The ligand-binding function of the porcine class Pi glutathione S-transferase.

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DECLARATION

I declare that this dissertation is entirely my own work and has not been presented for any degree at another University.

Paula C. G. Bico

ABSTRACT

Glutathione S-transferases are multifunctional intracellular proteins. They catalyse the conjugation of glutathione to endogenous or foreign electrophiles, and also bind non-substrate ligands.

Class Pi glutathione S-transferase (pGSTP1-1) was purified from porcine lung to a specific activity of 6.63μmol/min/mg. The homodimeric protein has a molecular weight of about 48.7kD and an isoelectric point of 8.6.

Anionic ligand-binding properties of this isoenzyme were investigated. Steady-state fluorescence methods were used to determine K_d values for 8-anilino-1-naphthalene sulphonic acid (K_d=17.1μM and 11.1μM using fluorescence enhancement techniques and quenching techniques respectively), bromosulphophthalein (K_d=1.1μM at pH 6.5 and 2.4μM at pH 7.5) and glutathione (K_d=120μM). The affinity of bromosulphophthalein for the enzyme, in the presence of 10mM glutathione was slightly enhanced (K_d=0.7μM at pH 6.5). The energy transfer between the protein's tryptophan residues and 8-anilino-1-naphthalene sulphonic acid was observed and found to be about 56% efficient. The impact of ligand binding on both protein structure and catalytic activity were assessed. Kinetic studies show that the active site of the enzyme is not the primary binding site for the non-substrate ligands, but that the binding of bromosulphophthalein and to a lesser extent 8-anilino-1-naphthalene sulphonic acid, does affect the active site of the enzyme, especially after saturating concentrations of the ligand. This may be the result of a small ligand-induced conformational change. Fluorescence studies also indicate that the primary site for anionic ligand binding is not in close proximity to either Trp28 or Trp38 in domain I. Competition studies indicated that the two anionic ligands bind the same site. Protein fluorescence, chemical modification and size-exclusion HPLC data indicate that ligand binding does not induce gross conformational changes in the protein.

This work is dedicated to my family;

António, Judite, Luísa, Mena, Tó and Sónia

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ABBREVIATIONS

A	absorbance
A_{280}	absorbance at 280nm
A_{260}	absorbance at 260nm
ANS	8-anilino-1-naphthalene sulphonic acid
BSP	bromosulphophthalein
[^{35}S]BSP	bromosulphophthalein labelled with isotope 35 of sulphur
CDNB	1-chloro-2,4-dinitrobenzene
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
GSH	reduced glutathione
GST	glutathione S-transferase
G-site	glutathione-binding site
GSO_3^-	glutathione sulphonate
HCl	hydrochloric acid
hGSTA1-1	human class Alpha GST with type 1 monomers
hGSTP1-1	human class Pi GST with type 1 monomers
hGSTT1-1	human class Theta GST with type 1 monomers
H-site	hydrophobic, electrophile binding site
K_d	dissociation constant
kD	kilodalton
K_i	inhibition constant
M	molar
mGSTM1-1	mouse class Mu GST with type 1 monomers
mGSTP1-1	mouse class Pi GST with type 1 monomers
Mr	relative molecular mass
NTB	2-nitro-5-thiobenzoate
NaCl	sodium chloride
NaOH	sodium hydroxide
pGSTP1-1	porcine class Pi GST with type 1 monomers
pI	isoelectric point
rGSTA1-1	rat class Alpha GST with type 1 monomers

rGSTA1-2	rat class Alpha GST with type 1 and 2 monomers
rGSTA2-2	rat class Alpha GST with type 2 monomers
rGSTM1-1	rat class Mu GST with type 1 monomers
rGSTM1-2	rat class Mu GST with type 1 and 2 monomers
rGSTM2-2	rat class Mu GST with type 2 monomers
rGSTP1-1	rat class Pi GST with type 1 monomers
rpm	rotations per minute
TEMED	N,N,N',N'-tetramethylethylenediamine
TNB	1,3,5-trinitrobenzene
vs	versus
v/v	volume per volume
W24	water molecule 24
W27	water molecule 27
x g	multiplied by gravity
σ-complex	1-(S-glutathionyl)2,4,6-trinitrocyclohexadienate

The IUPAC-IUBMB three letter codes for amino acids are used.
All abbreviations that are accepted without definition by the Biochemical Journal have been omitted from this table.

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CHAPTER 1

1.1 INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) were first discovered in 1960 by Booth and co-workers who partially purified and assessed the rat liver enzyme which catalysed the formation of glutathione derivatives from various compounds (Booth *et al.*, 1961). GSTs are a multigene family of isoenzymes found in most living aerobic organisms (Pemble and Taylor, 1992; Persson *et al.*, 1988) and are widely distributed among most mammalian tissues (Mannervik and Danielson, 1988; Mannervik, 1985).

1.1.2 Glutathione S-transferases as detoxification enzymes

These intracellular proteins form part of a specialized group of detoxification enzymes, the phase I/phase II enzymes, which have been well adapted to eliminate both foreign as well as endogenous toxic chemicals (Armstrong, 1987; Waxman, 1990; Hayes and Wolf, 1990; Coles and Ketterer, 1990; Sheehan and Casey, 1993).

Unlike most enzymes which catalyse metabolic reactions between specific substrates, detoxification enzymes have a broad substrate specificity. In addition, GST isoenzymes provide protection against a broad spectrum of toxic chemicals because of their individual and distinct structural, catalytic and non-catalytic properties.

Phase I of chemical detoxification is catalysed by enzymes which activate the substrate by hydrolysis, reduction or oxidation, exposing electrophilic or nucleophilic regions of the substrate. An example of such enzymes is the microsomal cytochrome P-450 system. Phase II enzymes catalyse the conjugation of xenobiotics to an endogenous substrate, like glutathione (GSH) for example, which may then be easily excreted from the cell. The products of phase I are often the substrates for phase II enzymes by which hydrophillic moieties such as glutathionyl-, glucoronyl-, or sulphuryl- groups are appended. However, not all xenobiotics pass through both phases, some only pass through phase I while others are substrates for phase II enzymes (Sheehan and Casey, 1993). Figure 1 illustrates how toxins are processed within a cell. Toxic chemicals may follow one of three pathways. If reactive, they may act directly upon cellular targets, such as nucleic acids, causing cellular damage, (Coles and Ketterer, 1990) or they may be substrates for phase I or for phase II detoxification enzymes. Subsequently, the highly reactive products of phase I, for example

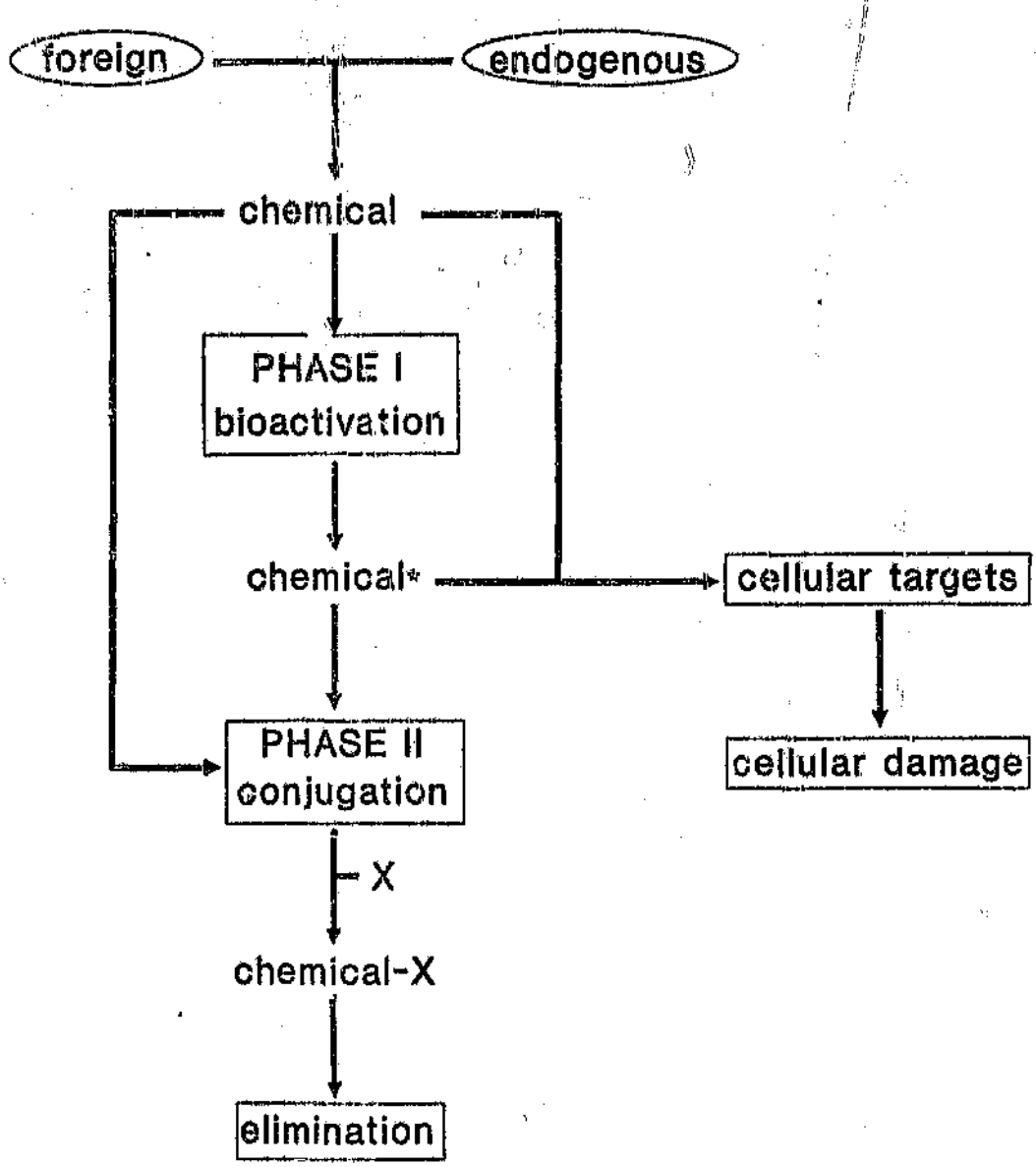


Figure 1. Detoxification pathway of cytotoxic chemicals within a cell.

*** represents an activated and potentially harmful compound; X represents endogenous substrates of phase II enzymes, eg. GSH.**

epoxides, which are often carcinogenic, may be the substrates of phase II enzymes or may also cause cellular damage.

GSTs are phase II detoxification enzymes and their primary function is the catalysis of the nucleophilic addition of glutathione to electrophiles, most of which are very apolar. The polar conjugated substrates are then excreted from the cell. This release from the cell also marks the first stage of GSH turnover. The released GSH-conjugate is transported by the blood plasma to the kidney. Here the degradation of GSH begins by the removal of the γ-glutamyl moiety. The γ-glutamyl moiety is transferred to an acceptor amino acid and relocated into the cell where it is involved in the resynthesis of GSH. The cysteinyl-glycine, however, is hydrolysed in a dipeptidase catalysed reaction. The remaining cysteine-conjugate is converted to a mercapturic acid, an excretion product, upon acetylation with acetylcoenzyme-A (Siegers and Younes, 1983; Mannervik, 1985) (see Figure 2).

1.2 STRUCTURE OF CLASS PI GLUTATHIONE S-TRANSFERASES

1.2.1 Domains and domain interactions

Class Pi glutathione S-transferase from porcine lung, pGSTP1-1, is a dimer of identical subunits (Dirr *et al.*, 1991). The tertiary structure of each subunit, determined by means of x-ray crystallography, is characterised by two different domains (Reinemer *et al.*, 1991; Dirr *et al.*, 1994; Dirr *et al.*, manuscript submitted). Domain 1 (residues 1-74), the N-terminal domain, is composed of both α helices as well as of ß strands (see Figure 3).
Its overall structure consists of a central four-stranded ß sheet, 3 α-helices, a 3_{10}-helix, 3 ß-turns and a cis-Pro bend. Strand ß2, which is located at the solvent exposed edge of the molecule, is parallel to strand ß1, whereas strands ß1, ß3 and ß4 run anti-parallel to each other. Strands ß1 and ß2 are connected to each other by a ß-turn and by helix αA which is situated at the rear of the ß-sheet, shielded from solvent. Strands ß2 and ß3 are connected by helix αB which runs almost perpendicularly to the ß-sheet. A hairpin bend connects strands ß3 and ß4, and helix αC extends from strand ß4 at the solvent-shielded side of the ß-sheet.

Domain 2 (residues 81-207) is covalently connected to the first domain by a short peptide

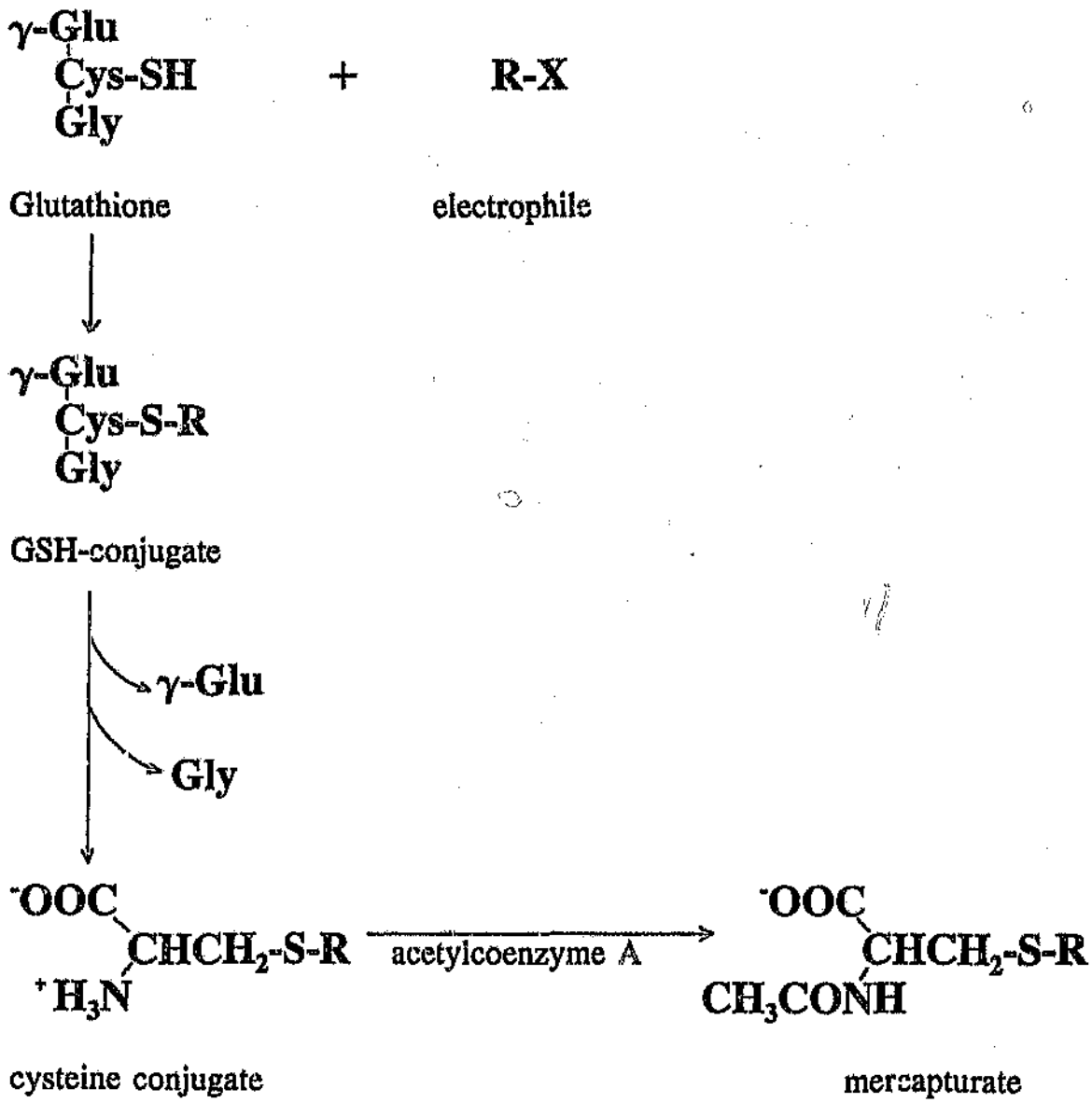


Figure 2. Mercapturic acid formation pathway. Once the glutathione-electrophile conjugate has been excreted from the cell, it loses its γ-glutamyl and glycine portion to become a cysteine conjugate. This conjugate is converted to a mercapturate upon acetylation.

Figure 3. Ribbon diagram showing a subunit of class Pi GST with glutathione sulphonate (thick line). View is perpendicular to the molecular two-fold axis. Domain 1 is shown in green and domain 2 is shown in red. Co-ordinates obtained from Reinemer *et al.*, 1991; Dirr *et al.*, manuscript submitted.

segment. It is composed of 5 α-helices, a 3_{10}-helix and 4 ß-turns (Figure 3). Helices αD, αE, αF are wound to form nearly one and a half turns of a right-handed superhelix. Helix αG which runs almost perpendicular to αF is connected to it by 2 ß-turns. These four helices αD, αE, αF and αG form a closely packed elongated structure. Helix αH however is slightly separated from this structure.

Each subunit has a total secondary structure content of about 54% α-helix and 8% ß-strands according to x-ray crystallography (Dirr *et al.*, manuscript submitted). Circular dichroism studies have, however, shown different values for α-helix and ß-sheet content. Nishihira *et al.* (1992a, 1993) observed 25.2% α-helix and 34% ß-sheet for a rat class Pi GST .

When domain 1 and domain 2 associate, about 18% of the total solvent-accessible surface area becomes buried. Interface contacts are mediated by polar and hydrophobic elements. In domain 1, the interacting regions are αA helix, the loop between strand ß1 and helix αA and αC helix. The interacting regions of the second domain involve helices αD, αF and αH and the C-terminal region of the domain. There are approximately 13 solvent molecules hydrogen-bonded along the domain interface.

1.2.2 Subunit interactions

The association of the two subunits causes a decrease of about 14% in solvent-accessible surface area. There are 27 contacts at the subunit interface, 14 of them apolar and 13 polar. The interactions are mostly between the ß-turn of residues 45-48, strand ß4 and helix αC of domain 1 in one of the subunits, and helices αD and αE of domain 2 in the other subunit. These interactions allow a V-shaped hydrophillic cleft to be created at the subunit interface of the molecule (Figure 4b). There are about 51 water molecules located at the subunit interface and in the hydrophillic cleft. Isothermal unfolding (Dirr and Reinemer, 1991; Erhardt and Dirr, unpublished results) and radiation studies (Boyer and Kempner, 1992) suggest that subunit interactions in the GSTs not only stabilize the association of subunits but are also a significant source of stabilization for the tertiary structures of the individual subunits.

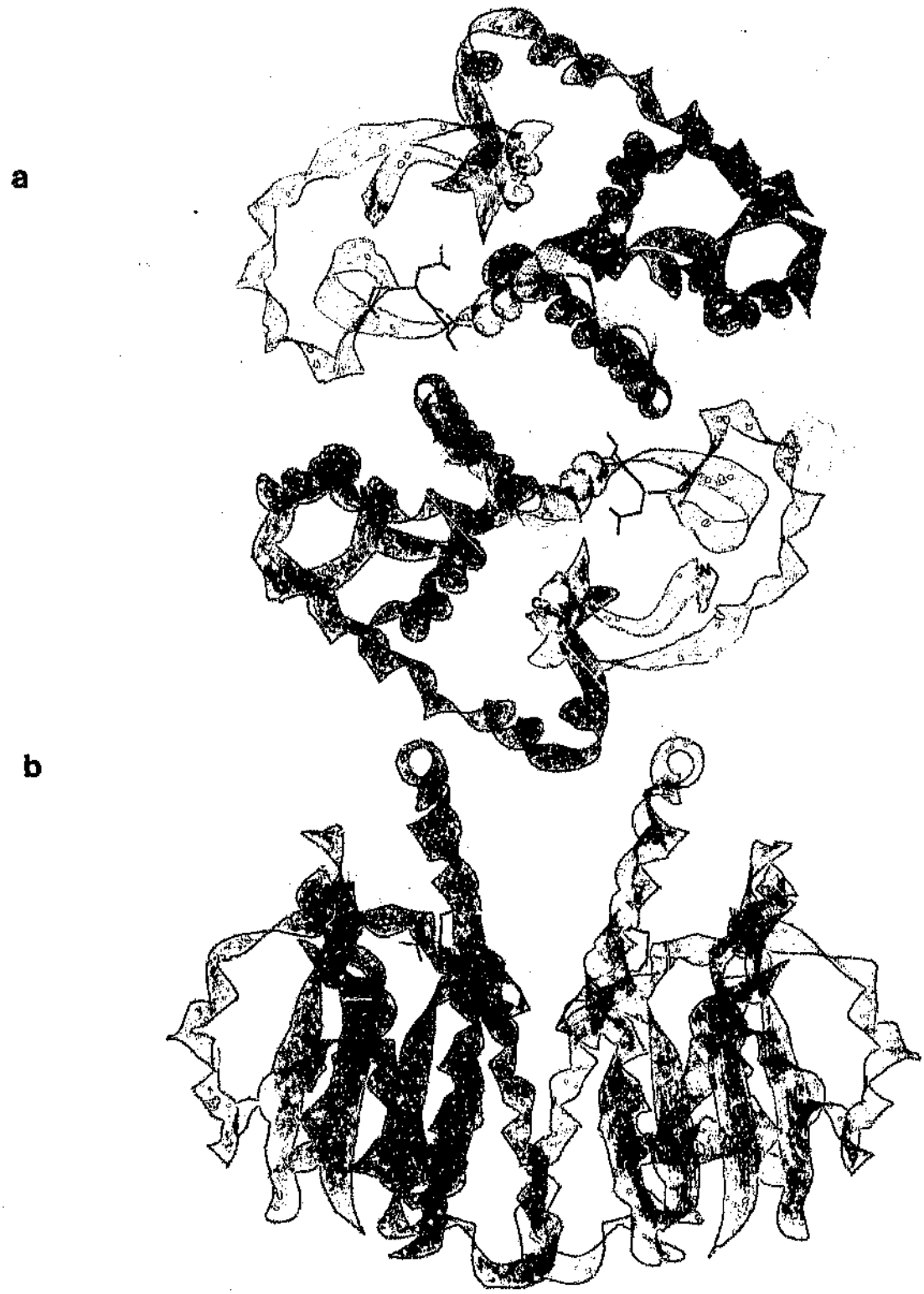


Figure 4. Ribbon drawing of the dimeric class Pi GST molecule with glutathione sulphonate (a) Along the local 2-fold axis (domain 1 shown in green; domain 2 shown in red) and (b) perpendicular to the local 2-fold axis (different subunits shown in red or blue). Co-ordinates obtained from Reinemer *et al.*, 1991; Dirr *et al.*, manuscript submitted.

1.2.3 Active site

The dimeric enzyme contains two kinetically independent active sites, one in domain 1 of each subunit. Each site is composed of two regions, the G-site or glutathione-binding site, and the H-site or hydrophobic electrophile-binding site (Figure 5).

