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Class pi glutathione S-transferase: unfolding and conformational stability in the absence and presence of G-site ligands

Julija Erhardt

A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 1996

DECLARATION

I declare that this thesis is entirely my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other University

Julija Erhardt

Julija Erhardt

4 day of october 1996

The results from the thesis have been published in the following articles:

Erhardt, J. and Dirr, H. (1995) Native dimer stabilizes the subunit tertiary structure of porcine class pi glutathione S-transferase. *Eur. J. Biochem. 230*, 614-620.

Erhardt, J. and Dirr, H. (1996) Effect of glutathione, glutathione sulphonate and S-hexylglutathione on the conformational stability of class pi glutathione S-transferase. *FEBS lett. 391*, 313-316.

This work is dedicated to my family:

Robert,

Filip,

Petra and

Luka.

ABSTRACT

The glutathione S-transferases (GST) are a supergene family of homo- or heterodimeric Phase II detoxification enzymes which catalyse the S-conjugation between glutathione and an electrophilic substrate. The active site can be divided into two adjacent functional regions; a highly specific G-site for binding the physiological substrate glutathione and a nonspecific H-site for binding nonpolar electrophilic substrates.

Unfolding of porcine class Pi isoenzyme (pGSTP1-1) was monitored under equilibrium conditions using different physicochemical parameters. The coincidence of unfolding curves obtained with functional and structural probes, the absence of thermodynamically stable intermediates such as a folded monomer, and the dependence of pGSTP1-1 stability upon protein concentration, indicate a cooperative and concerted two-state unfolding transition between native dimeric pGSTP1-1 and unfolded monomeric enzyme.

Equilibrium and kinetic unfolding experiments employing tryptophan fluorescence and enzyme activity measurements were preformed to study the effect of ligand binding to the G-site on the unfolding and stability of the porcine class pi glutathione S-transferase against urea. The presence of glutathione caused a shift in the equilibrium-unfolding curves towards lower urea concentrations and enhanced the first-order rate constant for unfolding suggesting a destabilisation of the pGSTP1-1 structure against urea. The presence of either glutathione sulphonate or S-hexylglutathione, however, produced the opposite effect in that their binding to the G-site appeared to exert a stabilising effect against urea. The binding of these glutathione analogues also reduced significantly the degree of cooperativity of unfolding indicating a possible change in the protein's unfolding pathway.

ACKNOWLEDGEMENTS

I would like to thank:

Prof Heini Dirr for all his help and support;

Prof Horst Klump for the DSC analysis of pGSTP1-1;

The members of the Department of Biochemistry, University of the Witwatersrand;

The University of the Witwatersrand and the South African Foundation for Research Development (FRD) for their financial support.

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ABBREVIATIONS

A	absorbance
A_{280}, A_{260}	Absorbance at 280 and 260nm, respectively
ANS	8-anilino-1-naphthalene sulphonic acid
ΔASA	change in solvent accesible surface area in proteins
CDNB	1-chloro-2,4-dinitrobenzene
ΔCp	heat capacity change
D	dimeric, folded, form of protein
DSC	differential scanning microcalorimetry
EDTA	ethylenediaminetetra-acetic acid disodium salt
F	fluorescence intensity
Fmax	maximal fluorescence signal reached
Fobs	fluorescence intensity at a particular time
ΔG	Gibbs free energy change
GSH	reduced glutathione
GST	glutathione S-transferase
GdnHCl	guanidinium chloride
G-site	glutathione-binding site
ΔH	enthalpy change
H-site	hydrophobic electrophile-binding site
GSO_3^-	glutathione sulphonate
Kd	dissociation constant
Keq	equilibrium constant
M	folded monomer
Mr	relative molecular mass

N	native dimeric form of protein
NaCl	sodium chloride
NaN_3	sodium azide
NMR	nuclear magnetic resonance spectroscopy
ΔS	entropy change
SEC-HPLC	size exclusion high pressure liquid chromatography
U	unfolded monomer
UV	ultraviolet

pGST P1-1, hGST P1-1 etc. are acronyms for the glutathione S-transferases (GST), the prefix indicating the species (p, porcine; h, human), while P indicates gene class pi; 1-1 indicates a dimer of two type-1 subunits.

The IUPAC-IUBMB three letter codes for amino acids were used.

Enzyme. Glutathione S-transferase (EC 2.5.1.18.)

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INTRODUCTION

1.1.Conformational stability and the (un)folding of proteins.

1.1.1.Conformational stability of proteins

1.1.1.1. Introduction

Conformational stability of proteins can be defined as the difference in the free energy between the folded and unfolded conformations under physiological conditions, and denoted as $\Delta G(H_2O)$. The conformational stability of almost all naturally occuring globular proteins is very low; between 5-15 kcal/mol, for monomeric proteins. It appears to be advantageous to living organisms to have proteins for which the folded, biologically active conformation is only marginally more stable than the unfolded inactive conformation (Baldwin and Eisenberg, 1987; Dill, 1987; Pace, 1990). The optimization in the course of evolution was obviously based on function instead of stability. Proteins from the organisms which live under extreme physical conditions show that evolution could have generated more stable proteins. Adaptation to these environments of extreme temperature, pH, salinity and hydrostatic pressure are not connected with big structural changes in the protein molecule. Enhanced intrinsic stability requires only small changes, so it is difficult to establish general strategies of stabilisation (Jaenicke and

Závodszky, 1990; Jaenicke, 1991).

The problem of protein stability has attained big industrial significance in the past ten years. Reasons in various fields of biotechnology are: preservation in food technology, long-term operation and regeneration of biological catalysts in enzyme technology, enzyme kits in medical diagnostics, biosensors in analytical chemistry, solubilization and nativation of recombinant proteins harvested from "inclusion bodies" etc. Studying protein stability has big importance, as from the point of view of improving the fundamental knowledge about processes in the cells, but also as a problem of great importance for the industry and the medicine (Jaenicke, 1988).

1.1.1.2. Forces that stabilise protein conformation

The thermodynamic stability of globular proteins is the sum of large contributions of diverse stabilizing and destabilizing interactions involved in the formation of the folded compact state (Pace, 1975; Privalov, 1979). The stabilising interactions are principally due to the hydrophobic effect, and destabilising due to loss of conformational entropy of the folded protein, but practically all types of molecular interactions are involved in the delicate balance of forces responsible for the native structure of the protein. The understanding of protein stability is complicated by size and complexity of macromolecules, but also by need to account for even the smallest energy change that can influence either folded or unfolded conformations (Lim and Sauer, 1991; Pace,

1992; Stickle et al., 1992; Rose and Wolfenden, 1993; Hendsch and Tidor, 1994).

The Gibbs free energy of unfolding can be separated into its entropic and enthalpic components. Loss of conformational entropy is the major force opposing protein folding. Although a parameter of principal importance for protein stability, many controversies remain regarding its estimation (Makhadatze and Privalov, 1996) The enthropy of protein unfolding in aqueous medium include two components, the first one being associated with increase of configurational freedom in the polypeptide chain.The rotation around the phi and psi bonds in a folded protein form is very limited, so unfolded protein form, where rotation about these bonds are much less restricted, should be favorable compared to the folded. Restricting the number of accessible side-chain conformers in the native structure, also results in a considerable loss of entropy. Second component of entropy of protein unfolding is associated with the hydration of groups that become exposed on unfolding (Dill, 1987; Dill and Shortle, 1991; Doig and Sternberg, 1995).

The driving force for protein folding was initially thought to be intramolecular hydrogen bonding. This was Pauling's view (see Dill, 1990) , but it was reappraised, and hydrogen bond came to be perceived as energetically neutral, or even unfavorable (Jaenicke, 1991). The most common secondary structures of folded proteins are hydrogen bonded alpha helices and beta sheets. The question is whether the ubiquity of hydrogen bonds in the folded proteins means that they stabilize the folded forms of proteins relative to the denatured, unfolded forms. In an unfolded protein, the groups capable of hydrogen bonding will generally

be completely hydrogen-bonded to water. When the protein folds, about half of the groups form intramolecular hydrogen bonds, most of the rest will remain hydrogen-bonded to water and only a few or none will be left with unpaired hydrogen-bond donors or acceptors. On the basis of experimental studies Fersht (1987) has suggested that intramolecular hydrogen bonds will be favoured over hydrogen bonds to water by 0.5-1.8 kcal/mol, depending on differences in the geometry and environment of a particular hydrogen bond. If hydrogen bonds fail to form intramolecularly, loss of stability in the protein molecule is about -4.2 kcal/mol. This is probably the reason why the extent of hydrogen bonding in globular proteins is so high and why they have such a high content of secondary structures like alpha helices and beta sheets in which the peptide N-H and C=O groups are completely hydrogen-bonded. Finally, hydrogen bonds are crucial in determining the secondary and tertiary structures of proteins. Because there are many such bonds, and because they tend to form cooperatively, their overall effect can be significant (Pace et al., 1991; Stickle et al., 1992; Rose and Wolfenden, 1993).

The interactions which are thought to be causing the folding of the proteins, are hydrophobic (Pace, 1992; Kellis et al., 1988). In the course of folding apolar residues tend to avoid contact with the solvent and to form an apolar core of the protein. In order to estimate the contribution of the hydrophobic interaction to the stability of globular proteins we need to know the free energy change for the transfer of a given nonpolar group from water to the interior of a globular protein. Measuring the free energy change for the transfer of the amino acid side chains from nonpolar environments such as n-octanol, hexane, or N-methylacetamide

to water are often chosen as models. The main opposition to these models is that no single solvent system can represent the heterogeneous interior of a globular protein. The isothermal Gibbs energy changes for the transfer of a hydrocarbon from an aqueous solution to a nonpolar solvent is negative in all cases, showing that it is a spontaneous process. Also, the hydrophobic effect contributes a large negative entropy to folding, because the unfolding of the protein leads to the ordering of water at the surface of the apolar groups in the protein. Recent studies suggest that each -CH2- group buried into the protein core contributes 1.3 (+/- 0.5) kcal/mol to the stability of a globular protein (Dill, 1990; Pace et al., 1991; Pace, 1992; Sandberg and Terwilliger, 1989).

Van de Waals interactions may play an important role in protein folding, but its magnitudes are very difficult to assess (Dill, 1990). Electrostatic interactions are another force which are important in protein structure. There are two different ways in which electrostatic interactions influence protein stability. Firstly, nonspecific repulsions arise when a protein is highly charged, for example at extreme pH values. The second way in which electrostatic interactions can affect the stability of the proteins is by specific charge interactions. When oppositely charged amino acid side chains are in close contact, ion pairing occurs, that can stabilize the folded protein Pace et al., 1991; Dill, 1990; Hendsch and Tidor, 1994).

Depending on their size and compartmentation, proteins may require covalent bonds such as disulfide bridges as additional stabilizing elements in order to maintain their functional state (Creighton, 1988; Creighton, 1992).

One further mechanism causing enhanced stability of proteins is the combination of polypeptide chains to form oligomers. In some cases quaternary structure make an important contribution to the conformational stability of the subunits (Garel, 1992; Jaenicke, 1991; Miller et al., 1987; Janin and Chothia, 1990) .

1.1.1.3. How can protein stability be affected?

Proteins that have evolved for the survival benefit of an organism may not exhibit features desirable for *in vitro* applications. Mutagenesis experiments continue to show that proteins are surprisingly tolerant of amino acid substitutions - only a fraction of the amino acids in a protein are critical for function, folding or stability. This sequence flexibility can be exploited to create proteins with features not previously developed for function *in vivo* (Jaenicke, 1988). Increasing stability by protein engineering is a approach that will extend the repertoire of protein activities as well as the environments in which they can be applied. Site-directed mutagenesis studies of diferent proteins have provided new insight into the nature and contribution of different forces to protein stability. As a result, it is becoming possible to construct proteins that are much more stable than the wild-type protein, (or even to construct the proteins with new features).

Different approaches can be used to construct more stable proteins, but it is important to distinguish the basis of the instability of the normal protein and to mutate the susceptible groups (Fágáin, 1995).

Protein stability might be enhanced either by increasing the stability of the folded protein, or by decreasing the stability of the unfolded form, or by a combination of both. Mutations that restrict or enhance conformational degrees of freedom can change the free energy of the unfolded state (Matthews, 1993). Introducing new S-S bridges into the protein molecule is the most obvious way to reduce the number of conformations possible for unfolded state. Some attempts to stabilize protein in that way have, however, been unsuccesful (Creighton, 1983). Main reason is because of covalent character of disulfide bond, there are strict stereochemical requirements for the relative positions and orientations of the two participating Cys residues. Introducing Cys residues by mutagenesis can thus, instead have a de-stabilizing effect because it can disrupt favorable interactions initially present (Clarke et al., 1995; Creighton, 1992).

Another way to decrease entropy of the unfolded state and in that way stabilize the protein is to replace amino acid residues which have high conformational flexibility (e.g. Gly, Ser, Ala) with more "rigid" amino acids (like, Thr, Val, Pro). Such mutations can only be made at positions that neither change the conformation of the main chain structure, nor introduce unfavorable contacts with neighbouring side chains (Fágáin, 1995).

Proteins can also be stabilized by affecting their hydrophobic core. Protein atoms in the protein core are very tightly packed (Dao-pin, 1991; Eriksson et al, 1993; Baldwin and Matthews, 1995). When a larger hydrophobic residue within the core replaced with a smaller one, many different effects can occur. The reduction in size of the residue in

question will reduce its contribution to the stability through a hydrophobic effect. At the same time the van der Waals interactions will be affected. These will be influenced by the way in which the surrounding protein structure relaxes in response to the amino acid replacement. The energetic change from this replacement should be proportional to the decrease in the number of hydrophobic contacts. Studies of destabilizing mutants in barnase, for eg., where cavities have been engineered into the hydrophobic core by mutations such as Ile to Val or Phe to Leu show that the introduction of a cavity size of one -CH2- group destabilizes protein by about 1 kcal/mol. Hence, to stabilize the protein by affecting the hydrophobic core, the cavities, if they are present, would need to be filled by non-polar groups, or some polar residues existing inside the molecule could be replaced with nonpolar ones (Eriksson et al., 1992; Lim et al., 1992; Mark, 1992; Lee and Levit, 1991).

Protein stability can be affected by influencing the electrostatic and polar interactions in the molecule. Unpaired polar or charged residues rarely occur inside the protein molecule. However, if found, they can be satisfied by the presence of polar or oppositely charged residues in the vicinity that have been introduced by amino acid replacement, to form a new, stabilizing hydrogen bond, or salt bridge . External electrostatic interactions contribute little to the overall stability (less than 0.5 kcal/mol per ionic par). Greater stabilization can be achieved if a charged residue is introduced in the vicinity of an α-helix to compensate for their unsatisfied dipole (Dill, 1990; Pace et al., 1991).

Mechanism of enhanced thermal stability involve improved

packing density, as well as specific local interaction (Mozhaev, 1993). Halophilic proteins differ from nonhalophilic in their increased number of acidic amino acids, and their decrease in hydrophobicity. But the molecular basis of acidophilic, alkalophilic and barophilic adaptation is still obscure (Jaenicke, 1991a).

1.1.2. Protein (un)folding

1.1.2.1 Introduction

Over 30 years ago Anfinsen and his colleagues demonstrated that the amino acid sequence is the primary determinant of the three-dimensional structure of a folded protein (see Baldwin and Eisenberg, 1987; Szulmajster, 1988; Moult and Unger, 1991). This observation stimulated numerous efforts to define the rules that govern the folding reaction. The importance of deciphering the folding code lies in the potential applications: Prediction of the three-dimensional structure of a protein from its amino acid sequence; Alteration of the amino acid sequence of naturally occuring proteins to increase stability, enzymatic activity, or to alter molecular recognition properties; Design of new proteins which catalyse reactions not found in nature; Optimization of the recovery of recombinant proteins from inclusion bodies by denaturation and renaturation under conditions favouring productive folding pathways, etc (Jaenicke, 1995).

The Levinthal paradox, i.e. how does the polypeptide chain search

through conformational space to find the lowest energy minima corresponding to the native state in a finite time, despite the immense number of conformations accessible to the unfolded polypeptide chain, remains a central issue in the protein folding problem (see Karplus and Shakhnovich, 1992). *In vitro*, many proteins fold to their native conformations spontaneously, without the additional input of energy or the presence of extrinsic factors. A complete search of all conformations is not possible at the physiological timescale even for the smallest proteins. It is clear that the search is simplified, suggesting that protein folding follows specific pathways.

Two views of the folding process have developed over the years (Baldwin and Eisenberg, 1987; Dill, 1987): In the first view, only thermodynamic stability determines the conformation of a folded protein. If this view is correct, it follows that the kinetic pathway of folding must be such that the most stable product is formed. Moreover, it should be possible in principle to predict the folding of a protein from its amino sequence by energy minimization. The second view of folding is that the conformation of the final product is determined by the kinetic pathway of folding. Folding occurs by the fastest route available, and the final folded conformation could be determined by the folding pathway without necessarily reaching the thermodynamically most stable structure (Baldwin and Eisenberg, 1987; Dill, 1987; Jaenicke, 1991; Matthews, 1991,1993a).

An understanding of protein folding pathways requires the identification and structural and energetical analysis of the all species on the pathway. Apart from the definition of native and unfolded states of

protein, the intermediates that define and direct the pathway must be identified, but these are usually thermodynamically unstable (Parker et al., 1995; Fersht, 1995).

1.2.2.The folded state

The native form of a protein is not a single state, but a collection of states that are structurally very similar, but are separated by measurable energy barriers (Figure 1) (Creighton, 1978). These conformational substates arise from: fluctuation that may involve the rotation of side chains, vibration of bonds and making/breaking of hydrogen bonds and Van der Waals contacts. As a result of these structural fluctuations a protein can be considered a macroscopic ensemble of a large number of closely related microstates. Proteins may also exist in different macroscopic structural states each macrostate having distinctly different folding pattern (in at least part of a structure). Different states of aggregation of a protein may also exist. In addition to these different structural states, a protein may also exist in various states of protonation. All different states (structural, aggregation, protonation) of a protein may be characterized by a different enthalpy level (Janin and Wodak, 1983; Richards, 1992).

Although the native state is the one that governs protein function, the reminder of the conformation space play an essential role as well, since it corresponds to the denatured state and the reaction path or paths between the denatured and the native state that lead to protein folding and

unfolding.

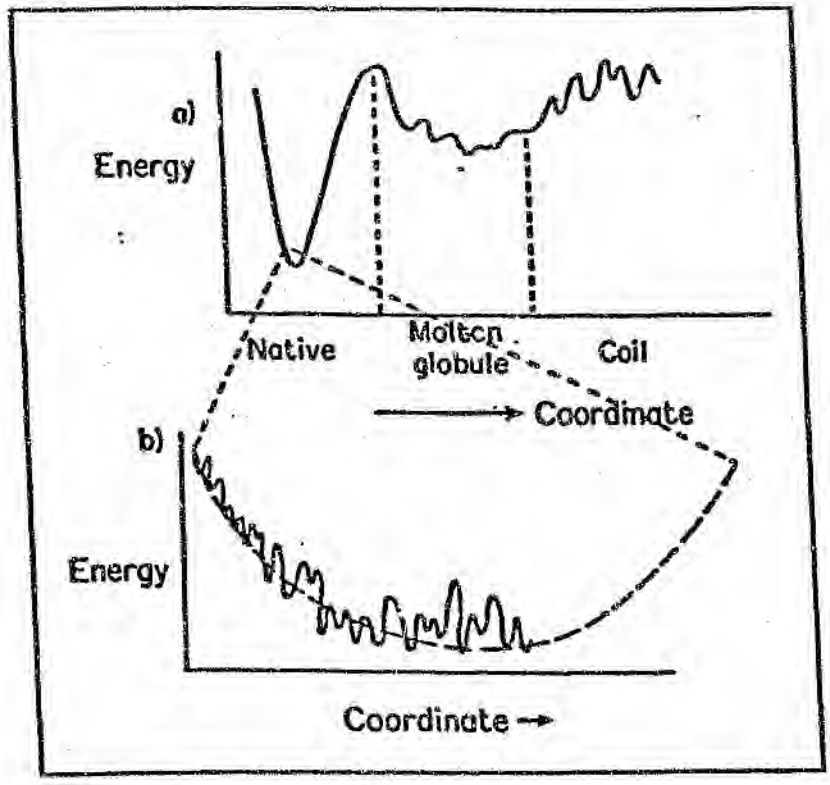


Figure 1. Schematic representation of the configuration space of a protein giving the energy as a function of a configurational coordinate: (a) complete space; (b) enlarged view in the vicinity of the native state (Karplus and Shakhnovich, 1992).

1.2.3. The unfolded state

The unfolded state comprises a specific subset of denatured states of protein with highly opened and solvent-exposed conformations with little or no residual structure (Dill and Shortle, 1991). Such states are generally obtained under strongly denaturing conditions. The NMR spectra under the various denaturing conditions shows that, what is called the "unfolded protein", has often persistence of structure. There is residual structure even under conditions leading predominantly to the random-coil state. Any such structure is only marginaly stable and is undoubtely rapidly fluctuating between conformational states. Even in these unfolded conformation, however, peptide bond isomerisation and disulphide interchange reactions (see below: folding *in vivo*) are much slower processes than the torsional conformational shifts (Richards, 1992; Creighton, 1978). Dill and Shortle (1991) defined denatured state under physiological conditions, D_0, as the most important denatured state which is in equilibrium with native state under physiological conditions. (Conformational stability of proteins being defined as the difference in free energy between the folded and unfolded conformations under physiological conditions). The structure of the D_0 state is far from random coil, but appear to be compact with extensive secondary structure. Just as with native state, structure of this physiologically important state seems to depend on the amino acid sequence.

The degree of departure from a two-state unfolding/refolding mechanism depends on the protein and on the denaturant. Unfolded stated obtained by using urea and guanidinium chloride are often more

completely unfolded than the products of pH and thermal denaturation (Matthews, 1993a).

The most frequently used protein denaturants are pH, heat, urea and guanidine hydrochloride. 6M GdnHCl and 8 M urea appear to be more effective in disrupting the native conformation of proteins, than pH or heat. Presently, it is still uncertain whether the action of these agents on proteins is direct and can be regarded as ligand binding, or if it is indirect and involves a change in the properties of solvent in their presence. Effects of urea and GdnHCl was initialy focused on their potential for hydrogen binding, and they were considered to act by breaking protein hydrogen bonds. Recent studies show that denaturation caused by these two denaturants are caused more because they decrease the magnitude of the hydrophobic interaction in the proteins (Makhatadze and Privalov, 1996; Kamoun, 1988).

Products of thermal and pH denaturation are very often referred to as compact denatured states, "A" states, molten globule states, or folding intermediates. Many proteins unfold at pH values less than about 5 or greater than 10. Unfolding at such extremes of pH usually occurs because the folded protein has groups buried in nonionized form that can ionize only after unfolding. The general electrostatic repulsion between the ionized groups on a surface of a protein might also tend to cause unfolding when the protein has substantial net charge because such repulsion would be minimized in the unfolded state (Yang and Honig, 1993; Yang and Honig, 1994). At elevated temperatures, the intensity of noncovalent forces which maintain the native structure of a protein become lower than increase entropy, and protein loses most of its ordered

structure (Dill, 1987; Mozhaev, 1993; Matthews, 1993a).

1.2.4. Intermediate states in protein (un)folding

Determination of the structure of the transition states is an essential step in the experimental analysis of the protein folding (Parker et al., 1995; Fersht, 1995). Even when the three-dimensional structure of a protein is solved (by X-ray crystallography or NMR methods) it is not straightforward to infer information about the folding pathway from final state of the protein. Thornton et al. (1995) compares protein folding problem ..."to the problem facing astrophysicist, who must deduce information about the origin of universe from the current state of galaxies". Analysing transition states in protein folding presents a puzzle which requires a different approach. Very often intermediates are just transiently present and in low stoichiometric amounts, so that they are difficult to study. Frequently, they only might be detectable as kinetic intermediates. The intermediates isolated during the folding process or at equilibrium under partially denaturing conditions may be side products or dead ends (Fersht, 1994). Some examples suggests that kinetic and equilibrium intermediates, when present, have similar structural features. The five-millisecond folding intermediate of myoglobin is found to be similar to the structure of equilibrium molten globule at low pH (Jennings and Wright,1993). Similar results were obtained for the acid-induced form of cytochrome c (Elove et al., 1988; Jeng et al., 1990).

Some intermediates on folding pathway has a relatively high

activation barrier that permits its isolation and study. Isomerisation of the peptide bond especially a preceding a proline residue or oxidation/reduction of the covalent disulfide bond are often processes that result in the accumulation of intermediate states (Freedman, 1991; Schmid et al., 1991).

A powerful method for analysing transition state structure at the level of individual residues is site-directed mutagenesis. By comparing the thermodynamics and kinetics of folding of proteins that differ by only one or a few amino acid residues, the roles of individual residues and interactions can be tested (Fersht, 1993,1994).

Two other experimental approaches offer the greatest potential to resolve the properties of kinetic intermediates (Parker et al., 1995). The first combines pulse protection studies with NMR (Radford et al., 1992), and the other involves electrospray mass spectrometry (Miranker et al., 1993)

Presently, many of the different intermediate states in protein folding are often referred as to the "molten globule" state. The term "molten globule" originated from Ohgushi and Wada (1983) who originally referred only to the acid-denatured state, but now is used to refer to compact denatured states that may arise from other conditions of denaturation. Molten globule is characterised with an almost native level of secondary structure, but with very little specific tertiary packing (Peng et al. 1995; Christensen and Pain, 1991; Ptitsyn, 1995).

One of the most important questions about the molten globule state is whether it is a specific thermodynamic state of a protein, or whether it is similar to either slightly disordered native state or a slightly ordered

unfolded state. The criterion of a specific thermodynamic state is the presence of phase transition between that state and other ones. The most important are the first-order phase transition ("all-or-none" transition) which are coupled with drastic change of at least one of the first derivatives of free energy, like enthalpy or number of "absorbed" solvent molecules etc. Molten globule is separated by intramolecular first-order phase transition from the native and unfolded states and therefore is a specific thermodynamic state of protein molecules (Ptitsyn, 1995; Ptitsyn et al., 1995).

