom of the active site with respect to the inner wall and the 3_{10}A helix 11−14. Arg11 has ϕ −74 and ψ −61, angles which on substitution by Pro with its fixed ϕ angle of −60 can be accommodated surprisingly well, when the C-terminal area 200−204 is moved away slightly. However, three out of the six hydrogen bonds are now lost, leading to weakened interaction with the inner wall, with the possibility of some compensation from hydrogen bonding of the hydroxyl group of Ser10 with the carbonyl atoms of 202 and 203.

The enzymes Gst p-2, P1-R11P, and P1-V10S-R11P, which contain a Pro instead of an Arg at position 11, were noticed to bind with significantly lower affinity to the GSH−agarose affinity column. This observation is consistent with the high K_m these two mutants have for GSH. The only residue close to residues 10−11 implicated in stabilizing the GSH is Arg13, which is near the terminal carboxyl group of the γ-glutamyl group of GSH. This suggests that there is a greater disturbance of the conformation than suggested by the simple model, in which no residues beyond Arg11 are affected. Residue Ser10 in Gst p-2 is positioned between two relatively rigid prolines, and the γ-turn 9−11 may not actually be present. It also cannot be excluded that the Pro has a cis conformation. Electrostatic calculations by Karshikoff et al. (1993) suggest that the Tyr 7 OH group is deprotonated by the influence of the protein charge constellation and the peptide dipoles, especially residues Gly12, Arg13, and Cys14. A drastic difference in the conformation of the 3_{10} helix 11−14 or a shift of residue Tyr 7 would have an influence on the catalytic activation.

The simultaneous replacement of Ser10 by Val and Pro11 by Arg in Gst p-2 (P2-S10V-P11R) results in an approximately 100-fold increase of k_{cat}. In contrast, an approximately 80-fold decrease in the k_{cat} of Gst p-1 is observed when Val10 and Arg11 are replaced by Ser and Pro (P1-V10S-R11P). These results provide strong evidence that the two N-terminal amino acid differences (Val/Ser, Arg/Pro) between Gst p-1 and Gst p-2 have a profound effect on the catalytic activities of these enzymes. In a recent paper by Xu and Stambrook (1994), evidence was also presented that residues 10 and 11 play an important role in determining the catalytic differences between these proteins. With both mutations present, there may be realignment of the bottom and inner wall of the active site, because of the shift of hydrogen bonding from helix αI toward the C-terminus, with possible effect on the positions of residues 202 and 205, which are close to the substrate. This might have an effect on dissociation when the GSH conjugate leaves the active site.

The single substitution of Met89 by Val has very little effect on the catalytic activity of Gst p-1. This is not surprising as this change is of a conservative nature. In addition, with the exception of mouse Gst p-1, all mammalian pi-class GSTs isolated so far have a Val residue present at position 89 (Figure 3). In the case of the *C. elegans* enzyme, this residue is conservatively replaced by Ile. Val89 in the structure of pig pi and Met89 in mouse Gst p-1 are virtually inaccessible to solvent. Although Met is slightly larger than Val, a comparison of both P1 structures does not indicate a difference in core packing. This difference, although situated in helix αD, is some distance from the active site and is therefore not expected to have a large effect on substrate binding on its own. This explains that a change from a Met to a Val in Gst p-1 leads to similar kinetic parameters.

The differences between mouse Gst p-1 and Gst p-2 do not include any of the residues involved in the contact areas within the GST dimer; therefore, Gst p-2 is expected to be a dimer. However, Val89 in mouse Gst p-2 is situated next to some residues involved in the dimer interface, Ala87, Leu88 (only marginally), Asp90, and Met91. Comparison of the mouse liver Gst p-1 and pig lung GST P1 structures does not indicate any significant differences in the dimer interface around residue 89. The only residue implicated in intersubunit stabilization of bound GSH, Asp98, is not affected.

Residues 104, 106, and 109 are in the C-terminal part of helix αD, close to residue Tyr108 which lines the H-site. The single substitution of Leu106 by Met in Gst p-1 has

very little effect on the catalytic activity. This is not surprising as the change is of a conservative nature. As for position 106, the only other pi-class GST which has a Met instead of a Leu present is the *C. elegans* enzyme. The replacement of Leu106 by a Met requires only small rearrangements to allow positioning with correct van der Waals distances in the core of the inner wall of the active site, which is unlikely to cause changes in substrate binding on its own. The nonconservative replacement of Thr109 by Arg has also little impact on the catalytic activity of Gst p-1. Thr109 is conserved in all pi-class GSTs, with the exceptions of Gst p-2 and the *C. elegans* enzyme, which both have an Arg present at this position. In the model, Arg 109 sticks out of the C-terminal part of helix αD into the large cavity between the two monomers making up the active GST dimer. It is accessible to the solvent. It is pointing away from the H-site and not directly interacting with substrates in the H-site. Because of its position it is unlikely to interfere with the substrate entering or the adduct leaving the active site. Although this difference is situated in helix αD, it is not expected to have a significant effect on substrate binding.