The G-site extends from residues 8-10 which connects strand β1 to helix αA, to Ser63 at the N-terminal of helix αC. One end of the site is open to the solvent while the N-terminal end of αC helix is situated near the cleft along the subunit interface. The side chains lining the G-site include those of Tyr7, Gly12, Arg13, Trp38, Lys42, Gln49, Pro51, Gln62, Ser63, Glu95 and Asp96. Glutathione sulphonate (GSO_3^-), an analogue of glutathione, has been chosen by a number of researchers (Reinemer *et al.*, 1991; Dirr *et al.*, manuscript submitted) for determining the G-site interactions of pGST1-1 to the tripeptide, because of its high affinity for the enzyme's active site (K_i=4μM) (Dirr *et al.*, 1991). GSO_3^- differs from glutathione by the replacement of the thiol moiety with a negatively charged sulphonate group (Figure 6).

The γ-glutamyl arm of GSO_3^- is orientated in the direction of the cleft along the subunit interface, its sulphonate moiety points toward domain 2 and the glycine portion of the peptide points away from domain 2 in the direction of the solvent (Figures 4 and 7).

The γ-glutamyl arm of the peptide locates itself in a polar pocket formed by the side chains of Arg13, Gln49, Gln62, Ser63 and Asp96 (a residue from domain 2 in the neighbouring subunit). Gln62 however has an unfavourable main-chain conformation. The necessity of Asp96 from the adjacent subunit in the active site possibly explains the loss of activity in isoenzyme monomers (Dirr and Reinemer, 1991). The sulphonate moiety interacts with the side chain of Tyr7, a fully conserved residue in all classes of cytosolic mammalian GST as well as in *Schistosoma japonicum* and in the maize GSTs. Gly12 which occurs in the vicinity of Tyr7 possesses a backbone conformation that would be unfavourable for non-glycine residues and which disallows the hydroxyl group of Tyr7 from hydrogen bonding to the main-chain carbonyl oxygen of Gly12. Instead, the hydroxyl group of Tyr7 forms a hydrogen bond with the sulphonate moiety of GSO_3^-. Any other residue in the same position would possibly cause a steric hinderance in the binding of glutathione.

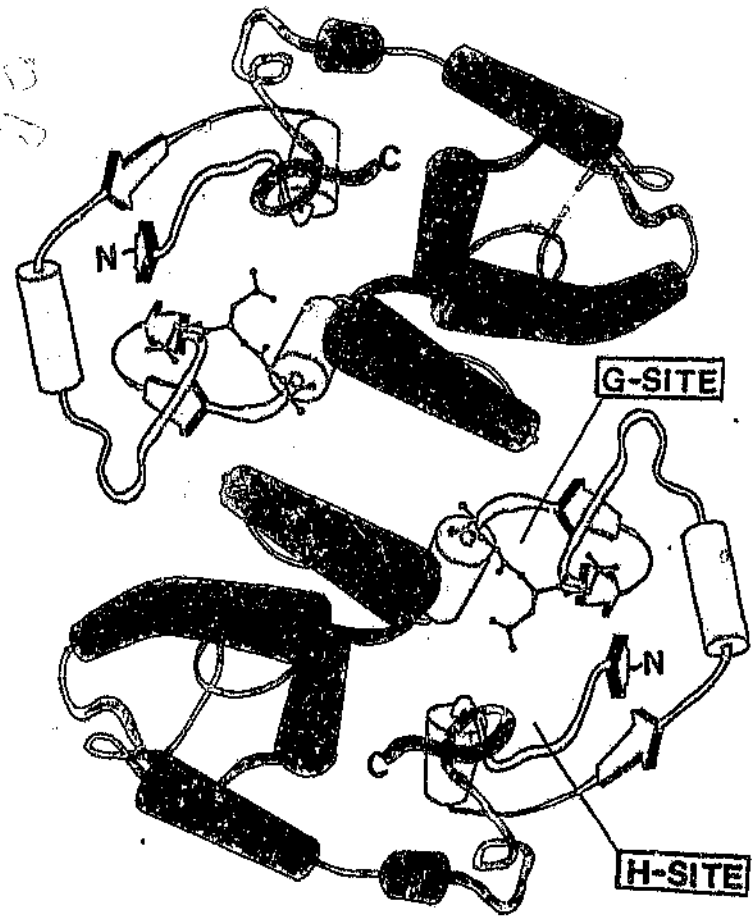
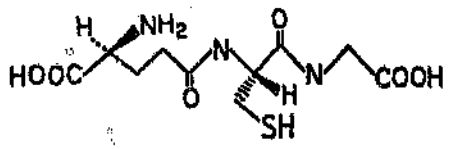


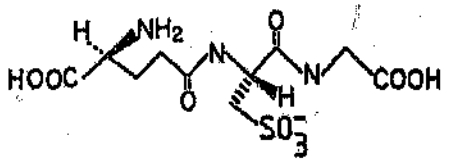
Figure 5. Schematic diagram of the dimeric GSTP1-1 isoenzyme showing the glutathione binding site (G-site), occupied by glutathione sulphonate (ball and stick model), and the electrophile binding site (H-site), which is unoccupied. Barrels represent α-helices and arrows ß-strands. Domain 1 is shown in green and domain 2 is shown in red.

a



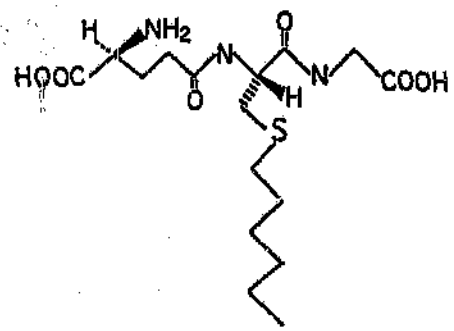
Glutathione (GSH)
[γ-Glu-Cys-Gly]

b



Glutathione sulphonate
(GSO_3^-)

c



S-hexylglutathione
(S-hexGSH)

Figure 6. A comparison between glutathione (a) and its analogues, glutathione sulphonate (b) and S-hexylglutathione (c).

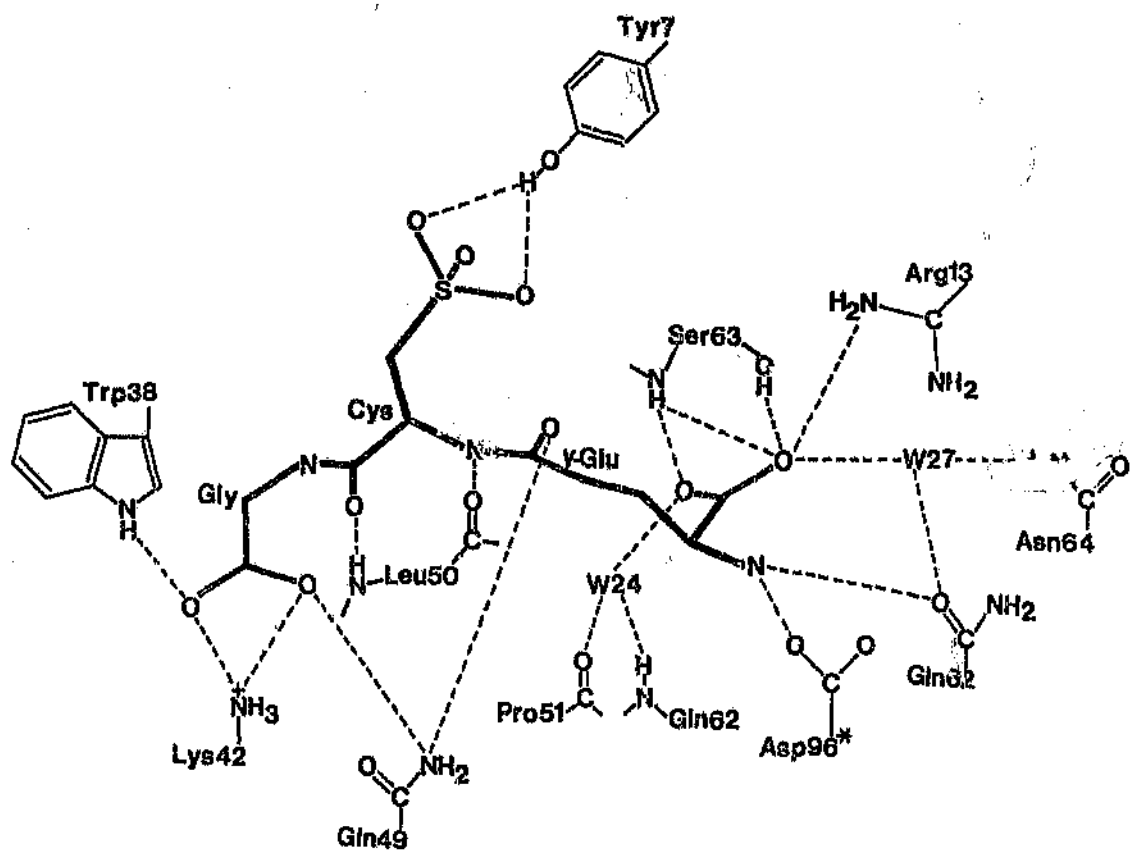


Figure 7. Active-site interactions of class Pi GST with glutathione sulphonate (thick lines). All residues represented are from domain 1 of one subunit except for Asp 96 which is of domain 2 of the adjacent subunit. W24 and W27 represent water molecules 24 and 27 respectively.

The α-carboxyl group of the glycine portion of GSO_3^- interacts with the side chains of Trp38 and Lys42. The disruption of these interactions in Pi class GST by site-directed mutagenesis (Kong *et al.*, 1992a; Widersten *et al.*, 1992) and chemical modification of the enzyme (Xia *et al.*, 1993) have shown a drastic decrease in both the enzymes affinity for GSH and in its catalytic activity. However, structure-activity studies with glycyl-modified GSH analogues, suggest that the glycine moiety of the tripeptide is not absolutely essential for the binding of the peptide (Adang *et al.*, 1990). The principle binding determinant of glutathione, is the γ-glutamyl arm.

Site-directed mutagenesis and chemical modification studies have shown that Arg13, Gln 49, Gln62 and Asp96 are all essential for the binding of GSH to GST from the Pi class, (Manoharan *et al.*, 1992 a,b; Kong *et al.*, 1992a; Xia *et al.*, 1993; Stenberg *et al.*, 1991a; Widersten *et al.*, 1992; Kong *et al.*, 1993; Wang *et al.*, 1992a) and that replacement or chemical alteration of these residues also result in a significant loss of enzymatic activity.

The H-site of pGSTP1-1 is located in an equivalent position to that of the human class Pi enzyme H-site (Reinemer *et al.*, 1992) and occurs between the G-site and the loop between strand β1 and αA helix. The side chains lining the site include Tyr7, Phe8, Pro9, Val10, Met35, Trp38 of domain 1 and Tyr106, Gly203 of domain 2 (Reinemer *et al.*, 1992; Dirr *et al.*, manuscript submitted).

The location of the H-site was first determined using glutathione analogues, S-hexylglutathione and S-benzylglutathione in the crystal structures of hGSTP1-1 (Reinemer *et al.*, 1992) and hGSTA1-1 (Sinning *et al.*, 1993) respectively in which the H-sites were occupied by the hydrophobic hexyl or benzyl moieties of the analogues. Overlayed models of the bound inhibitors in each subunit showed superimposable peptide regions but different orientations of the hexyl or benzyl groups. This was a clear indication of the greater conformational freedom allowed in the H-site.

1.2.4 Structural comparison with other GST classes

Class Pi, class Alpha and class Mu are remarkably similar in their tertiary and quaternary structures, even though the aligned amino acid sequences show very little identity between the gene classes (Reinemer *et al.*, 1991, 1992; Ji *et al.*, 1992; Dirr *et al.*, 1994; Dirr *et al.*, manuscript submitted; Sinning *et al.*, 1993). Sequence identities between classes are as follows: Alpha-Pi 32%; Pi-Mu 30%; Alpha-Mu 20%. There are however structural features

that are specifically characteristic of a gene class. The most prominent difference between the Mu class and the other gene class tertiary structures is the presence of an extended and mobile loop (the mu loop) between strand ß2 and helix αA which interacts with the C-terminus of the enzyme forming part of the H-site (Sinning *et al.*, 1993). The mu loop is obviously a consequence of an insertion sequence and is necessary if the rest of the protein is to fold correctly.

In class Alpha, helix α8 (or αH in the Pi class) is followed by an additional α helix, helix α9 which is created by the folding of the C-terminus region of the polypeptide chain and forms a part of the Alpha class domain 1. Furthermore, helix α9 is an integral part of the H-site and provides an additional hydrophobic wall, making the H-site less open to solvent. The existence of different H-site topologies in the various gene classes, as a result of sequence differences, explains the relatively distinct specificity for substrates shown by the various classes. However, because of the hydrophobicity of the site and the non-specific binding of substrates a number of structurally different hydrophobic substrates can be accommodated.

1.2.5 Catalytic function of GST

The kinetic mechanism for the nucleophilic addition of electrophiles to glutathione catalysed by GST is sequential, the enzyme binding either GSH or the electrophile and the conjugation of the two substrates occurring within the ternary complex (Armstrong, 1991) (Figure 8).

The enzymes have a reasonable binding affinity for GSH (Pi class: Kd≈120μM (Bico and Dirr, unpublished results); Mu class: Kd≈20μM (Graminski *et al.*, 1989; Ji *et al.*, 1992). At physiological conditions, GSH is present in 1-10mM concentrations and therefore the catalytic mechanism is likely to be ordered with GSH binding first (Armstrong, 1991). Upon GSH binding, the enzyme deprotonates the tripeptides thiol group resulting in the formation of a highly reactive thiolate anion which readily attacks electrophilic substrates to form water-soluble glutathione conjugates (Figure 9). The thiolate anion is up to 10^9 more reactive than its conjugate acid (Armstrong, 1991; Roberts *et al.*, 1986).

The deprotonation of GSH by the enzyme, previously led many researchers to speculate the occurrence of base catalysis in the enzyme, possibly involving the basic residue histidine in

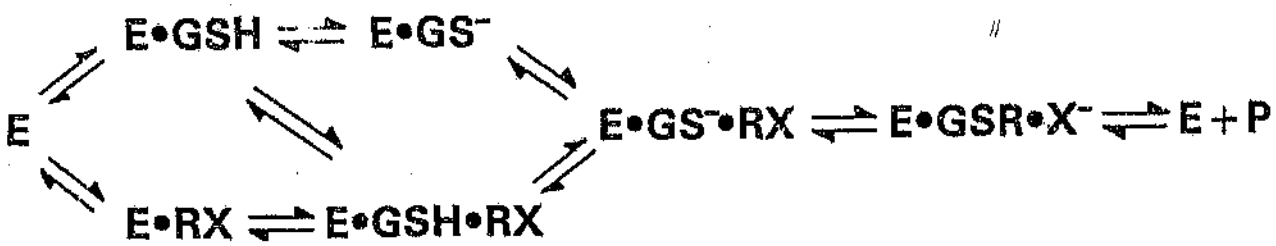


Figure 8. Schematic diagram of sequential kinetic mechanism for glutathione S-transferase. E represents the enzyme GST, GS^- represents the thiolate anion, RX represents the electrophilic substrate where R is the electrophilic moiety and X is the leaving group, GSR represents the GSH-conjugate in the enzyme and P is the product.

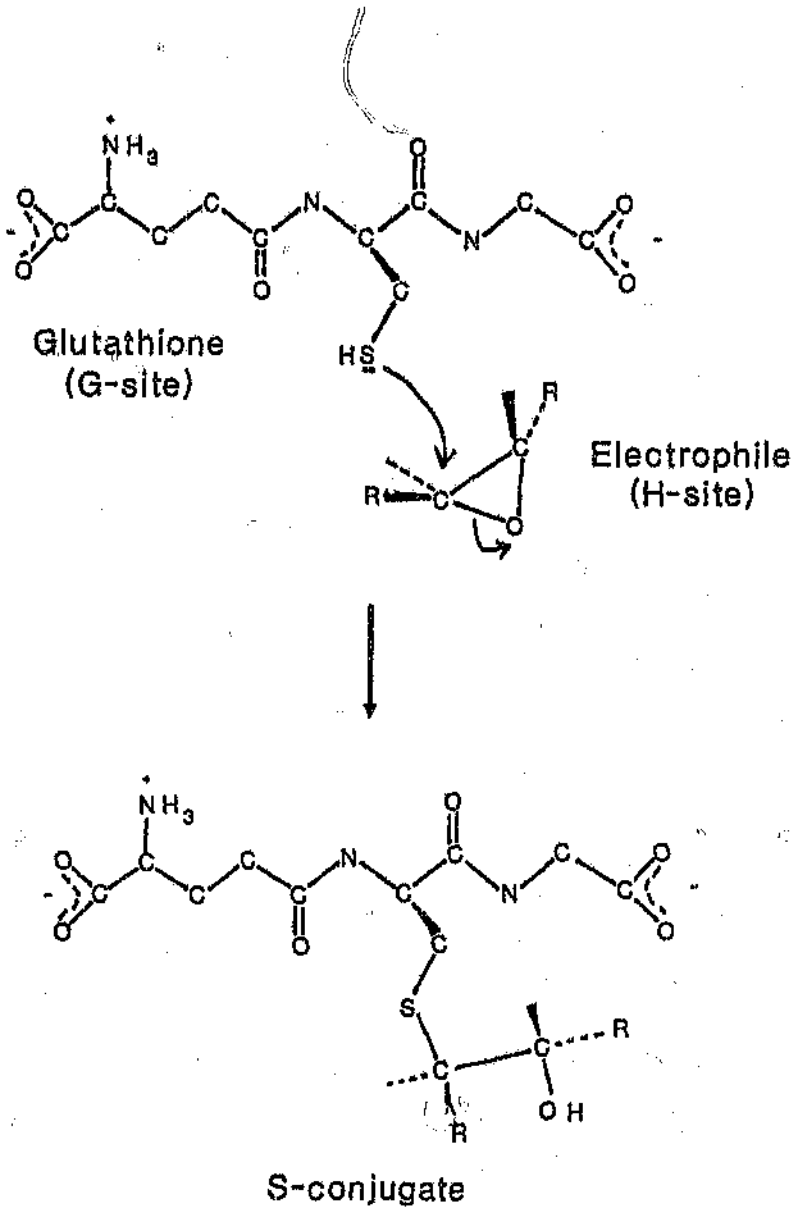


Figure 9. Glutathione S-transferase catalysed reaction. The electrophile represented is an epoxide. The GSH conjugate is formed by the nucleophilic addition of the thiolate anion to the electrophile.

the protein's G-site. X-ray crystallography has shown, however, that histidine is not part of, nor is it near the active site of GSTs. It is not conserved throughout the GST family and site-directed mutagenesis has revealed a non-essential role for histidine in glutathione binding as well as in the catalytic activity of the enzyme (Zhang *et al.*, 1991; Wang *et al.*, 1991; Chang *et al.*, 1993; Widersten and Mannervik, 1992). An unexpected structural feature that was first observed at the active site of pGSTP1-1 is a conserved hydrogen bond interaction between the thiol group of glutathione and the hydroxyl group of Tyr7 at the G-site. Tyrosine 7 is a conserved residue in all classes of GST (equivalent residues in Mu:Tyr6; Alpha:Tyr8). It is located in a hydrophobic environment at or near the C-terminus of strand ß1 in domain 1.

Replacement of Tyr7 with phenylalanine by site-directed mutagenesis reduces enzyme activity to less than 1% of the wild type, although little change is observed in affinity of the enzyme for glutathione (Wang *et al.*, 1992a; Kolm *et al.*, 1992; Kong *et al.*, 1992b; Manoharan *et al.*, 1992b; Stenberg *et al.*, 1991a,b; Liu *et al.*, 1992; Meyer *et al.*, 1993; Penington and Rule, 1992).

It has previously been shown that the pKa values for the thiol group of GSH bound to the enzyme (Pi: 6.3; Kong *et al.*, 1992c), (Mu: 5.7-6.9; Liu *et al.*, 1992), (Alpha: 6.7-7; Wang *et al.*, 1992a) are at least 2 pH units below the pKa for glutathione in aqueous solution (pKa ≈ 9; Graminski *et al.*, 1989; Liu *et al.*, 1992). In the mutant enzyme, where Tyr7 is replaced with phenylalanine, the pKa of the bound GSH in class Pi however, is no longer 6.3 as in the wild type, but increases to at least 8.7 which is close to the pKa of GSH in aqueous solution. Tyrosine 7 is, therefore, definitely essential in enhancing the nucleophilicity of the GSH thiol group (Kong *et al.*, 1992c; Liu *et al.*, 1992; Manoharan *et al.*, 1992b). It seems to act as a hydrogen bond donor, promoting thiolate formation by decreasing the pKa of GSH in the enzyme complex from pKa 9 to pKa 6.3 thereby also stabilizing the thiolate anion (Kolm *et al.*, 1992; Liu *et al.*, 1992) (Figure 10).

An alternative mechanism for the role of Tyr7 has however been proposed more recently for the class Pi enzyme (Meyer *et al.*, 1993; Karshikoff *et al.*, 1993). The chemical modification of the enzyme by diethylpyrocarbonate (Meyer *et al.*, 1993) suggests that a significant amount of tyrosinate ion occurs in the enzyme which may function as a base or proton donor. Furthermore, Karshikoff *et al.*, (1993), by analysing the electrostatic potential

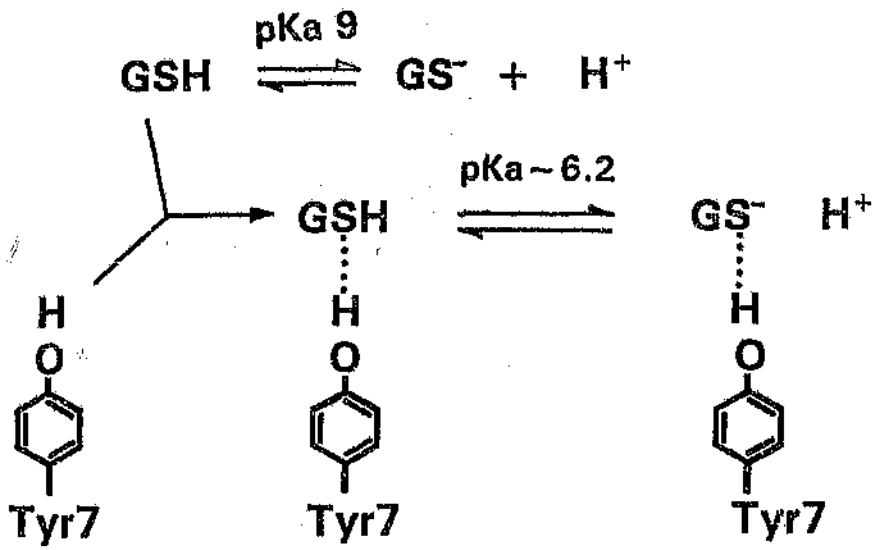


Figure 10. A proposed catalytic role for tyrosine7. Tyr7 hydrogen bonds to the thiol portion of GSH promoting the formation of a highly reactive thiolate anion in the GSH-enzyme complex by lowering the pKa of GSH from 9 in the aqueous solution to 6.2 in the complexed form.

in the active site of the enzyme, proposed that the hydroxyl group of Tyr7 is deprotonated by the influence of the charge at the active site, suggesting also that Tyr7 acts as a general base, promoting proton removal from GSH creating a reactive thiolate anion (Figure 11).