It was suggested that "molten globule" state can exist in a living cell and can be involved in a number of a physiological processes like transport of proteins, transport and release of big non-polar ligands, etc (Bychova et al., 1988; Bychova and Ptitsyn, 1993; O'Brien Gress et al., 1994).

1.2.5.Cooperativity of folding transition and unfolding pathways

Proteins are usually sufficiently stable to retain their native properties when the external conditions vary slightly from optimal. If the deviation exceeds some critical value, the protein denatures within a relatively narrow range of temperature, pressure, pH, denaturant concentration, etc., suggesting that this process involves a breakdown of the entire native protein structure. In the case of small globular proteins, all these properties change simultaneously, suggesting that the native structure breaks down in an "all-or-none" manner. Thermodynamically,

this means that denaturation process can be considered as a simple transition between the two macroscopic states: the native state and the denatured state (Privalov, 1992).

This cooperativity results from the simultaneous presence in the folded state of many favourable interactions whose total contribution to stability may be much greater than the sum of their individual contributions. Breaking the small number of interactions destabilises the others causing a complete unfolding of the protein (Horovitz and Fersht, 1992).

Although, the experimental consequence of cooperativity is always a sigmoidal behaviour of observed parameter, cooperativity may be one of essentially two different types; namely, two-state (first order) and one-state (higher order) (Chan et al., 1995; Mirny et al., 1996). The discrimination between these two cooperative processes determines fundamental information about the underlying molecular mechanism of the cooperative process (Figure 2). The definitive way to distinguish experimentally between these two types of transition is by analysis of the population of protein states at the transition midpoint. A two-state transition will have two identifiable states, while one-state transition will have only one broad peak, involving high populations of intermediates near the transition midpoint. A two-state cooperativity, or first-order phase transition, implies a free energy surface with two minima separated by a barrier, i.e. two stable species, native and unfolded state, separated by a free energy barrier. One-state cooperativity, or higher-order phase transition implies a single free energy minimum, without a free energy barrier between native and denatured state. (Dill and Shortle, 1991; Chan et al., 1995; Fersht, 1995).

It was believed (Ptitsyn et al., 1990) that metastable products on the folding pathway, i.e. intermediates or molten globule states, play a key role in the minimizing the search problem. The major class of mechanisms for protein folding, where the concept of intermediates naturally fit invokes the formation of a protein in stages, with hierarchical addition of stable, smaller elements of structure (Fersht, 1995). Two major models for protein folding of this type are: 'framework' (Ptitsyn et al., 1995) and 'diffusion-collision' model (Karplus and Weaver, 1976) (Figure 3). Recently, it was found for few small monomeric proteins to fold and unfold via simple two-state kinetics, without the accumulation of any stable intermediate (Jackson and Fersht, 1991; Alexander et al., 1992; Schindler et al., 1995).

It was recently suggested by Fersht, (1995), Mirny et al., (1996) that accumulation of the stable intermediates on the folding pathway are not desirable for the optimization of the rate of the pathway. According to above authors, the existence of intermediates does not facilitate folding, but rather makes it slower and considerably decreases the thermodynamic stability of the native state. They suggested nucleation-condensation mechanism as mechanism which fulfills the criteria for fast folding. The nucleation sites occurs flickeringly in the denatured state without accumulation of folding intermediates. The 'nucleus' become stabilised by long-range interactions that are formed as the rest of the protein collapses around it (Fersht, 1995).

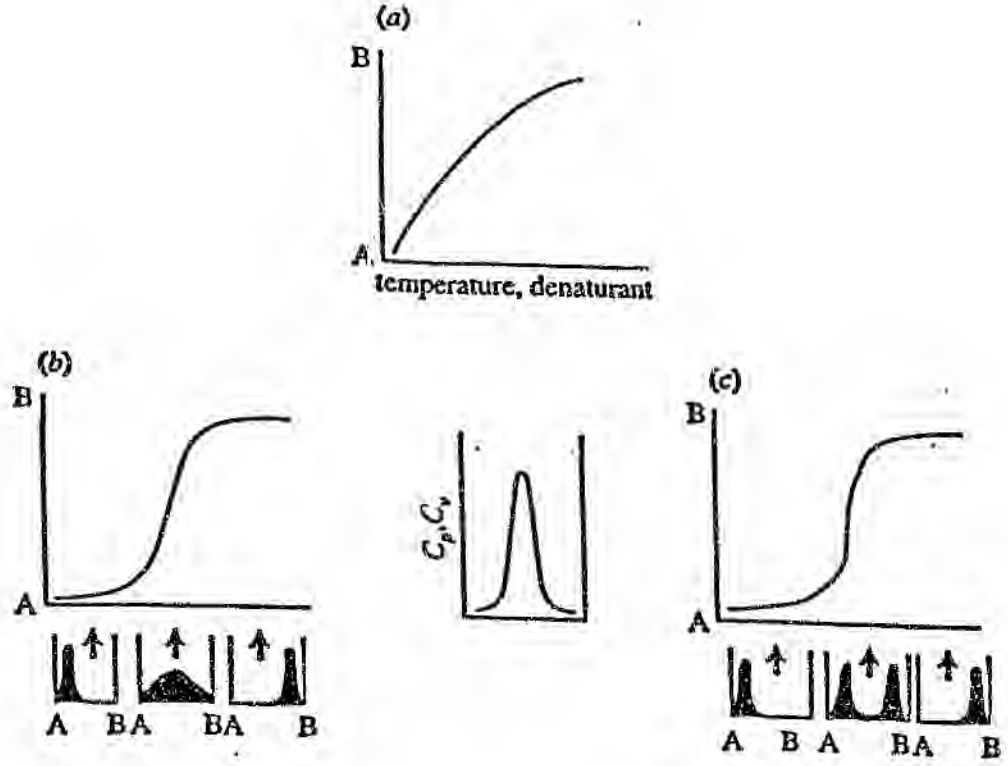


Figure 2 Models of cooperativity in protein folding. (a) gradual change, no cooperativity, (b) cooperative transition of the one-state type; (c)Cooperative transition of the two-state type. Both one-state and two-state transitions can have sigmoidal behaviour and heat absorption (a peak in the Cp or Cv plot); they can not be distinguished on these bases or from steepnes of the sigmoidal curve. The main distinction is whether there is one broad peak involving high population of "intermediates" near the denaturation midpoint (one-state) or whether there are two populated states and less intermediate population (small plots at the bottom of (b) and (c)). (Chan et al., 1995)

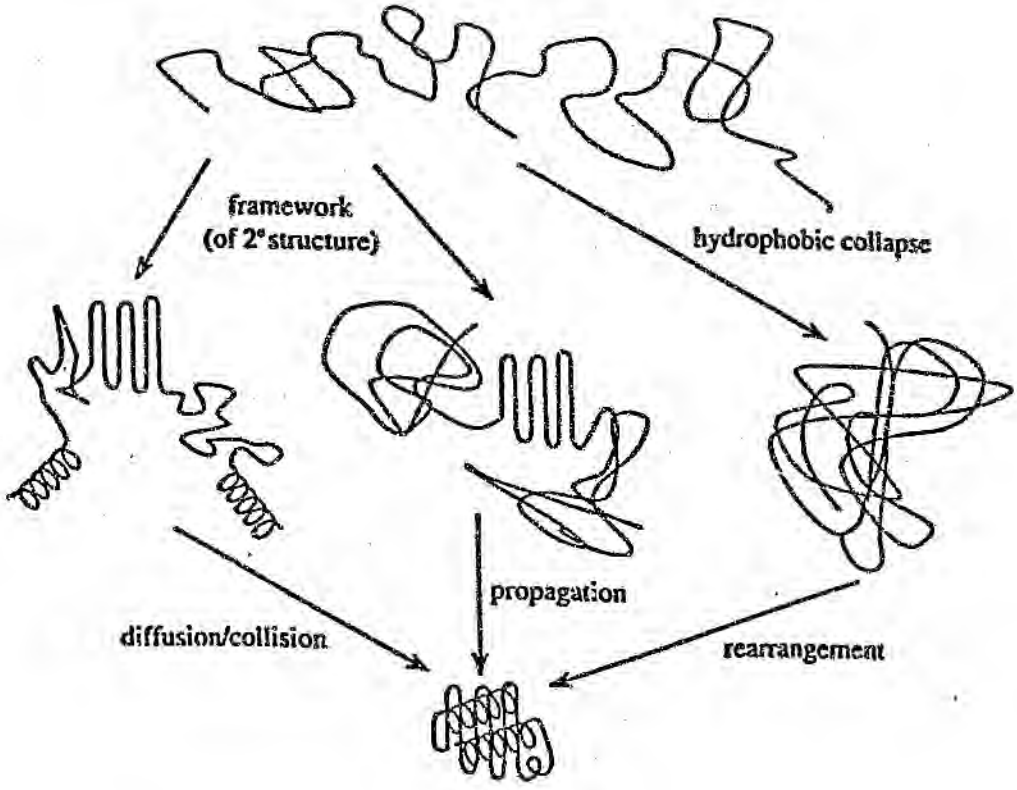


Figure 3. Pathways of folding. (Fersht, 1995)

1.2.6. Protein folding of large (multidomain) molecules

The "folding problem" adds another dimension when the protein in question has a multidomain and/or multimeric structure. A domain is a part of the chain that forms a compact globular substructure with more interactions within itself than with other part of the chain (Janin and Wodak, 1983). The independent domains (un)fold like single domain proteins that can lead to complex unfolding curves. Although stability of isolated structural domains and/or subunits, are often as when they are in the intact protein, not markedly modified by the rest of the protein, the rest of the chain influence rate of domains folding. The slowest step in the folding of large multidomain proteins is pairing of already folded domains (Garel, 1992). In some cases domains are interdependent with mutually stabilizing interactions as to become single cooperative folding units. In the case of multidomain/multimeric proteins proper folding of each subunit together with the association of the monomers is required. These two processess must be properly coordinated, otherwise, specific recognition by a subunit may not be achieved during folding (Jaenicke, 1991) (Figure 4). At equilibrium, many oligomeric proteins display essentially a two-state behaviour upon folding/unfolding for which thermodynamically stable folding intermediates are not detected (Bowie and Sauer, 1989; Gitelmann and Matthews, 1990; Liang and Terwillinger, 1991; Perry et al., 1992; Grant et al., 1992; Kwon et al., 1993). Other mechanisms do result in the formation of stable intermediates in the unfolding of oligomers. The intermediates are either

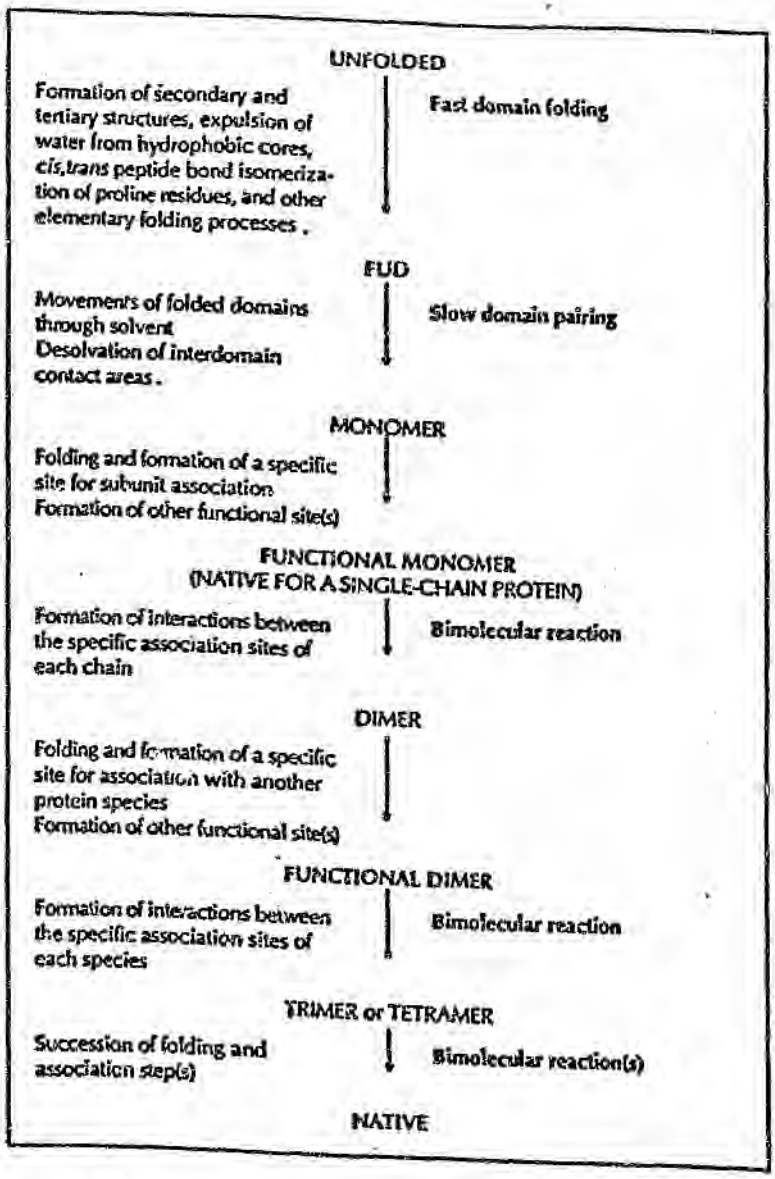


Figure 4. The overall folding pathway of an oligomeric "average" protein. Bold letters indicate the species that have been detected in several cases because they precede slower steps (Garel, 1992).

partially folded oligomeric states or monomeric states. These monomeric intermediates sometimes have preserved tertiary structure or appear as molten globules. This type of behaviour is also proposed for many proteins (Herold and Kirschner, 1990; Mei et al. 1992; Bhattacharyya, 1993; Wilkins et al., 1993; Eftink et al., 1994).

1.2.7. Theoretical studies on protein folding and unfolding

Theoretical methods to predict protein properties can be classified as follows:

1. Methods to assess the correctness of a given protein structure (Novotny et al., 1984; Lűthy et al., 1992) and for the prediction of protein structure. Dill 1985; Unger and Moult, 1993; Kolinski and Skolnick, 1994)
2. Methods to determine the relative stability of protein mutants or different protein conformations in terms of relative free energies (Shi Yun-yu et al., 1993)
3. Methods to simulate the process of protein folding or denaturation. (Levitt and Warshel, 1975; Šali et al., 1994)

The properties of proteins, as of all molecules, are governed by their potential energy surfaces. Strategies for predicting protein structure employ the description of these energy functions for describing the conformational space for the given polypeptide chain. Prediction of the fully folded tertiary structure for even small proteins has not been possible to date, because of the present limitations on computing power.

It would take centuries of the computer time to solve a single folding problem by evaluating all possible conformations. Most theoretical studies introduce simplifications to the potential of the mean force. These simplifications omit structural details of the protein chain (e.g., only backbone is included), the nature of interactions (only hydrophobic/hydrophilic terms for spatially near neighbours are included), and minimisation of the conformational space (e.g., only positions on a cubic or other lattice are allowed). Without these simplifications treating a sufficient amount of conformational space of a polypeptide chain to examine the large-scale changes involved in the transition between unfolded and native protein would not be possible. More realistic energy function will always lead into more accurate predictions, but the energy functions used to predict structure from sequence will always be approximations of the true energy function (van Gunsteren et al., 1995; Bornman, 1995; Shortle et al., 1996).

1.2.8. Protein folding *in vivo*

Cellular factors and cellular conditions might make folding in the cell significantly different from conventional folding studies. Although information of the native structure of the protein is contained in amino-acid sequence, in the cell proper folding of many proteins, especially multi-domain and multi-subunit, need the assistance of chaperones and folding catalysts (Freedman, 1992). The three most important late events

isomerization and subunit assembly are catalysed or directed by specific helper proteins (Jaenicke, 1995). Rather than promoting folding, they prevent aggregation by helping to guide the nascent protein to its final form.

Cis-trans isomerisation of peptide bonds preceding Pro residues is an intrinsically slow process (kinetic barrier in folding), and extensive formation of ordered secondary and tertiary structure is possible prior to the reversal of incorrect proline isomers. Proteins capable of catalysing such cis-trans isomerisation have been detected and are known as *cis-trans* prolylpeptide isomerase (Brandts et al., 1975; Schmid et al., 1991).

Another rate-determining process in the folding of some proteins is the formation and isomerisation of disulfide bonds. *In vivo* there is an enzyme which facilitate these rearrangements, protein disulfide isomerase (Freedman, 1991)

Chaperons are another big group of proteins which facilitate protein folding *in vivo*, by preventing off-pathway reactions leading to aggregation. Molecular chaperons occur in all cell types and comprise several protein families that are structuraly unrelated. Many of them are classified are stress- or heat-shock proteins. Chaperons binds unstable non-native conformation of proteins, predominantly by shielding hydrophobic surfaces exposed to solvent, and facilitate correct folding by releasing the bound polypeptide in a controlled way. Conditions in a cell, with high amount of total protein and high amount of nascent non-native protein are conditions that would increase the propensity of proteins to aggregate (Figure 5).

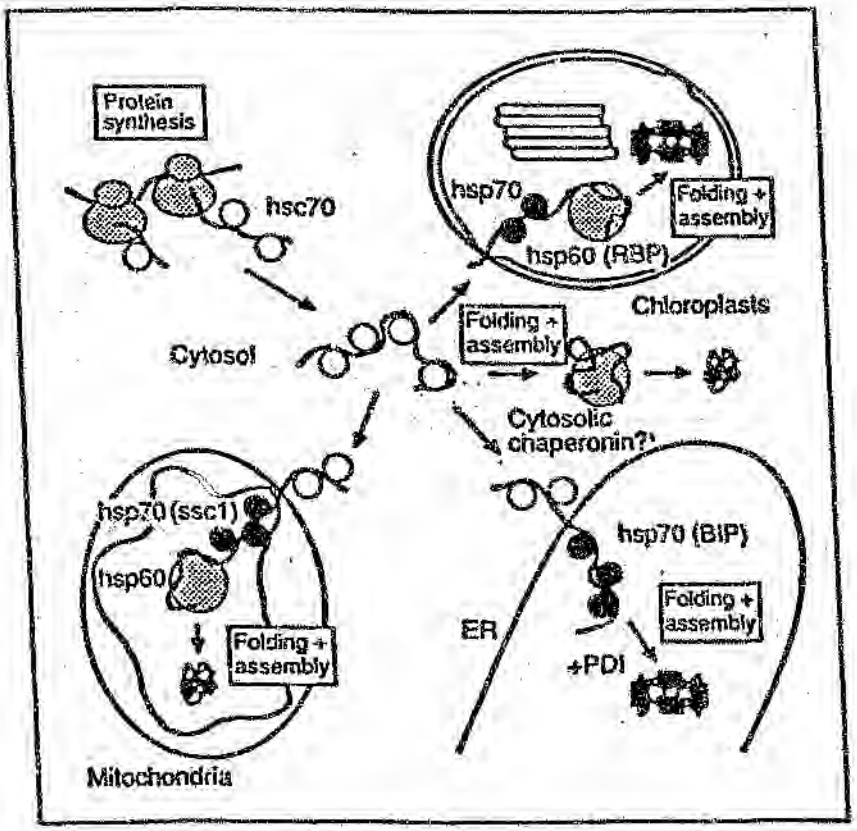


Figure 5. The cellular compartments where chaperone-mediated protein folding occurs (Hlodan and Hartl, 1994).

Chaperons do not have steric information that would effect the folding pathway, but rather by preventing unproductive intra- and intermolecular interactions, they enable the unfolded protein to reach the defined structure as specified by its amino acid sequence (Craig, 1993; Agard, 1993; Jaenicke, 1995; Hartl and Martin, 1995; Shinde and Inouye, 1993; Hartl. et al., 1994).

Although "solvent conditions" *in vivo* are very much different than in the experimental models of protein folding, the protein folding models studied *in vitro* provide essential background against which the cellular events can be understood (Freedman, 1992).

1.3. Influence of ligands on the conformational stability and unfolding of proteins.

The ability of proteins to interact with other molecules allows them to realize their biological function. Interaction with ligands, substrates etc. can change protein stability and affect metabolic control in the cell (Creighton, 1993). Increase in the free energy upon ligand binding to a protein could translate into specific conformational changes that may have either stabilising or destabilising effects on the protein (Ruvinov and Miles, 1994). Since native proteins are only marginally more stable than denatured proteins, ligand-mediated de/stabilisation can influence protein turnover. Stabilisation of a protein by ligand binding was first observed one hundred years ago when O'Sullivan and Tompson reported: "We

have shown that invertase when in the presence of cane sugar will stand without injury a temperature fully 25°C higher than in its absence. This is a striking fact, and, as far as we can see, there is only one explanation of it, namely, the invertase enters into combination with the sugar" (see Pace, 1990).

Ligand-mediated stabilisation occur as a consequence of preferential interaction of ligand with the native form of a protein (Mach and Middaugh, 1994). Conversely, ligand-induced destabilisation of proteins is usually result of the ligand bound less strongly to the native protein than to the either unfolded or partially folded states of protein (Edge et al. 1988; Bromberg et al., 1994).

Interactions between proteins and ligands demonstrate both steric and physical complementarity between the two. The interface between protein and ligand is usually as closely packed as the protein interior, with interactions that follow structural rules similar to those in the protein themselves (Janin and Wodak, 1983; Janin and Chothia, 1988).

Understanding of molecular complexes requires a description of three separate aspects: structure, energetics and dynamics. The most information about the physical nature of the protein-ligand interactions comes from the crystallographic determination of the structure of the complex. In the case of smaller proteins, the two-dimensional NMR is the most promising method for studying the structure of a protein-ligand complex in solution, therefore, closer to biological conditions, thus providing information about protein dynamics (MacArthur et al., 1994).

Under appropriate conditions (temperature, solvent) a given amino acid sequence has a unique average structure corresponding to the native

state and significant motions occur relative to that average structure at room temperature. Although these fluctuations are small, they often play an essential role in protein function (Huber, 1979). Perturbation of the average structure by the binding of ligands are made possible by the existence of the rapid fluctuations. If different states of a protein exist it is likely that they will have different abilities to interact with ligand. The ligand by preferentially binding to some specific state(s) of the protein, may affect a shift in a distribution of the states of the protein. The binding of a ligand will be critically dependent on the proper alignment of the amino acid residues that constitute the binding pocket of the protein, and slight alterations in the position of these residues would be expected to alter the affinity of the ligand (Weber, 1992). The structure of a protein domain generally does not change substantially when it binds a ligand, but small adjustments are probably important in general to allow rapid rates of association and dissociation (Creighton, 1993; Stanfield and Wilson, 1995).

The observed affinity of the interaction of a protein with a ligand depends on the relative free energies of the complex and of the components. This includes the interactions between the two in the complex but also changes in their average conformations and their flexibilities produced by complex formation. Differences in their various interactions with solvent, the loss of translational and rotational freedom of each component, and displacement of solvent are all factors that influence final energy of binding (Weber, 1992).

Water may be involved in a number of ways (solvent, proton source, hydration) in the transition between states of the protein. In any

ligand binding reaction, the formation of the complex will require the displacement of first-layer solvent molecules from both the protein and the ligand and, perhaps, the reorganisation of some solvent not directly in the binding site. The amount and thermodynamic parameters of such solvent must be known in any serious attempt to calculate binding constants, even where the structures of the two components and the complex are known in detail.

The magnitude of the affinity determines whether a particular interaction is relevant under a given set of conditions. Whether or not any particular affinity of a protein for a ligand is significant depends on the concentration of the ligand that the protein is likely to encounter. Weaker affinity can always be overcome by a higher concentration of that ligand, so binding affinities should always be considered relative to the concentration of the ligand. The free energies of formation of enzyme-substrate complexes are on the lower range, as the enzyme-product complex has to dissociate in very short time for efficient catalytic activity (Creighton, 1993).

The effect of ligation on thermal denaturation of multimeric proteins has been studied in a number of systems (Schwarz, 1988; Zolkiewski and Ginsburg, 1992; Mach and Middaugh, 1994,) and has been much better documented than influence of ligation on unfolding induced by chaotropes. It is interesting to establish whether the binding of ligands only shift the unfolding pathway to a higher value of denaturant, or if it alters the way the protein responds to denaturant, i.e. alters the unfolding pathway. Such information would be of interest in attempts to design proteins with improved stability.

1.2. STRUCTURE AND FUNCTION OF GST's

1.2.1. Definition, classification and function of GSTs

1.2.1.1.Definition of GSTs

The glutathione S-transferases (EC 2.5.1.18) are a supergene family of multi functional intracellular proteins that participate in wide spectrum of very important biological functions. They function primarily as detoxification enzymes protecting cells against chemical-induced toxicity and stress (Armstrong, 1991). GSH and GSTs first appeared in Prokariotes as a part of protective mechanism against oxygen induced toxicity. GSTs are found in vertebrates, arthropods, mollusca, nematodes, platyhelmintes, also in plants and some bacteria (Pemble and Taylor, 1992). GSTs realize their catalytic function by catalysing the nucleophilic addition of the reduced tripeptide glutathione to wide variety of lipophilic molecules having electrophilic functional groups, which may be carbon, oxygen, nitrogen or sulphur (Ketterer et al., 1988; Mantle et al., 1990; Armstrong, 1991). In addition to their catalytic property, they can function as ligand-binding proteins that can facilitate intracellular transport of nonsubstrate apolar compounds. By binding xenobiotics, GSTs are also involved in the development of resistance of cells to drugs, herbicides, pesticides, etc (Tsuchida and Sato, 1992; Beckett amd Hayes, 1993; Listowsky, 1993).

1.2.1.2. Detoxification system in cell

Metabolism of endo- and exotoxins generally proceed via Phase I detoxification system, which is mediated mostly by cytochrome P-450 enzymes. Toxins bioactivated in Phase I become substrates for the Phase II detoxification system (Figure 6). In the Phase II detoxification system activated hydrophobic xenobiotics are converted into more hydrophilic forms by conjugation with glutathione. GSH-conjugates generated in Phase II system are secreted from cell via ATP-dependent GSH-conjugate export pump, considered as the Phase III system (Ishikawa, 1992; Zimniak et al., 1993). Export from the cell in metabolism of xenobiotics is extremely important, because accumulation of GSH-conjugates in cell can decrease activity of Phase II enzymes and decrease effectivity of cell detoxification. Phase I , II and III system are also involved in synthesis and release of biologically active endogenous substances like leukotriene C4 (Saxena et al., 1992).