In contrast, the nonconservative replacement of Val104 by Gly results in an approximately 55% decrease in the k_{cat} value of Gst p-1 and a 2- and 3-fold increase in the K_m for GSH and CDNB, respectively. This finding is initially somewhat surprising, as Val104 is not conserved in the members of the pi class. Human P1-1, which has an Ile at position 104, has a similar specific activity for CDNB as mouse Gst p-1 (Mannervik & Danielson, 1988). Compared with human P1-1, the human isoform of P1-1, which has a Val at position 104, has about half the specific activity for CDNB, identical K_m for GSH, and a 4-fold increase of K_m for CDNB (Zimniak et al., 1994), data which are in very good agreement with our results. The rat pi enzyme also has a Gly present at position 104 and has much lower specific activity for CDNB than mouse Gst p-1 but not as low as mouse Gst p-2. On the basis of a comparison of human P1-1 and its Val104 isoform, Zimniak et al. (1994) have proposed that the residue at position 104 helps in defining the geometry of the H-site, with bulkier residues resulting in a smaller more restrictive H-site, while the residue may also influence activity by interacting with Tyr108 directly involved in substrate binding. Tyr108 is located within 4 Å of the Ile (or Val) at position 104 in human P1-1, suggesting that apart from size also hydrophobicity may play a role in influencing the function of 108. Since for both human P1-1 and mouse Gst p-1 the K_m for CDNB increases as the size of residue 104 decreases, a similar interpretation appears to be applicable. As in the human P1-1 structure, in the mouse Gst p-1 structure the aliphatic side chain of Val at position 104 is within van der Waals range of the ring of Tyr108, possibly keeping it in a relatively fixed position with respect to the lipophilic substrate. In mouse Gst p-2 and rat P-1 where 104 is replaced by the much smaller Gly, this stabilization will not be present at all, and the Tyr will be more free to move, which may both affect its position and ability to aid in controlling stereoselectivity, as well as its steric hindrance of dissociation of the product. CDNB may therefore be held less firmly, explaining the 3-fold increase in K_m of the mutant P1-V104G. Another possibility which needs to be considered is that because Gly can have main-chain conformations, which other residues cannot accommodate because of their size, the replacement causes a change in direction in helix αD, changing the position of Tyr108 in the active site and the size of the active site itself. In mouse Gst p-2 this might bring 108 closer to Ser10. Since no residues implicated in binding GSH are directly affected when replacing Val104 by Gly in Gst p-1, a similar K_m for GSH is expected. Ile104 in human P1-1 is at roughly van der Waals distance to the carbonyl group of the glutamic acid in GSH, possibly aiding in the catalytic reaction by stabilizing the position of the GSH in the G-site. Val in the human P1-1 isoform is at a similar distance, thus not causing a difference in K_m for GSH. In contrast, Val104 in mouse Gst p-1 is already at a distance of 6.5 Å, making such stabilization less effective. There is no possibility of such an interaction with the much smaller Gly, possibly explaining the small increase in K_m for GSH when replacing Val by Gly in Gst p-1.

The hybrid enzyme P2-S10V-P11R, which can be interpreted as a mutated Gst p-1 carrying the four mutations Met89Val, Val104Gly, Leu106Met, and Thr109Arg simultaneously, displays kinetic parameters remarkably similar to those found for the mutant P1-V104G. This finding strongly suggests that the four amino acids present in Gst p-2 at positions 89, 104, 106, and 109 do not affect the catalytic activity in a combined way.

In conclusion, primary sequence analysis, catalytic activity, and kinetic analysis clearly identify Gst p-1 as the major pi-class GST isolated from mouse liver. Gst p-2 displays very low conjugating activity of various electrophilic substrates with glutathione, very low selenium-independent peroxidase activity, and no isomerase activity toward Δ^5-androstene-3,17-dione. Three out of the six amino acid differences occurring between the two enzymes are responsible for the loss of activity of Gst p-2; these are Ser10, Pro11, and Gly104. To determine the significance of these changes will require further structural studies. Whether Gst p-2 has a specific function in relation to Gst p-1 also remains to be determined.

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