1.2.6 Cys45 and glutathione binding

Class Pi GST has four cysteine residues in each subunit at positions 14, 45, 99 and 167. Replacement of each of these residues with serine, by site-directed mutagenesis, have indicated a non-essential role in the catalytic function of the enzyme (Kong *et al.*, 1991). All replaced cysteine residues except for Cys45 also showed no effect in the binding of glutathione to the protein (Kong *et al.*, 1991). Cys45 has a highly reactive thiol located about 1.2nm from the glutathione's thiol group. In the uncomplexed enzyme, the Cys45 thiol group is exposed to the solvent, however it seems to become inaccessible upon glutathione binding and hence unreactive in the complexed enzyme. Chemical modification of the reactive thiol group of Cys45, by 5,5'-dithiobis(2-nitrobenzoate), spin-labelled maleimide,7-fluoro-4-sulfamoyl-2,1,3-benzodiazole,N-etylmaleimide,chloro-dinitrobenzene or by mild oxidation resulted in an almost complete loss of glutathione binding and subsequent loss of enzyme activity (Dirr *et al.*, 1991; Desideri *et al.*, 1991; Nishihira *et al.*, 1992b; Tamai *et al.*, 1990; Caccurri *et al.*, 1992a,b). X-ray crystallography and NMR studies have shown that Cys45 is not in the active site (Reinemer *et al.*, 1991; Nishihira *et al.*, 1992b). The data from the chemical modification, mutagenesis and solvent-accessibility studies have indicated a local structural change occuring at or near the G-site during GSH binding (Dirr *et al.*, 1994).

The G-site of GSH-free enzyme does not seem to be in the ideal configuration for catalysis and the binding of GSH seems to occur via an induced-fit mechanism in order to obtain the catalytically functional conformation (Adang *et al.*, 1989). The conformational change occurring upon GSH binding appears to conceal the reactive Cys45 thiol group in the protein making it inaccessible for modification (Lo Bello *et al.*, 1993). Its chemical modification in the free enzyme prevents the necessary structural change form occurring and consequently the binding of GSH is prevented.

Cys45 is, however, not found in class Alpha and Mu and thus a similar structural change upon GSH binding has not yet been observed (Chen *et al.*, 1992; Tamai *et al.*, 1990; Wang *et al.*, 1992b; Widersten *et al.*, 1991).

Figure 11. Tyrosinate ion of GST as a general base in the formation of the thiolate anion. EO⁻ represents the tyrosinate ion, HSG represents the substrate, glutathione and ⁻SG represents the thiolate anion.

1.2.7 Nomenclature

The nomenclature for the isoenzymes belonging to the different gene classes has undergone many changes. This has been a problem in the past since various authors referred to the same enzyme by using different terminologies. A new nomenclature system for the human glutathione S-transferases has been proposed by Mannervik (1992), and seems to be the most suitable to this date (see Table 1). This system can also be extended to include GSTs from other species.

In the new system, superscripts, subscripts and Greek letters are not acceptable, instead, Arabic numerals are used to show the subunit composition, lower case Roman lettering is used to designate the species from which the enzyme comes and upper case lettering (A, M, P, T) indicates the gene class of the enzyme. Thus the acronym pGSTP1-1, is the new nomenclature for porcine GST from class Pi consisting of two identical type-1 subunits. The monomer would be represented as pGSTP1.

1.2.8 Export of glutathione-conjugates

The export of GSH-conjugates from cells is not only essential for mercapturic acid formation, but is also needed for the maintenance of GST efficiency. Recently, an ATP-dependent glutathione S-conjugate export pump has been described (Ishikawa, 1993, 1990; LaBelle *et al.*, 1986a,b; Kon[illegible] *et al.*, 1980). It appears to be a protein molecule of about 37kD situated in the canalicular plasma membranes (Figure 12). The negative charge of GSH-conjugates appears to be important for the functioning of this carrier protein.

The GSH-conjugate trans-membrane carrier has also been found to be distinct from the multiple drug resistance gene product (P-glycoprotein) which is also an ATP-dependent export system found in plasma membranes (Ishikawa, 1993, 1990).

1.3 LIGAND-BINDING FUNCTION OF CLASS PI GST

1.3.1 Ligand-binding properties

In addition to their enzymatic function, GSTs also act as ligand binding proteins. They are able to bind substrate as well as lipophilic non-substrate ligands such as haem, bilirubin, hormones, bile acids, fatty acids, leukotrienes, drugs, dyes and other xenobiotics including

TABLE 1 Nomenclature examples of cytosolic glutathione S-transferase

Previous nomenclature	New nomenclature
Class Pi	
Rat	
Y_pY_p, Y_fY_f, P, 7-7	rGSTP1-1
Class Alpha	
Human	
ϵ, B_1B_1, GST2-type 1, H_a(subunit 1), $\alpha_x\alpha_x$,I	hGSTA1-1
Class Mu	
Mouse	
GT-8.7, C1-1, F_3, D1-1, MIII, N1-1	mGSTM1-1
Class Theta	
Human	
GST Θ	hGSTT1-1

mutagens and carcinogens (Litwack et al., 1971; Listowsky, 1993b). The binding of the non-substrate ligands (endogenous and exogenous compounds) to GST, prevents the build up of apolar molecules at lipophilic sites such as membranes, and directs the transport of these compounds either for metabolism or to their target sites. GSTs, for example, rGSTA1-1 and rGSTA1-2, have a greater binding affinity for organic anions such as bilirubin than does the extracellular ligand-binding protein, albumin (Listowsky et al., 1978). It is therefore possible that for this reason and because of the high GST concentrations within a cell (about 10^{-6}M), GST could, to a certain extent, regulate the net flux of certain organic anions from the plasma into the cells (Listowsky et al., 1978). Glutathione S-transferases, have however been previously shown to have access only to substrates in the aqueous phase and not the ability to bind the membrane bound substrates in liposomes (Boyer et al., 1983). Substrates such as bromosulphophthalein (BSP) and 1-chloro-2,4-dinitrobenzene (CDNB) nevertheless, have rapid rates of release into the aqueous phase in which they can bind the soluble GSTs (Boyer et al., 1983; Tipping et al., 1979 a,b). However, White and Plisher (1983) did observe a calcium-dependent association of GST with the human erythrocyte membrane. The calcium dependency of GST was not tested by Boyer et al. (1983) in his investigation of liposome-GST interactions.

Listowsky and co-workers have proposed a model accommodating the possible functions of GST in the intracellular binding and transport of non-substrates (Listowsky et al., 1988, Listowsky, 1993a,b).

On entering a cell, a ligand may assume one of two pathways. It may bind to its specific receptor with high affinity and be transported to its target site, from which induction of the phase I and/or phase II detoxification system, which include, GSTs could occur. Alternatively, the ligands may bind to GST, when receptor levels are limited or absent. If neither of these pathways are assumed, cytotoxicity within the cell may ensue.

GSTs may transport the bound ligand along several pathways; they may, for example, transfer the hormones to their specific receptors, when and if these are available, or to phase I enzymes for metabolic conversion. They may also catalyse the conjugation of the ligand to glutathione.

Although the affinities of ligands for GST are low in comparison to the specific receptor (Kd of 10^{-6}-10^{-7}M compared to 10^{-9}-10^{-11}M) (Listowsky et al., 1988), GSTs are often present in

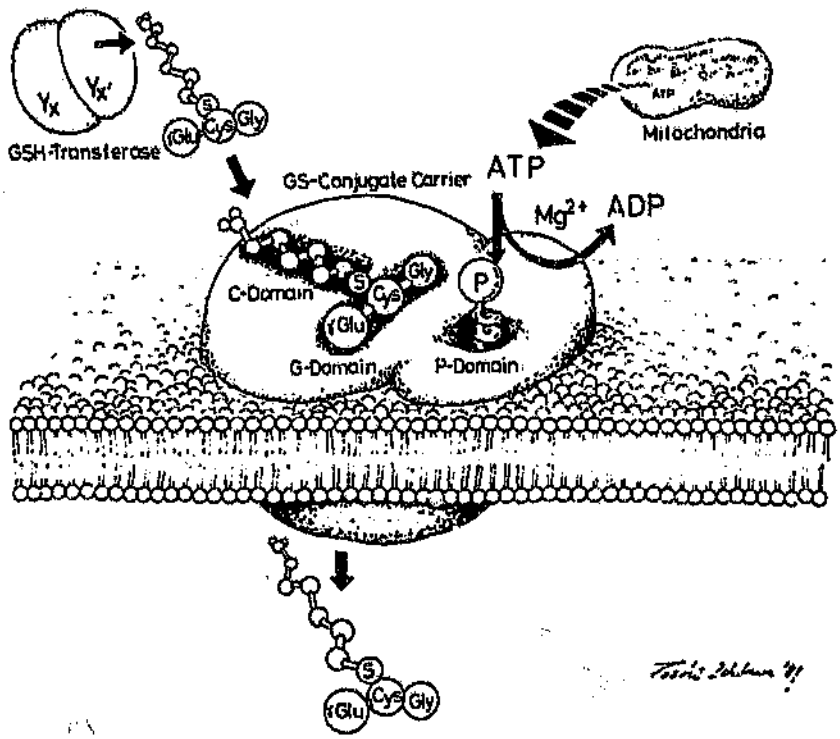


Figure 12. Proposed scheme for ATP-dependent GSH-conjugate efflux system. C-domain: aliphatic carbon chain domain; G-domain: recognizes GSH portion of GSH-conjugates; P-domain undergoes phosphorylation (Ishikawa, 1990).

abundance, up to 10% of the total cellular protein, whereas receptor molecules are found in the nanomolar concentration range (Listowsky, 1993a). As a result, it is possible for GSTs to play a significant physiological role within the cell. The extent of a cell's response to thyroid or steroid hormones, for example, may depend on GST levels and high GST concentrations could render cells insensitive to hormones (Listowsky et al., 1988; Listowsky, 1993 a,b).

1.3.2 GST and drug resistance

Over-expression of GSTs would also increase the resistance of a cell to cytotoxicity, not only because of its catalytic function but also as a result of non-substrate binding (Morrow and Cowan, 1990).

The expression of GSTs in a cell may be constitutive, in other words, various cells are intrinsically resistant to cytotoxic drugs and their GST content is sufficient to detoxify the cell, or it may be induced (acquired), in other words, cells which are initially sensitive to drugs but become resistant after prolonged exposure to the cytotoxins (Hayes and Wolf, 1988, 1990). This is seen often in human tumours which may initially be highly sensitive to chemotherapy but after a short period of treatment become highly resistant due to increased GST expression. The elevated levels of GST found in these tumours, serve as tumour markers. Rat liver tumours for example have an over-expression of class Pi GSTs (Kitahara et al., 1983; Morrow and Cowan, 1990). This class of GST is not usually expressed in hepatocytes and thus serves as a tumour marker. Acquired drug resistance represents one of the major reasons why many tumours cannot be treated with success (Hayes and Wolf, 1988). The resistance of a cell is often not only a result of the increased expression of phase II detoxification enzymes such as GSTs, but rather the combined effect of other factors such as the reduced expression of phase I enzymes, causing a reduction in the formation of activated and potentially carcinogenic compounds, and the increased expression of drug efflux systems such as the P170-glycoprotein pump (Hayes and Wolf, 1990; Morrow and Cowan, 1990; Waxman, 1990; Thorgeirsson et al., 1987; Batist et al., 1986). If the catalytic and drug-binding properties were inhibited in some way, possibly by substrate or non-substrate competitive inhibitors, chemotherapeutic drugs may find an opportunity to function in the cell. Unfortunately, the available three dimensional structure for the GSTs do not enable us to identify those sites on the protein to which these non-

substrate ligands bind, because stable non-substrate ligand-GST crystal complexes have not yet been developed.

Extensive research has been done on the ligand-binding properties of GST. The affinities of various compounds for the different isoenzymes, their effect on the enzymes catalytic efficiencies and the location of the primary binding sites for the non-substrates are but a few areas under study.

1.7.3 Bromosulphophthalein, bilirubin, 8-anilino-1-naphthalene sulphonic acid and haem as ligands for GSTs

Bromosulphophthalein (BSP), is one of several anionic dyes which are used to test liver function (Figure 13). Upon administration, it is eliminated from the blood and excreted in the bile. Failure to enter the hepatocytes, is indicative of liver dysfunction (Schwenk et al., 1976). The binding of BSP to GST isoenzymes has been extensively studied (Table 2). In rat class Alpha, the homodimer, rGSTA1-1, appears to have two high affinity binding sites, whereas the heterodimer, rGSTA1-2, appears to have one high affinity binding site and one low affinity binding site (Bhargava et al., 1980 a,b). This seems to suggest that one monomer has a greater affinity for the ligand than does the other. Photoaffinity coupling of [^{35}S]BSP to rGSTA1-1, has revealed a region in domain 1, to be the primary binding site for BSP (Bhargava and Dasgupta, 1988). The general region of BSP binding, extends from amino acid residues 16-49 and includes helices α1 and α2 and strand ß2 (Sinning et al., 1993). This area, is in close proximity to both the G-site and H-site of the molecule (Sinning et al., 1993). However, the exact location of the binding site within these structures has not been identified (Bhargava and Dasgupta, 1988). In the rat class Mu isoenzyme, rGSTM1-1, BSP appears to bind a high affinity site on each monomer at low concentrations of the dye, however at higher concentrations, a low affinity binding site appears to be present (Jakobson et al., 1979). Jakobson et al. (1979) found that BSP was able to displace a GSH analogue, S-(2-chloro-4-nitrophenyl)GSH in rGSTM1-1, indicating it bound at least partially to the same site (G-site), however she also observed that this was not altogether reciprocal. This suggested that BSP possibly also binds to other areas on the enzyme inaccessible to the GSH derivative. The different areas could account for the high or low affinity sites observed. BSP has previously also been shown to be a substrate for GST isoenzymes (Satoh et al., 1991; Kosower, 1976). The catalytic activity of the protein with BSP as a substrate is

however very low in comparison to the activity using other substrates (Satoh et al., 1991) (Figure 14).

Two high affinity sites are also observed in rGSTA1-1 for the endogenous compound bilirubin, these sites are absent or undetected in rGSTA2-2 (Boyer, 1986; Kamisaka et al., 1975, Bhargava et al., 1980 a,b). The heterodimer, rGSTA1-2, appears to possess only one high affinity site for bilirubin on monomer rGSTA1. Boyer (1986) labelled the heterodimer, rGSTA1-2, with a [^{14}C]Bilirubin-Woodward's reagent K label, and observed that both monomers were labelled equally under saturating conditions of the protein. He observed that at low concentrations of bilirubin the high affinity site on monomer rGSTA1 was preferentially occupied, but that monomer rGSTA2 also possessed a low affinity site. Interestingly, Boyer also observed that the catalytic site was not blocked by the covalently bound label, and that catalytic activity was retained. However, others have observed inhibition of catalytic activity for the Alpha isoenzymes by bilirubin (Bhargava *et al.*, 1980b). Covalent-labelling with bilirubin as well as competition studies, suggest that the organic anions BSP and bilirubin share a common binding site (Bhargava, 1988; Boyer, 1986).

8-Anilino-1-naphthalene-sulphonic acid (ANS), is another hydrophobic compound that typically binds hydrophobic regions of proteins non-covalently (Lakowicz, 1983). ANS is essentially nonfluorescent in hydrophillic media, but becomes highly fluorescent when dissolved in non-polar solvents or when bound to apolar regions on macromolecules (Lakowicz, 1983). For this reason, it is a useful probe for detection of apolar regions on GSTs. ANS has also been reported to bind to the same site as bilirubin and as fatty acids. Nishihira and coworkers (1992a,c) suggested that GST has a nonsubstrate ligand binding site in domain 2 which is a site for bilirubin, ANS and fatty acid binding.
The putative binding site for fatty acids was determined by the covalent labelling of a class Pi enzyme with 12-(9-anthroyloxy) stearic acid conjugated with Woodward's reagent K (Nishihira *et al.*, 1992c). The thiol group of Cys169 of GST was suggested to react covalently with the carboxyl group of the fatty acid. This residue is located in a peptide loop connecting helices α6 to α7 in domain 2 of the molecule. It is located in a hydrophobic region which is about 10% exposed to solvent. The lipid conjugate bound to GST inhibited the enzymatic activity in a non-competitive manner as did ANS. Inhibition by ANS was

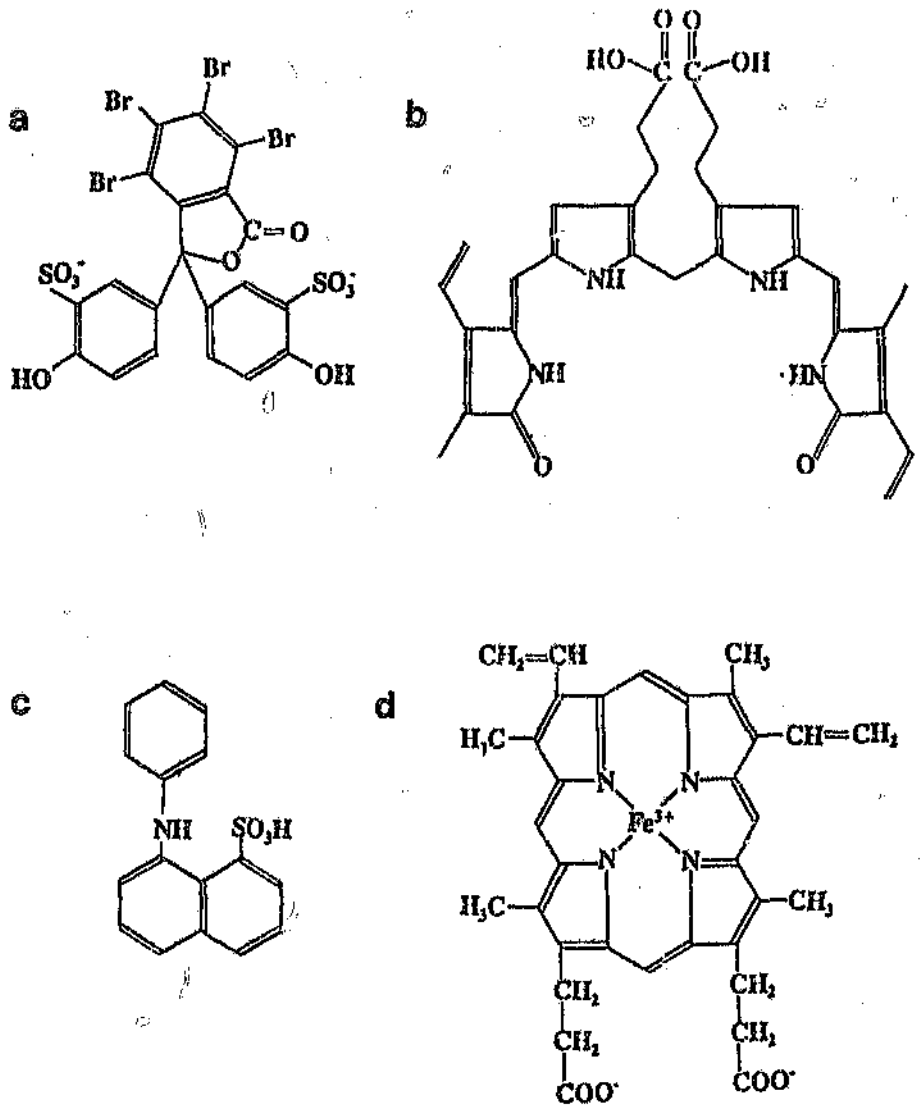


Figure 13. Structures of anionic ligands for GST: (a)Bromosulphophthalein, (b)Bilirubin, (c)8-Anilino-1-naphthalene sulphonate, (d)Haem.

TABLE 2 Dissociation Constants, K_d (μM), for GSTs with Anionic Ligands

Isoenzyme	BSP	Bilirubin	Haematin	ANS
rGSTA1-1	0.79[a],0.23[b]	0.42[a],0.053[b], 0.34[d]	0.16[a],0.02[b]	
rGSTA1-2	1.27[a],0.37[b] 0.1[f],1[e],18[c]	1.47[a],2[h],0.13[b], 0.02[c],0.13[c],0.68[d]	0.60[a],0.1[h], 0.068[b],0.03[c]	95[h]
rGSTA2-2	3.76[b]	0.94[a],100[b],	0.49[a],4[h]	700[h]
rGSTM1-1	0.63[a],0.44[g],28[a] 0.94[b]	0.22[a],15[h],0.69[b], 0.11[c]	0.12[a],2[h] 0.2[b],0.03[c]	330[h]
rGSTM1-2	1.21[a],0.83[b]	0.26[a].2[h],0.78[b] 0.41[b]	0.83[a],7[h]	400[h]
rGSTM2-2	0.67[a],1.07[b]	0.35[a],1.1[b]	0.78[a],0.25[b]	
rGSTP1-1	1.19[b]	0.36[c]	0.26[a]	15[j]
hGSTP1-1			0.02[i]	

[a]Satoh *et al.*, 1991, [b]Sugiyama, 1984; [c]Van der Jagt *et al.*,1982; [d]Boyer *et al.*,1983; [e]Kamisaka *et al.*,1975; [f]Tipping *et al.*,1976; [g]Jakobson *et al.*,1979; [h]Ketley *et al.*,1975, [i]Caccuri *et al.*, 1990; [j]Nishihira *et al.*,1992.

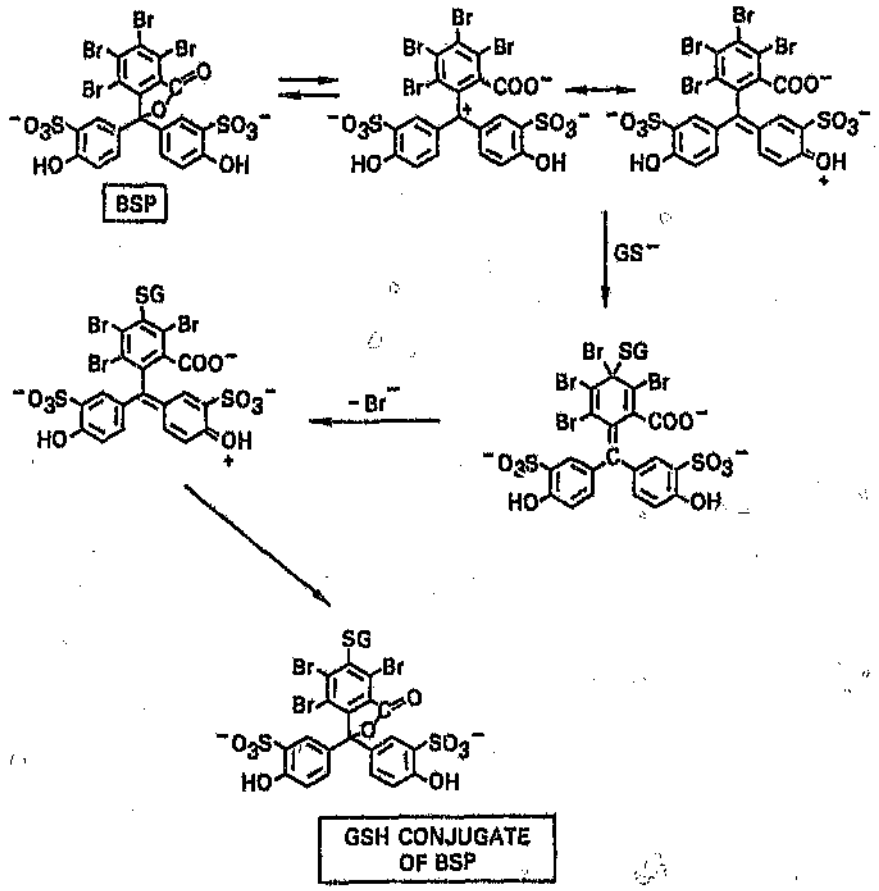


Figure 14. Catalytic interaction of GSH and BSP (Kosower, 1976).

proposed to be as a result of a massive structural change occurring in domain 1 due to the disruption of most of the protein's ß-sheet structure upon ligand binding. More recently, Nishihira *et al.* (1993) has proposed the hydrophillic/amphipathic V-shaped cavity formed at the subunit interface to be the hydrophobic ligand primary binding site. Cys169, however, does not occur in this cavity.