There are a small number of GST substrates that either are not detoxified by conjugation with GSH or become more toxic when they are conjugated with GSH. For example, conjugates such as 2-bromoethylglutathione are directly toxic through spontaneous formation of reactive episulfonium ions. Some others GSH conjugates require further metabolism to realize its toxicity (Boyer, 1989; Tsuchida and Sato, 1992; Beckett and Hayes, 1993).

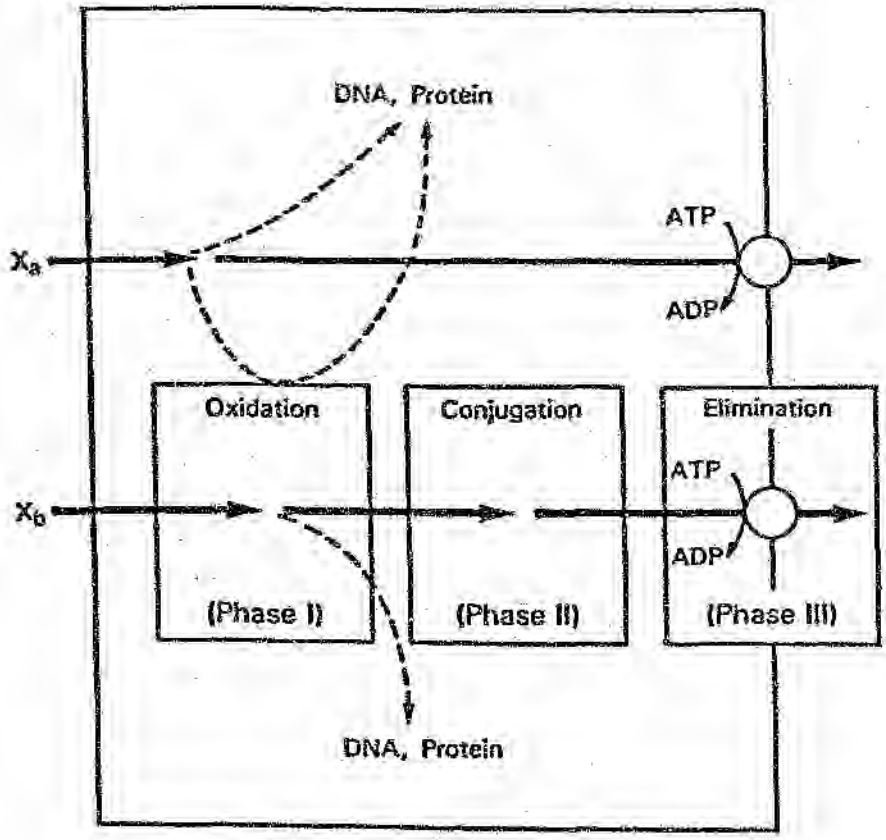


Figure 6. Metabolism and elimination of xenobiotics via two distinct pathways. Xa: xenobiotics that are exported by P-glycoprotein. Xb: xenobiotics that are bioactivated by monooxygenase in phase I and conjugated with glutathione in the phase II system. The metabolites bioactivated in the phase I system can react with cellular proteins or DNA. The glutathione S-conjugates formed in the phase II system are eliminated from the cell by the GS-X pump (phase III) (Ishikawa, 1992)

1.2.1.3. Classification and nomenclature

GSTs have been highly studied in different mammalian species. They are present in the wide variety of tissue at concentration of up to 10% of total protein in the cell. Different cell types and different tissues have different isoenzyme patterns. According to amino-acid sequence, physical properties, substrate specificity, immunological reactivities and subunit assembly patterns, cytosolic GSTs are classified into five species-independent gene classes: Alpha, Mu, Pi, Theta and Sigma, (Mannervik et al., 1985, Meyer et al., 1991; Ji et al, 1995) each represented by a large number of cytosolic isoenzymes.

Nomenclature for the GST's has undergone many changes in the past, causing problems with referring to the same isoenzyme with different terminology. Mannervik et al. (1992) suggests nomenclature for cytosolic human GSTs, which can easily extended to the other species as well. For example, the acronym hGSTA1-1 denotes human GST (small letter in front of the GST denote species) from class A (the capital letter after the GST denote class) consisting of two identical 1 type subunits (the arabic numerals after the class define subunit composition).

1.2.1.4. Non-cytosolic GSTs

In addition to the cytosolic glutathione S-transferases, there is a membrane-bound microsomal enzyme which can be categorised into the same enzyme superfamily (Kraus and Gross, 1979). It catalyses the same

reaction but shares little resemblance with the cytosolic enzymes. Microsomal GST is a trimeric protein (Hebert et al., 1995), which probably conjugate very hydrophobic substances, and participate in the protection of cells against lipid peroxidation (Anderson et al. 1993).

Leukotriene C_4 Synthase is another membrane bound member of GST superfamily and plays a role in cellular metabolism by catalysing the conjugation of GSH with the leukotriene A_4 epoxide. That reaction presents the first step in the formation of the biologically active cysteinyl leukotrienes. Leukotriene C_4 synthase may, therefore, be a controlling enzyme in the biosynthesis of these potent inflammatory mediators (Nicholson, 1993).

1.2.1.5. Regulation of GST expression

The regulation of GST is complex; they are subject to developmental control, their expression is tissue specific, and they are inducible by many drugs. Evolution of catalytic diversity of detoxification enzymes is one of the most fascinating issues with respect to their function. The fact that the expression of the genes encoding the cytosolic GSTs is regulated by xenobiotic signals strongly suggests that enzymes primary role is to respond to chemical insult (Benson et al., 1979; McLellan et al., 1991). The mechanisms responsible for the induction of GST by treatment with foreign compounds currently represent an active area of research (Hayes and Pulford, 1995).

1.2.1.6. Evolution of the cytosolic GSTs

An analysis of the gene sequences and exon-intron boundaries provide some interesting clues about the evolutionary relationships between glutathione S-transferases. The correlation between individual or groups of secondary structure elements and the exons encoding the GSTs is quite extensive (Armstrong, 1994).

GSTs appeared very early in the course of evolution. Theta class, highly conserved from bacteria to mammals, has little sequence identity with alpha, mu and pi class and seems to represent separate multigene family, which is representative of the ancient progenitor GST gene (Pemble and Taylor, 1992). Alpha-, Mu- and Pi-class enzymes, not present in plants, derive from a Theta-class gene duplication so early that the time and the order of divergence cannot be reliably assessed with the available data (Wilce et al., 1995). The three-dimensional structure of squid sigma enzyme suggests that sigma class enzyme diverged from the ancestral gene prior to the divergence of pi/mu/alpha precursor, which originally encoded a protein that lacked the loop and hydrophobic lock for subunit dimerisation (Ji et al., 1995) (Figure 7). Alpha/mu/pi type subunit interface developed later in the course of evolution, can be considered as the structural feature what contribute in strengthening the dimeric structure. Pemble and Taylor (1992), based on highly conserved 3'-non-coding sequences of the mu and theta class, propose that the mu gene diverged from this precursor before the alpha/pi precursor gene. Crystal structure of *Schistosoma japonica* GST has GSH and xenobiotic binding site the most similar to mu GST (McTigue et al., 1995).

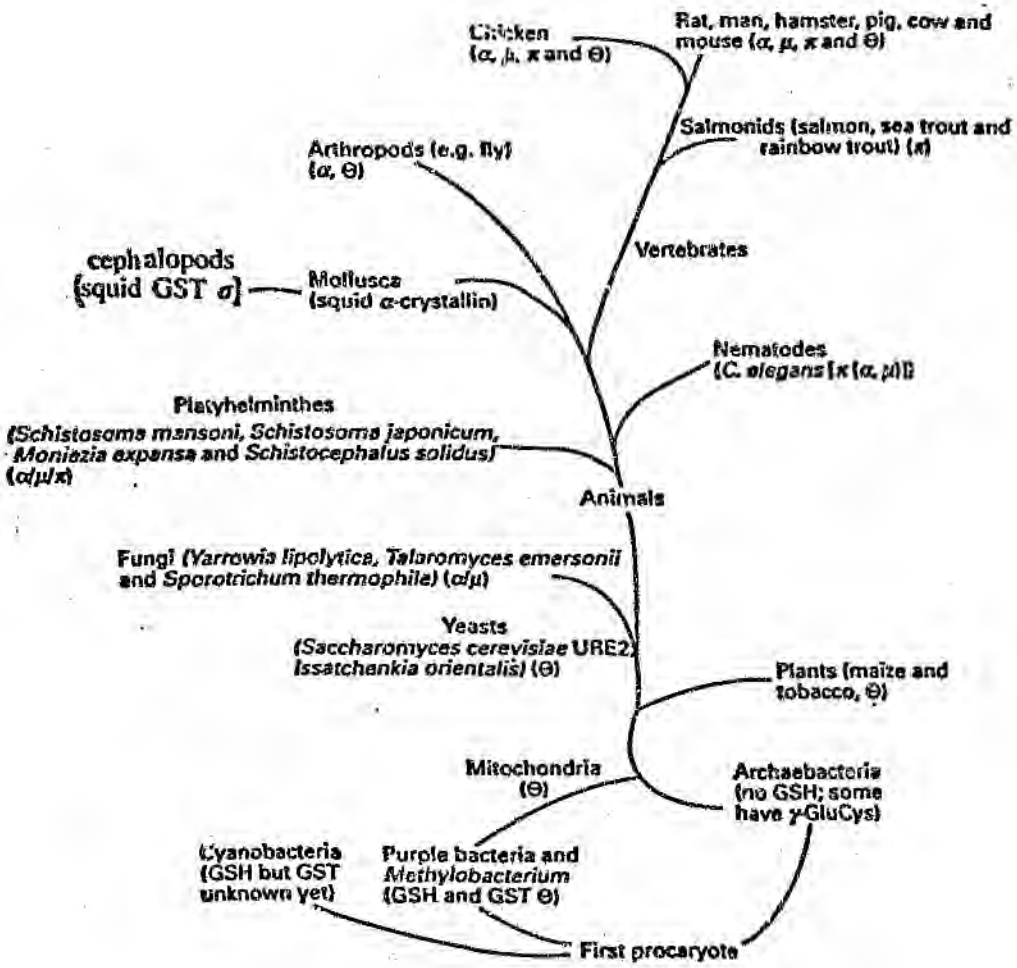


Figure 7. Evolutionary relationship of GSTs (for convinience greek letters indicate class of GST in the species) (adapted from Pemble and Taylor, 1992)

1.2.1.7. Catalytic function

The active site of GSTs is composed of a hydrophylic G-site for binding glutathione and an adjacent hydrophobic H-site able to accommodate a diversity of electrophilic compounds, with certain isoenzymes preferably binding certain types of compounds (Jakoby, 1978) (for structural details see further 1.2.2.3.).

The nucleophilic addition of the thiol group of glutathione to a wide range of structurally diverse nonpolar electrophilic compounds (Armstrong, 1991) is a central reaction of GSTs catalysis (Figure 8). Any compound with reasonably high electrophilicity can be substrate for GST. In general, a variety of products of oxidative metabolism such as alkenes, epoxides,organic hydroperoxides, quinones etc. appear to be substrates for GSTs (Ketterer et al., 1988; Listowsky, 1993). Many of reactions catalysed with GSTs can occur in the absence of enzyme, but with much lesser efficiency (Boyer, 1989). The glutathione thiol group becomes thiolate upon binding the enzyme and is up to 10^9 times more reactive than its conjugated acid (Figure 9). Thiolate anion also bind much more tightly to the enzyme than the conjugated acid, suggesting that the thiolate is the preferred ionization state of GSH (Douglas, 1987; Graminski et al., 1989). Therefore, converting and stabilising of glutathione thiol to its thiolate form appears to be essential for the enzyme function. The pK of free GSH in water is about 9, but the binding to enzyme lowers the pKa value to about 6.2-6.7 ensuring that bound GSH is in its most reactive form at physiological pH.

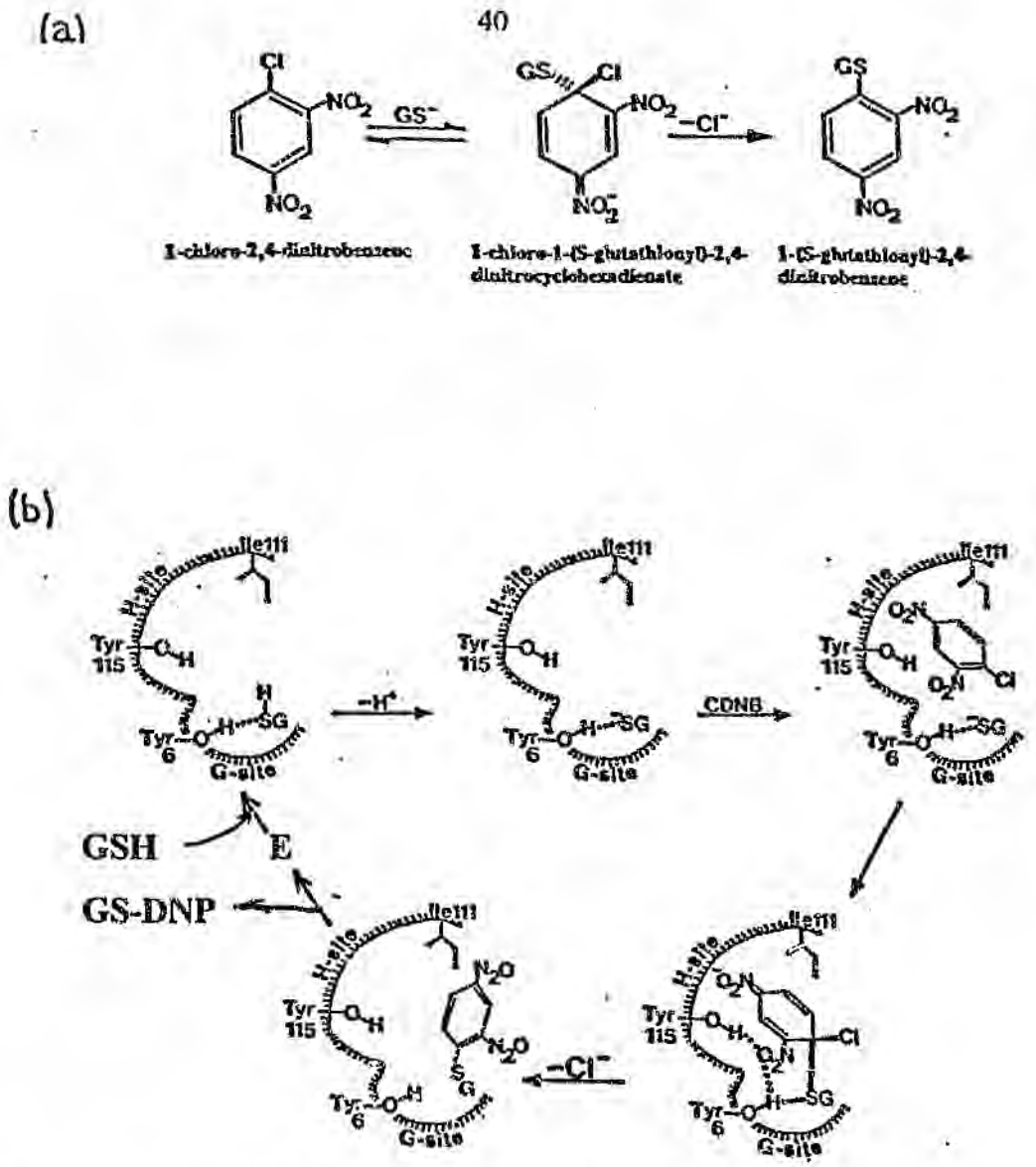


Figure 8. Glutahione S-transferase catalysed reaction.

(a) Conjugation reaction between 1-chloro-2,4-dinitrobenzene and glutathione in the enzyme activity assays.

(b) Proposed mechanism for stabilisation of the transition state in the catalytic pathway of mu class GST.

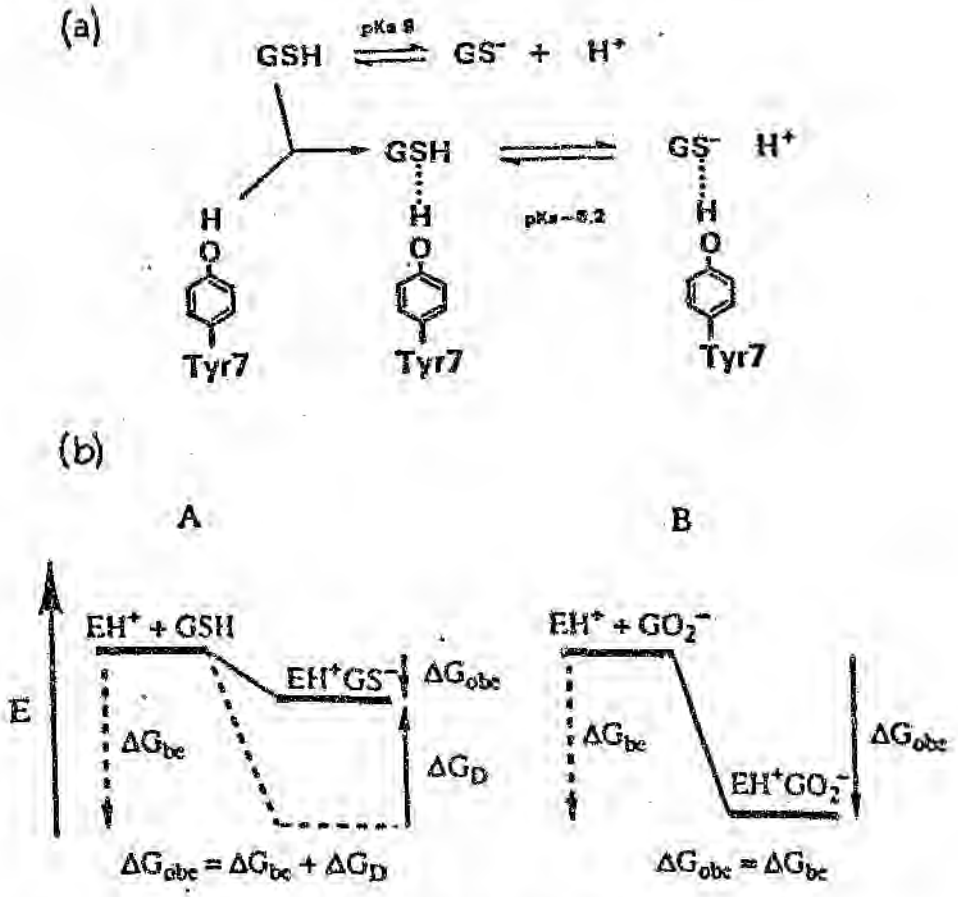


Figure 9. (a) A proposed catalytic role for Tyr 7

(b) (A) Utilisation of binding energy ΔG_{be} for the deprotonation of GSH in the active site of the enzyme. The actual observed binding energy for the protonated peptide ΔG_{obe} is smaller than ΔG_{be} by the energy required to destabilise the thiol, ΔG . (B) Realisation of ΔG_D as observed binding energy for the resonance-stabilised carboxylate analogues of GSH. The amount of additional binding energy $\delta\Delta G_{obe}$ actually realised with the carboxylate analogues can be calculated from $\delta\Delta G_{obe} = -RT\ln (K_d^{GSH}/K_d^{analogue})$ (Armstrong, 1991).

reaction is random. Under physiological conditions, the reaction is probably ordered by addition of GSH first because of its high concentration in the cell (1-10mM), about three orders of magnitude higher than its dissociation constant for the enzyme (Bico et al., 1995).

Isoenzymes have overlapping substrate specifities so it can be difficult to accurately determine tissue distribution and to identify certain isoenzymes. In addition to the substrate specificity studies and electrophoretical mobility, inhibition studies can be useful and help to distinguish among the different isoenzyme forms (Armstrong, 1994).

1.2.1.8. Ligand binding function

The another function of members of this super-gene family is storage and transport of some nonsubstrate electrophilic compounds such as bilirubin, haem, hormones, bile acids, fatty acids, drugs, etc.(Listowsky, 1993).

Most ligands are hydrophobic and are bound noncovalently to GST. However, a number of reactive metabolites formed from carcinogens are bound covalently by GST (Tsuchida and Sato, 1992). It has been proposed that GSTs may serve to sequester/store such compounds or, alternatively, they may be involved in the transport of ligands, either across cells or between compartments/organells. Being very abundant in the cell, with 1-2 binding sites per dimer, GSTs can account for a considerable portion of intracellular ligand binding. Binding of ligands in the cell can proceed either by binding to a highly specific

receptor or to an unspecific but highly abundant GST. By binding to GST some ligands induce its catalytic function and conjugation of ligands with GSH. If capacity of specific receptor is exceeded, or Phase II detoxification pathway is not induced, cytotoxic effects can follow.

Structural data about the location and nature of non-substrate binding site on GST's is becoming available. Crystal structure of *Schistosoma japonica* GSTcomplexed with the non-substrate drug praziquantel has been solved (McTigue et al., 1995). It is first crystal structure which reveals the location of a non-substrate binding site in GSTs. It binds one praziquantel molecule per dimer and appeared to be located at the subunit interface connecting the two catalytic sites. It has been previously reported that GST possess a single high-affinity binding site per dimer for the haem, bilirubin and bile acids (Ketley et al., 1975). For the porcine class pi isoenzyme, on the basis of fluorescence resonance energy transfer data done with ANS, a proposed location for the non-substrate binding site is also at the subunit interface (Sluis-Cremer et al., in press). Bico et al. (1995) found that bromosulphophthalein and ANS compete for the same ligand binding site but binding induces a microstructural changes that impacts on the functional conformation of the G-site of pGSTP1-1. That the ligand binding site is distant from the glutathione binding site was also shown by Caccuri et al. (1990) who found that haemin is still capable of binding hGSTP1-1 in the presence of the G-site inhibitor, S-methylglutathione. Truncation of the C-terminus in alpha class isoenzyme inhibits substrate binding but does not alter the binding of the anionic ligand bromosulphophthalein (Board et al., 1991).

1.2.2. Structure of cytosolic GSTs

1.2.2.1. Structure analysis and protein architecture of the cytosolic glutathione S-transferase

The cytosolic glutathione S-transferase have stable dimeric structures composed of identical or nonidentical subunits. No spontaneous exchange of subunits occur between enzyme molecules. The first diffraction-quality crystals of a GST were described in 1987 for the mu class isoenzyme 3-3 from rat (Sesay et al 7), and subsequently for other isoenzymes (Schaeffer et al., 1988; Cowan et al., 1989; Parker et al., 1990; Dirr et al., 1991). But the first solution of the three-dimensional structure of a GST only appeared in 1991, when Reinemer et al. (1991) reported a structure of the porcine class pi isoenzyme. Today, there are representative of three-dimensional structure for each known cytosolic GST class.

The three-dimensional structure for the porcine class pi turned out to be the structural archetype for the family. It was solved in complex with substrate analogue, glutathione sulfonate (Reinemer et al., 1991; Dirr et al., 1994a). Human class pi was solved after in complex with S-hexylglutathione (Reinemer et al., 1992). Mouse liver pi enzyme was solved as a complex with S-(p-nitrobenzyl)glutathione (García-Sáez et al., 1994).

Class mu was the first isoenzyme whose structure was solved in complex with the physiological substrate GSH (Ji et al., 1992). The first class alpha structure appeared in 1993 (Sinning et al., 1993), and was

solved in complex with S-benzylglutathione. The crystal structure of *Schistosoma japonica* GST has been solved without ligands, in complex with the non-substrate drug praziquantel, (McTigue et al., 1995), and in complex with the physiological substrate glutathione (Lim et al. 1994). Recently reported structure of a *Lucilia cuprina* class theta isoenzyme, was the first crystal structure presented on a non-mammalian enzyme (Wilce et al., 1995). Subsequently, a three-dimensional structure was solved for *Arabidopsis thaliana* GST another theta class GST (Reinemer et al., 1996). According to the amino acid sequence, gene structure and three-dimensional structure (solved in complex with 1-(S-glutathionyl)-2,4-dinitrobenzene), the GST from the squid digestive glands is placed into the separate, sigma gene class (Ji et al., 1995).

Cytosolic GSTs are roughly globular proteins with molecular dimensions of about 6.2nm x 5.1nm x 4.6 nm. Although the aligned primary structures show little identity between the gene classes, the overall protein architectures are remarkably similar (Dirr et al., 1994b) (Figure 10).

Subunits are characterized by two distinct domains. The N-terminal domain, domain I, comprise approximately 80 amino acid residues and has four β-strands and three α-helices arranged in a βαβαββα fold, typical for many GSH binding proteins, like thioredoxin, glutaredoxin and glutathione peroxidase (Gilliland, 1993; Martin, 1995). The C-terminal domain comprise about 150 amino acid residues, all arranged in α helices, which are packed in a right-handed spiral.

Cytosolic GSTs of the same class show identities, even across the species, of about 65-80%, while sequence identities between classes are

not more than 25-35%. Subunits of the same gene class dimerize forming either homodimers or heterodimers, but interclass dimerization is not possible, indicating existence of class specific interface interactions. Each class has unique features, particularly about the active site and at the C-terminus (Wilce and Parker, 1994). In domain I, residues 36-42 of class mu enzyme forms a loop ("mu-loop"), which serves as a lid on the active site shielding it from the surrounding medium. Class alpha enzymes have an additional helix at the C-terminus (α9), and also a short β-strand at the end of C-terminal segment not present in other classes.

Class pi lacks all of these structural features and has the active site cleft which appears more open and accessible to the environment. Similarly, class theta and class sigma enzymes have an open active site, which is a deep V-pocket, and is even more exposed to the solvent than the pi class enzyme.

By association into dimers about 14% of surface area become buried. Interactions at the subunit interface are mediated primarily between secondary structural elements in domain I of one subunit with domain II of the adjoining subunit. Although at first sight the interactions responsible for holding the subunits together look quite similar in different classes, the detailed interactions are quite different. The interface is roughly V-shaped at the centre of the molecule. In the middle of the interface, an intrasubunit salt link is created by the stacking of two symmetrically equivalent arginine groups (alpha, R69; mu, R77; pi, R68; sigma, R 68; *S. japonicum*, R72; theta, not conserved). Aspartate or glutamate side chains, depending on isoenzyme, participate in balancing the charge (Dirr et al., 1994a; Wilce et al., 1995; Ji et al., 1995).

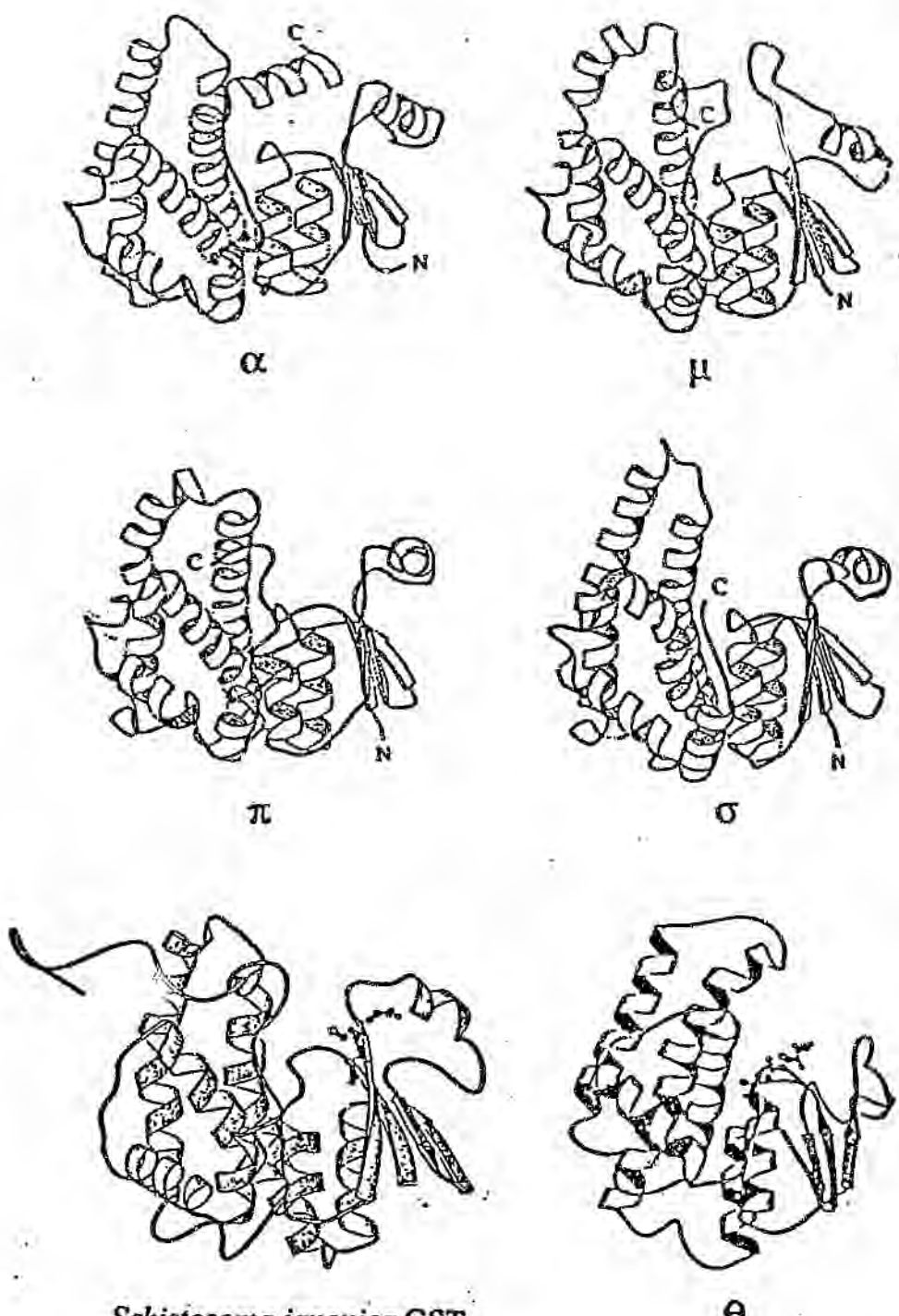


Figure 10. Ribbon representation of GST crystal structures.

Extent of hydrophilic or hydrophobic interactions involved in the dimerisation, vary from class to class. Sigma class dimer interface appear to be more hydrophilic than the interfaces of other classes. Alpha/mu/pi class isoenzymes has a prominent "lock-a-key" type hydrophobic interaction at the subunit interface not present in the class theta and sigma isoenzymes (Dirr et al., 1994a; Wilce et al., 1995; Ji et al., 1995). Alpha/mu/pi type interface developed later in the course of evolution, can be considered as the structural feature what contribute in strengthening the dimeric structure.

1.2.2.2.. Structure of pGSTP1-1

Porcine pi class GST is built of 207 amino acids with no disulphide bonds present (Figure 11)(Dirr et al., 1991). Domain I is built of 74 amino acid residues, with overall structural topology βαβαββα. The centrally located four β-strands is arranged in the order $\beta_2\beta_1\beta_3$ and β_4 and form a β-sheet with a right-handed twist. The β-sheet is surrounded by 3 helices: 2 towards domain II (αA and αC) are almost parallel with the direction of the β-strands, forming about 22 contacts with the β-sheet and with each other. Helix αB is located on the opposite side exposed to the solvent and is almost perpendicular to the β-strands, making 6 hydrophobic contacts and 3 hydrogen bonding pairs with neighboring β-sheet. The only two residues in the pGSTP1-1 molecule assumming a cis configuration are Pro2 and Pro51, both in domain I. The cis-Pro bend at position 51 is essential for a correct backbone

conformation at the glutathione binding site (Figure 12).

Domain II (81-207) is built of residues 81-207 and is composed of six α-helices. The main body of domain II and hydrophobic core form αD, αE, αF and αG. αD (27 residues) and αE (24 residues) are quite long, αE being curved as a consequence of the disruption of main chain hydrogen bonds by Pro121 and Pro126. C-terminal helices αH (8 residues) and αI (4 residues) are much shorter and separated from the main body of domain II. Arg11 from domain I penetrates domain II and prevents these two helices to pack with the main body of domain II, forming a channel filled with water molecules. The C-terminus itself turns back making contacts with C- and N-terminal region of αD and αE (Figure 12).

In domain II, helices αH and αI are a region with above-average main-chain thermal factors. Other regions with increased mobility in domain II include the C-terminal region of αD, the C- and N-terminal region of αE, the C-terminal region of αF and the C-terminus itself. Higher than average mobility are also seen in regions which connect domains and segments which connect secondary structure elements in domain I: residues between αA and β2, β2 and αB, residues 54-57 and turn between β3 and β4.

About 80% of the nonpolar amino acid side chains are buried in compact hydrophobic cores, thus removing these residues from contact with water and stabilizing the tertiary structure. Total secondary structure content is about 65%. The crystal structure suggests that the side chain of Asp96 in one subunit is hydrogen bonded to glutathione bound at the G-site of the other subunit (Reinemer et al., 1991; Dirr et al., 1994a).

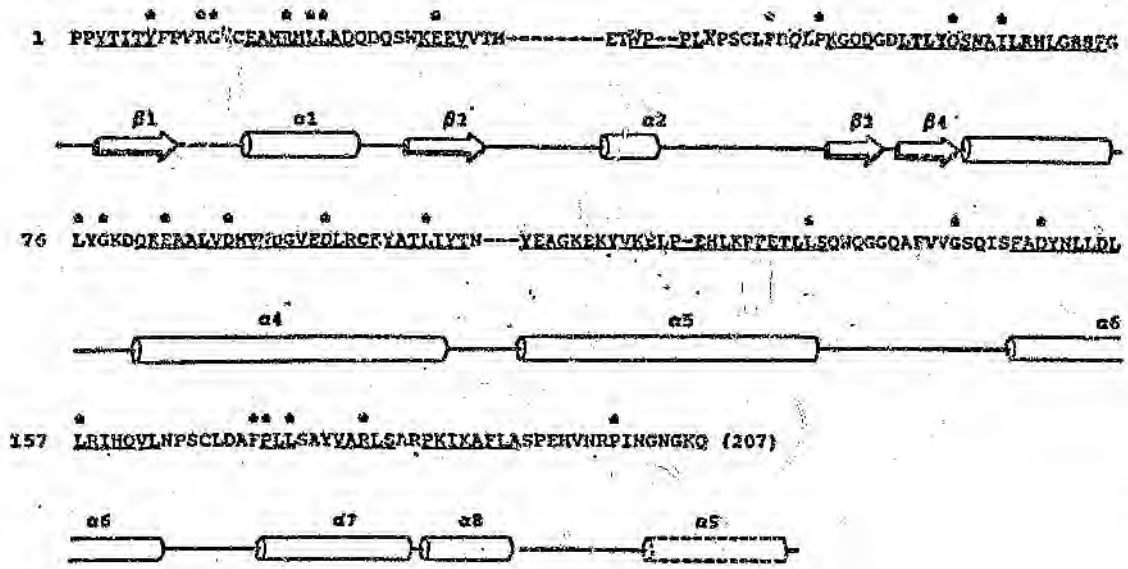


Figure 11. Amino acid sequence of pGSTP1-1 with aligned secondary structure elements. An asterisk (*) denotes residues conserved in most of GST classes.

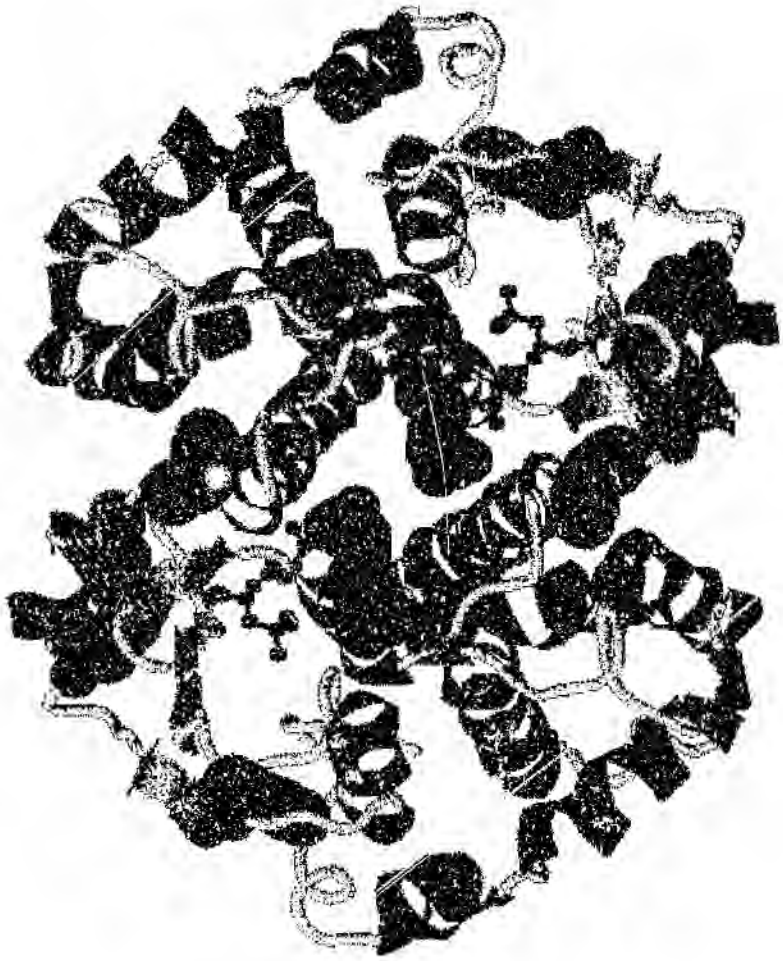


Figure 12. Dimeric pGSTP1-1 structure: Cartoon drawing of the molecule with glutathione sulphonate along the local 2-fold axis; Tryptophan residues are shown in purple, Cys45 in yelloworange, Phe47 in cyan and Arg72 in green.

This was confirmed by site-directed mutagenesis studies with human GST P1-1 indicating that the disruption of the hydrogen bond markedly reduces glutathione binding and consequently enzyme activity (Kong et al., 1993). The dimeric quaternary structure is, therefore, a prerequisite for the formation of a fully functional active site.

1.2.2.3. Structural basis for catalysis

Each molecule of GST has two functionally independent active sites, one on each subunit. The position and architecture of the active site is clear from the crystal structure. Each subunit has an active site that appear as a cleft along the domain interface. Between the two active sites in the dimer and extending along the subunit interface is a hydrophylic and solvent accessible furrow with a prominent cavity in the centre. Each site can be separated into two distinct functional regions: a hydrophilic G-site for glutathione binding and H-site for binding structurally diverse electrophilic substrates. Catalytically independent, fully functional active sites are formed by structural elements from both subunits of the dimer, with the conserved core of domain I providing major structural framework. For all classes except theta, the active site is structurally dependent of the neighbouring subunit and require structural elements from both subunits to be fully functional (Dirr et al., 1994a; Wilce et al., 1995; Ji et al., 1995).

Structure of glutathione, glutathione sulphonate and S-hexyl glutathione is presented in Figure 13. Glutathione/analogues peptide

backbone assume an extended conformations upon binding to the active site. Molecular recognition of glutathione or its analogues bound at the G-site involves a network of specific polar interactions between the tripeptide and a number of protein moieties in domain I of one subunit and one amino acid residue from domain II of the other subunit (Figure 14). Most functional groups in the glutathione molecule are sequestered by protein. Crystallographic temperature factors indicate that the glutathione backbone is rigidly bound at the G-site. Not all glutathione-contacting residues are conserved between the GST classes, although substitutions have tended to be conservative.

The G-site extends from residues 8-10 which connects strand β1 to helix αA, to Ser 63 at the N-terminal of helix αC. The residues lining the G-site include Tyr7, Gly12, Arg13,Trp38, Lys42, Gln49, Pro51, Gln62, Ser63, Glu95 and Asp96.

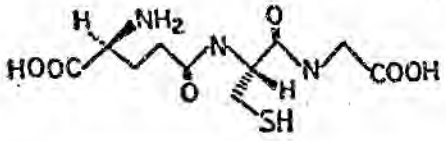
Interactions with the γ-glutamyl arm of bound glutathione are considered to be of greatest importance for substrate recognition. It interacts extensively with a hydrophylic complementary pocket formed by the side chains of conserved residues Arg13, Gln49, Gln62, Ser63 and Asp96 from domain 2 of the neighbouring subunit. A salt link betweeen Arg13 and Glu95 stabilises the protein conformation at the γGlu site, disruption of which decreases the termostability of the porcine pi GST (Widersten et al., 1992).

The N-terminus of the two α helices A and C in domain I are situated close to the G-site and their helix dipoles might make an electrostatic contribution towards attracting to and orienting glutathione at the G-site. Structure and activity studies with truncated or glycyl-

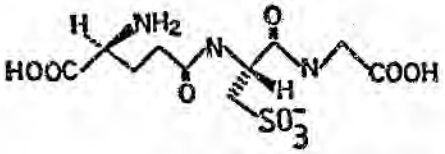
modified analogues of glutathione suggest that the tripeptide's glycine moiety might be the least restrictive and thus less important group for GSH recognition (Adang et al., 1990).

One of the roles of the enzyme is to remove the proton from the thiol group and form the much more reactive thiolate anion. Reinemer et al. (1991) first suggested the role of Tyr7 in catalysis. Tyr 7 acts as a hydrogen bond donor, promoting thiolate formation by decreasing the pKa of glutathione in the enzyme complex from pKa 9 to pKa 6.3 and also stabilizing the thiolate anion, which is up to 10^9 times more reactive than its conjugate acid (Armstrong, 1991; Parsons and Armstrong, 1996; Liu et al., 1993). Numerous site-directed mutagenesis studies showed that the replacement of Tyr7 does not influence significantly the affinity of the enzyme for glutathione, but reduces enzyme activity to less than 1% of the wild type (Wang et al., 1992; Kolm et al., 1992; Kong et al., 1992; Manoharan et al., 1992; Meyer et al., 1993). Further stabilisation of thiolate comes from the helix dipole of αA which is positioned so that it can contribute to stability. Liu et al. (1992) suggested that the tyrosine may also play a role in the correct orientation of glutathione in the active site. In the alpha class, Arg15, which is within hydrogen bonding distance of the sulphur atom, is another source of stabilisation for the thiolate. The mutation of Asp 98 (Asp 96, pi; 105, mu) to alanine increases the pK of the thiol group by about 0.8 pH units, which supports a role for Asp98 in facilitating formation of the thiolate anion (Kolm et al., 1992).

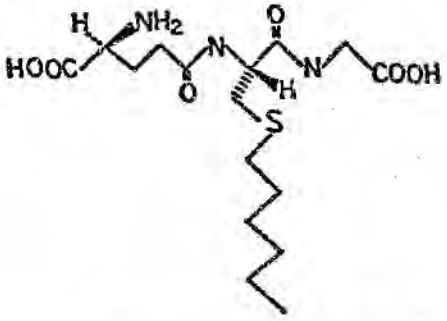
The binding of glutathione or its analogues at the G-site involves a network of specific polar interactions between the tripeptide and GST



Glutathione (GSH)
[γ-Glu-Cys-Gly]



Glutathione sulphonate
(GSO_3^-)



S-hexylglutathione
(S-hexGSH)

Figure 13. Structure of glutathione, glutathione sulphonate and S-hexyl glutathione.

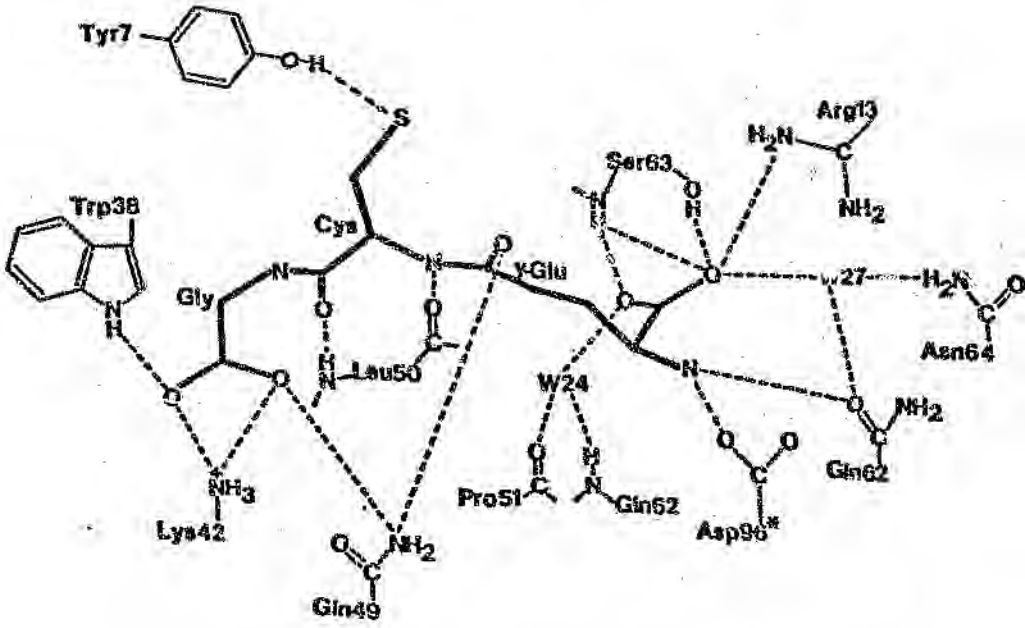


Figure 14. Active site interactions of pGSTP1-1 with glutathione (thick lines). An asterisk (*) indicates a residue from the neighbouring subunit. W24 and W27 represent water molecules 24 and 27 respectively. The hatched lines indicate hydrogen bonding interactions (Dirr et al., 1994b).

Although there is no direct evidence about conformational changes which occur in pGSTP1-1 upon ligand binding, these changes must be small (Dirr et al., 1994b). Comparison of human class alpha GST in apo form and in complexes with glutathione conjugate (Cameron et al. 1995) and the structure of *Schistosoma japonicum* glutathione S-transferase solved without ligands (McTigue et al., 1995) and in the complex with reduced glutathione (Lim et al., 1994) reveals that no significant conformational changes occur at the G-site upon ligand binding in these enzymes.

The active site does not seem to be in ideal conformation for binding glutathione and its analogues, and binding seems to occur via induced fit mechanism (Dirr et al., 1994b). The data from the chemical modification, mutagenesis and solvent-accessibility studies have indicated a local conformational change at or near the G-site which is translated through the protein causing Cys 45 to become buried in the molecule (Dirr et al., 1991,1994a). The absence of a Stoke's shift in the maximum emission wavelength of the protein tryptophans seems to eliminate the possibility of any major conformational change in pGSTP1-1 upon glutathione binding. Wang et al. (1993) shows that rat alpha class 1-1 isoenzyme undergo microconformational rearrangement when S-hexyl glutathione binds to it. Glutathione does not induce the same changes, implying that the occuppation of the H-site is required for conformational changes. Structure of porcine isoenzyme was solved in complex with glutathione sulphonate, and human pi class enzyme with S-hexyl glutathione. No global domain movement or dramatic reorganisation takes place when the S-hexyl moiety is added to the H-site.

1.2.3. Conformatioanal stability and unfolding of GSTs

Dirr and Reinemer, (1991), in a preliminary equilibrium un/folding study of the porcine class pi GST, suggested a two-state unfolding pathway with only folded dimer and unfolded monomers significantly present in equilibrium. In addition, studies on class alpha and mu GSTs (Dirr et al., unpublished results) and *Schisostoma japonicum* GST (Kaplan, W., Husler, P., Klump, H., Erhardt, J., Sluis-Cremer, N. and Dirr, H., submitted) also demonstrate a two-state unfolding mechanism. However, studies on the human class pi isoenzyme(Aceto et al., 1992), and *Proteus mirabilus* isoenzyme (Sacchetta et al., 1993), show multistep unfolding pathways, suggesting unfolding processes with a stable monomeric specie in equilibrium.

OBJECTIVES

Porcine class pi glutathione S-transferase (pGST P1-1) is a homodimeric protein, with two active sites kinetically independent, but structurally dependent on the neighbouring subunit. Conformational stability studies have been performed in order to understand more clearly the dynamics and the folding and assembly pathway of the protein, particularly to address the question about the existence of a thermodynamically stable GST monomer. The experimental data presented by this work substantiates preliminary finding (Dirr and Reinemer, 1991) that the monomeric state of the class pi enzyme is unstable under equilibrium conditions. Solvent denaturation experiments under equilibrium conditions suggest a two-state unfolding mechanism for unfolding of free pGSTP1-1, with only folded dimer and unfolded monomer significantly presented in equilibrium.

Conformational change induced upon binding of physiological substrate glutathione and its analogues, glutathione sulphonate and S-hexyl glutathione, on glutathione S-transferase, does not seem to be significant, but nothing is known about how they influence the conformation stability and unfolding of protein. As small changes in stability of proteins can result in a big changes in the steady-state concentration of proteins *in vivo*, the question how glutathione/analogues influence the conformationl stability and unfolding of porcine GSTP1-1 is addressed here. Both, equilibrium and kinetic studies suggest destabilising effect of glutathione and stabilising effect of its analogues on glutathione S-transferase.

Chapter 2

EXPERIMENTAL PROCEDURES

1. Materials.

GdnHCl (Aristar) and urea (Aristar) were purchased from BDH. 1,4-Dithiotreitol and reduced glutathione were purchased from Boehringer Mannheim. ANS and glutathione sulphonate were obtained from Sigma and 1-chloro-2,4-dinitrobenzene was purchased from Merck. S-Hexylglutathione was prepared according to the method of Vince et al. (1971) and washed with ethanol.

Molecular mass markers were obtained from Sigma.

All other chemicals were of analytical grade quality.

2.2. Methods

2.2.1. Purification of GSTs

pGSTP1-1 was purified from porcine lung according to the procedure described by Dirr et al. (1991). About 450 g of frozen pig lung was processed at a time, yielding about 20-30 mg of electrophoretically

pure protein. Enzyme was stored in 20 mM sodium phosphate, 0.1 M NaCl, 1 mM EDTA, 0.02% NaN_3, pH6.5, at 4°C and used within a month.

2.2.2. Protein determination

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard, or according to the semiquantitative method of Layne (1957):

Protein concentration (mg/ml) = $1.55\ A_{280} - 0.76\ A_{260}$

where A_{280} and A_{260} represent absorbance at 280 and 260 nm, respectively.

Another method for determining protein concentration was by measuring the absorbance, A_{280} and using the Beer-Lambert Law. The molar absorption coefficient, ϵ, for pGSTP1-1 was calculated according to Perkins (1986):

$$\epsilon(280)(M^{-1}cm^{-1}) = (\#Trp)(5500\ M^{-1}cm^{-1}) + (\#Tyr)(1340\ M^{-1}cm^{-1}) + (\#Cys)(150\ M^{-1}cm^{-1})$$

where # denotes number of particular residue in the protein molecule.

2.2.3. Homogeneity of the protein

The purity of enzyme samples were checked by SDS-polyacrylamide gel electrophoresis, according to Laemmli (1970) as described by Robyt and White (1990). The gel electrophoresis was done on 15% acrylamide separating and 3.75 % stacking gels, run for about 2.5 hours at 150-180V. The molecular weight markers used were α-lactalbumin (Mr 14000), trypsin inhibitor (Mr 20000), trypsinogen (Mr 24000), carbonic anhydrase (Mr 29000) glyceraldehyde-3-phosphate dehydrogenase (Mr 36000) and egg albumin (Mr 45000). The gels were stained for about 1 hour (0.25% Coomassie brilliant blue R250 in 45.4% methanol and 9.2% glacial acetic acid) and destained in 7% methanol, 7% glacial acetic acid as it was required.

2.2.4. Enzyme activity measurements

Because of its sensitivity to subtle structural changes in a protein molecule, measuring enzyme activity is a useful and widely used functional probe to monitor protein unfolding (Herold and Kirschner, 1990; Perry et al. 1992; Kwon et al. 1993; Zhuang et al. 1994).