GSTs are the proteins involved in the transport of haem from the mitochondria to the acceptor apoprotein on the endoplasmic reticulum for the formation of cytochrome b_5 (Senjo *et al.*, 1985, Ketterer *et al.*, 1976). Further it has been found that haemin and bilirubin share a common binding site on class Pi GST (Caccuri *et al.*, 1990). This class of GSTs are homodimers. Caccuri and coworkers (1990) in fact noted the presence of two binding sites on the enzyme by fluorescence spectroscopy. One being of high affinity and the other of low affinity for haem. Interestingly, in the presence of GSH, the high affinity site increased its affinity for haem, whereas the low affinity site reduced its affinity for haem. It is possible that a conformational change upon GSH binding (Caccuri *et al.*, 1990), changes the binding affinities of the two sites for haem. GSH also interfered with the ability of haem to inhibit the catalytic activity of the enzyme.

1.4 OBJECTIVES

This work involved investigating some aspects of the ligand-binding function of the porcine class Pi enzyme. The non-substrate ligands used included bromosulphophthalein (BSP) and 8-anilino-1-naphthalene sulphonic acid (ANS). Certain aspects of the investigation also included glutathione and its sulphonate analogue, GSO_3^-. Generally, the binding of the ligands and their affinity for the enzyme were determined. This was accomplished using fluorescence quenching and fluorescence enhancement.

It was of importance to observe whether or not the primary binding site for the non-substrate ligands was the active site of the enzyme. Enzyme activity assays were performed in the presence of the ligands and the displacement of the Meisenheimer complex by the non-substrate ligands as well as by GSO_3^- were investigated. Competition studies between the non-substrate ligands were performed using fluorescence spectroscopy to observe whether or not these ligands bind the same primary site on the enzyme.

To determine whether the overall conformation of the protein crystal structure differed much from the active conformations in the aqueous solution, the formation and subsequent disappearance of the Meisenheimer complex were investigated. As previously mentioned, ANS has been thought to disrupt the overall conformation of the enzyme upon binding (Nishihira *et al.*, 1992a). Overall conformational changes in the protein, as a result of the binding of either of the non-substrate ligands were investigated using size-exclusion HPLC, chemical modification of the enzyme by Ellman's reagent and acrylamide quenching techniques.

Ligand-GST interactions are very important in the medical field where inhibition of this detoxification enzyme is essential for the optimization of chemotherapeutic drugs.

CHAPTER 2 EXPERIMENTAL PROCEDURES

2.1 MATERIALS

Bromosulphophthalein, 8-anilino-1-naphthalene sulphonic acid, and glutathione sulphonate were obtained from Sigma Chemical company.

Reduced glutathione was obtained from Boehringer Mannheim.

1-chloro-2,4-dinitrobenzene was obtained from Merck. 1,3,5-Trinitrobenzene was obtained from TCI.

S-hexylglutathione was prepared according to the method of Vince *et al.* (1971) and was subsequently washed in ethanol.

All other chemicals used were of analytical reagent grade.

2.2 PURIFICATION OF PORCINE LUNG CLASS PI GST

The purification was performed according to the procedures described by Dirr *et al.* (1991). Fresh pig lung was obtained from the slaughter house and frozen immediately at -20°C. When required, about 300g of pig lung was cut into small pieces and thawed in a beaker kept at 40-50°C. Two volumes of buffer A (20mM Tris, 100mM NaCl, 1mM EDTA, 5mM dithiothreitol, 0.02% sodium azide, pH 7.5) were added to the lung, and the mixture was homogenised at 4°C in a Waring blender for 2 minutes at maximum setting. The homogenate was then centrifuged at 17000xg (9000rpm in a Sorvall superspeed RC2-B, GS3 rotor) for 60 minutes at 4°C. The supernatant was filtered through glass wool and subsequently ultracentrifuged at 105000xg, (35000rpm in a Beckman Preparative Ultracentrifuge L8-55, 45Ti rotor) for 60 minutes at 4°C. The supernatant was carefully removed and applied onto a S-hexylglutathione Sepharose column equilibrated in buffer B without 1mM S-hexylGSH (20mM Tris, 200mM NaCl, 1mM EDTA, 5mM dithiothreitol, 0.02% sodium azide, pH 7.8) at a rate of about 10ml/hour. The column had been prepared according to the method of Mannervik and Guthenberg (1981). Once all the residual protein had been washed off the column, the protein still remaining on the column, including glutathione S-transferase was eluted off with buffer B containing 1mM S-hexyl-GSH. Protein fractions containing enzyme activity were collected, pooled and concentrated by ultrafiltration through a PM10 43mm membrane (Amicon). The concentrated protein was loaded onto a Sephadex G-25 column

equilibrated with buffer C (20mM Tris/HCl, 5mM dithiothreitol, pH 7.8). The enzyme containing fractions were pooled and subsequently loaded onto a DEAE cellulose (Whatman DE52), anion exchange column equilibrated in buffer C. Glutathione S-transferase, eluted with the flow-through, was adjusted to pH 6.0-6.5 with 1M Mes. The GST fractions were concentrated by ultrafiltration and passed through a Sephadex G-25 column equilibrated with buffer E (20mM Mes/NaOH, 0.1M NaCl, 1mM EDTA, 0.02% sodium azide, pH 6.5).

2.3 PROTEIN DETERMINATION

Protein determination assays were performed according to the semiquantitative method of Layne (1957). This method is represented by the following equation:

Protein concentration (mg/ml) = $1.55\ A_{200} - 0.76\ A_{260}$

2.4 ENZYME ACTIVITY ASSAYS

The enzyme activity assays were performed according to the method of Habig and Jakoby (1981). 0.02μM pGSTP1-1 was added to each assay mixture. The final substrate concentrations in the assay were 1mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol and 1mM reduced glutathione. The ethanol concentration in the assay did not exceed 3%(v/v), or inactivation of the protein would have occurred. The assay was performed in 0.1M potassium phosphate buffer, pH 6.5. The formation of S-(2,4-dinitrophenyl)glutathione was monitored spectrophotometrically at a wavelength of 340nm (Figure 15). The extinction coefficient of this conjugate is $9600M^{-1}cm^{-1}$ (Habig and Jakoby, 1981). All absorbance readings were made on a Hewlett Packard Vectra CS model 8452A Diode array spectrophotometer at room temperature (20-24°C).

2.4.1 Enzyme activity assays in the presence of BSP

Enzyme activity assays were also performed in the presence of increasing amounts of BSP, to a final concentration of 100μM. These were performed at pH 6.5 or pH 7.5 in a 0.1M potassium phosphate buffer. Enzyme activity assays in the presence of ANS were not performed because of the high absorbances of ANS at 340nm.

GSH + 1-chloro-2,4-dinitrobenzene --> S-(2,4-dinitrophenyl)glutathione + HCl

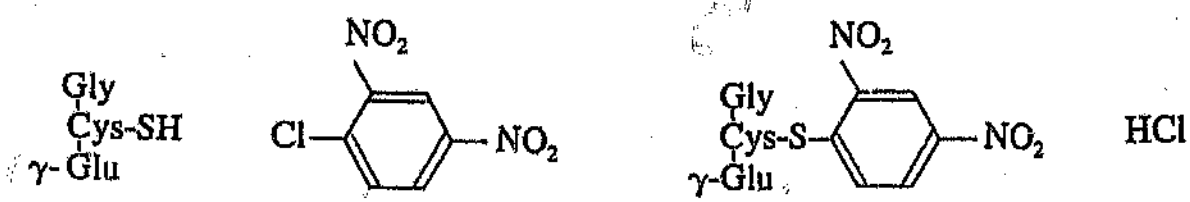


Figure 15. Conjugation reaction between 1-chloro-2,4-dinitrobenzene and glutathione forming S-(2,4-dinitrophenyl)glutathione in the enzyme activity assays.

2.5 HOMOGENEITY OF THE PROTEIN

The homogeneity of glutathione S-transferase was evaluated by SDS-polyacrylamide gel electrophoresis and isoelectric focusing. SDS-polyacrylamide separating gels of 15% acrylamide and 0.4% bisacrylamide and stacking gels of 3.75% acrylamide and 0.1% bisacrylamide were prepared. The gel electrophoresis was performed according to the method of Laemmli (1970) as described by Robyt and White (1990). The molecular weight markers used were egg albumin (Mr 45kD); glyceraldehyde-3-phosphate dehydrogenase (Mr 36kD); carbonic anhydrase (Mr 29kD); trypsinogen (Mr 24kD); trypsin inhibitor (Mr 20kD) and α-lactalbumin (Mr 14kD). All molecular weight markers were obtained from Sigma. The gels were run at 150-180V for about 2½ hours. The gels were stained for at least 1 hour (0.25% Coomassie brilliant blue R250, 45.4% methanol and 9.2 % glacial acetic acid) and destained for at least 2 hours (7% methanol and 7% glacial acetic acid).

Isoelectric focusing was performed according to the instructions of the BioRad Model 111 Mini-IEF gel kit. The monomer concentrate consisted of 24.25% acrylamide and 0.75% bisacrylamide. The monomer-ampholyte solution was prepared by mixing 2ml monomer concentrate with 0.5ml 3/10 ampholyte solution (BioRad) and 2ml 25% glycerol. This was brought to 10ml with water and degassed for 5 minutes. The 3/10 ampholyte has a pH range of 3.8 to 9.2. The catalyst solution was prepared by mixing 15μl 10% ammonium persulphate with 50μl 0.1% riboflavin and 3μl TEMED. It was subsequently added to the monomer-ampholyte mixture and swirled gently. The solution was pipetted between the glass plate and the casting tray and allowed to photopolymerise for 45 minutes. Once removed from the casting tray, the gel was irradiated with light for a further 15 minutes. A template for sample application was placed onto the gel and 2μl of undiluted sample was loaded into each groove. The samples were allowed to diffuse into the gel afterwhich the template was removed and the gel placed directly onto the graphite electrodes of the cell.

The pI markers used were trypsinogen from bovine pancreas (pI 9.3); L-lactate dehydrogenase from rabbit muscle (pI 8.6); myoglobin from horse heart (pI 7.2); carbonic anhydrase I from human erythrocytes (pI 6.6); carbonic anhydrase II from bovine erythrocytes (pI 5.9); ß-lactoglobulin A from bovine milk (pI 5.1) and trypsin inhibitor from soybean (pI 4.6). The pI markers were obtained from Sigma.

The gels were focused at 100V for 15 minutes after the samples had been applied to the gels.

The gels were subsequently run at 200V for 15 minutes and then at 450V for 1 hour. After the run, the gel was placed into a fixative solution (4% sulphosalicyclic acid, 12.5% trichloroacetic acid and 30% methanol) for 30 minutes. The gels were subsequently stained and destained by the same procedure as described for the SDS-polyacrylamide gels.

2.6 FLUORESCENCE SPECTROSCOPY

Fluorescence is a very useful technique in protein biochemistry because of its sensitivity and because of the favourable lifetime of the fluorophore (about 10^{-8}s) which allows a range of molecular processes to occur which can affect the spectral characteristics of the fluorophore (Lakowicz, 1983).

The absorption and emission of light may be illustrated by the energy level in Figure 16. The ground, first and second electronic states are depicted by S_0, S_1 and S_2 respectively. At each electronic level, the fluorophore can exist in different vibrational levels, shown as 0, 1, or 2. Following light absorption, a number of processes can occur. A fluorophore is usually excited to a higher vibrational level of energy levels S_1 or S_2. These rapidly relax to the lowest vibrational level in the energy level (internal conversion). Fluorescence emission therefore generally occurs only when the fluorophore is at a thermally equilibrated excited state and emits the energy gained as light. The absorption spectrum of the molecules reflects the vibrational levels of the electronically excited states and the emission spectrum reflects the vibrational levels of the ground electronic state which occurs at the electronic transition down to the lowest electronic level. The excitation wavelength absorbed by the molecule possesses greater energy than does the emitted light, the latter thus having a longer wavelength. Molecules that possess significant fluorescence properties, usually have delocalised electrons formally present in conjugated double bonds.

To detect interactions between proteins and ligands, it is, therefore, possible to measure some fluorescence parameter that changes upon the binding of the ligand. Either the fluorescence of the intrinsic tryptophan fluorophores or that of an extrinsic fluorophore can be monitored. 8-Anilino-1-naphthalene sulphonic acid (ANS) is an extrinsic fluorophore which fluoresces strongly when in apolar surroundings. Its affinity for the hydrophobic site on the protein can be measured by monitoring the enhanced fluorescence of ANS upon binding. However, the

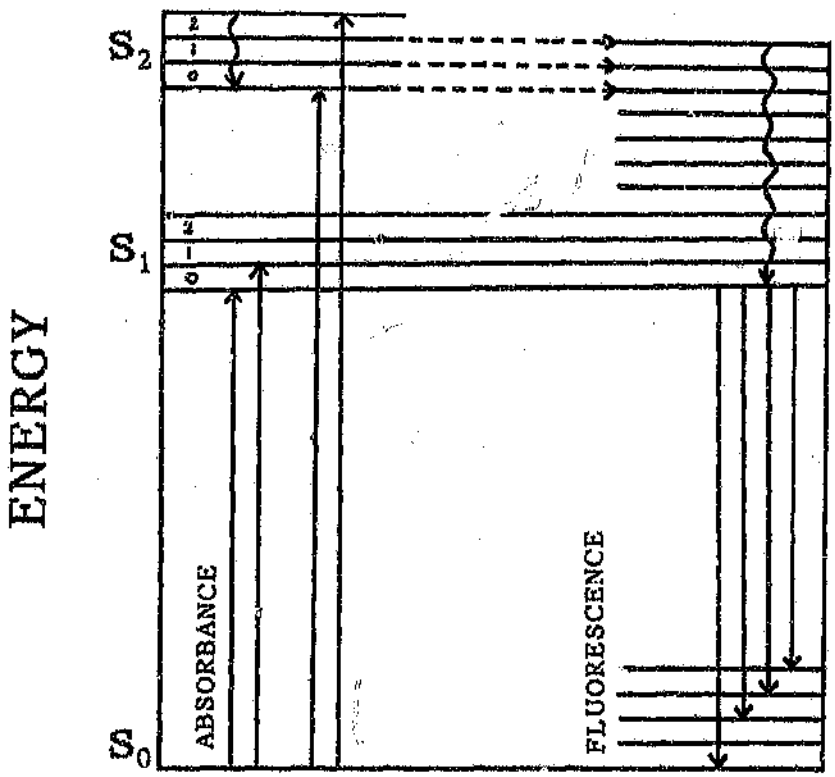


Figure 16. Energy level diagram showing absorption and emission of energy by electrons. S_0, S_1 and S_2 represent the ground, first and second electronic states respectively (adapted from Lakowicz, 1983).

Decrease or quenching of the intrinsic protein fluorescence can also be measured upon ANS binding. Bromosulphophthalein (BSP) does not fluoresce and hence its interaction with the protein can only be monitored by observing the quenching of the protein's intrinsic fluorescence (Lakowicz, 1983; Casey *et al.*, 1981).

All fluorescence measurements were performed using a Hitachi model 850 Fluorescence Spectrophotometer. Conditions for measurements were as follows: bandpass for excitation wavelength, 5nm, and for emission wavelength, 15nm; Scan speed, 60nm/min; response time, 2 seconds; normal photomultiplier gain; performed at room temperature (20-24°C).

2.6.1 Fluorescence enhancement

The affinity of 8-anilino-1-naphthalene sulphonic acid (ANS) for pGSTP1-1 was determined by measuring the fluorescence enhancement of ANS at an excitation wavelength of 400nm and at an emission wavelength of 480nm as performed by Nishihira et al. (1992a). 10μM ANS was titrated with increasing amounts of pGSTP1-1 to a final protein concentration of 2μM in buffer E. The dilution factor did not exceed 10% of the initial volume.

The dissociation constant was determined using the following procedures:

$$L_f = L_t - LP \qquad (1)$$

where L_f is the free ligand, L_t is the total ligand and LP is the protein-bound ligand.

For the bimolecular interaction:

$$L_f + P <--> LP \qquad (2)$$

The bound ligand, LP, is in equilibrium with free protein, P, and free ligand, L_f.

Substituting equation 1 into equation 2

$$LP <--> P + (L_t - LP) \qquad (3)$$

From the law of mass action:

$$K_d = \frac{[P][L_t-LP]}{[LP]} \quad (4)$$

By rearranging equation 4 we obtain:

$$[LP] = \frac{[L_t][P]}{[P] + K_d} \quad (5)$$

where L represents the ligand, ANS, and P represents the protein, GST.

The fluorescence intensity is proportional to the concentration of the ligand-protein complex as shown:

$$F = c[LP] \quad (6)$$

where c is a constant.

Therefore the maximum fluorescence observed can be represented by:

$$F_{max} = \lim_{L \to \infty} F \quad c[L_t] \quad (7)$$

The relative fluorescence intensity may be represented by:

$$V = \frac{F}{F_{max}} \quad (8)$$

By substituting equations 6 and 7 into equation 8:

$$V = \frac{[LP]}{[L_t]} \quad (9)$$

Thus by substituting equation 5 into equation 9:

$$V = [P]/([P] + K_d) \quad (10)$$

Equation 10 can be rearranged into:

$$\frac{1}{V} = \frac{1}{P} K_d + 1 \quad (11)$$

The slope obtained from the straight-line graph of equation 9 is the dissociation constant, K_d.

2.6.2 Fluorescence quenching

The affinity of bromosulphophthalein (BSP), 8-anilino-1-naphthalene sulphonic acid (ANS) and glutathione (GSH) to pGSTP1-1 was determined by measuring the quenching of the intrinsic tryptophan fluorescence of the protein. The excitation wavelength was set at 295nm and the emission wavelength at 335nm. A solution of 1μM pGSTP1-1 in buffer E was titrated with increasing amounts of the ligand to final concentrations of 10μM BSP, 100μM ANS or 5mM GSH. Controls were prepared in the same manner but in the absence of pGSTP1-1. The dilution factor did not exceed 10% of the initial volume.

The dissociation constants were determined using the following procedures:

$$P_f = P_t - LP \quad (12)$$

where P_f is the free protein, P_t is the total protein and LP is the protein complexed with ligand.

For the bimolecular interaction:

$$L + P_f <--> LP \quad (13)$$

The complexed protein, LP, is in equilibrium with free ligand, L, and free protein, P_f. Substituting equation 12 into equation 13:

$$LP <---> L + (P_t - LP) \quad (14)$$

From the law of mass action:

$$K_d = \frac{[L][P_t-LP]}{[LP]} \quad (15)$$

By rearranging equation 15 we obtain:

$$[LP] = \frac{[P_t][L]}{[L]+K_d} \quad (16)$$

Where L represents the ligand and P represents the protein, GST.

The enhanced fluorescence intensity is proportional to the concentration of the complexed protein, as shown:

$$F=c[LP] \quad (17)$$

where c is a constant.

Therefore the maximum fluorescence can be represented by:

$$F_{max}=\lim_{P\to\infty} F \; c[P_t] \quad (18)$$

By dividing equation 17 by equation 18:

$$\frac{F}{F_{max}} = \frac{[LP]}{[P]} \qquad (19)$$

Substituting equation 16 into equation 19:

$$\frac{F}{F_{max}} = \frac{[L]}{[L]+K_d} \qquad (20)$$

Equation 20 can be rearranged into:

$$\frac{1}{F} = \frac{K_d}{F_{max}[L]} + \frac{1}{F_{max}} \qquad (21)$$

By plotting 1/F versus 1/[L], K_d is determined directly by extrapolating the straight line through the 1/[L] axis where the intercept is equal to $-1/K_d$.

The concentration of the non-substrate ligands bound to pGSTP1-1 were determined, according to Lee (1982), using:

$$L_b = \frac{(K_d+nP_t+L_t) - \sqrt{(K_d+nP_t+L_t)^2 - 4nP_tL_t}}{2} \qquad (22)$$

Where L_b is the ligand bound, K_d is the dissociation constant for the ligand, L_t is the total ligand and n is the number of sites on the enzyme to which the ligand binds. One binding site per monomer is assumed.

2.6.3 Competition between BSP and ANS for the same binding site

To determine whether BSP and ANS compete for the same primary site on the protein, BSP's concentration was kept constant at either 0μM, 5μM, 15μM or 30μM while increasing amounts of ANS were titrated into the solution up to a final concentration of 20μM in buffer

E containing 1μM pGSTP1-1. Controls were prepared in the same manner but in the absence of the enzyme. The fluorescence enhancement of ANS was measured at an excitation wavelength of 400nm and an emission wavelength of 480nm.

2.6.4 Effect of glutathione on BSP binding

To determine the effect of GSH binding on BSP's affinity for the protein, 1μM GST in the absence or presence of 10mM GSH were titrated with increasing amounts of BSP to a final concentration of 10μM. Controls were prepared in the same manner but in the absence of the protein. Fluorescence quenching of the protein was measured at an excitation wavelength of 295nm and an emission wavelength of 335nm.

2.6.5 Energy transfer of ANS

In 1948, Förster described how electronic excitation energy can be efficiently transferred between a fluorescent energy donor and a suitable energy acceptor (Stryer, 1978). He postulated, that the rate for the dipole-dipole energy transfer depends on the inverse sixth power of the distance between the donor and the acceptor:

$$E \text{ (energy transfer)} \propto 1/r^6 \qquad (23)$$

where r is the distance between the donor and the acceptor.

This observation made it possible for energy transfer to be used as a spectroscopic ruler in biological macromolecules.

Efficient energy transfer requires that the energy donor and acceptor be in resonance, in other words, the fluorescence emission spectrum of the donor must overlap the absorption spectrum of the acceptor. Furthermore, the spectral characteristics of the donor and acceptor must be sufficiently distinctive so that the number of photons released and absorbed can be measured (Stryer, 1978).

The absorption spectrum of ANS overlaps the emission spectrum of the protein's intrinsic fluorescence and re-emits this energy at 480nm when bound to the protein (Figure 17).