The catalytic activity of pGST P1-1, without or previously equilibrated with denaturant (urea or GdnHCl), was determined by adding the enzyme to a reaction mixture containing 1 mM 1-chloro-2,4-dinitrobenzene, 1 mM reduced glutathione in 20 mM sodium phosphate, 0.1 M NaCl, 1 mM EDTA, 0.02% NaN_3, pH6.5, at room temperature.

All reactions were followed for 1 min at 340 nm. Activities were corrected for the corresponding blanks. The final concentration of enzyme was 6 nM. The highest residual denaturant concentration (106mM) and ligand concentration did not interfere with the assay. Linear progress curves and less than 10% reactivation of the enzyme were observed during the assay indicating that the distribution between active and inactive enzyme species was not significantly affected by the assay conditions. Reactivation of denatured glutathione S-transferases following the removal of denaturant by dilution is not instantaneous but requires at least 20 min for pGSTP1-1.

2.2.5. Unfolding/refolding studies.

If unfolding follow two-state mechanism (D⇌N), thermodynamic parameters for the process can be extracted from the unfolding data. The following basic strategy was used to study (un)folding equilibrium of pGSTP1-1:

(1) conditions were used which promote unfolding,

(2) changes which occur in the protein molecule upon unfolding were monitored by different techniques, and

(3) unfolding data were analyzed.

2.2.5.1. Conditions for promoting unfolding

All unfolding/refolding experiments were performed at room temperature in 20 mM sodium phosphate buffer, 0.1 M NaCl, 1mM

EDTA, 0.02% NaN_3, pH 6.5. Dimeric pGST P1-1 ranged from 0.1 to 100μM. Solutions of 10 M urea and 8 M GdnHCl were prepared in 20 mM sodium phosphate buffer containing 0.1 M NaCl, 1mM EDTA and 0.02% NaN_3, pH 6.5, according to Pace et al. (1989). Protein samples without or with different concentrations of denaturant were prepared by adding aliquots of a concentrated stock solution of denaturant to an eppendorf tube containing protein. In the refolding studies, a solution of the unfolded protein (6μM incubated in 2 M GdnHCl for 1h, or 8 M urea for 2h) was then diluted ten-fold with buffer without denaturant. All spectroscopic studies and enzyme activity measurements were performed after equilibrium was attained.

Unfolding/refolding studies in the presence of glutathione, glutathione sulphonate and S-hexyl glutathione were done by adding 1 mM or 5 mM glutathione, and 5 mM glutathione sulphonate and S-hexyl glutathione in the assay mixtures.

2.2.5.2. Monitoring changes which occur in the protein molecule upon unfolding by different techniques

It is important to monitoring unfolding with technique which shows significantly different signal for folded and unfolded protein, and signal-to-noise ratio to be as great as possible. Observed signal is then plotted vs denaturant concentration to generate unfolding curve. It is essential that the unfolding reaction has reached equilibrium before measurements are made, and that the unfolding reaction is reversible.

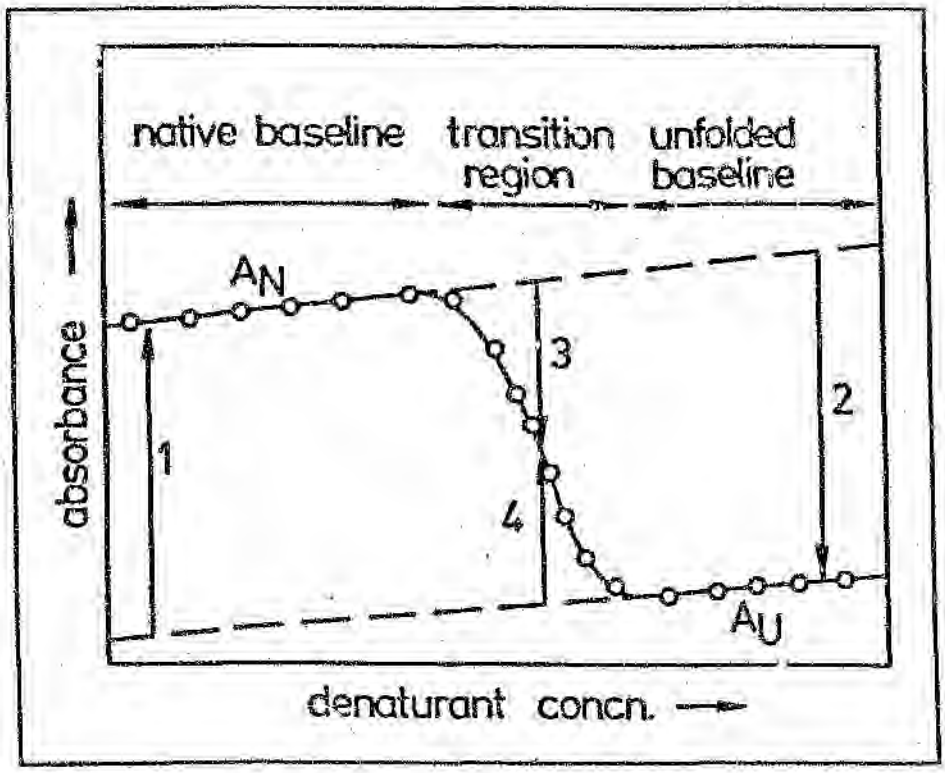


Figure 15. Schematic representation of a denaturant-induced unfolding transition of a protein that obeys the two-state approximation. The absorbance is selected as a property that change in the course of the transition. The extrapolated baselines for the native (A_N) and for unfolded (A_U) protein are given as dashes lines. The approximate division of the unfolding conditions into various regions is indicated on top of the figure (Schmid, 1992).

Equilibrium unfolding curves are usually sigmoidal type (figure 15) and can be divided into three regions (Pace et al., 1989):

1. The pre-transition region, which shows how value of the signal (Y) for the folded protein Y_F, depends upon the denaturant.
2. The transition region, which shows how Y varies as unfolding occurs.
3. The post-transition region, which shows howY for the unfolded protein, Y_U, varies with the denaturant.

All three regions are important for analysing unfolding curve and should have as many points as possible, at least 4 in pre- and post-transition and at least 5 in the transition. Unfolding should be monitored with as many different probes as possible, and coincidence of unfolding curves followed by different probes are indication of two-state unfolding process. It is important to emphasise that the simple observation of sigmoidal behaviour does not prove or disprove the two-state of unfolding (see Chapter 3 for further discussion) (Dill and Shortle, 1991).

2.2.5.2.1. Fluorescence spectroscopy

Fluorescence spectroscopy is a very useful for monitoring conformational transitions in a macromolecule, because the fluorescence signals are extremely sensitive to the microenvironment of a fluorophore (Lakowicz, 1983 ; Eftink, 1994). In studies with proteins, the fluorophore can be either intrinsic (tryptophan, tyrosine) or extrinsic (different covalently or noncovalently attached probes). Fluorescence is intrinsically a multi-dimensional method and enables measurements to be made as a

function of wavelength, time, polarization angle or solvent conditions (changing chaotrope concentration in (un)folding studies). The favourable lifetime of fluorophores allow different molecular processes to occur and affect the spectral characteristics of the fluorophore. Thermodynamic and kinetic information about transitions of macromolecules, such as a protein folding reaction, can be extracted from fluorescence measurements. In order to extract thermodynamic data from fluorescence it is critical to use method in which the signal is proportional to the population of macrostates, and satisfy the equation (Eftink, 1994):

$$S=\Sigma X_i S_i$$

where Si is the intrinsic signal for a given macrostate, and X_i is the mole fraction of molecules in the macrostate i.

The most straightforward signal that satisfies this equation, is fluorescence intensity, measured at some pair of excitation and emission wavelenghts. Anisotropy does not linearly track the mole fraction of macrostates and signal is weighted by both the fraction of states and by the fluorescence quantum yield of each state. In this work anisotropy was used to monitor protein unfolding, as it was done by several other researchers (Timm and Neet, 1992; Banik et al., 1992; Dufour et al., 1994; Tominaga et al., 1994).

Steady-state fluorescence methods.

All fluorescence measurements were performed at room temperature using a Hitachi model 850 Fluorescence Spectrophotometer.

The excitation bandwidth was set to 5 nm and the emission bandwidth to 10 nm. Samples were irradiated at 295 nm to selectively excite tryptophan residues and the emission monitored at 335 nm (for folded protein) and 350 nm (for unfolded protein). Spectra from 300-400nm were collected at a scan speed 60 nm/min.

Anisotropy decay were measured using Hitachi polarisation accessories. The fluorescence intensities I_{vv}, I_{vh}, I_{hh} and I_{hv}, were monitored, where subscripts refer to horisontal (h) or the vertical (v) positioning of the excitation and emission polarizers. Steady-state anisotropy (A) were calculated as follows (Lakowicz, 1983):

$$A = (I_{vv} - G*I_{vh})/(I_{vv} + 2G*I_{vh}).$$

where

$$G = I_{hv}/I_{hh}$$

G is the factor that corrects for the different efficiencies of the excitation and emission monochromators for horizontally and vertically polarized light, and in this case has a value of 1.02. Anisotropy measurements were made with excitation and emission wavelengths of 295 nm and 350 nm, respectively.

Kinetic fluorescence methods

Because of the great conformational heterogeneity of the unfolded state, protein folding is a special kinetic phenomenon in which every molecule of a typical population is likely to have a unique conformation at every moment. It is not clear at what stage different molecules start to

follow the same folding pathway (Schmid, 1992). For protein folding to be two-state kinetically, intermediates in folding must not be populated and the structural fluctuations within the unfolded states must be rapid, relative to the rate-limiting step in folding, so that all the unfolded protein molecules can follow the same pathway (Fersht, 1995; Schindler et al., 1995).

Unfolding kinetic studies were performed by mixing 200µl of pGSTP1-1 in buffer with ' µl of 10M urea, to yield a desired final concentration of glutathione, glutathione sulphonate and S-hexyl glutathione (0M - 0.5M) and 1µM pGSTP1-1. The samples were stirred continuously and maintained at 18°C. After an initial mixing period (dead-time ca. 10 sec), the appearance of the unfolded enzyme form was monitored with time by measuring the increase in fluorescence intensity at 350nm. All data were averaged over at least three experiments.

2.2.5.2.2. ANS binding.

The change in the spectral characteristics of 1-anilino-8-naphthalene sulfonic acid (ANS) upon binding to hydrophobic surfaces make the dye a valuable tool for monitoring conformational changes induced in the proteins during folding/unfolding (Lindsay and Pain, 1990). The binding of ANS to pGST P1-1 was studied by fluorescence at 25°C as a function of urea concentration (0-8 M). Excitation wavelength was set at 295 nm for measuring the transfer of excitation energy from Trp to ANS or excitation was at 400 nm for the direct

excitation of ANS. Emission from ANS was monitored at 480 nm. The concentrations of ANS and pGST P1-1 were 50μM and 1μM, respectively. Protein samples containing different amounts of urea were allowed to equilibrate for 2 hr before the addition of ANS and measurement of ANS binding.

2.2.5.2.3.Second-derivative spectroscopy

Second-derivative spectroscopy has been effectively used to resolve overlapping bands in the normal spectrum. Its ability to resolve complex protein absorption spectrum into the individual contributions of the three aromatic acids and to detect conformational changes involving the microenvironments of aromatic acids, makes it a useful tool in protein chemistry (Butler, 1979). The mutual interference between the second-derivative bands of tyrosine and tryptophan, evaluated in terms of the ratio between two peak to peak distances, has been utilised in detection of the exposure of tyrosyl residues even in the presence of a relatively high content of tryptophanyl residues. The second-derivative spectrum of N-AcTrpNH$_2$ (Fig. 16) in the spectral region between 280 and 300 nm shows two maxima centered around 287 and 295 nm and two minima at 283 and 290.5 nm, the position of which is only slightly affected by changing the polarity of the solvent.

Degree of exposure of tyrosyl residues (α) has been calculated as follows:

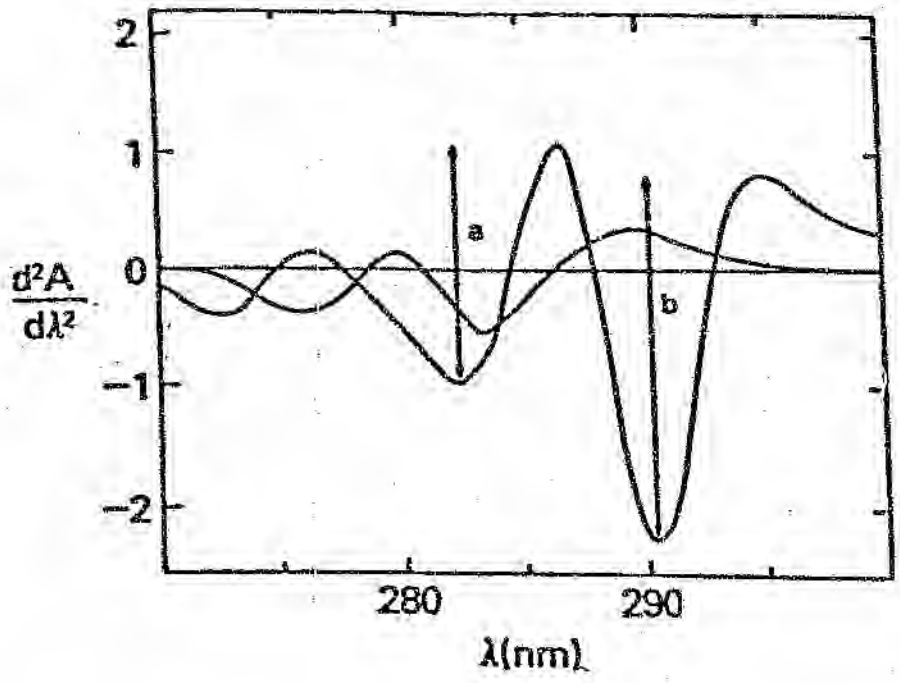


Figure 16. Second derivative spectra of equimolar solutions of N-AcTrpNH$_2$ and N-AcTyrNH$_2$ dissolved in 6.0 M Gdn Hcl - 0.05 M phosphate, pH 6.5. The spectrum of N-AcTrpNH$_2$ is identified by the two arrows a and b, which indicate the peak to peak distances between the maximum at 287 nm and the minimum at 283 nm and the maximum at 295 nm and the minimum at 290.5 nm, respectively (Ragone et al., 1984).

$\alpha = [(a/b)_n - (a/b)_a]/[(a/b)_u - (a/b)_a]$,

where a represents the peak to peak distance between the maximum at 287 nm and the minimun at the 283 nm,

b represents the peak to peak distance between the maximum at 295 nm and the minimun at the 290.5 nm.

$(a/b)_n$ presents a and b ratio for native protein,

$(a/b)_u$ presents a and b ratio for unfolded protein, and

$(a/b)_a$ presents ratio for the mixture which contain the same molar ratio of aromatic amino acids dissolved in a solvent possessing the same characteristics of the interior of the protein matrix. In this study ethylene glycol was used as a reference solvent (Ragone et al., 1984).

Second-derivative spectra were done on Varian DMS200 spectrophotometer, with slit 1 nm, smoothing 0.2 sec and speed of 200 nm /min.

2.2.5.3. Analysis of the unfolding curves

The unfolding data were fitted assuming the two-state unfolding mechanism. Consequently, for any points in the unfolding curve, only the folded and unfolded conformations are present at significant concentrations, and $f_F + f_U = 1$, where f_F and f_U represent the fraction of protein present in the folded and unfolded conformations, respectively. Therefore, the observed value of Y at any point is

$$Y = Y_F f_F + Y_U f_U$$

where Y_F and Y_U represent the values of Y characteristic of the folded and unfolded state, calculated as follows:

$$Y_F = mx + c,$$
$$Y_U = mx + c$$

from these values fraction of unfolded protein (F_u) at specific denaturant concentration was calculated according to:

$$F_u=(Y_F - Y_{obs})/(Y_U - Y_F),$$

where Y_{obs} is the observed signal (fluorescence intensity ratio F350/F335, or % residual enzyme activity).

2.2.6. Measuring the dependence of pGSTP1-1 stability on protein concentration.

The protein concentration dependence of the stability of the pGSTP1-1 in the presence of 4.5M urea (within unfolding transition) was monitored using both structural (tryptophan fluorescence, and second-derivative spectroscopy) and functional (enzyme activity) probes. The concentration of pGST P1-1 ranged from 0.1 to 100 μM in 20 mM sodium phosphate buffer, 0.1 M NaCl, 1 mM EDTA, 0.02% NaN_3, pH

6.5. Fluorescence, second-derivative absorption spectra and activity measurements were made as described above.

2.2.7. Differential scanning calorimetry (DSC)

Temperature-induced changes in proteins always proceed with a corresponding change of enthalpy (Privalov & Potekhin, 1986). The temperature dependence of the enthalpy can be determined experimentally by calorimetric measurements of the heat capacity of the protein solution over the temperature range of interest. The heat capacity of the protein in the denatured state is significantly higher than in the native state (Makhatadze and Privalov, 1996).

Temperature dependence of the heat capacity was determined by scanning pGSTP1-1 over a temperature range from 45 to 80°C using the differential adiabatic scanning microcalorimeter DASM-4 (Mashpriborintork, Moscow). 0.5 mg/ml of pGSTP1-1 in 20 mM sodium phosphate buffer, 0.1 M NaCl, 1 mM EDTA, 0.02% NaN_3, pH 6.5, was heated at a constant heating rate of 1°C/min. The baseline determined by scanning the sample buffer was subtracted from the data files before analysis. The area under the heat capacity curve represents the total enthalpy change resulting from the thermal unfolding of the dimeric protein into the denatured monomers.

Data were fitted to the two-state reaction scheme:

$$N \overset{K}{\rightleftharpoons} 2U,$$

where K is the equilibrium constant which is defined as a function of ΔH, ΔS and the partial specific heat capacity ΔCp. The equilibrium constant can be expressed as:

$$K = \exp(-\Delta G/RT) \qquad \text{(Eq. 1)}$$

$$\Delta G = \Delta H + \Delta cp(T-T_R) - T\{\Delta S + \Delta Cp \ln(T/T_R) - R \ln(2Ct)\} \qquad \text{(Eq. 2)}$$

ΔH and ΔS presents enthalpy and entropy changes at reference temperatures T_R.

The enthalpy changes (Δhcal) were calculated by integrating Δcp vs temperatur over the transition interval, taking the protein concentration into account. Δcp is the apparent heat capacity change from the initial state $F_{initial}$ to the final state F_{final}, and Ct is the protein concentration molarity (M). The equilibrium constant K differs from a conventional equilibrium constant as the molar concentration term Ct has been included.

The molar enthalpy and entropy changes for the unfolding process was obtained by fitting the equations (3 and 4) to the experimental data.

$$\alpha_2 = (-K = (K^2 + 4K)^{1/2})/2 \qquad \text{(Eq. 3)}$$

α_2 is the fractional amount of species $F_{final} = F_{initial}/2Ct$. The fitted molar

heat capacity function (Cp) which represents the DSC data can be expressed in terms of
α_2, ΔH and ΔCp as follows:

$$Cp = \Delta H (d\alpha_2/dT) + \alpha_2 \Delta Cp \qquad \text{(Eq. 4)}$$

The midpoint of the transition (Tm) values were defined as:

$$Tm = \Delta H / [\Delta S - R \ln(2Ct)] \qquad \text{(Eq. 5)}$$

where R is the gas constant (8.314 J/mol/K).

The Van't Hoft enthalpy was determined from the Van't Hoft plot of UV absorbance at A280 ($\ln A_{280}$) vs temperature ($1/T_{1/2}$), which have a slope

$$S = \Delta H_{vH}/(n - 1)R \qquad \text{(Eq. 6)}$$

where R is the gas constant and n the number of monomers for oligomeric proteins.

2.2.8. Size-exclusion HPLC.

Size-exclusion chromatography is often used for study unfolding of proteins because of the good correlation between the retention times and the Stokes radii of the proteins (Uversky, 1993).

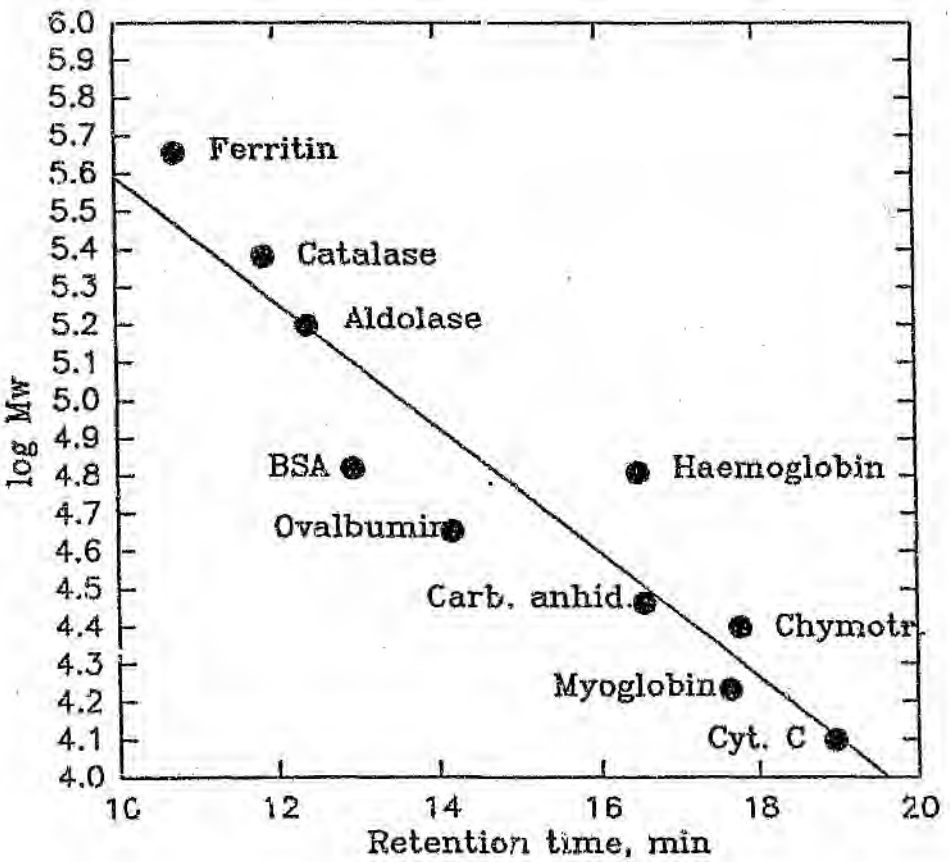


Figure 17. Calibration curve for BioSep SEC-HPLC S3000 column in 6 M GdnHCl in 20 mM sodium phosphate buffer, pH 6.5.

The hydrodynamic volume of pGST P1-1 at different GdnHCl concentrations was assessed at room temperature by analytical SEC-HPLC using a BioSep SEC-HPLC S3000 column (Phenomenex) (30 x 0.75 cm) at a flow rate of 0.5 ml/min. All buffer solutions prepared for HPLC were degassed and passed through a 0.45-µm nylon filter (Phenomenex). pGST P1-1 samples containing 12 µmoles of protein at different GdnHCl concentrations were prepared 15 min before injection onto the column that was pre-equilibrated with the same concentration of GdnHCl, and detected at 280nm. Column was calibrated with following molecular weight standards: catalase (Mr 240000), aldolase (Mr 158000), bovine serum albumin (Mr 68000), ovalbumin (Mr 45000), carbonic anhydrase (Mr 28800), chymotrypsinogen (Mr 25000), lysozyme (Mr 14300), cytochrome C (Mr 11700) (Figure 17). All markers were obtained from Boehringer Mannheim except for carbonic anhydrase and lysozyme which were obtained from Sigma.

Chapter 3

RESULTS AND DISCUSSION

3.1.CONFORMATIONAL STABILITY AND UNFOLDING OF pGSTP1-1

3.1.1 Fluorescence properties and reversibility of unfolding of pGSTP1-1

The first prerequisite for studying protein unfolding at equilibrium is to demonstrate reversibility of the unfolding reaction (Pace et al., 1989). Reversibility of pGST P1-1 unfolding was tested by a ten-fold dilution of denatured protein followed by enzyme activity (functional) and fluorescence (structural) measurements. Refolding was not instantaneous but required about 20 min to achieve equilibrium. Refolded pGSTP1-1 shows a tryptophan fluorescence spectrum with an emission maximum at 337 nm (native protein at 335nm) and a fluorescence intensity that is almost identical to the intensity for the native protein (Figure 18). This data indicates the presence of a significant amount of refolded protein. Furthermore, the recovery of enzyme activity was 70-80%, indicating that the refolding of pGST P1-1 observed by fluorescence is mainly to its catalytically functional form. Refolding of pGST P1-1 was not diminished after prolonged periods (18h) of exposure to 2M GndHCl.

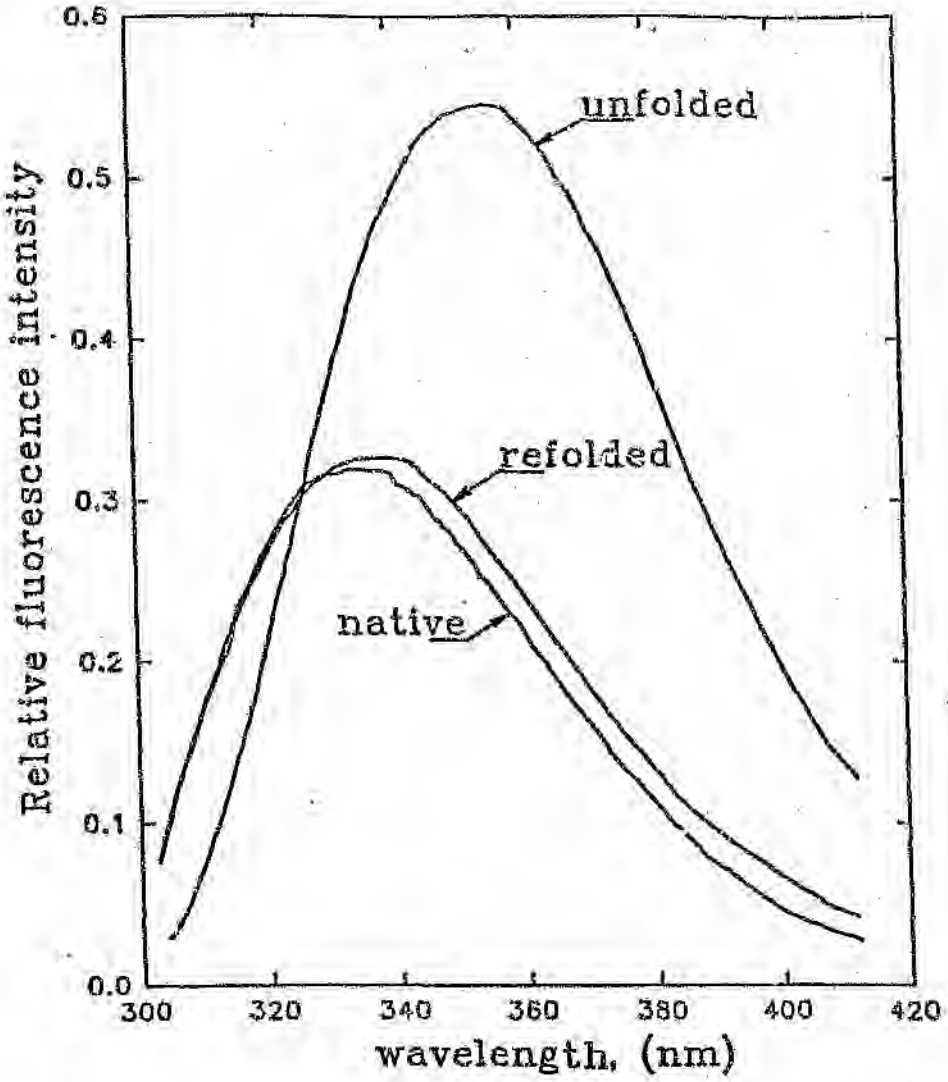


Figure 18. Reversibility of pGST P1-1 unfolding. Fluorescence emission spectra of 0.6μM pGSTP1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5. Excitation was at 295nm. Native pGST P1-1 was in 0.2M GdnHCl; unfolded pGST P1-1 in 2M GdnHCl; refolded pGST P1-1 in 0.2M GdnHCl after a ten-fold dilution of 6μM pGST P1-1 in 2M GdnHCl. All spectra were corrected for solvent blanks.

The intrinsic tryptophan fluorescence of pGST P1-1 arises from the partially exposed Trp28 and Trp38, both of which are located in domain I (Dirr et al., 1994a). As the concentration of denaturant is increased the fluorescence emission spectrum of pGST P1-1 red shifts from 335nm to 352nm, indicating the complete exposure of the tryptophans to solvent. In addition, unfolding is accompanied by a 2- to 3-fold increase in the fluorescence intensity indicating a quenching environment for the Trp residues in the folded protein. According to the crystal structure (Dirr et al., 1994a), the side chains neighbouring onto Trp28 include Ile5, Arg18, Leu21, Glu30, Phe190, Glu195 and Arg199, while those for Trp38 include Phe8, Val33, Met35, Pro39, Leu41, Cys45 and Leu50. Quenching of Trp28 in the folded protein can be explained by the hydrogen bond interaction between the fluorophore's indole ring and the carboxylate side chain of Glu30 (interatomic distance between Trp28 OE1 and Glu30 OE2 is 2.9Å). The situation for Trp38, however, is not clear since the crystal structure is of a binary enzyme-inhibitor complex and some conformational change occurs about Trp38 upon ligand binding (Dirr et al., 1994b).

High refolding recoveries following urea- or GdnHCl-denaturation were also reported for hGSTP1-1 (Aceto et al., 1992), *Proteus mirabilus* glutathione S-transferase (Sacchetta et al., 1993) as well as for rat liver class mu and alpha isoenzymes and *Schisostoma japonicum* GST (Hayes et al., 1981; Hayes, 1993; Dirr et al., unpublished results).

3.1.2. The equilibrium unfolding transition of pGSTP1-1

Equilibrium unfolding of pGSTP1-1 was followed by monitoring catalytic activity and fluorescence of tryptophan residues (Trp28 and Trp38, located in domain I) induced with urea (Figure 19) and anisotropy using guanidinium hydrochloride to promote unfolding (Figure 21). Transition midpoints obtained for urea and guanidine hydrochloride induced unfolding were 4.6 M and 1.4 M, respectively. Both figures shows conformational transition of the enzyme from folded to unfolded state, with coincident unfolding curves obtained for fluorescence and activity. All unfolding curves exhibit characteristic sigmoidal shape, implying cooperative interactions within the native state, result of free energy barrier which prevent individual residues to unfold rapidly. By "cooperative" is usually meant that that the state of protein is observed to have sigmoidal dependence on parameter which cause denaturation. Further, "sigmoidal", actually reffer to the S-shaped nature of a curve and not to specific mathematical functional form. In that regard, "cooperative" is very nonspecific and can refer to many different types of molecular behaviour (Dill and Shortle, 1991).

In a preliminary study, Dirr et Reinemer (1991) shows as well that the unfolding reaction of pGSTP1-1 is highly cooperative, with coincident individual curves obtained monitored with the different probes. They also suggest absence of any thermodynamically stable intermediates in equilibrium.

The minimal hypothesis for analysing folding and unfolding of globular proteins assumes that the protein exist in only two states, native

folded state, and the unfolded state. Most small monomeric proteins (Pace, 1990; Fersht, 1995) and many oligomeric (Liang and Terwillinger, 1991; Timm and Neet, 1992; Gittelman and Matthews,1990) obey a two-state unfolding model and the analysis of unfolding curve for obtaining $\Delta G(H_2O)$ in these cases is straightforward (Pace, 1986). If the denaturation curve shows steps, it is an indication that the denaturation is not a two-state process and the above mentioned method for estimating the free energy of unfolding is, therefore, not applicable. Most denaturation curves, however, show a single step, but this is no guarantee that denaturation follows a two-state mechanism. A standard qualitative test for the two-state unfolding mechanism is to compare the transition curves obtained by using different techniques for following denaturation. Coincident curves are an indication, but not the absolute proof for two-state unfolding. Noncoincident unfolding curve in some cases also can resulted from two-state unfolding (Dill and Shortle, 1991).

Proof that the unfolding reaction follows two-state mechanism is considered to be agreement between the Van't Hoff and calorimetrically determined enthalpy (dealt with in chapter 3.1.7.) (Sturtevant, 1987) and the existence of two distinct populations near the transition midpoint (dealt with in chapter 3.1.6.) (Chan et al., 1995).

$\Delta G(H_2O)$ of guanidine hydrochloride unfolding was found to be 25.2kcal/mol, while value for urea unfolding was 21kcal/mol.

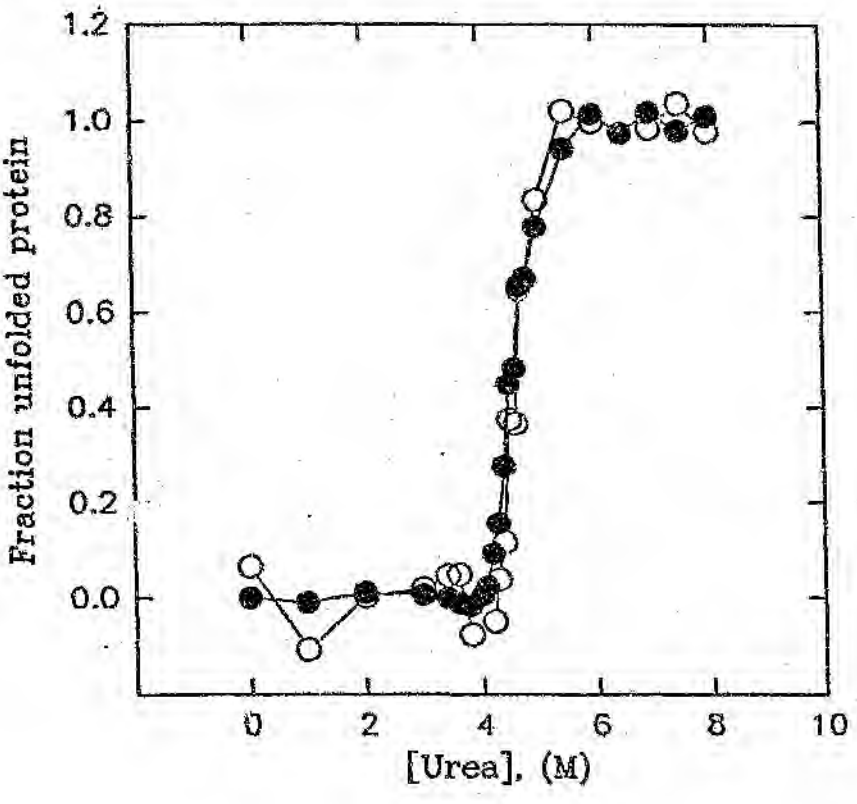


Figure 19. Equilibrium unfolding of pGSTP1-1. Fraction of unfolded protein determined from (◦) enzyme activity data, (•) fluorescence data.

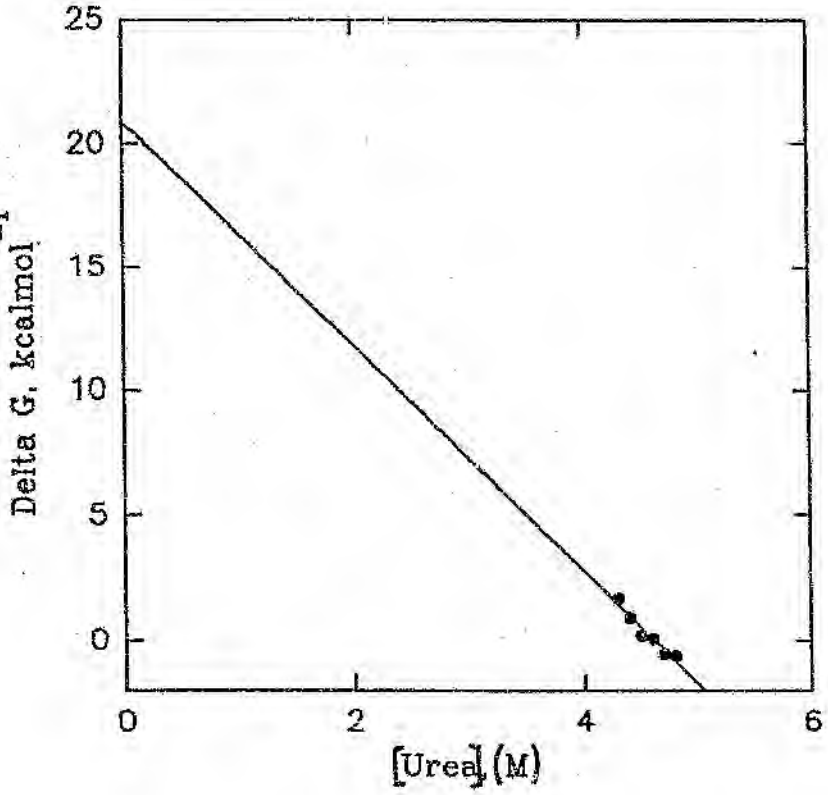


Figure 20. Free energy change for pGSTP1-1 unfolding as a function of urea concentration (solid line represent equation $\Delta G = \Delta G(H_2O) - m[\text{denaturant}]$.

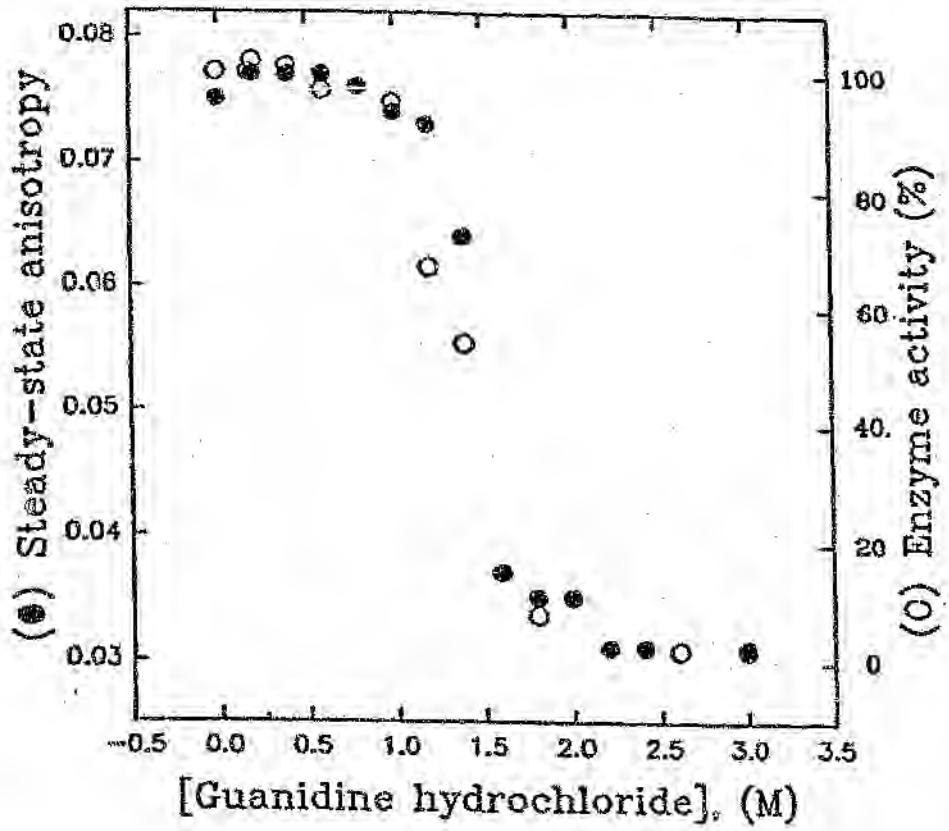


Figure 21. Fluorescence anisotropy and enzyme activity profiles for pGST P1-1 during unfolding. 1μM pGST P1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5, was equilibrated at different GdnHCl concentrations. For anisotropy, excitation was at 295nm and emission measured at 350nm.

Unfolding reaction of pGST1-1 is reversible and had reached equilibrium before measurements were made. Assuming the two-state model with the native and denatured states as the only relevant species, we are interested in the free energy change between the native and the denatured state of the protein:

$N \rightleftharpoons 2D$

Apparent equilibrium constant for dimeric protein is calculated from:

$Keq=2Pt[Fu2/(1-Fu)]$,

Pt being total dimeric concentration, and Fu, fraction of unfolded protein (as it is described in Methods, Chapter 2).

The difference in free energy, ΔG, is calculated:

$\Delta G=-RTlnKeq$.

From figure 20 is evident that ΔG varies linearly with denaturant concentration in the limited region in the transition. The simplest method of estimating the conformational stability in the absence of urea, $\Delta G(H_2O)$, is to assume that this linear dependence continues to zero concentration, and to use a least-squares analysis to fit the data to the equation:

$\Delta G_{app}(H_2O)=\Delta G-m[\text{denaturant}]$,

where *m* is the slope of the straight line, and indicates dependence of ΔG on denaturant concentration.

Other extrapolation methods also can be used to estimate $\Delta G(H_2O)$. At present, there is no good reason for using these more complicated procedures. In general, the estimates resulting from the other methods do not differ significantly when urea is the denaturant, but they may have some curvature with GdnHCl (Pace, 1975; Makhadatze and Privalov, 1994).

The *m* value determined from urea-unfolding curve, 4.52kcal/mol per M, was found to be in the excellent agreement with expected value (4.51kcal/cal per M) calculated according to Myers et al., (1995). From the study on 45 proteins gathered from literature, they found that the amount of area buried in each protein correlates very strongly (R=0.99) with the number of residues in each protein. They calculated change in solvent-accessible surface area (ΔASA) upon unfolding on the number of residues and found following correlation:

$$\Delta ASA = -907 + 93(\#res)$$

where #res denotes number of residues in particular protein.

Further, *m* value correlates very strongly with the amount of protein surface exposed to solvent upon unfolding, with linear correlation coefficients (R) of 0.84 for urea and 0.87 for guanidine hydrochloride yielding:

$$m = 368 + 0.11(\Delta ASA)$$

A difference in the *m* values can result from differences in the mechanism of folding (Myers et al., 1995). The presence of the stable, partially folded intermediate states at equilibrium will generally lead to a decrease in the *m* value. The larger m value must reflect a larger increase in the extent of folding (Carra and Privalov, 1995, Myers et al., 1995). For a two-state mechanism, the *m* value depends on the amount and composition of polypeptide chain that is freshly exposed to solvent upon unfolding. The larger *m* value must reflect a larger increase in the extent of folding. Changes in the *m* value arise from changes in the cooperativity of folding. This conclusion suggest that the two-state assumption is invalid and should not be applied, in any case where mutations are found to result in significant changes in the *m* value (Shortle, 1995; Carra and Privalov, 1995). For the proteins that undergo a simple two-state unfolding mechanism, the amount of surface exposed to solvent upon unfolding is a main structural determinant for *m*. Agreement of experimental and calculated *m* values confirm that the amount of pGSTP1-1 surface area exposed to solvent upon unfolding is the major structural determinant for its m-value.

3.1.3. Binding of ANS to pGST1-1 during unfolding

ANS has been widely used as a sensitive probe for monitoring structural changes in proteins and in detecting the presence of folding intermediates with hydrophobic patches such as the molten globule (Lindsay and Pain, 1990, De Young et al., 1993). Favourable sites for

ANS binding are clustered hydrophobic sites of defined geometry present in the protein folding intermediates. Usually, native proteins exhibit very little or no ANS binding due to the absence of the accessible hydrophobic binding sites. The unfolded states of protein exhibits very weak ANS signal exhibit as well even though all hydrophobic sites are exposed to the solvent, due to the absence of defined hydrophobic patches (Ptitsyn, 1995). Chaotropic agents such as urea directly competes with the interaction between ANS and the protein. This can be another reason of low ANS binding of unfolded states of protein. High concentrations of chaotrope are used for complete unfolding and this leads to the dye-displacement effect. The high hydrophobicity of intermediate states of folding seem to be sufficient to over-ride the dye-displacement effect at the lower chaotrope concentration used to promote forming of these states (Kumar et al., 1996).

As a ligand-binding protein, pGST P1-1 has a hydrophobic nonsubstrate binding site which also binds ANS weakly. Sluis-Cremer et al. (1996) on the basis of fluorescence resonance energy transfer data, shows that ANS binding site is at the subunit interface. Bico et al. (1995) found that ANS binding induces a microstructural changes that impacts on the functional conformation of the G-site of pGSTP1-1. In the absence of pGST P1-1, ANS showed low fluorescence and a maximum emission wavelength at 530 nm. When bound to the hydrophobic surface of the native protein the fluorescence intensity increases markedly accompanied by a blue shift in emission maximum to 480 nm (Bico et al., 1995).

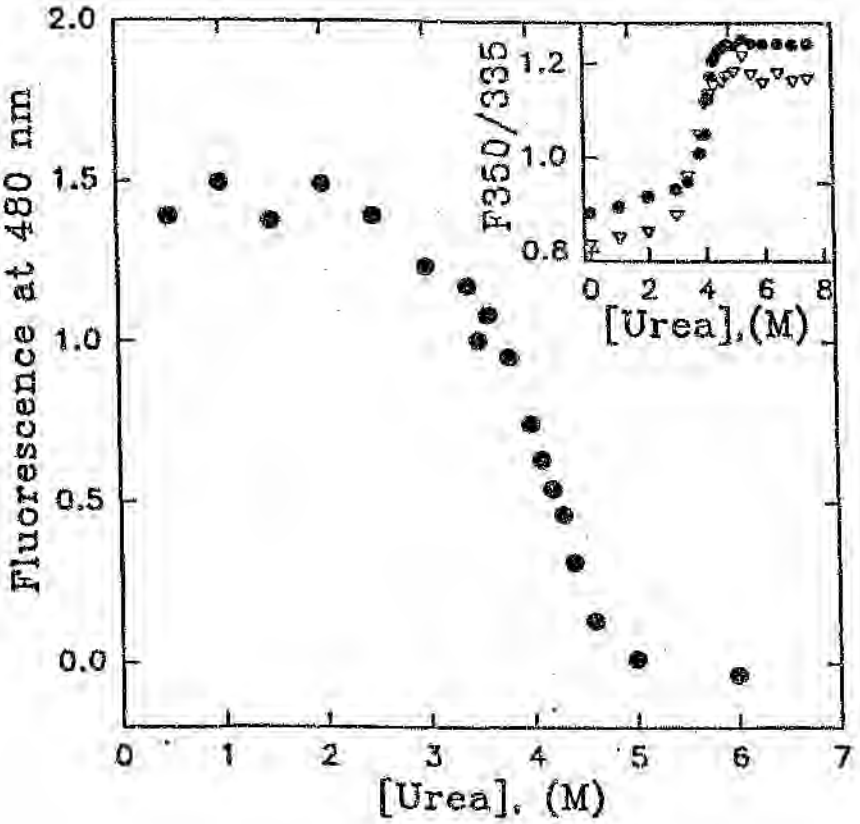


Figure 22. Binding of ANS to pGST P1-1 during unfolding. 50μM ANS was added to solutions of 1μM pGST P1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5, containing different urea concentrations. Excitation was at 400nm and the emission measured at 480nm. The insert shows unfolding curves for 1μM pGSTP1-1 in the same buffer as above without ANS (●) or with 50μM ANS (▽) by monitoring tryptophan fluorescence at 350nm and 335nm (excitation at 295nm).

Figure 22 illustrates the ANS fluorescence profile at 480nm during urea-induced unfolding of pGST P1-1. As the concentration of denaturant increases, the fluorescence intensity (and, therefore, ANS binding) decreases via a single transition indicative of a cooperative two-state process. The appearance in the unfolding transition of an intermediate with hydrophobic patches would have resulted in a marked increase in ANS binding by the intermediate followed by a decrease as the intermediate unfolds. Similar results were observed when ANS was either excited directly at 400nm or when excited by the transfer of excitation energy from tryptophan residues (excitation at 295nm). Furthermore, the binding of ANS does not appear to influence the unfolding mechanism of pGSTP1-1. This is indicated by the similar unfolding curves for the protein (measuring tryptophan fluorescence) in the absence and presence of ANS (Figure 22, insert). The slightly lower fluorescence values in the presence of ANS are as a result of the quenching of tryptophan fluorescence by ANS (Bico et al., 1995).

3.1.4. Protein-concentration dependence studies on pGSTP1-1.

Protein concentration dependence were monitored with enzyme activity as a functional probe, and fluorescence spectroscopy, anisotropy and second-derivative spectroscopy as structural probes, at denaturation midpoint (4.5 M Urea) in the range of 0.1 - 100 μM pGSTP1-1. All examined probes (Figure 23a, b, c) shows dependence of pGSTP1-1 unfolding of protein concentration, i.e. higher amount of folded protein

in the assay mixture as protein concentration increase.

Several descriptions for the unfolding pathway for the pGSTP1-1 are possible, depending on the relative stabilities of dimer and folded monomers. It is reasonable to assume two basic models:

Model 1, a three-state pathway in which dissociation of the active dimer (D) to inactive but structured monomers (M) occurs prior to the formation of the unfolded state (U):

$$D \overset{K_1}{\rightleftharpoons} 2M \overset{K_2}{\rightleftharpoons} 2U$$

where $K_1 = [M]^2/[D]$ is the equilibrium constant for the bimolecular dissociation reaction, and $K_2 = [U]/[M]$ is the equilibrium constant for the unimolecular unfolding reaction. It is assumed that the structure for M is similar to that for the dimer's subunits (also see Aceto et al., 1992).

Model 2, a two-state model with only the active dimer and denatured monomer significantly populated at equilibrium:

$$D \overset{K}{\rightleftharpoons} 2U$$

where $K = [U]^2/[D]$ is the equilibrium constant for the concerted and bimolecular unfolding reaction.

According to the law of mass action, the bimolecular reactions (and, therefore, the probes monitoring these reactions) described above by K_1 (model 1) and K (model 2) should be protein concentration dependent. This dependence has been used as a diagnostic tool for

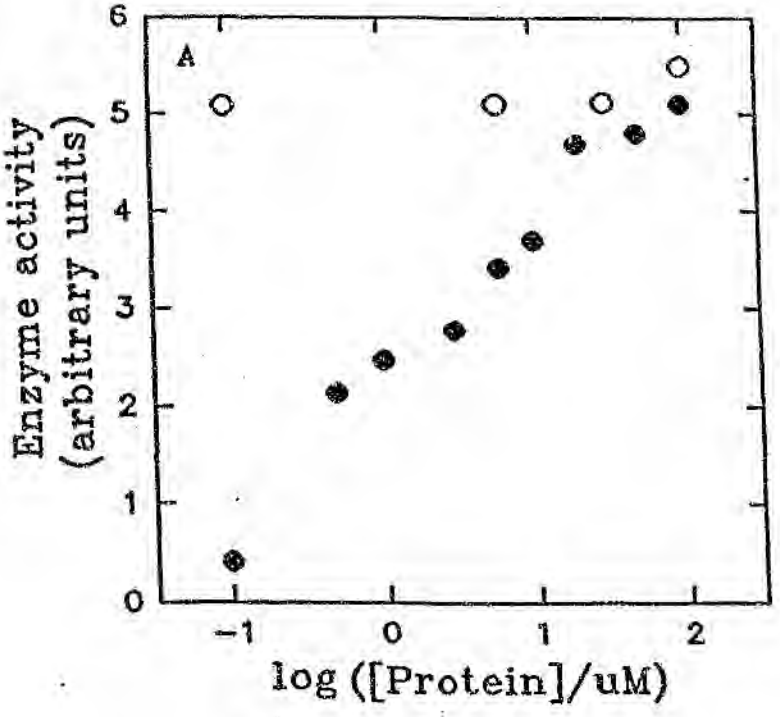


Figure 23a. Protein-concentration dependence of pGST P1-1 stability in 4.5M urea. Enzyme activity in arbitrary units was measured for pGST P1-1 (0.1- 100μM) incubated in the absence (o) or presence of 4.5M urea (●).