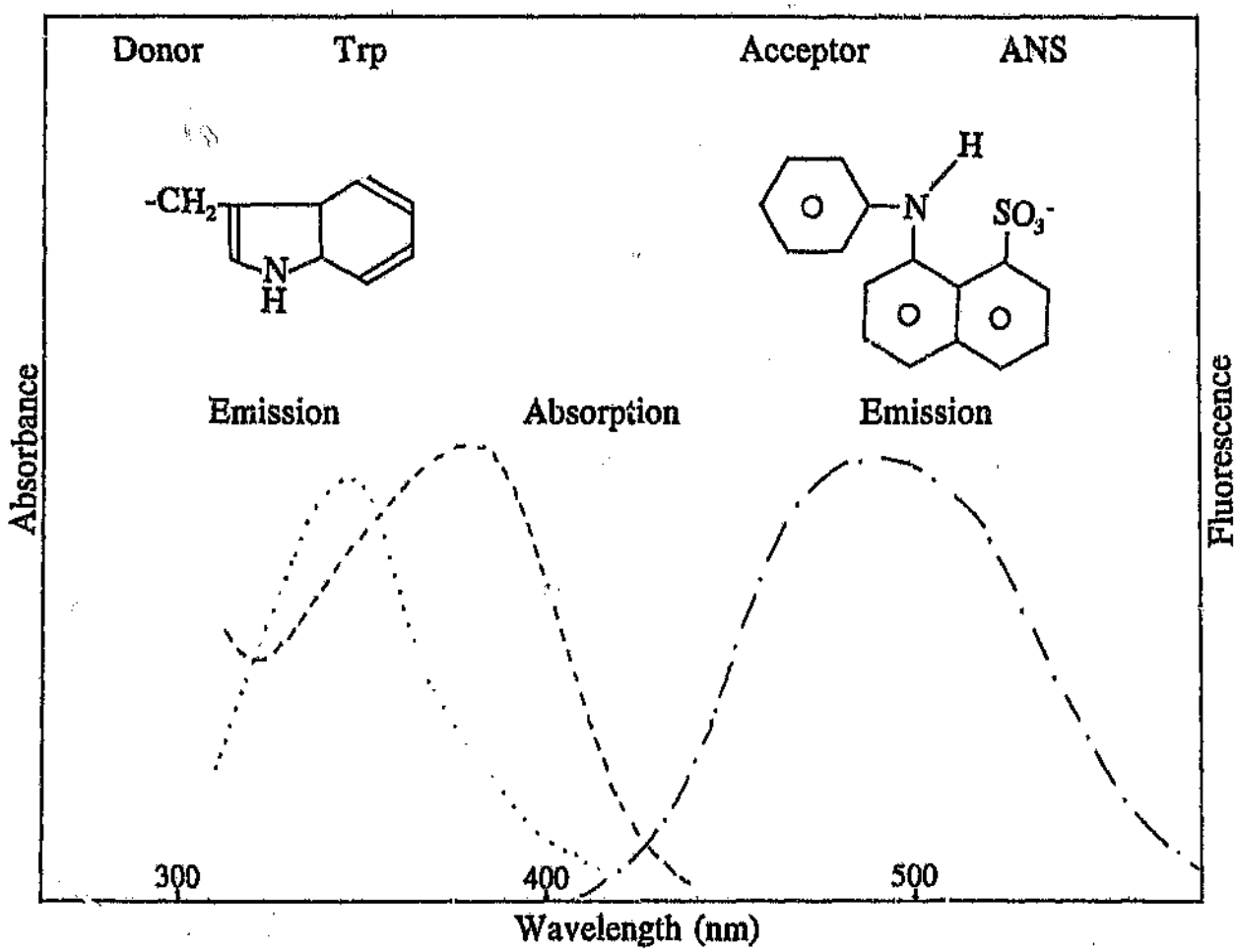


Figure 17. Spectral properties of the energy donor tryptophan and the energy acceptor ANS. Tryptophan has an emission wavelength maximum of 335nm. The absorbance spectrum of ANS overlaps the emission spectrum of tryptophan. ANS has an emission wavelength maximum of 480nm (Shepherd and Hammes, 1976).

The efficiency of the energy transfer, E, can be determined, according to Stryer (1978), using:

$$E = 1-(Q_T/Q_o) \quad (24)$$

Where Q_o is the relative fluorescence intensity of energy donor (pGSTP1-1) in the absence of the energy acceptor (ANS) and Q_T is the relative fluorescence in the presence of the energy acceptor.

The quenched protein fluorescence (1μM pGSTP1-1) was measured in the presence of increasing amounts of ANS to a final concentration of 50μM with an excitation wavelength of 295nm and an emission wavelength of 335nm.

To determine the maximum efficiency of energy transfer (E_{max}) a double-reciprocal curve of 1/E vs 1/[ANS] was plotted, where the y-intercept is $1/E_{max}$.

2.6.6 Corrections for Fluorescence measurements

A number of variables affect the accuracy of fluorescence measurements, of which the most important in the context of equilibrium constant determinations is the absorption of the excitation beam, referred to as the primary absorption effect (Birdsall *et al.*, 1983). If a large part of the fluorescence is absorbed by the compound whose concentration is varied in the course of the experiment, the primary absorption effect will alter the concentration dependence of the fluorescence, resulting in incorrect dissociation constants (Birdsall *et al.*, 1983).

Corrections on fluorescence measurements were made as follows:

1) Corrections for protein controls: $F_1 = F_{obs}-F_{con}$

2) Corrections for dilutions: $F_2 = F_1 \times V_f/V_i$

3) Correction for primary absorption effect: $F_{act} = F_2 x 10^{(Aex+Aem)/2}$

(Birdsall *et al.*, 1983).

where F_1 and F_2 represent fluorescence values after corrections 1 and 2 respectively. F_{act} = actual fluorescence; F_{obs} = observed fluorescence; F_{con} = control fluorescence; V_f and V_i are the final and initial volumes respectively. A_{ex} and A_{em} = absorbances at excitation and emission wavelengths.

The correction for the primary absorption effect can only be used for sufficiently low absorbances of the ligand, at the excitation and emission wavelengths ($A < 0.2$).

2.7 MEISENHEIMER COMPLEX FORMATION

2.7.1 Meisenheimer complex formation in aqueous solution

The formation of the Meisenheimer or σ-complex intermediate, [1-(S-glutathionyl)-2,4,6-trinitrocyclohexadienate) between GSH and 1,3,5-trinitrobenzene (TNB) (Figure 18) occurs in the active site of the protein (Graminski *et al.*, 1989). TNB does not possess a good leaving group and therefore the GSH conjugation reaction does not proceed to completion. The Meisenheimer complex can be observed spectrophotometrically by measuring the absorption spectrum of the complex from 400nm to 650nm. By observing the interaction of the Meisenheimer complex with the active site spectrophotometrically, it is possible to also observe whether or not other ligands displace the complex either by interaction with the active site or by causing a conformational change in the protein that would affect the active site. In other words, the Meisenheimer complex may provide useful information regarding the binding sites of the ligands.

An assay mixture of 4mM GSH in buffer E and 10μM pGSTP1-1 was titrated with 2μl increments of 0.1M TNB in acetonitrile to a final concentration of 0.99mM. ANS, BSP or GSO_3^- were added after σ-complex formation, in increasing quantities to the assay mixture to final concentrations of 100μM, 100μM or 500μM, respectively. Controls were prepared in the same manner but in the absence of the protein. The displacement of σ-complex from the active site of the enzyme by the ligands was monitored at 456nm.

The formation constant for the σ-complex was determined according to Benesi and Hildebrand (1949).

$$\frac{1}{A_{456}} = \frac{1}{K_f \epsilon [E.GS^-]} \times \frac{1}{[TNB]} + \frac{1}{\epsilon [E.GS^-]} \qquad (25)$$

Where A_{456} is the absorbance of the complex at 456nm, K_f is the formation constant, ϵ is the

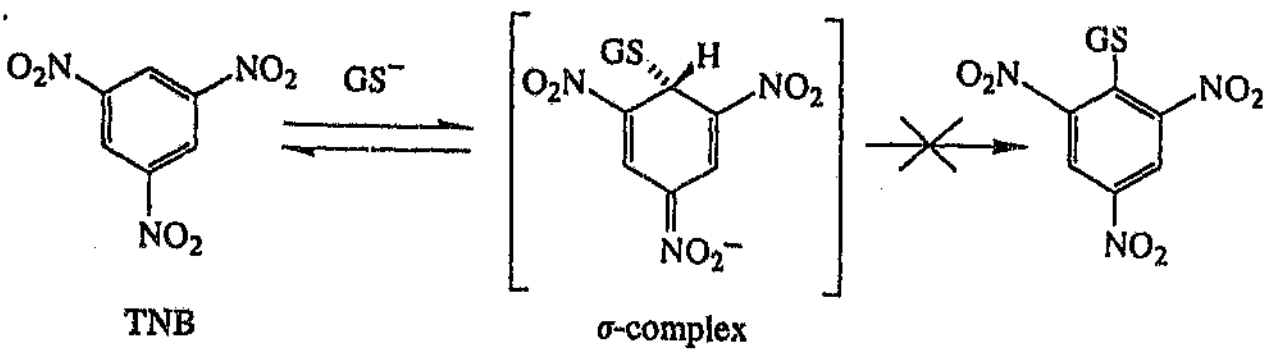


Figure 18. Formation of the Meisenheimer complex. The reaction between TNB and glutathione (or rather thiolate anion GS^-) does not proceed to completion because TNB does not possess a good leaving group. The red-orange σ-complex accumulates in the active site and inhibits the enzyme (Graminski *et al.*, 1989).

extinction co-efficient, [TNB] is the concentration of TNB added and [E.GS⁻] is the concentration of enzyme-thiolate anion in solution.

By plotting $1/A_{456}$ versus 1/[TNB], the y-intercept can be determined and subsequently substituted into the slope equation to obtain the formation constant (Graminski *et al.*, 1989).

2.7.2 Meisenheimer complex formation in the protein crystal

Orthorhombic crystals which had been previously prepared as described by Dirr *et al.* (1991) were placed in fresh harvesting solution (25% poly(ethylene glycol) 4000, 100mM Mes, 0.02% sodium azide, pH 6.5) and allowed to equilibrate for an hour in this solution. GSH was subsequently added to the mixture to a final concentration of 5mM. The solution was allowed to reach equilibrium for 1 hour. TNB was then added to a final concentration of 1mM. Equilibrium was once again reached. The crystals were photographed after each step, and any colour changes observed. Finally, GSO_3^- was added to the mixture to a final concentration of 1mM and the solution allowed to equilibrate for a further hour. Controls were performed in the same manner but in the absence of the enzyme crystal. All work was viewed under an Olympus SZ-40 stereo microscope.

2.8 SIZE-EXCLUSION HPLC

High performance liquid chromatography is a useful technique for monitoring gross changes in the disruption of the protein's structure, such as the dissociation of the dimer upon ligand binding. It is however, not sufficiently sensitive to monitor slight changes in the conformation of the protein upon ligand binding. An isocratic HPLC system with a Spectra series UV100 detector and an LKB model 2150 pump was used to determine whether or not ligand binding to pGSTP1-1 causes large conformational changes and the dissociation of the dimer. It was also used to confirm the homogeneity of the purified protein.

10μM pGSTP1-1 was run through a Biosep SEC-S3000 column (Phenomenex) equilibrated with 0.02M sodium phosphate buffer pH 6.5 at a flow rate of 0.5ml/min. A 10μM pGSTP1-1, 50μM BSP mixture was subsequently run through the same column equilibrated in 0.02M sodium phosphate buffer pH 6.5 plus 50μM BSP. Molecular weight standards used for calibrating the column included: ferritin (Mr 450kD); catalase (Mr 240kD); aldolase (Mr 158kD); bovine serum albumin (Mr 68kD); haemoglobin (Mr 64.5kD); ovalbumin (Mr 45kD); carbonic anhydrase (Mr 28.8kD); chymotrypsinogen (Mr 25kD); myoglobin (Mr

16.9kD); lysozyme (Mr 14.3kD) and cytochrome C (Mr 11.7kD). All markers were obtained from Boehringer Mannheim except for carbonic anhydrase and lysozyme which were obtained from Sigma and haemoglobin and myoglobin which were obtained from Serva. Proteins were detected at a wavelength of 280nm.

SEC-HPLC of the protein in the presence of ANS was not performed, because of the high absorbance of ANS at 280nm.

2.9 REACTION WITH ELLMAN'S REAGENT

5,5'-Dithiobis(2-nitrobenzoic acid), DTNB, is a sulphydryl reagent able to react with aliphatic thiols by an exchange reaction to form a mixed disulphide of the protein and a molecule of the yellow aromatic thiol, 2-nitro-5-thiobenzoate (NTB) per mole of protein sulphydryl group (Habeeb, 1972; Creighton, 1993) (Figure 19).

It is possible to determine the protein sulphydryl groups accessible for reaction with DTNB by monitoring the formation of the yellow NTB compound at a wavelength of 412nm.

The rate of the reaction of sulphydryl groups with DTNB can be used to detect conformational changes in the protein upon ligand binding (Habeeb, 1972; Creighton, 1993). Either 5μM BSP, 100μM ANS or 100μM GSO_3^- were added to 1μM pGSTP1-1 in 0.1M Tris-1mM EDTA buffer pH 7.5. DTNB was subsequently added to the mixture to a final concentration of 150μM. Controls were prepared in the same manner, but in the absence of pGSTP1-1. The reaction was observed spectrophotometrically at 412nm for 2 minutes.

2.10 ACRYLAMIDE QUENCHING OF TRYPTOPHAN FLUORESCENCE

Acrylamide is an uncharged polar quencher that is very sensitive to the exposure of tryptophans in proteins. It has been found to quench tryptophan fluorescence predominantly by a collisional process (Eftink and Ghiron, 1976 a,b).

The relationship often employed to describe the collisional quenching process is the Stern-Volmer equation (Lehrer, 1971).

$$F_o/F = 1 + K_{sv}[Q] \qquad (26)$$

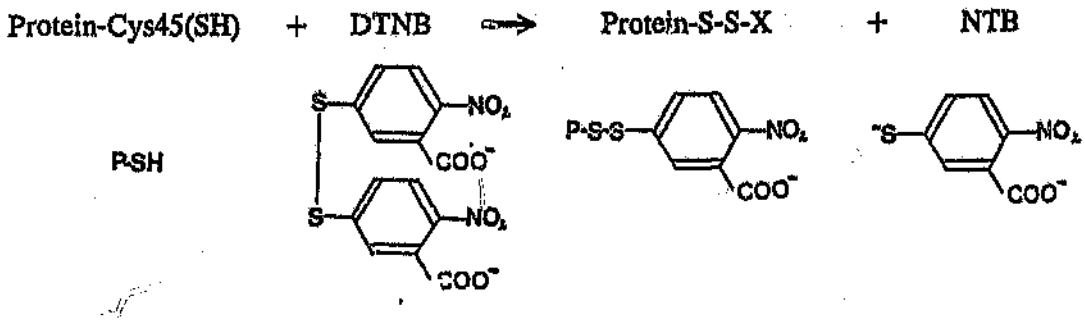


Figure 19. Reaction of Ellman's reagent with protein sulphydryl groups. P-SH represents the thiol group of the protein, P (Creighton, 1993).

Where F_o and F are the fluorescence intensities in the absence and presence of quencher respectively; K_{sv} is the collisional quenching constant; [Q] is the concentration of the quencher. By plotting F_o/F vs [Q], K_{sv} can be obtained from the slope.

When the plot observed is not linear but instead curves downwards or has a negative deviation this suggests that the fluorescence of certain tryptophans in the protein is selectively quenched before others. This can only be detected however if the quenching constants for each tryptophan is quite different. If not, an apparently linear plot will be observed.

Not all the excited states are quenched by the collisional process, however, and some are deactivated almost immediately after becoming excited because a quencher molecule happens to be present at the time of excitation. This is referred to as static quenching (Eftink and Ghiron, 1976a,b). Static quenching causes the plot to curve upwards and would oppose any negative deviations due to selective quenching.

It is possible to loosely categorize multi-tryptophan proteins such as pGSTP1-1. An upward curving plot would indicate that one fluorophore dominates the fluorescence. A downward curving plot would indicate that the fluorophores have very different accessibilities to the quencher. A linear plot indicates that the tryptophans differ only slightly in accessibility. This quenching technique can detect very subtle conformational changes which may accompany the binding of ligands due to increased or decreased exposure of the tryptophans (Eftink and Ghiron, 1976a,b).

A modified Stern-Volmer relationship has been described to include both the collisional and the static quenching processes as well as considering proteins with more then one fluorescing tryptophanyl residue (Lehrer, 1971).

$$\frac{F_o}{\Delta F} = \frac{1}{f K_{sv}} \cdot \frac{1}{[Q]} + \frac{1}{f} \qquad (27)$$

Where ΔF is the fluorescence change observed and f is the fraction of accessible fluorescence quenched. From the above equation, a plot of $F_o/\Delta F$ vs $1/[Q]$ will yield a straight line of slope $1/fK_{sv}$ and intercept $1/f$ (Lehrer, 1971; Eftink and Ghiron, 1981).

The quenching of 1μM pGSTP1-1 was observed by increasing amounts of acrylamide (0-0.6M) in buffer E at different concentrations of BSP (0, 2, 10, 20 or 50μM) or ANS (0, 20 or 40μM). Fluorescence measurements were made at an excitation wavelength of 295nm and

emission wavelength of 335nm. Controls were performed in the same manner but in the absence of the protein. $F_o/\Delta F$ was plotted against 1/[acrylamide] and the fraction of fluorescence quenched observed under the increasing ligand concentrations.

CHAPTER 3 RESULTS AND DISCUSSION

3.1 PURIFICATION OF PORCINE LUNG CLASS PI GST

The class Pi GST isolated from porcine lung was purified 643.2- fold to a specific activity of 6.63μmol/min/mg (Table 3). A 33% yield and 11.2mg pure enzyme was obtained from about 250g wet tissue.

The homogeneity of the protein was confirmed using SDS-polyacrylamide gel electrophoresis (Figure 20), isoelectric focusing (Figure 21) and size exclusion HPLC (Figure 22). The molecular weight of the dimer was found to be about 48700 by SEC-HPLC (Figure 22), each monomer being about 24kD (SDS PAGE) (Figure 23). The molecular weight of the protein is in accord with previously reported values (Dirr *et al.*, 1991; Mannervik and Danielson, 1988). The pI of the protein was found to be 8.6 (Figure 21). Nishinaka *et al.* (1991) has also purified pGSTP1-1 from pig lens, and found its pI to be 8.5 and Warholm *et al.* (1986) reported a Pi class isoenzyme from mouse liver, mGSTP1-1, with a pI value of 8.7. The human class Pi enzyme, although sharing an 82% sequence identity with the class Pi pig enzyme (Dirr *et al.*, 1991, Reinemer *et al.*, 1992)) has an isoelectric point of 4.6 (Widersten *et al.*, 1992). The different isoelectric points for these isoenzymes are obviously as a result of their different amino acid compositions.

3.2 GLUTATHIONE BINDING

The binding of glutathione (GSH) or S-analogues thereof to pGSTP1-1, quenches about 20% of the protein's tryptophan fluorescence. The dissociation constant for the binding of GSH was found to be 120μM (Figure 23). Philips and Mantle (1991) determined a K_d value of 190μM for GSH when working with mGSTP1-1. These values are high in comparison to the K_d for glutathione sulphonate (K_d=4μM) (Dirr *et al.*, 1991) for example. This is probably because glutathione sulphonate possesses a negative charge in its sulphonate moiety which provides the ligand with additional binding strength. It is, however, undesirable for glutathione to have such a strong affinity for the enzyme, as this could interfere with its efficiency by lowering the turnover rate of GSH-conjugate formation. Ji *et al.* (1992) determined a tighter binding of GSH for the active site of a Mu class isoenzyme, rGSTM1-1, (K_d=20μM).

Table 3 : Purification Table for pGSTP1-1 from porcine lung

Step	Volume (ml)	Total Protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Yield (%)	Purification factor (x)
Extract	450	21825	225	0.010	100	1
Cytosol	335	13534	167.5	0.012	74	1.2
S-hexylGSH	16.5	22.1	106.2	4.81	47	467
DEAE 1	16	11.2	74.2	6.63	33	643.2

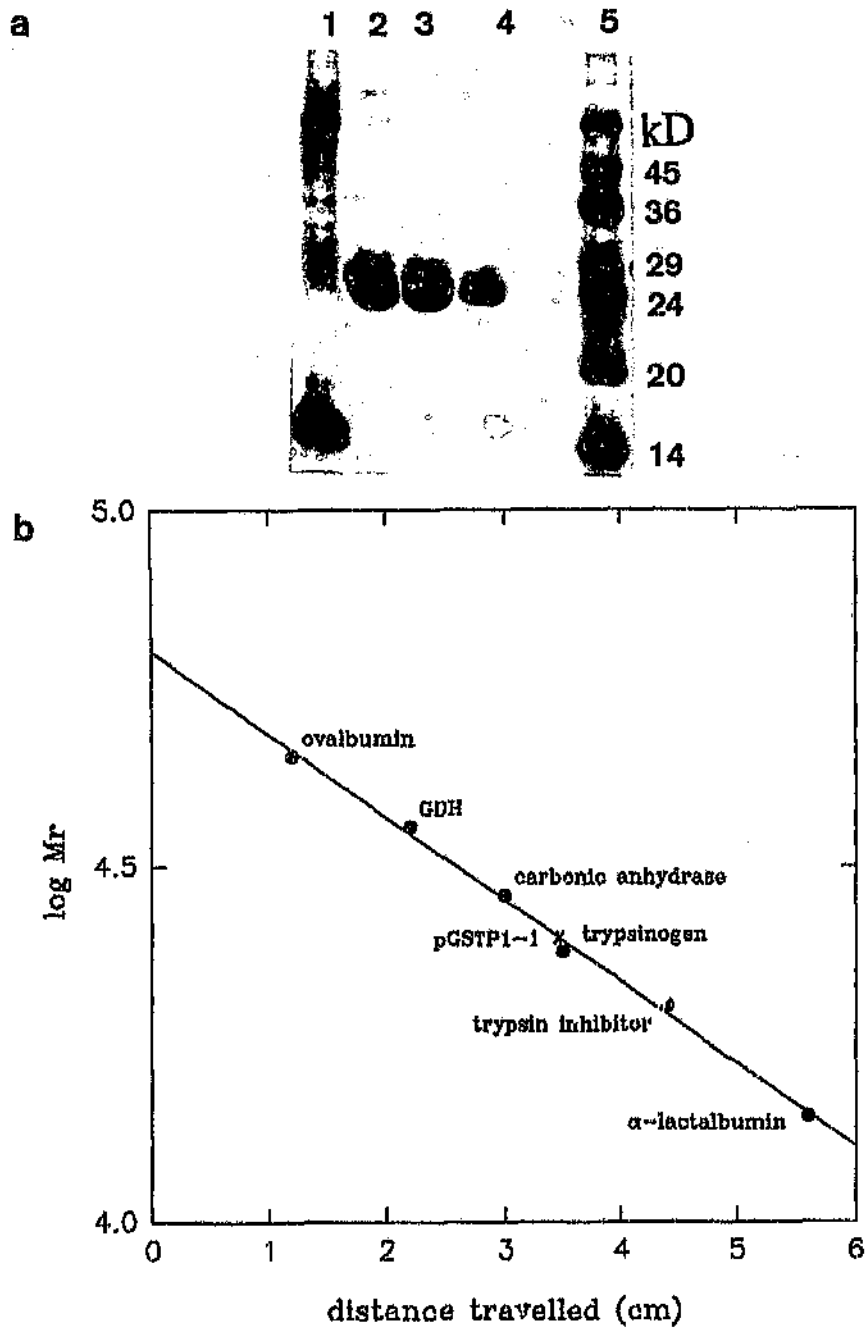


Figure 20. Molecular weight determination of the pGSTP1 monomer, by SDS PAGE. (a) 15% SDS gel showing the migration of the protein, lanes (1) cytosol; (2) S-hexylGSH; (3) and (4) purified pGSTP1-1 and of molecular weight markers (lane 5) (b) standard curve for molecular weight markers of SDS gel, molecular weight for the pGSTP1 polypeptide is about 24kD (marked with an x).