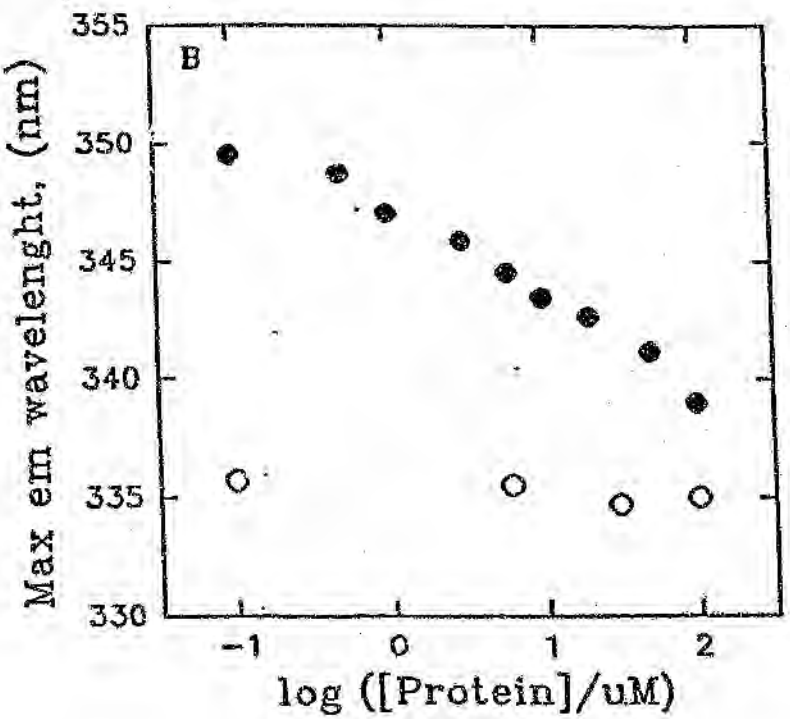


Figure 23b. Protein-concentration dependence of pGST P1-1 stability in 4.5M urea. Changes in the fluorescence maximum emission wavelength was monitored (excitation at 295nm) for 0.1-100μM pGST P1-1 incubated in the absence (O) or presence of 4.5M urea (●).

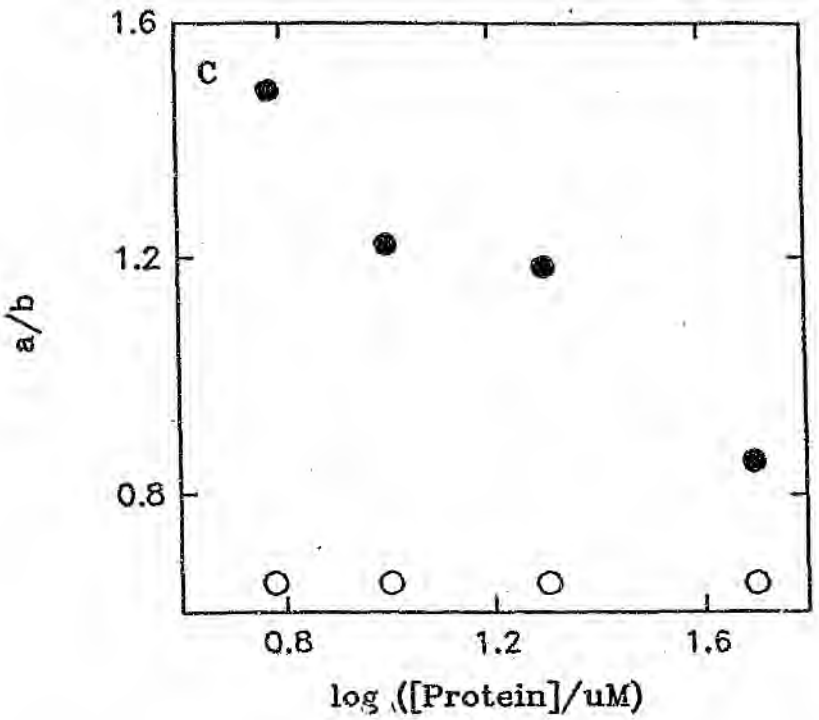


Figure 23c. Protein-concentration dependence of pGST P1-1 stability in 4.5M urea.Changes in the second-derivative spectroscopy signal for 0.1-100μM pGST P1-1 incubated in the absence (O) or presence of 4.5M urea (●).

Since the active enzyme requires a dimeric structure, the dependence illustrated in Fig. 23a of enzyme activity or protein concentration is suggestive of either model 1 or model 2 for the unfolding pathway. For Model 1, the stability of the folded monomer (M) as monitored by structural probes should not change with protein concentration. Fig. 23b, however, indicates a protein-concentration dependence for fluoresence (a structural probe) as expected from a two-state transition between native dimer and unfolded monomer (Bowie and Suaer, 1989). Each subunit of pGSTP1-1 contains ten tyrosine residues distributed throughout the polypeptide; three in domain I, six in domain II and one in the peptide linker joining the two domains. The same conclusion could be drawn from stability data when the degree of tyrosine exposure was monitored by second-derivative spectroscopy (fig. 23c). Protein-concentration dependence of unfolding reaction was also confirmed by using fluorescence anisotropy as a probe. As both functional and structural probes show a protein-concentration dependence, model 1 involving a stable folded monomer can be excluded for describing the unfolding of pGST P1-1.

3.1.5. Fluorescence anisotropy studies of pGSTP1-1

Fluorescence anisotropy is a sensitive probe for monitoring different conformational states of proteins and has been widely used to monitor protein unfolding (Timm and Neet, 1992; Banik et al., 1992; Dufour et al., 1994, Tominaga et al., 1994). Steady-state anisotropy of a

spherical molecule is dependent upon temperature, viscosity, fluorescence lifetime and molecular volume (Lakowicz, 1983). Angular displacement of a fluorophore resulting in reduced anisotropy is dependent upon the rate and extent of rotational diffusion during the lifetime of the fluorophore's excited state. Since neither of the two tryptophans in pGST P1-1 are located at or near the subunit interface we assume that the fluorescence lifetimes of these residues will not be affected by a possible dissociation of dimer to folded monomer. Therefore, under conditions of constant temperature, low viscosity and dilute protein concentration rotational diffusion (which is dependent on molecular volume) should be the only significant process which would affect anisotropy. If a structured monomer could exist at equilibrium, the dissociation of the dimer to monomers should be observed by anisotropy. Fig. c illustrates a monophasic and cooperative decrease in anisotropy with increasing concentrations of denaturant. The anisotropy curve is also coincident with the curve obtained when monitoring enzyme activity (Fig. 21). A relatively low anistropy value of 0.075 in the absence of urea indicates partially mobile indole fluorophores with a degree of rotational freedom. This would be consistent with the partial exposure of the residues. Of significance is the absence of steps or shoulders in the pretransition region suggesting that the dimer does not dissociate into structured monomers prior to the unfolding transition (see Tominaga et al., 1994). The low anisotropy value of 0.03 in post-transition region suggests high mobility of unconstrained tryptophan fluorophores in the unfolded protein.

3.1.6. Size-exclusion HPLC of pGST P1-1 during unfolding.

Size-exclusion chromatography is a widely used technique for monitoring protein (un)folding (Shalongo et al., 1987; Zerovnik et al., 1992; Philo, et al. 1993; Palleros et al., 1993; O'Brien Gress et al. 1994). It is able to resolve changes in the hydrodynamic properties along the denaturation pathway, and to detect the presence of intermediate states, provided they are kinetically stable within the time scale of the chromatographic run (Corbett and Roche, 1984). Figure 24 shows the elution profiles of pGST P1-1 in the presence of different GdnHCl concentrations. Only two species can be observed; one at the retention time for the native dimeric protein, and another at the retention time for the unfolded monomer. Both species appeared in the transition.The presence of two distinct population near the midpoint of the transition, obtained with methods such as size-exclusion chromatography or gel electrophoresis is considered as another strong evidence for the two-state unfolding (Uversky, 1993; Chan et al, 1995). Some swelling of the unfolded specie was observed with increasing GdnHCl concentration, evident as slight increase in the retention time, after transition. This is most likely due to the solvation of the unfolding protein with denaturant already observed for many other proteins (Uversky and Ptitsyn, 1994). The midpoint for the unfolding transition in Figure 22 is at 1.35 M GdnHCl and is similar to a value of 1.4 M obtained by other methods (this work; Dirr and Reinemer, 1991). The presence of folded monomer was not observed in the elution profiles suggesting an "all-or-none" transition between the folded dimer and unfolded monomers.

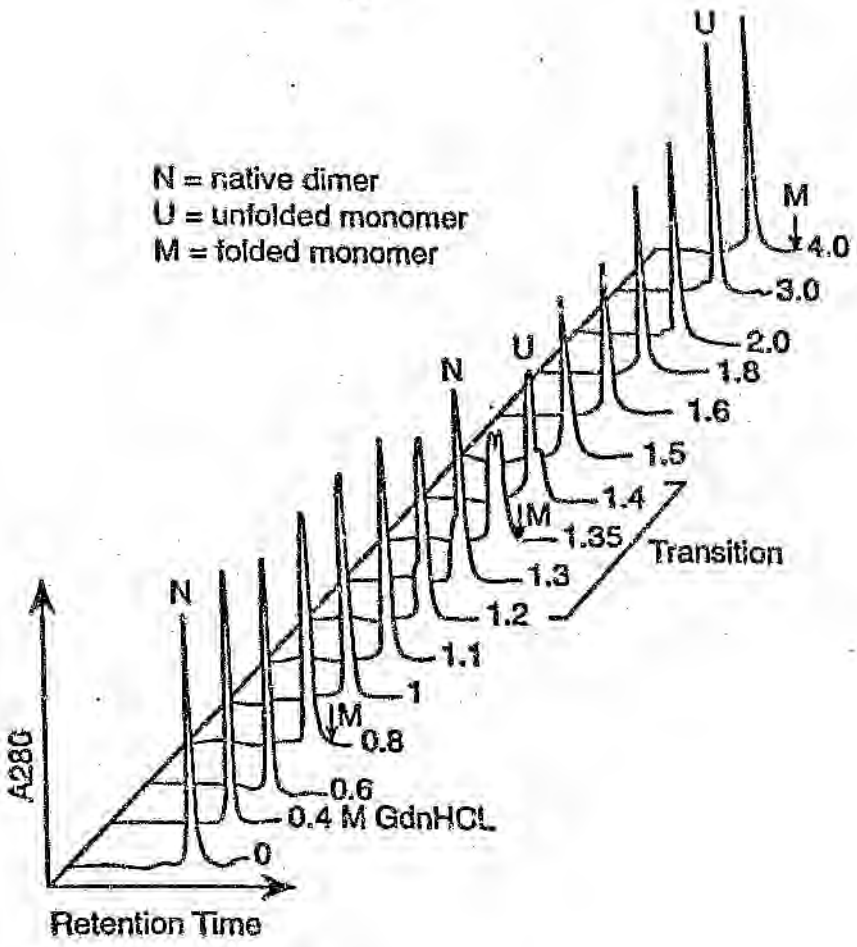


Figure 24. SEC-HPLC elution profiles of pGST P1-1 incubated with GdnHCl. pGST P1-1 samples containing 12 μmoles of protein at different GdnHCl concentrations (0-4M) were injected onto a BioSep SEC-HPLC S3000 column pre-equilibrated at the same concentration of GdnHCl. The peaks of native dimer and unfolded monomer are marked N and U, respectively. The unfolding transition region is also indicated.

3.1.7. Differential scanning microcalorimetry of pGSTP1-1

DSC has been extensively used as a technique to monitor unfolding processes since it allows one of the most direct thermodynamic analysis for the process (Privalov and Potekhin, 1986; Griko et al., 1994; Liu and Sturtevant, 1996).

The profile for the heat capacity change due to the thermal unfolding of pGSTP1-1, can be deconvoluted into two independent, cooperative, but partially overlapping transitions, with maximum heat capacity at 59°C for the first transition and at 62°C for the second (Figure 25). The ratio of these two transitions is 1 : 2. Heating the protein to 59°C and then cooling it down rapidly to the room temperature does not effect the scan of the second transition. Both steps are irreversible, and rerun after heating beyond the upper transition shows almost baseline values. At this stage we cannot identify the molecular basis for the two processes. Light scattering measurements at 350 nm, as a function of temperature increase with denaturation, indicating that aggregation is taking place. The interpretation of equilibrium unfolding data with the purpose of extracting thermodynamic parameters are usually based on the reversibility of the process, but relevance of using data from irreversible proccesses have also been demonstrated (Sturtevant, 1987; Privalov, 1982).

The calorimetric enthalpy is a model-independent quantity, determined directly from experiment, while Van't Hoff enthalpy is based on certain thermodynamic assumption. The agreement of these two enthalpies is considered as strong evidence for an overall two-state

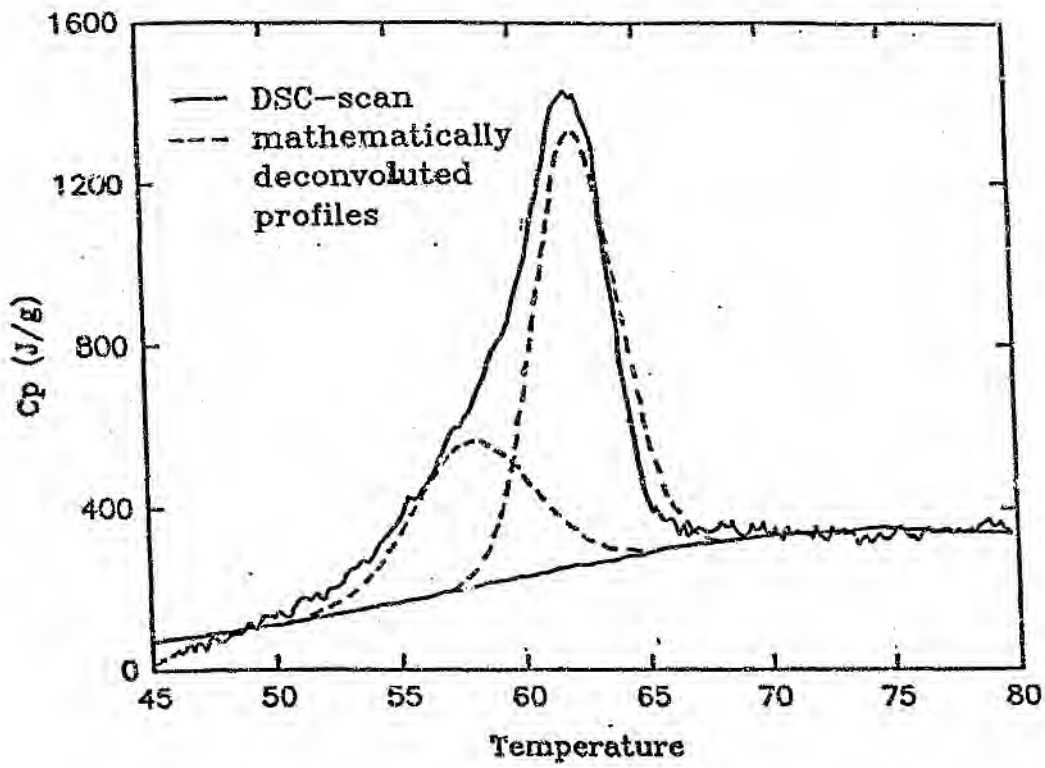


Figure 25. DSC-scan for pGSTP1-1

mechanism of unfolding (Privalov, 1979; Privalov and Gill, 1988, Chan et al., 1995).

The transition enthalpy per mol of dimer was found to be 166.8 kcal, for the sum of pre- and main transition. The Van't Hoff enthalpy determined from the slope of the UV absorbance at A280 vs temperature scan was 167 kcal/mol of dimer. The ratio of Van't Hoff (167 kcal/mol of dimer) and calorimetrically determined enthalpy value (166.8 kcal/mol of dimer) obtained was very close to unity, again indicating a two-state process of unfolding.

3.1.8. Discussion

All experimental data presented here and in the preliminary study (Dirr and Reinemer, 1991) are in full support of a highly cooperative and concerted two-state pathway for the equilibrium unfolding of pGST P1-1 (i.e., dimer $\rightleftharpoons$ unfolded monomer). A folded monomer could not be detected implying that it is thermodynamically unstable. Subunit interactions in the protein, therefore, not only stabilise the association of subunits but are a significant source of stabilisation of the subunit tertiary structure. This phenomenon has been observed for an increasing number of dimeric proteins (Kwon et al., 1993, Grant et al., 1992, Timm & Neet, 1992). Complementary interactions at the subunit interface of pGST P1-1 are mediated primarily between structural elements in domain I of one subunit and in domain II of the adjacent subunit (Dirr et al., 1994a).

Furthermore, about 14% of the subunit's solvent-accessible surface area is buried in the assembled dimeric pGST P1-1 molecule. This value is larger than those observed at the interface for protein-protein complexes involving proteins that are thermodynamically stable separately (Janin and Chothia, 1988).

However, two studies with the human class pi orthologue hGST P1-1 (Aceto et al., 1992) and a glutathione S-transferase from *Proteus mirabilus* (Sacchetta et al., 1993), suggest multistep unfolding processes in which the dimeric molecule first dissociates to structured monomers before unfolding. hGST P1-1 and pGST P1-1 display a sequence identity of about 83% and have almost identical overall structures (Reinemer et al., 1992; Dirr et al., 1994b), it is not clear at present why their unfolding pathways should be so different.

Furthermore, conformational stability studies with a Schistosomal glutathione S-transferase also demonstrate a two-state process for urea-induced unfolding (Kaplan, W., Hüsler, P., Klump, H., Erhardt, J., Sluis-Cremer, N. and Dirr, submitted). Unpublished results from our research group for some other isoenzymes also indicate two-state unfolding mechanism (Mu1-1 and Mu2-2, human Alpha1-1, rat Alpha 2-2, while sigma isoenzyme shows multi-state unfolding mechanism (Dirr et al., unpublished results).

3.2. INFLUENCE OF GLUTATHIONE/ANALOGUES ON CONFORMATIONAL STABILITY AND UNFOLDING OF pGSTP1-1

3.2.1. Fluorescence properties of pGSTP1-1 in the presence of glutathione, glutathione sulphonate and S-hexyl glutathione and reversibility of unfolding

The binding of GSH or its S-analogues to pGSTP1-1 quenches about 20% of the protein's tryptophan fluorescence. The maximum emission wavelength remained unchanged at 335 nm indicating that the polarity of tryptophan microenvironment was not changed significantly. Fluorescence quenching by GSH/analogues occurs most likely as a consequence of a direct interactions between GSH and the indole fluorophore of Trp 38 which is located at the active site (Dirr, 1994a). Unfolding of pGSTP1-1 in the presence of ligands results in a red shift in the emission maximum from 335nm to 352nm accompanied by a 3-fold increase in the fluorescence intensity, similar to that observed for the uncomplexed enzyme (Fig 26a, 26b, 26c).

To determine any influence G-site ligands might have on pGSTP1-1 stability, it is essential to check reversibility of unfolding in the presence of each of these ligands (Pace et al., 1989). Refolded pGSTP1-1 (following a 10-fold dilution in non-denaturing buffer) shows a tryptophan fluorescence spectrum with an emission maximum being the

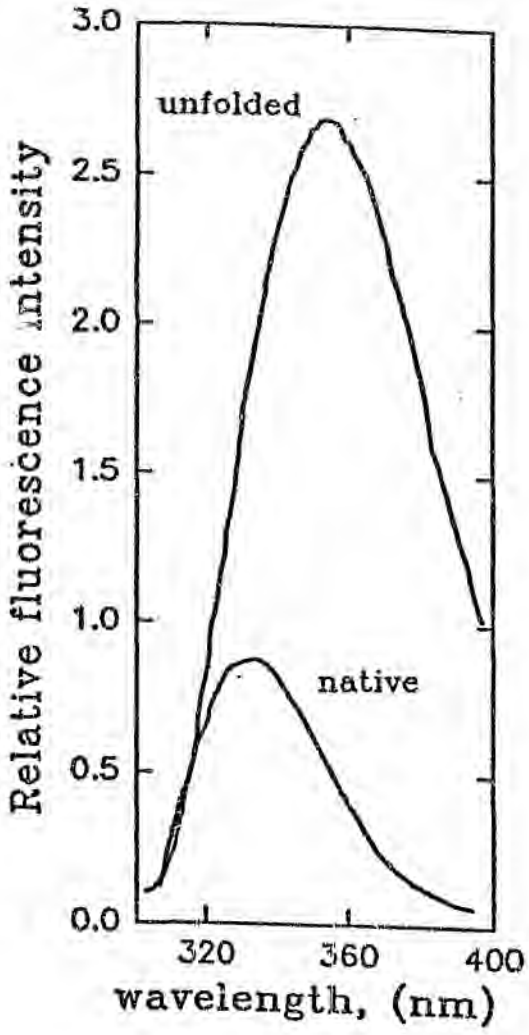


Figure 26a. Fluorescence emission spectra of 1 μM pGSTP1-1 during unfolding (excitation was at 295 nm) in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5 in the presence of gluthatione (5 mM). Excitation was at 295nm. All spectra were corrected for solvent blanks.

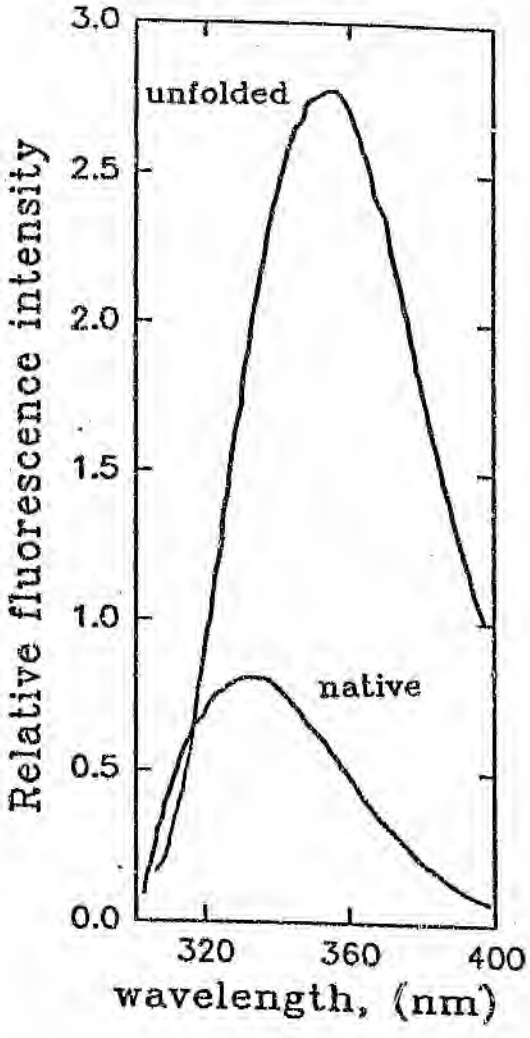


Figure 26b. Fluorescence emission spectra of 1 μM pGSTP1-1 during unfolding (excitation was at 295 nm) in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5 in the presence of glutathione sulphonate (0.5 mM). Excitation was at 295nm. All spectra were corrected for solvent blanks.

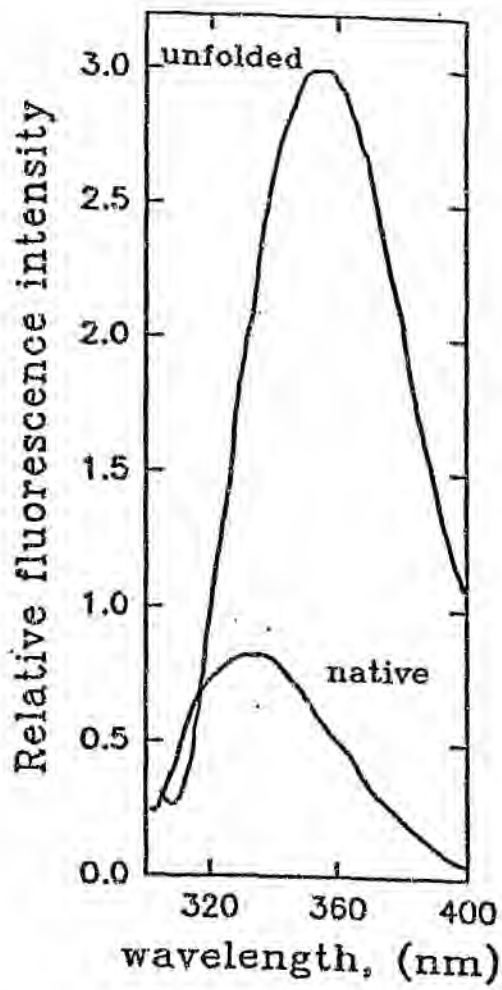


Figure 26c. Fluorescence emission spectra of 1 μM pGSTP1-1 during unfolding (excitation was at 295 nm) in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5 in the presence of S-hexyl glutathione (0.5 mM). Excitation was at 295nm. All spectra were corrected for solvent blanks.

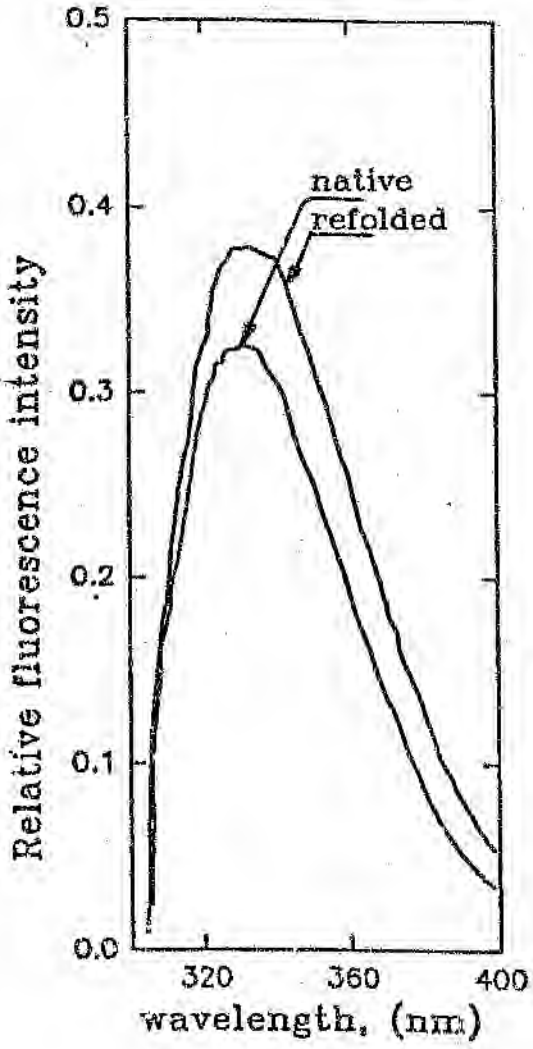


Figure 27a. Reversibility of pGSTP1-1 in the presence of 5 mM glutathione. Fluorescence emission spectra of 0.4μM pGSTP1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5. Excitation was at 295nm. Unfolded pGST P1-1 in 8M urea; refolded pGST P1-1 in 0.8 M urea after a ten-fold dilution of 4μM pGST P1-1 in 8M urea. All spectra were corrected for solvent blanks.