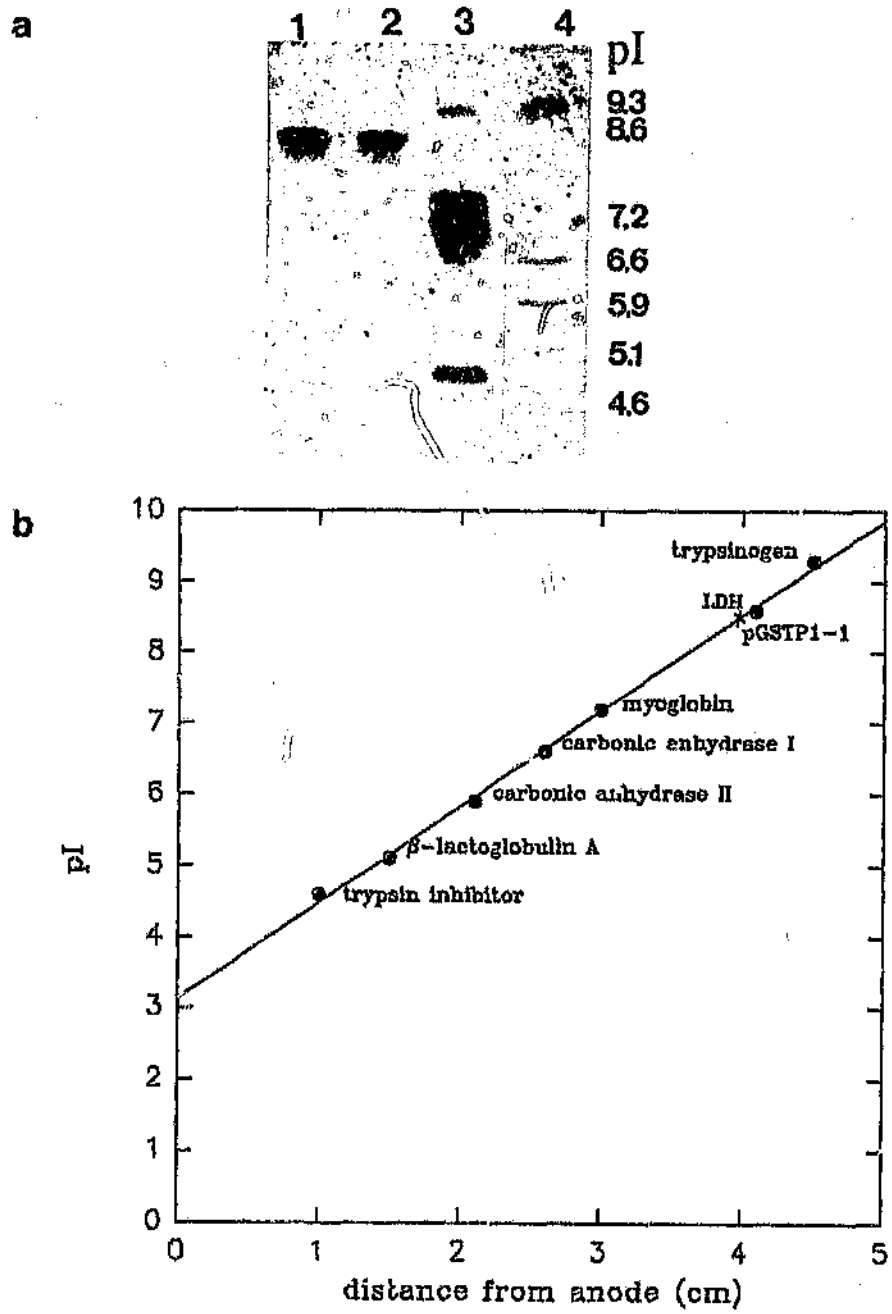


Figure 21. Determination of the isoelectric point of pGSTP1-1. (a) Gel showing the migration of the protein, lanes (1) and (2) purified pGSTP1-1; (3) cytosol and of pI markers (lane 4) (b) standard curve for pI markers, pI of pGSTP1-1 is 8.6 (marked with an x).

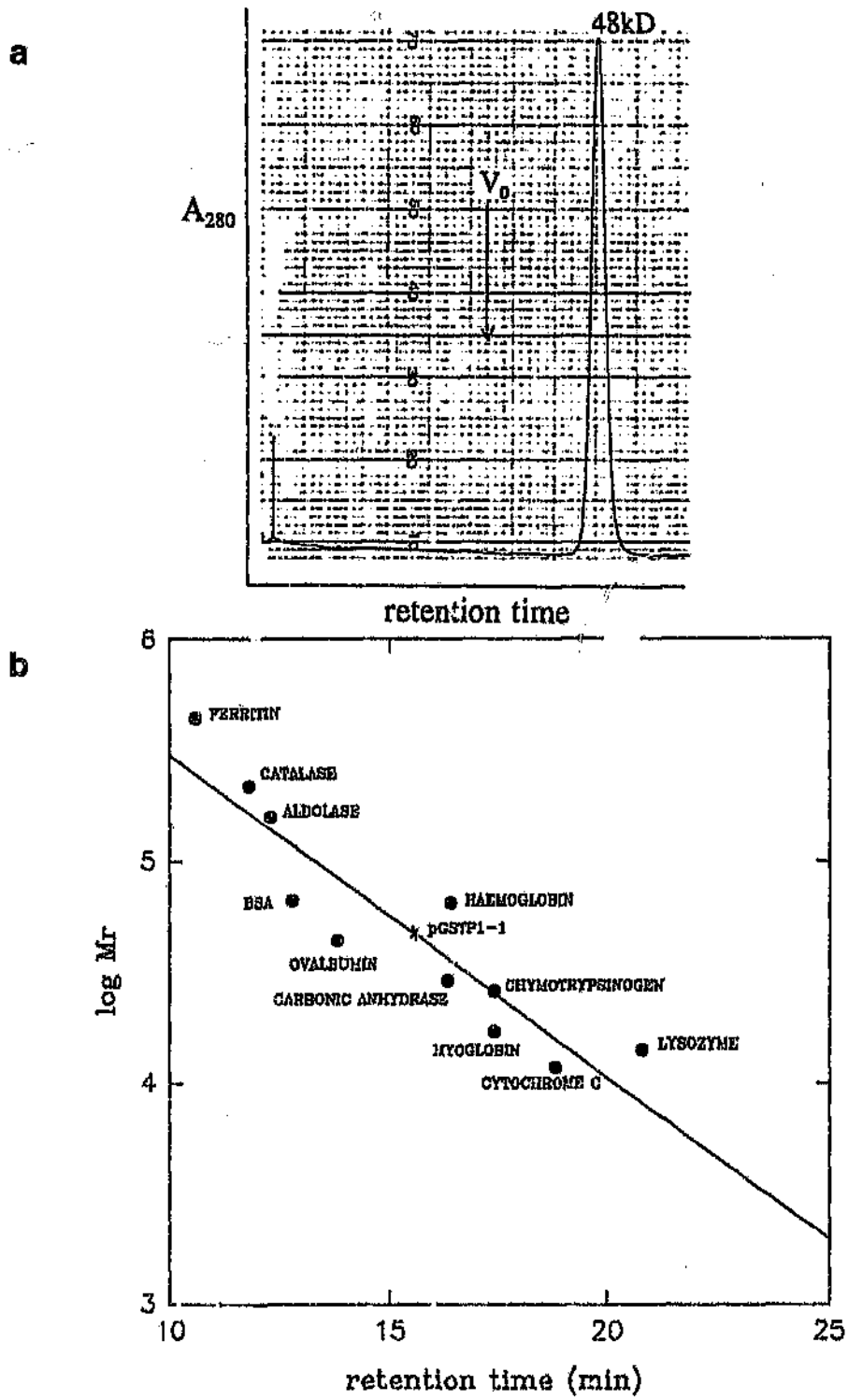


Figure 22. Molecular weight determination of the native pGSTP1-1 dimer by SEC-HPLC. (a) Elution profile of pGSTP1-1, V_0 indicates the void volume of the column (b) standard curve for molecular weight markers eluted from the column; molecular weight of pGSTP1-1 dimer is about 48700 (marked with an x).

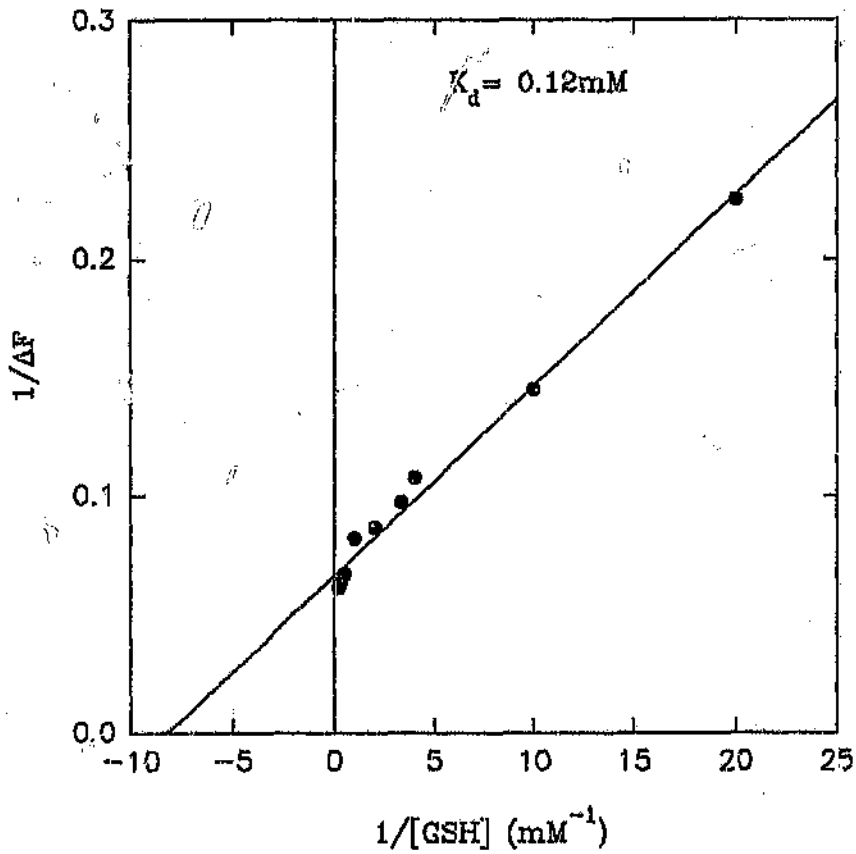


Figure 23. Determination of the dissociation constant, K_d, for glutathione using the fluorescence quenching method (section 2.6.2).

Fluorescence quenching by GSH occurs most likely as a consequence of a direct interaction between GSH and the indole fluorophore of Trp38 which is located at the active site (see Figure 7).

The absence of a stoke's shift in the maximum emission wavelength of the protein seems to eliminate the possibility of any major conformational change in pGSTP1-1 upon GSH binding. However, as mentioned above in section 1.5, GSH binding does appear to induce a local conformational change which is translated through the protein causing Cys45 to become buried in the molecule.

3.3 BROMOSULPHOPHTHALEIN AS A LIGAND FOR pGSTP1-1

3.3.1 Bromosulphophthalein binding

BSP binding to GSTs have been extensively studied (Satoh *et al.*, 1991; Bhargava and Dasgupta, 1988; Clark and Carrol, 1986; Jakobson *et al.*, 1979). It has been found to behave as both a non-substrate as well as a substrate for certain isoenzymes (Kosower, 1976; Habig *et al.*, 1974; Satoh *et al.*, 1991).

Do these ligands bind the active site of the protein as their primary site? Do they inhibit the enzyme? Is there a common binding site for non-substrate ligands in pGSTP1-1? Does the binding of this ligand affect the conformation of the protein in any manner? These are but a few questions, concerning the binding of this ligand to GSTP1-1, that remain unanswered.

BSP binds the protein rather tightly (K_d=1.1μM at pH 6.5; Figure 24). Its binding to the protein was monitored by observing the quenching of the intrinsic tryptophan fluorescence of the protein upon addition of the ligand. It is interesting to note that at pH 7.5, BSP's affinity for pGSTP1-1 decreases two-fold (K_d=2.4μM at pH 7.5; Figure 24).

Satoh *et al.* (1991) also reported a pH dependence of BSP in its inhibition of the rGSTP1-1.

3.3.2 Acrylamide quenching in the presence of BSP

Acrylamide quenching studies show a very small decrease in the quenching constant for pGSTP1-1 with increasing concentrations of BSP (Figure 25). The fraction of tryptophan fluorescence in the enzyme available for quenching by acrylamide is also not altered

significantly upon addition of BSP. Each monomer has only two tryptophan residues, Trp38 at the active site and Trp28 close to the active site in domain 1. Therefore, the above results indicate that BSP does not bind close enough to either of the tryptophan residues in order to markedly reduce its accessibility to acrylamide.

Wang *et al.* (1992b) has also observed that the anionic non-substrate ligand, haem, which may bind to the same primary site as BSP, bound to a mutant form of rGSTA1-1 where Trp21 had been replaced by phenylalanine. They concluded that the tryptophan residue was thus not involved in the binding of haem. Bhargava and Dasgupta (1988) however, found the primary binding site of rGSTA1-1 to be in domain 1 in a region extending from residues 16-49. It is therefore possible that the primary binding site for BSP to pGSTP1-1 may also be in domain 1 but not in close proximity to either of the tryptophan residues.

3.3.3 Enzyme activity in the presence of BSP

The activity of the enzyme with GSH and CDNB as substrates, was assayed in the presence of BSP at pH 6.5 and at pH 7.5 (Figure 26). Initially, BSP had no effect on the activity of the enzyme, however, after about 10μM (at pH 6.5) or 20μM (at pH 7.5) of BSP, the enzymes activity decreased rapidly. At these concentrations of BSP, the enzyme is about 88% saturated with ligand according to the dissociation constants of BSP at the different pH values.

These studies show firstly that the active site of the enzyme is not the primary binding site for BSP since only after a certain concentration of ligand is the activity of the enzyme affected. They also confirm the previous finding in this study of decreased affinity of BSP for pGSTP1-1 at pH 7.5. Therefore, more ligand is required to inhibit the enzyme at pH 7.5 than at pH 6.5.

Finally, the results suggest that pGSTP1-1 has more than one binding site for BSP and that after saturation of the primary site, BSP binding partially interferes with the access of one of the substrates to the active site or induces a conformational change at the active site of the protein. As mentioned above in section 1.3.3, Jakobson *et al.* (1979) found that at low concentrations of BSP, it bound to a high-affinity primary binding site on rGSTM1-1, whereas at higher concentrations of the ligand a second low-affinity site appeared to be present. Satoh *et al.* (1991) has also previously reported two anion binding sites per

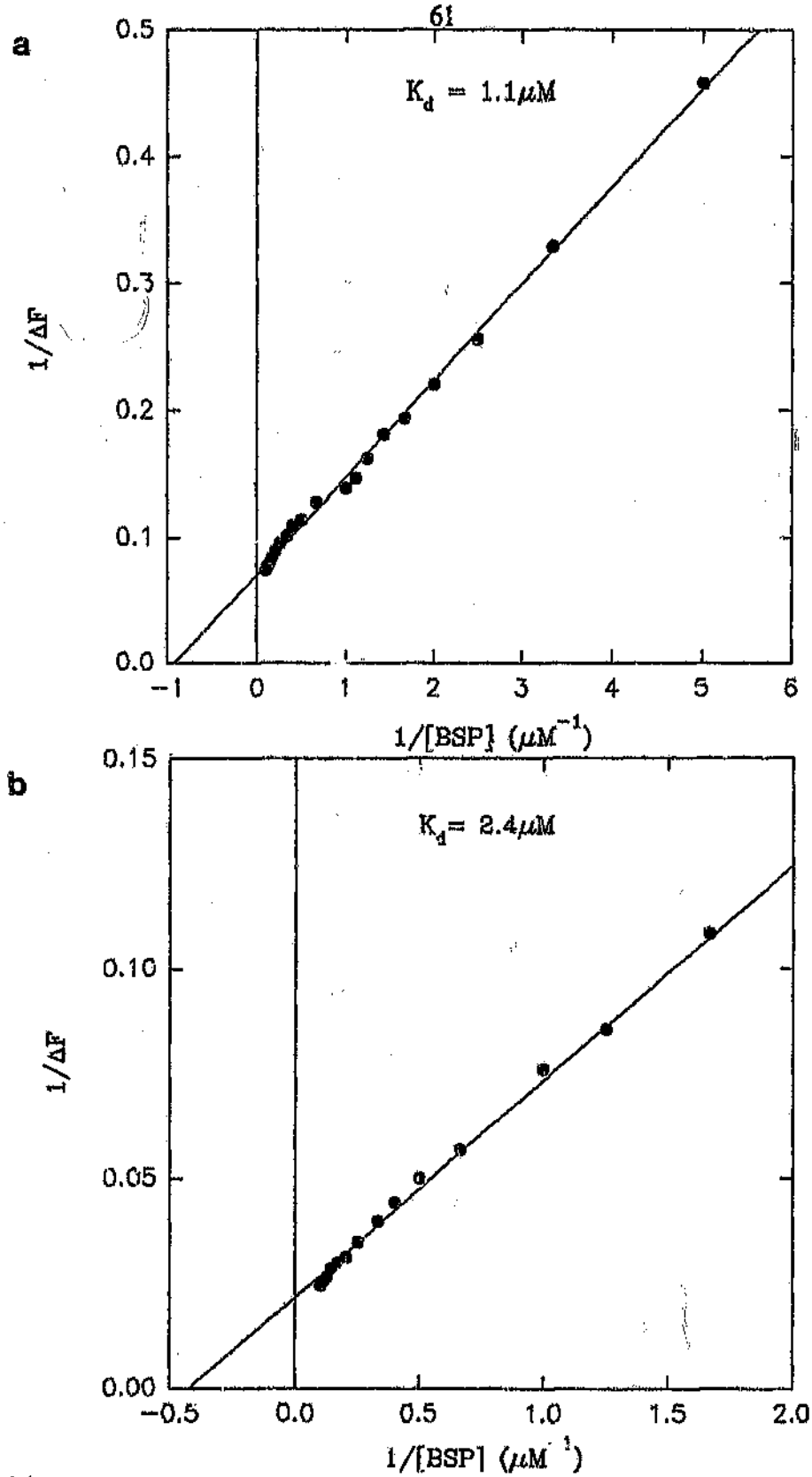


Figure 24. Determination of the dissociation constant, K_d, for BSP at (a) pH 6.5 and (b) pH 7.5; using the fluorescence quenching technique (section 2.6.2).

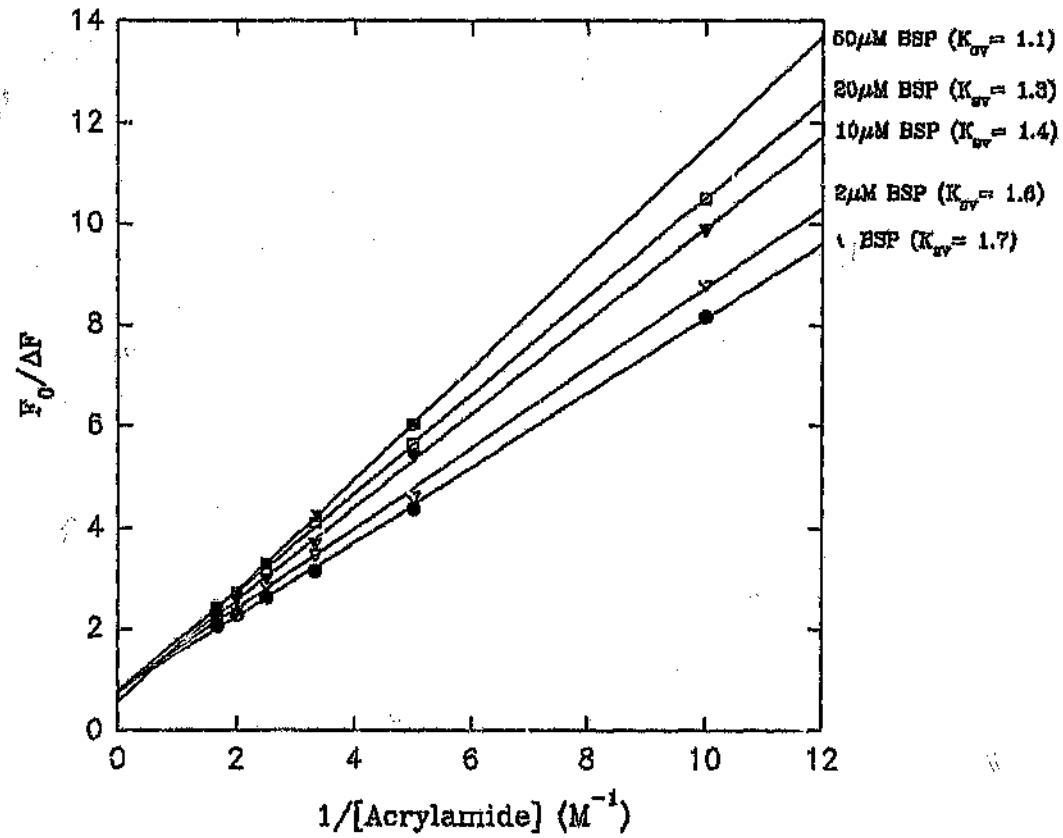


Figure 25. Acrylamide quenching of pGSTP1-1 intrinsic tryptophan fluorescence in the absence or presence of BSP. The y-intercept 1/f (inverse of fractional fluorescence) essentially remains constant and the quenching constant K_{sv} decreases only slightly.

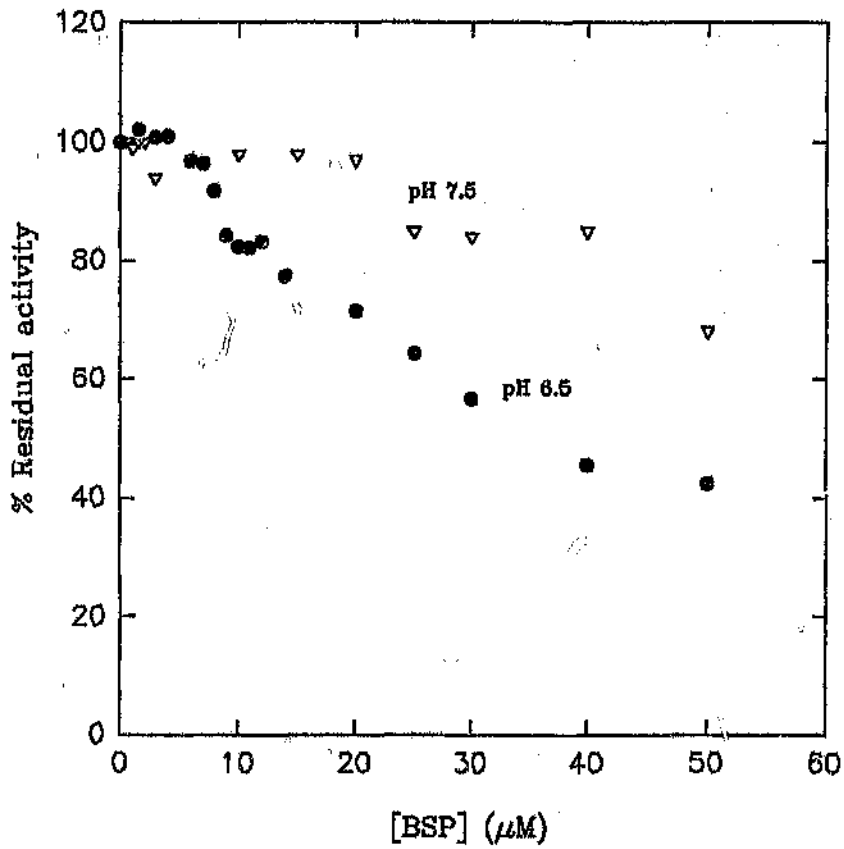


Figure 26. Percentage residual activity of pGSTP1-1 with increasing concentrations of BSP at pH 6.5 (•) and at pH 7.5 (▽).

monomer of rGSTP1-1, one being of high affinity and unrelated to the active site and the other being of low affinity but having a close relationship with the active site. In addition, he suggested that the insensitivity of the enzyme to, yet high affinity for, various dye inhibitors, such as BSP, bilirubin and haematin are the same properties responsible for resistance in cancer cells. They provide for the scavenging of non-substrate ligands especially upon the administration of drugs and carcinogens. It is also possibly for this reason that the Pi class is the isoenzyme form often selected for and found in elevated quantities in tumours, serving as preneoplastic cell markers (Coles and Ketterer, 1990; Satoh *et al.*, 1991).

As mentioned above in section 1.3.3, BSP not only behaves as a non-substrate ligand to GST, but has also been used as a substrate for class Pi and class Mu isoenzymes (Satoh *et al.*, 1991). Very low activity, however, was observed in these enzymes when using BSP as a substrate whereas no detectable activity could be observed for the Alpha class isoenzymes. This is possibly a result of the extra helix αI in the Alpha class enzymes which forms an additional wall in the H-site of the enzyme and possibly becomes an obstacle to the binding of BSP to the H-site.

These results strongly suggest that the active site is indeed not the primary binding site, but is possibly a second low affinity binding site. The latter has however not been experimentally established.

3.3.4 Meisenheimer complex formation

The alteration of the Meisenheimer or σ-complex levels upon ligand binding serves to show whether or not a ligand affects the active site of the enzyme. The dissociation constant of the σ-complex for pGSTP1-1 in the aqueous solution is about 547μM (reciprocal of the formation constant (Figure 27). A decrease in the Meisenheimer complex, for example, would suggest either that the ligand itself binds to the active site, displacing the σ-complex, or induces some sort of conformational change in the protein that would force the σ-complex out of the active site.

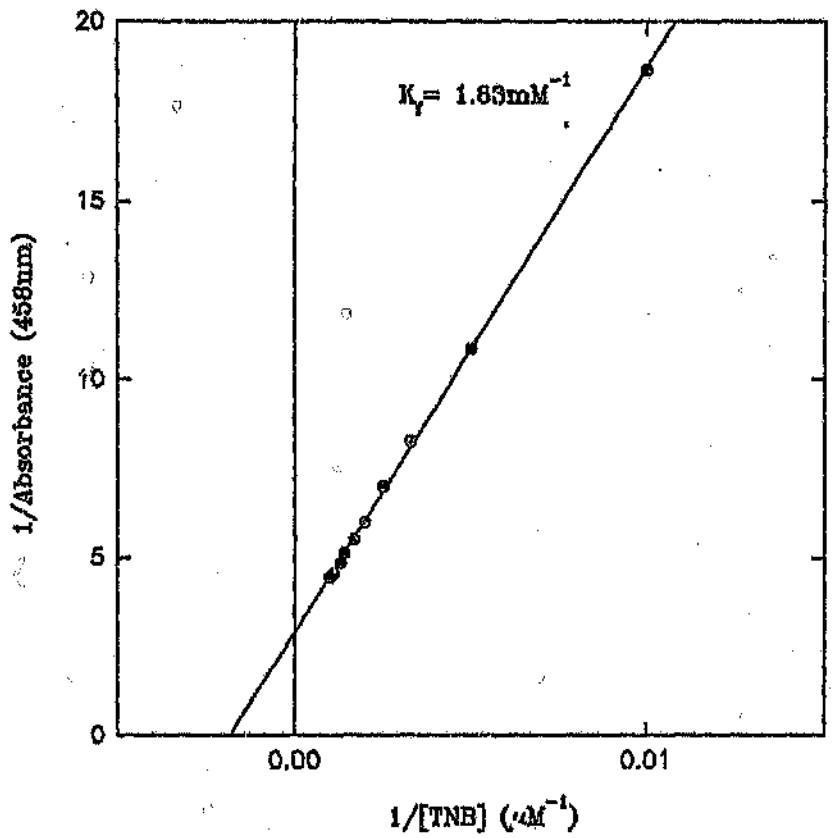


Figure 27. Determination of the formation constant, K_f, for the Meisenheimer complex at the active site of pGSTP1-1.

Glutathione sulphonate, a GSH analogue, displaces the Meisenheimer complex because it binds to the G-site on the enzyme.

The formation of the σ-complex in the pGSTP1-1 crystal was demonstrated when the colour of a crystal, soaked with GSH and TNB, changes from colourless to red-orange (Figure 28a). Upon the addition of 1mM glutathione sulphonate to the crystal the σ-complex was displaced and the crystal once again became colourless. Previous researchers (Nishihira *et al.*, 1992a) have suggested that the conformation of the protein in solution and in the crystal may be different. However, the above results show that the crystallized form of the enzyme is catalytically competent and, therefore, is highly likely to be in the same conformation as that of the enzyme in the aqueous solution.

Figure 28b shows how increasing amounts glutathione sulphonate reduces the absorption spectrum for the σ-complex in aqueous solution. The addition of 100μM of GSH sulphonate (96% saturation of the enzyme), displaced about 40% of the σ-complex from the active site (Figure 29).

3.3.4.1 σ-complex and BSP

The interaction of 100μM BSP with the enzyme in solution (98-99% saturation of the enzyme) resulted in a 35% decrease of the σ-complex from the active site at pH 6.5 and a 20% decrease at pH 7.5 (Figure 29). A surprising observation, is the steady decrease in the σ-complex which occurs from the lowest ligand concentration.

If there were two unrelated sites on the enzyme for BSP, one would expect a decrease in the Meisenheimer complex only after the primary site was saturated as observed in the activity assays with GSH and CDNB in the presence of BSP. The conclusion that can be derived from this study is that BSP does have an effect on the active site of the enzyme using the σ-complex as a probe, whether it be an induced conformational change or its secondary binding site. It is also interesting to note that at pH 7.5 the effect of BSP on the active site is reduced. This is in agreement with the ligands reduced binding affinity at this pH.

3.3.5 Size-exclusion HPLC

The elution profiles of pGSTP1-1 in the absence or presence of BSP from the size-exclusion column were identical (Figure 30).

This is a clear indication that a gross conformational change, such as the dissociation of the

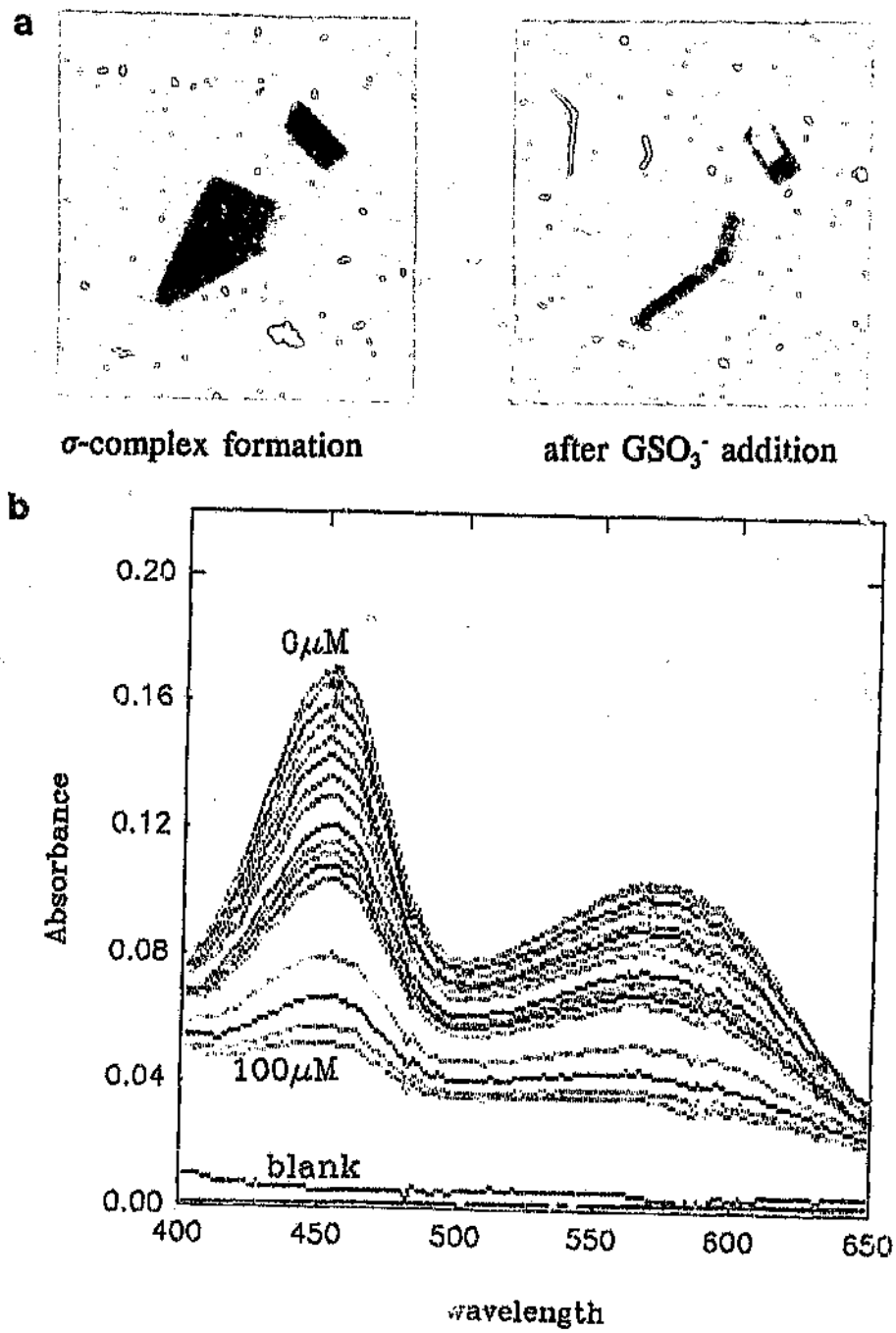


Figure 28. Disappearance of the Meisenheimer complex at the active site of pGSTP1-1 in the presence of increasing concentrations of glutathione sulphonate. (a) σ-complex in the crystal structure of pGSTP1-1 before and after the addition of 1mM CSO_3^- (b) Absorption spectrum of σ-complex with increasing concentrations of GSO_3^- (0-100μM).

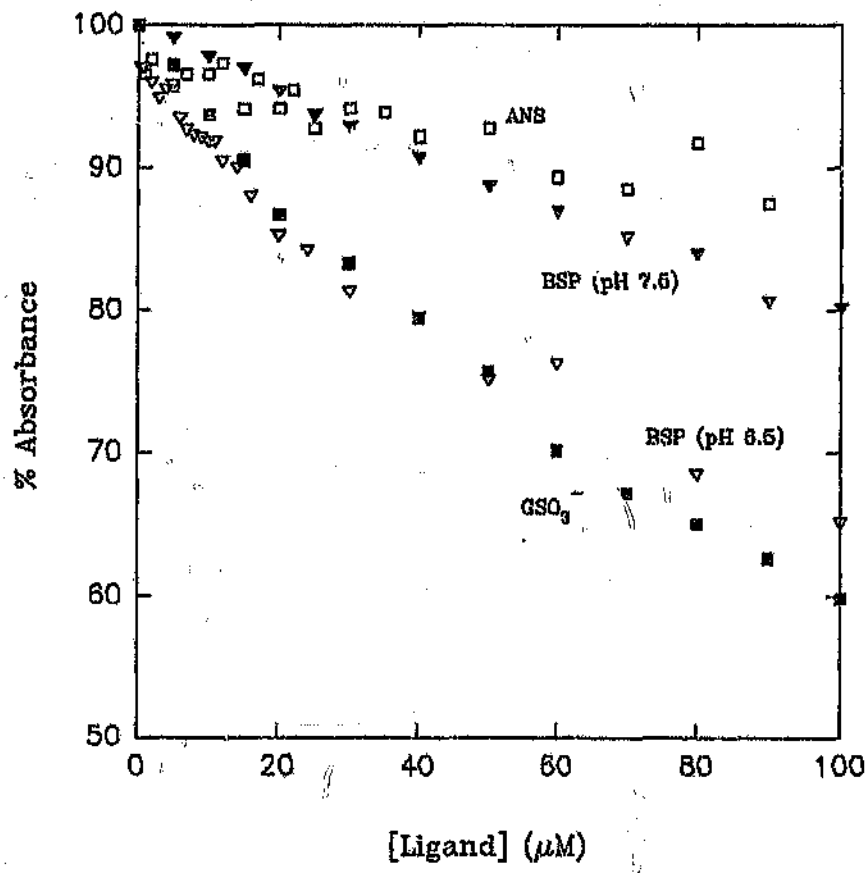


Figure 29. Percentage decrease of absorbance of σ-complex at 458nm at increasing concentrations of GSO_3^- (▪), BSP at pH 6.5 (▽) and at pH 7.5 (▼), and of ANS (□).

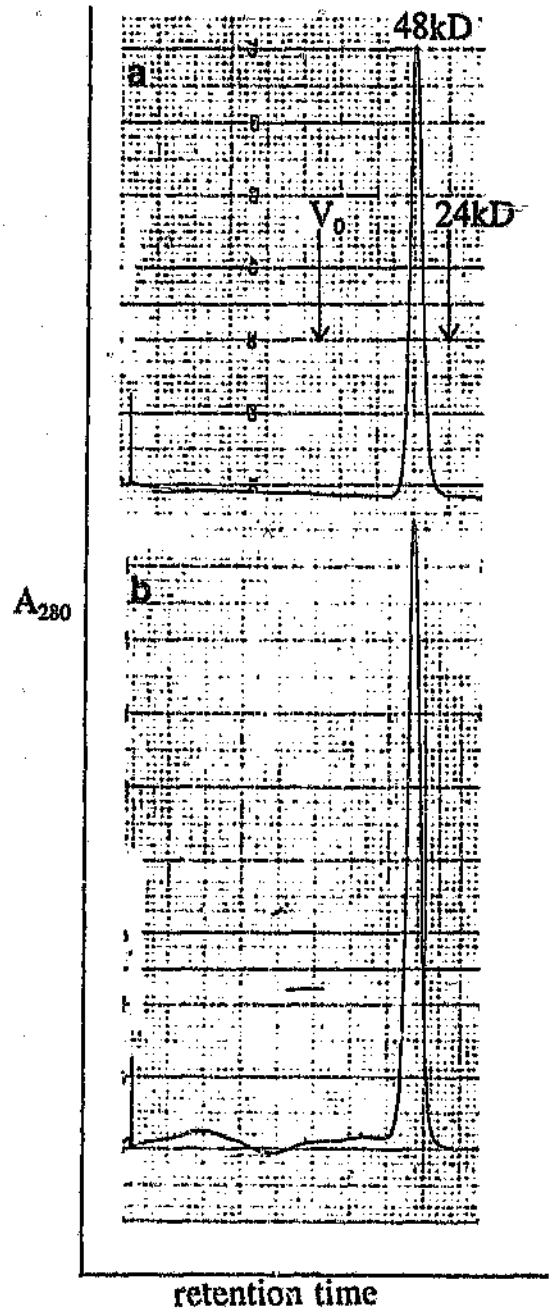


Figure 30. Elution profile of pGSTP1-1 from the SEC-HPLC column in the absence (a) or presence (b) of 50μM BSP.

dimeric protein, did not occur when BSP bound to the protein.
Furthermore, the absence of a shift in the maximum emission wavelength of the protein's fluorescence upon addition of saturating concentrations of BSP also suggests that no major conformational change occurs when BSP binds the enzyme.

3.3.6 Cys45 modification by DTNB

As mentioned above in section 1.2.6, when GSH binds to pGSTP1-1 it appears to induce a small local conformational change which is translated through the protein and results in the burial of Cys45 into the protein molecule. Cys45 is located in domain 1 between helix α2 and strand ß3. In the uncomplexed enzyme, the thiol group of Cys45, is exposed to the solvent and is highly reactive. It can therefore be chemically modified by 5,5'-dithiobis(2-nitrobenzoate) (DTNB) as shown in Figure 19. In the presence of GSH analogues (such as GSO_3^-), however, the reaction with DTNB is retarded because of the burial of Cys45. However, because the entire system is in a dynamic equilibrium with the GSH analogue, Cys45 is continuously fluctuating between buried and exposed states. When it is exposed, and is irreversibly chemically modified, the adduct becomes too large to be buried again and the enzyme loses its activity because it loses its ability to change into the enzymatically active conformation.
As shown in Figure 31, the presence of glutathione sulphonate (GSO_3^-) retards the reaction between the thiol group of Cys45 and DTNB. This is to be expected assuming that GSO_3^-, a competitive inhibitor, binds the protein molecule by a similar induced-fit mechanism as does GSH.

In the presence of 5μM BSP (76% saturation of the enzyme) however, the reaction rate for the chemical modification of Cys45 remained unchanged (Figure 31). It is thus clear that BSP binding to the protein does not have any influence on the exposure of Cys45 and does not induce the same conformational change on the protein as does GSH.

3.3.7 BSP binding in the presence of GSH

It is interesting to note that in the presence of GSH, the affinity of BSP for the protein increases about two-fold (Figure 32). A similar observation was also made by Caccuri *et al.* (1990) when they observed that the affinity of haem for the class Pi placental isoenzyme also

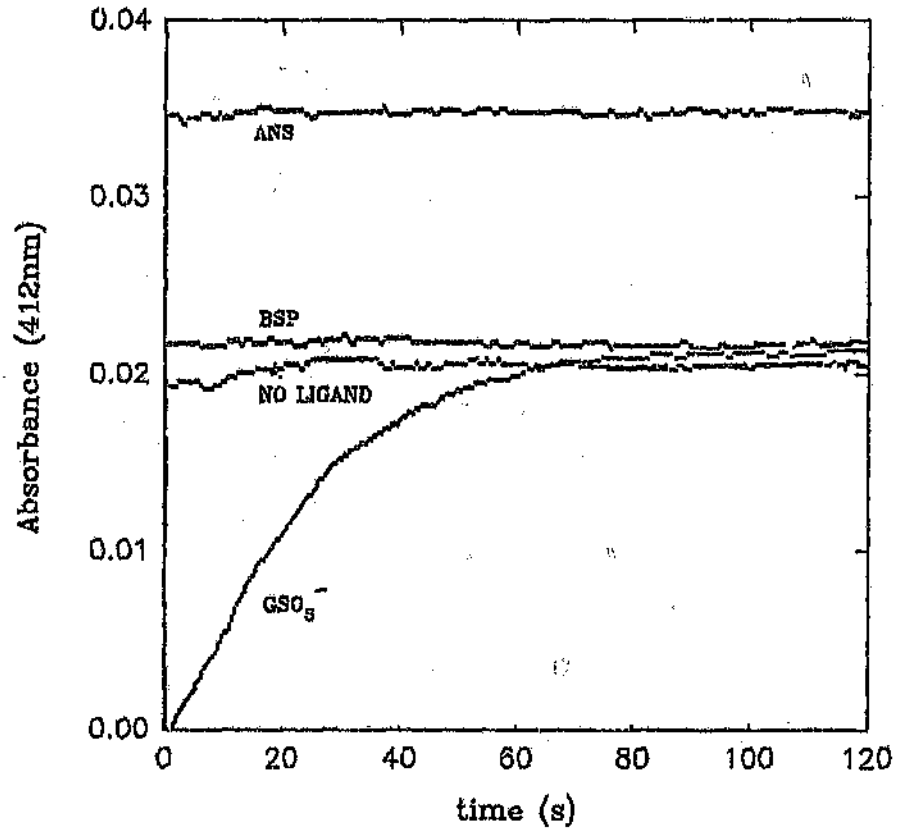


Figure 31. Reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with Cys45 in pGSTP1-1 (1μM) in the absence or presence of 5μM BSP; 100μM ANS or 100μM glutathione sulphonate.

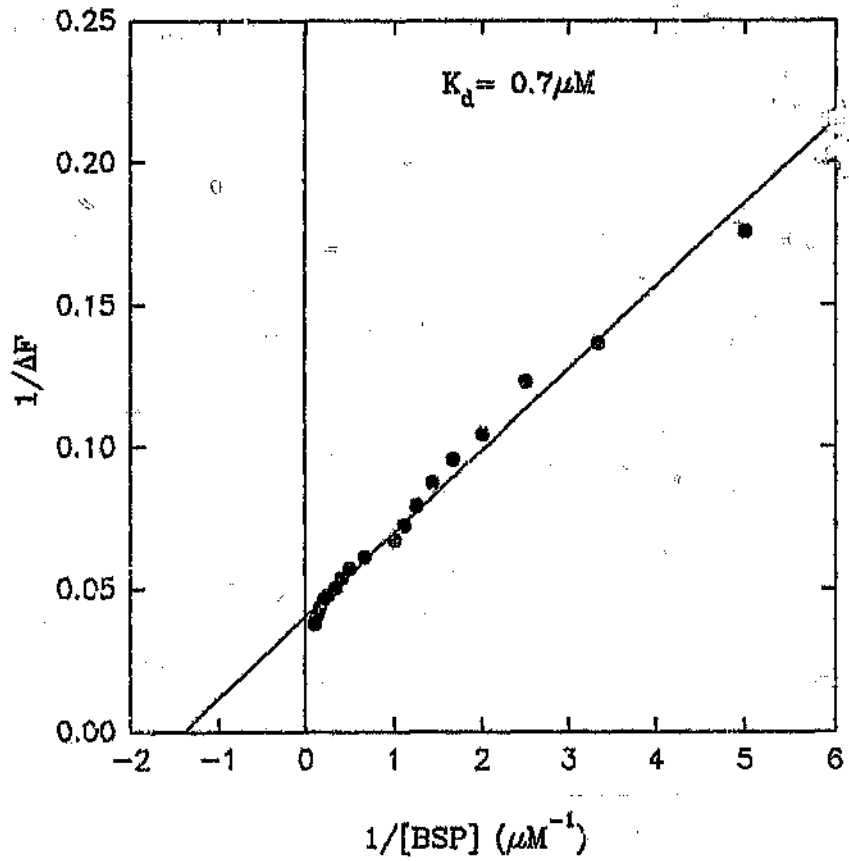


Figure 32. Determination of the dissociation constant, K_d, of BSP in the presence of 10mM glutathione using the fluorescence quenching technique (section 2.6.2).

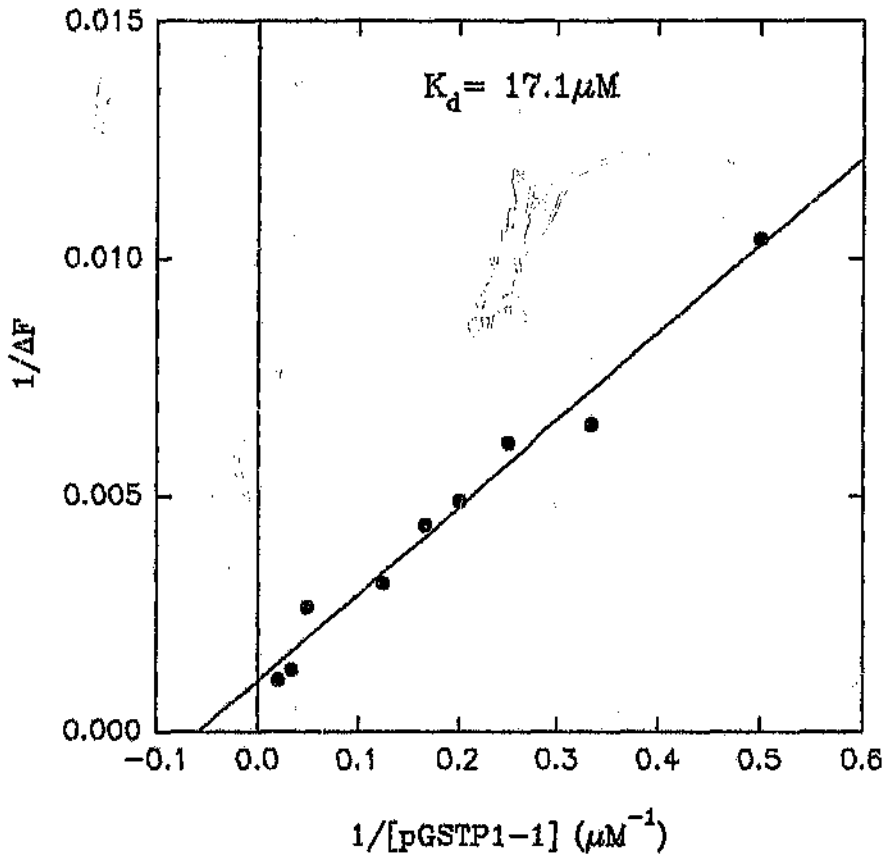


Figure 33. Determination of the dissociation constant, K_d, of ANS using the fluorescence enhancement technique (section 2.6.1).

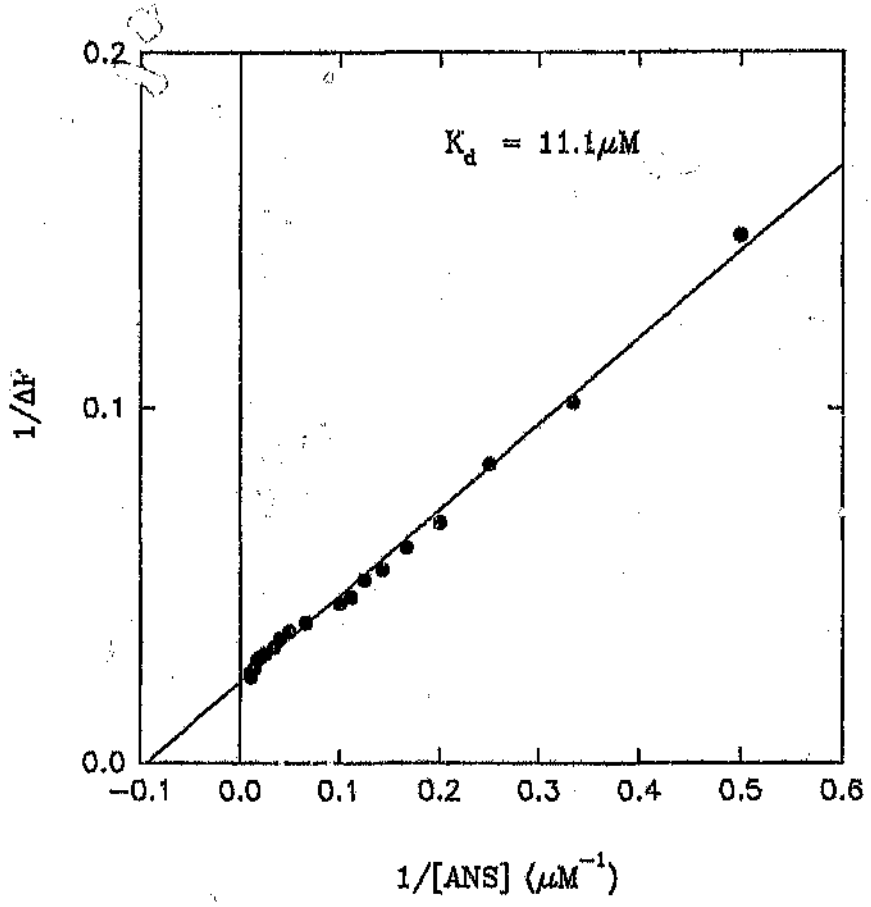


Figure 34. Determination of the dissociation constant, K_d, of ANS using the fluorescence quenching technique (section 2.6.2).

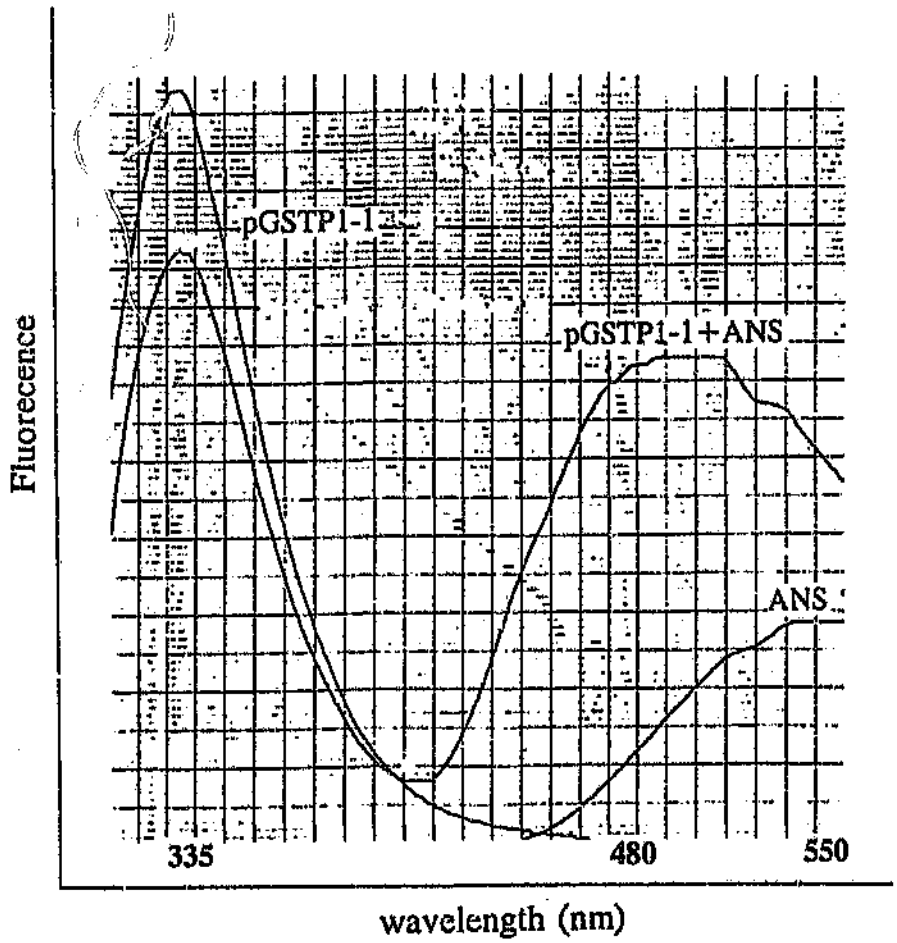


Figure 35. Energy transfer from pGSTP1-1 tryptophan residues to bound ANS. The maximum emission wavelength of pGSTP1-1 is at 335nm and is not affected by the presence of ANS, whereas the maximum emission wavelength of ANS is blue-shifted from 550nm to 480nm upon binding to the protein.

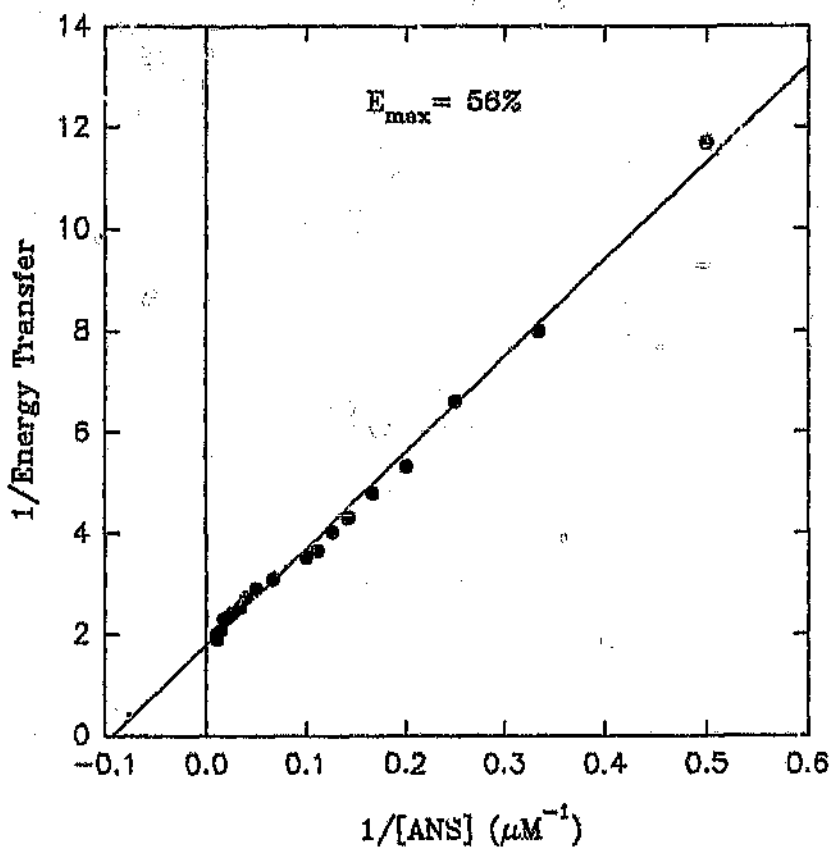


Figure 36. Efficiency of energy transfer between pGSTP1-1 tryptophan residues and bound ANS. The efficiency of transfer, determined from the y-axis intercept, is about 56%.

increased in the presence of GSH. These results are in accord with the fact that the GSTs are detoxification proteins with the ability to bind a wide range of non-substrate ligands and yet are always saturated by GSH in the cell due to the high intracellular concentrations of GSH (1-10mM). It is possible that the induced-fit mechanism for GSH binding which results in the catalytically functional conformation of the protein is also the preferred conformation for the binding of various non-substrate ligands.

3.4 8-ANILINO-1-NAPHTHALENE SULPHONIC ACID AS A LIGAND FOR pGSTP1-1

3.4.1 ANS binding

The binding of ANS to pGSTP1-1 was observed by two different fluorescence methods. Firstly, the enhanced fluorescence of ANS upon binding to the enzyme was monitored (section 2.6.1) and secondly the quenching of the intrinsic tryptophan fluorescence of the protein was monitored (section 2.6.2) upon ANS binding. The dissociation constants obtained for the different methods were similar (17.1μM for the fluorescence enhancement procedure (Figure 33), and 11.1μM for fluorescence quenching (Figure 34)). Using the fluorescence enhancement procedure, the ligand : protein ratio decreased progressively at increasing concentrations of the protein. This procedure is therefore used in monitoring ligand binding to the primary binding site of the enzyme exclusively. Nishihira *et al.* (1992a) obtained a dissociation value of 15μM for a class Pi isoenzyme, also using fluorescence enhancement of ANS as a technique.

ANS emits at a maximum wavelength of 550nm when it is not bound to GST. However, when it does bind a hydrophobic region in the protein, a blue shift to 480nm is observed in the maximum emission wavelength of ANS (Figure 35).

Quenching of tryptophan fluorescence by ANS occurs as a consequence of a transfer of excitation energy from tryptophan to ANS. This is possible because of the overlap of the emission spectrum of the protein's tryptophan residues (donor) and the absorption spectrum of ANS (acceptor) (Figure 17). The efficiency of the energy transfer was estimated to be about 56% (Figure 36). Should tryptophan be involved in the binding of ANS to its primary site, then the energy transfer would be expected to be much greater.

increased in the presence of GSH. These results are in accord with the fact that the GSTs are detoxification proteins with the ability to bind a wide range of non-substrate ligands and yet are always saturated by GSH in the cell due to the high intracellular concentrations of GSH (1-10mM). It is possible that the induced-fit mechanism for GSH binding which results in the catalytically functional conformation of the protein is also the preferred conformation for the binding of various non-substrate ligands.

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Therefore, the decrease in the fluorescence of the protein is not the result of a major conformational change induced by the binding of ANS, as suggested by Nishihira *et al.* (1993) and described below, but rather as a result of an energy transfer between the protein and the ligand. Furthermore, the absence in a shift of the maximum emission wavelength of the protein in the presence of ANS also indicates that no gross conformational changes occur when ANS binds the enzyme.

3.4.2 Acrylamide quenching in the presence of ANS

The quenching by acrylamide of the protein's tryptophan fluorescence was not affected by the presence of ANS (Figure 37). The quenching constant as well as the fraction of accessible tryptophan fluorescence remained essentially unchanged in the presence of ANS. This result suggests that ANS, like BSP, does not bind to a region in close proximity to either of the tryptophan residues.

3.4.3 σ-complex and ANS

The interaction of 100μM ANS with the enzyme complexed with the Meisenheimer complex (85% saturation of the enzyme) resulted in less than a 10% decrease in the σ-complex (Figure 29). This result indicates that ANS has a very small effect on the displacement of the σ-complex and hence on the active site.

3.4.4 Cys45 modification by DTNB in the presence of ANS

The presence of 100μM ANS (85% saturation of the enzyme) did not affect the rate of the reaction between DTNB and Cys45 (Figure 31). ANS binding to the protein, therefore, does not influence the exposure of Cys45 and does not induce the conformational changes on the protein as does GSH or its analogues.

Nishihira *et al.* (1993) reported that upon the addition of ANS to a class Pi GST, rGSTP1-1, the ß-sheet content decreased from 34.0% to 5.2% and that the α-helix content increased from 25.2% to 38.1%. If this were true, then a near-complete disruption of domain 1 would occur. This would be observed in a shift in the maximum emission wavelength of the protein upon binding to ANS. Addition of ANS would also result in an increased or decreased exposure of the tryptophan residues found in domain 1, which would be detected using

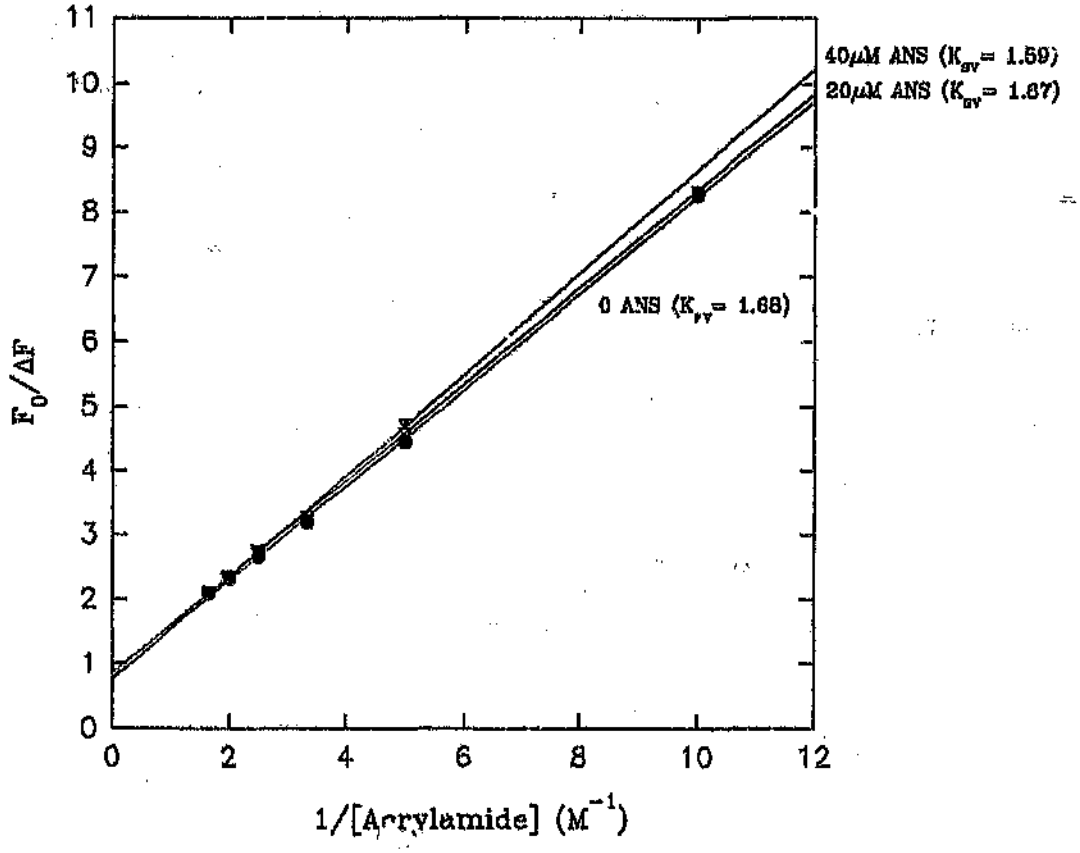

Figure 37. Acrylamide quenching of pGSTP1-1 tryptophan fluorescence in the absence or presence of ANS. The y-intercept 1/f (inverse of fractional fluorescence) and the quenching constant K_{sv} essentially remained constant.

acrylamide quenching. Furthermore, the σ-complex in the active site of the enzyme would be completely displaced in the presence of ANS. ANS, however, was found to have very little, if any, effect on the protein's structure at saturating concentrations of the ligand.

3.4.5 ANS and BSP competition for the same primary binding site

Fluorescence studies have shown that BSP and ANS share a common primary binding site in pGSTP1-1 (Figure 38). Previous researchers have also shown a common site for BSP and bilirubin in rGSTA1-2 (Bhargava and Dasgupta, 1988; Boyer, 1986), and for haem and bilirubin in the class Pi placental enzyme (Caccuri *et al.*, 1990). It is thus possible that GSTs have a single site which accommodates a wide variety of anionic non-substrate ligands. Nishihira *et al.* (1992 a,c) determined the primary binding site to be the same as that for fatty acids and bilirubin in a class Pi enzyme. They determined the site is in domain 2 in the region of Cys169. They then proposed a different location for the primary binding site for ANS as the hydrophillic/amphipathic V-shaped cavity at the subunit interface of the protein molecule (Nishihira *et al.*, 1993).

However, the results found in this study, which seem to be in agreement with those of Bhargava and Dasgupta (1988) in that primary binding site shared by ANS and BSP, appears to be in domain 1 of pGSTP1-1 but not in the vicinity of either of the tryptophan residues.

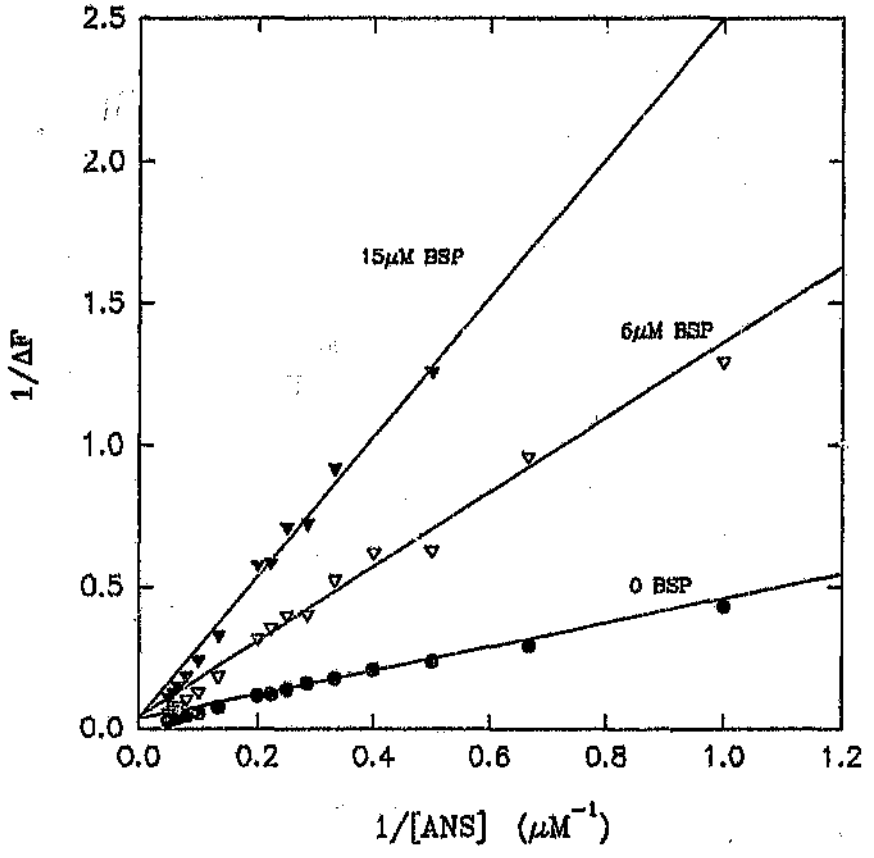

Figure 38. Competition studies between BSP and ANS for the binding to a common primary site, at increasing concentrations of ANS and at 0, 5 or 15μM BSP.

3.5 CONCLUSIONS

According to the data described in this work, it can be concluded that neither BSP nor ANS induce gross conformational changes in pGSTP1-1 upon binding to the protein. The two anionic ligands share a common primary binding site which is not the active site, but is probably also located in domain 1 of the protein. ANS does not appear to exert any significant effect on the active site. On the other hand, BSP binding to the enzyme does have an effect on the active site of the protein molecule, especially after saturating the primary site for the ligand. It has not been established whether this effect is a result of a local conformational change in the active site or whether this site is a secondary binding site for BSP.

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