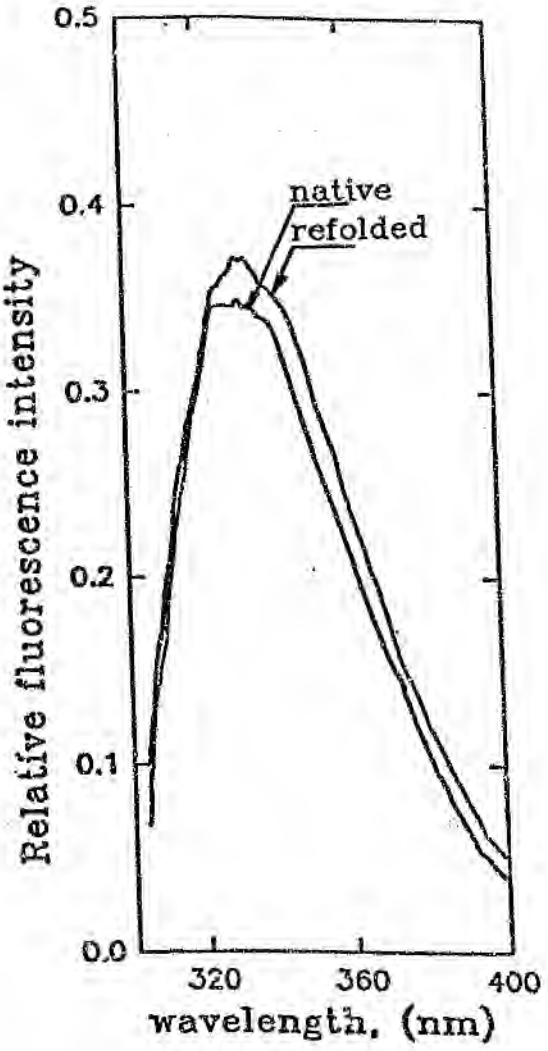


Figure 27b. Reversibility of pGSTP1-1 in the presence of 0.5 mM glutathione sulphonate. Fluorescence emission spectra of 0.4μM pGSTP1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5. Excitation was at 295nm. Unfolded pGST P1-1 in 8M urea; refolded pGST P1-1 in 0.8 M urea after a ten-fold dilution of 4μM pGST P1-1 in 8M urea. All spectra were corrected for solvent blanks.

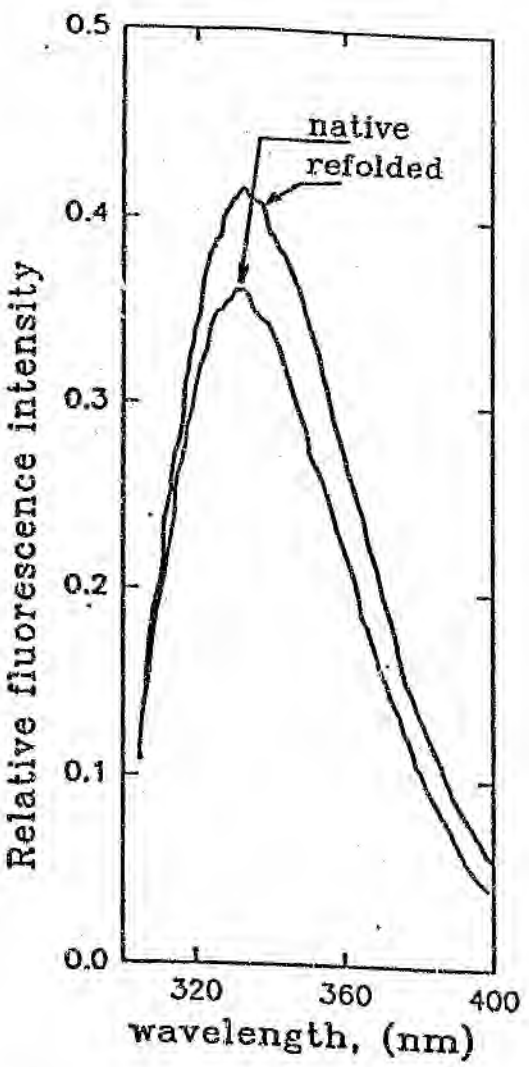


Figure 27c. Reversibility of pGSTP1-1 in the presence of 0.5 mM S-hexyl glutathione. Fluorescence emission spectra of 0.4μM pGSTP1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5. Excitation was at 295nm. Unfolded pGST P1-1 in 8M urea; refolded pGST P1-1 in 0.8 M urea after a ten-fold dilution of 4μM pGST P1-1 in 8M urea. All spectra were corrected for solvent blanks.

Fluorescence intensity is slightly higher than for the native protein indicating that ligands had not bound back to refolded state with the same extent as to the native state, causing quenching of protein fluorescence of refolded state to be lower. That further can indicate that not all protein refolded to native like conformation, but overall, the estimated amount of refolded protein is at least 85% in the presence of glutathione/analogues (Fig. 27a, 27b, 27c), even slightly larger than for uncomplexed enzyme.

3.2.2. Influence of GSH and its analogues on equilibrium unfolding of pGSTP1-1

Equilibrium unfolding of pGSTP1-1 in the absence and presence of glutathione, glutathione sulphonate or S-hexylglutathione was monitored using Trp fluorescence and m asuring enzyme activity. Data are illustrated in the Figure 28. The unfolding transition curves obtained from fluorescence (l ure 28A) and activity measurements (Figure 28B) are monophasic and essentially coincident

Glutathione has a relatively low affinity for the pGSTP1-1 (Kd = 120 μM; Bico et al., 1995), while gluthathione sulphonate and S-hexyl glutathione bind much tighter to the enzyme (Kd = 4 μM; Kd of 2 μM, respectively; Dirr et al., 1991).

The unfolding curves for the pGSTP1-1-glutathione complex are shifted towards the lower urea concentration compared to the free enzyme indicating a destabilising effect on the pGSTP1-1 molecule.

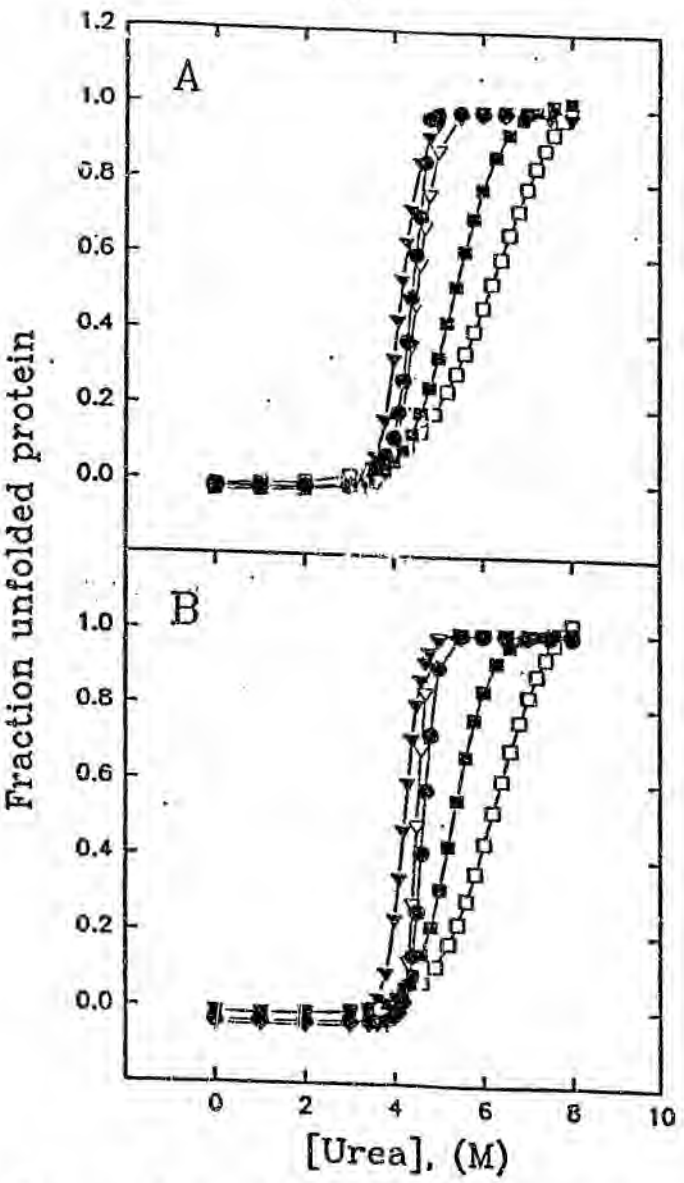


Figure 28. Influence of glutathione, glutathione sulphonate and S-hexylglutathione on the equilibrium unfolding of pGSTP1-1. Fraction of unfolded protein determined from (A) fluorescence data and (B) enzyme activity data. (●) without ligands; (▽) with 1 mM GSH; (▾) with 5 mM GSH; (■) with 0.5 mM S-hexGSH; (□) with 0.5 mM glutathione sulphonate.

At a saturating concentration of 5mM glutathione, the transition midpoint of pGSTP1-1 is shifted from 4.6M to 4.1M urea. Contrary to the destabilising effect of GSH, the saturating concentration of glutathione sulphonate and S-hexyl glutathione seems to have stabilising effect on the pGSTP1-1 molecule. The transition midpoints are shifted towards the higher urea concentration, from 4.6 M up to 5.4 M urea for S-hexyl glutathione and 6M urea for glutathione sulphonate.

The slope of the unfolding transition (or denaturant m-value; see 3.1.2.) for the free enzyme is 4.5 kcal/mol, and is decreased to 4.4 and 3.8 kcal/mol/M, in the presence of 1mM and 5mM GSH, respectively. According to these *m*-value data, the binding of glutathione appears not to affect significantly the way the protein responds to urea-induced unfolding. Glutathione sulphonate and S-hexylglutathione unfolding transitions exhibit much lower slopes than observed for the free enzyme causing a significant decrease in the *m* value. The *m* values observed for glutathione sulphonate and S-hexyl glutathione are 1.07 and 1.35 kcal/mol respectively, indicating that the bound ligands significantly altered the protein's unfolding pathway.

Assuming a two-state equilibrium unfolding/refolding process in the absence and presence of glutathione, conformational stabilities, i.e., $\Delta G(H_2O)$, of 21 kcal/mol, 20 kcal/mol and 16 kcal/mol were determined from the unfolding data (Figure 20) for pGSTP1-1 without and with 1mM and 5mM glutathione, respectively. Free energy difference values for pGSTP1-1 in the presence of the glutathione analogues were not determined in light of the large lowering of the m-value suggesting that a two-state model is no longer valid (Pace, 1986; Carra and Privalov,

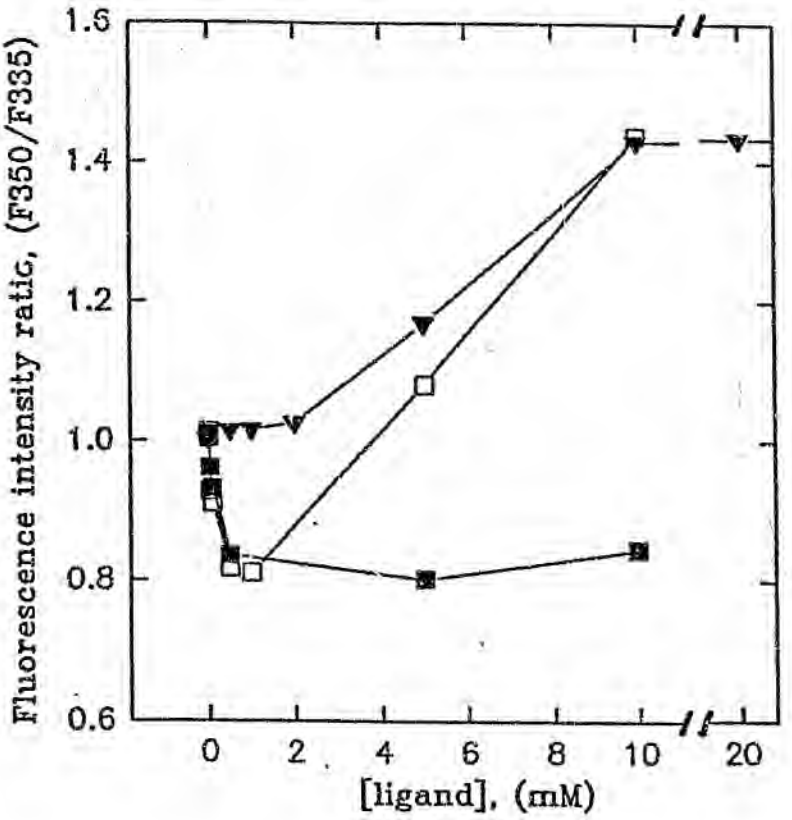


Figure 29. Dependence of fluorescence intensity ratio of pGSTP1-1 in 4.4 M urea upon the concentration of (▾) glutathione, (■) S-hexylglutathione, and (□) glutathione sulphonate. F350 and F335 are the fluorescence intensities at 350nm (unfolded protein) and 335nm (folded protein), respectively.

1995).

The dependence of the ratio of unfolded to folded pGSTP1-1 in 4.4M urea (i.e., urea concentration near midpoint of pGSTP1-1 without ligand) upon the concentration of glutathione and its analogues, is shown in Fig. 29 and is an indication of the specificity of ligand effect on the pGSTP1-1 structure. Fluorescence ratio values (intensity at 350nm for unfolded protein to intensity at 335nm for folded protein) of about 0.8 and 1.43 represent fully folded and unfolded pGSTP1-1, respectively. There is very little effect observed up to a concentration of 1 mM GSH which is considered to be the lowest GSH concentration *in vivo*. From 1 mM to about 10 mM GSH the fraction of folded GST is significantly lowered. Increasing the concentration of reduced glutathione to 3mM causes a gradual increase in unfolded pGSTP1-1 followed by a greater increase in the fraction of unfolded protein at higher concentrations. pGSTP1-1 in 4.4M urea becomes fully unfolded at glutathione concentrations of 10mM or greater. The glutathione analogues exerted the opposite effect of glutathione in that both glutathione sulphonate and S-hexylglutathione up to a concentration of about 1mM increased markedly the fraction of folded protein (i.e., pGSTP1-1 in 4.4M urea is fully folded at 1mM ligand). At concentrations greater than 1mM, S-hexylglutathione does not exert any further effect on the protein whereas glutathione sulphonate begins to increase the fraction of unfolded protein to fully unfolded at 10mM. Reasons for the destabilising effect of glutathione sulphonate observed at high concentrations are not known at present but could be due to it stabilising unfolded states through nonspecific interactions via its negatively charged sulphonate. The

maximum stabilising effect is reached close to the saturation concentration of the each ligand, calculated on the basis of apparent Kd obtained for the protein without denaturant present.

3.2.3. Influence of GSH and its analogues on the unfolding kinetics of pGSTP1-1

Although uncomplexed GST seems to exist in either completely folded or unfolded state at equilibrium, it is possible that, during unfolding in the presence of ligands, there are intermediate states that become transiently highly populated, and can be revealed only by kinetic studies (Schmid, 1992).

Dissociation and unfolding of oligomeric proteins are unimolecular processes and first-order kinetics data were obtained from the unfolding progress curves as follows:

Log Fmax/(Fmax-Fobs) is plotted vs. time,

where Fobs represents the fluorescence intensity at a particular time and Fmax the maximal fluorescence signal reached

Values for the first-order rate constants (in arbitrary fluorescence units per sec) were obtained from the slope of the linear plots.

Slope (m) of straight line, m=k/2.303,

where k is 1st order rate constant.

Linear plots of log Fmax/(Fmax-Fobs) against time of free and complexed pGSTP1-1 obtained are indication of the first order unfolding kinetics (Figures 30, 31, 32).

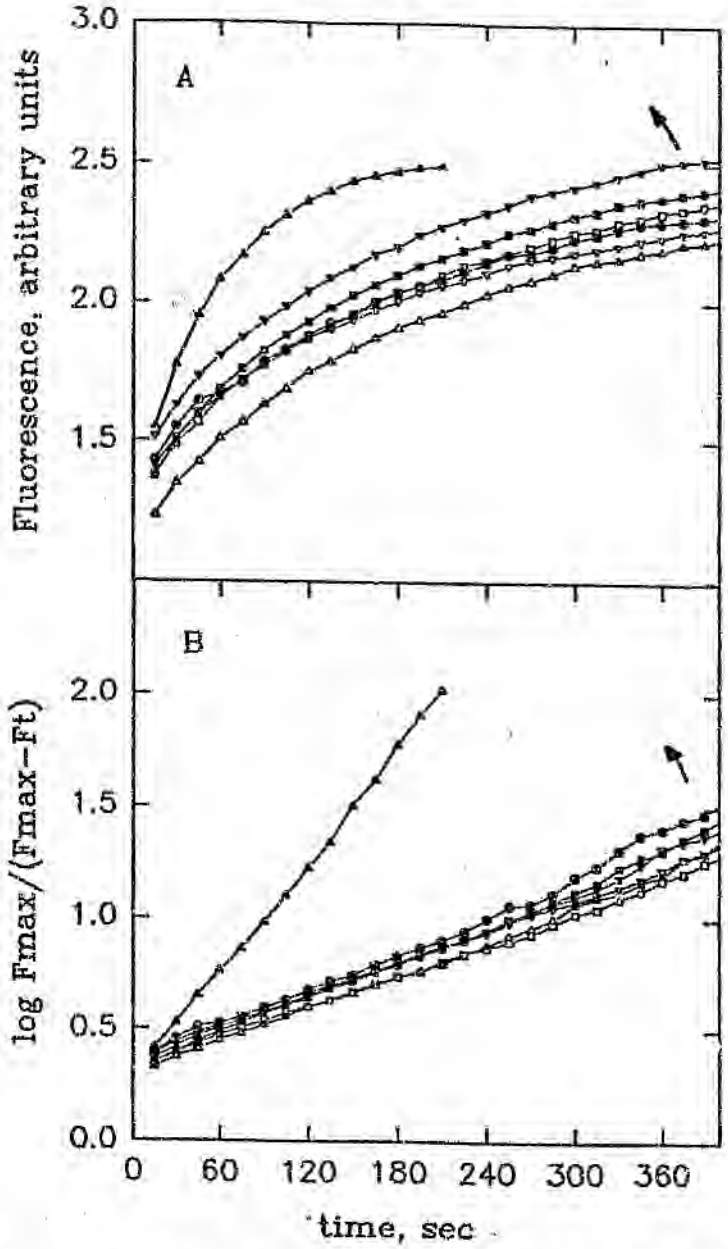


Figure 30. Unfolding rate of 1μM pGSTP1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5 in 8 M urea at 18°C in the presence of 0.5-3 mM glutathione. (a) A raw data; (b) straight line plots of kinetic equation for first-order reaction (slope = k/2.303; k, first-order rate constant).

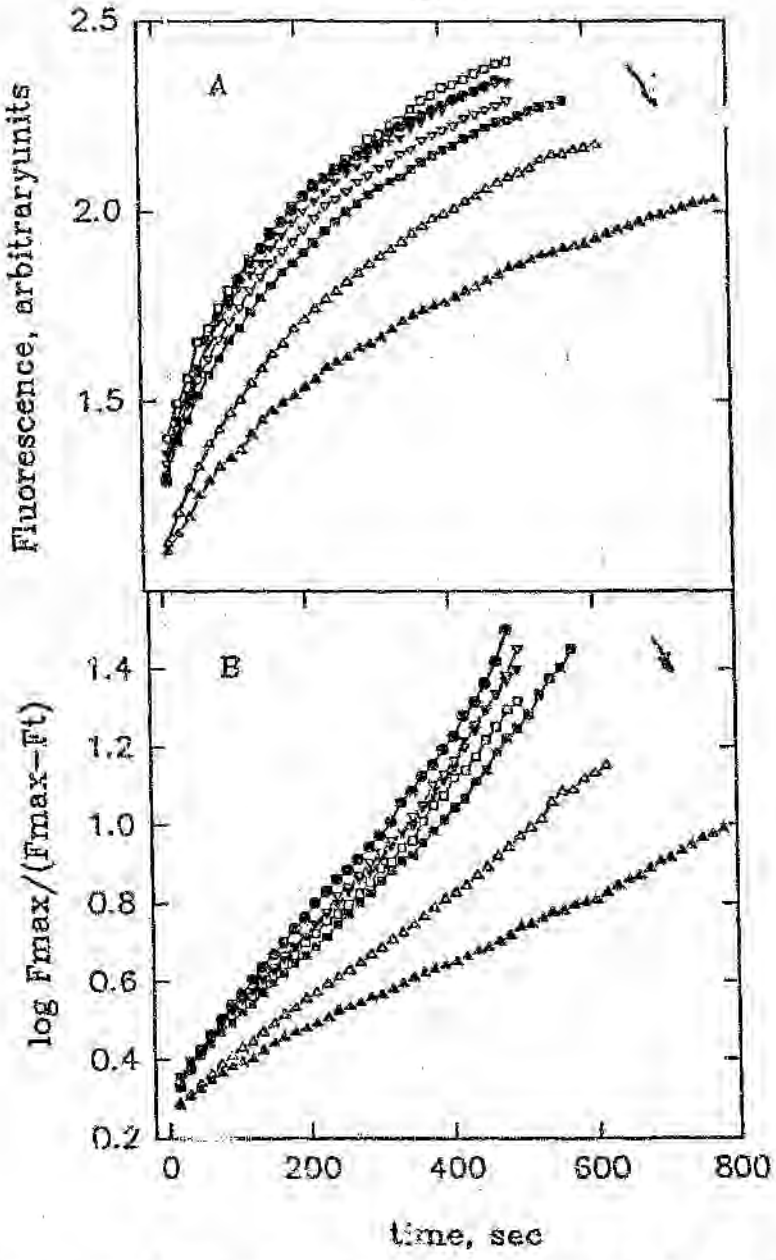


Figure 31. Unfolding rate of 1μM pGSTP1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5 in 8 M urea at 18°C in the presence of 2μM-0.1 mM glutathione sulphonate. (a) A raw data; (b) straight line plots of kinetic equation for first-order reaction (slope = k/2.303; k, first-order rate constant).

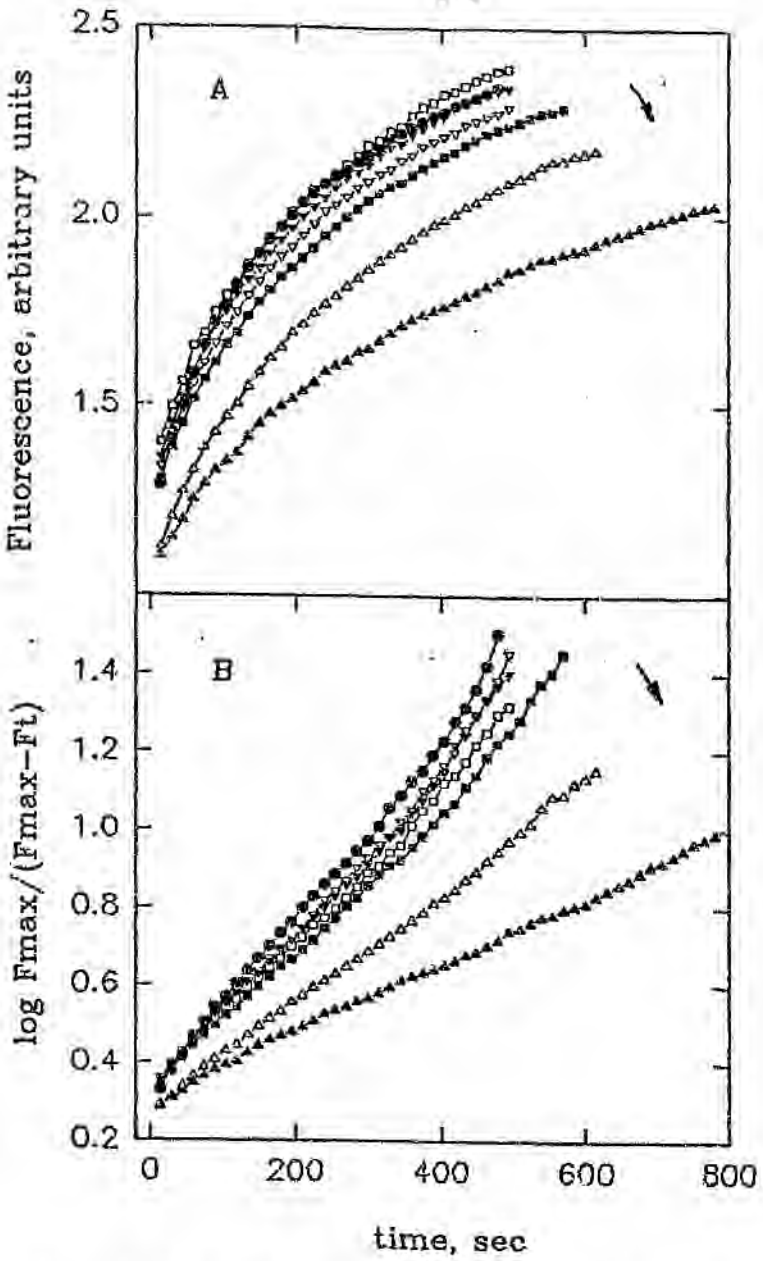


Figure 32. Unfolding rate of 1μM pGSTP1-1 in 20mM sodium phosphate buffer, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 6.5 in 8 M urea at 18°C in the presence of 1μM - 0.5mM S-hexyl glutathione. (a) A raw data; (b) straight line plots of kinetic equation for first-order reaction (slope = k/2.303; k, first-order rate constant).

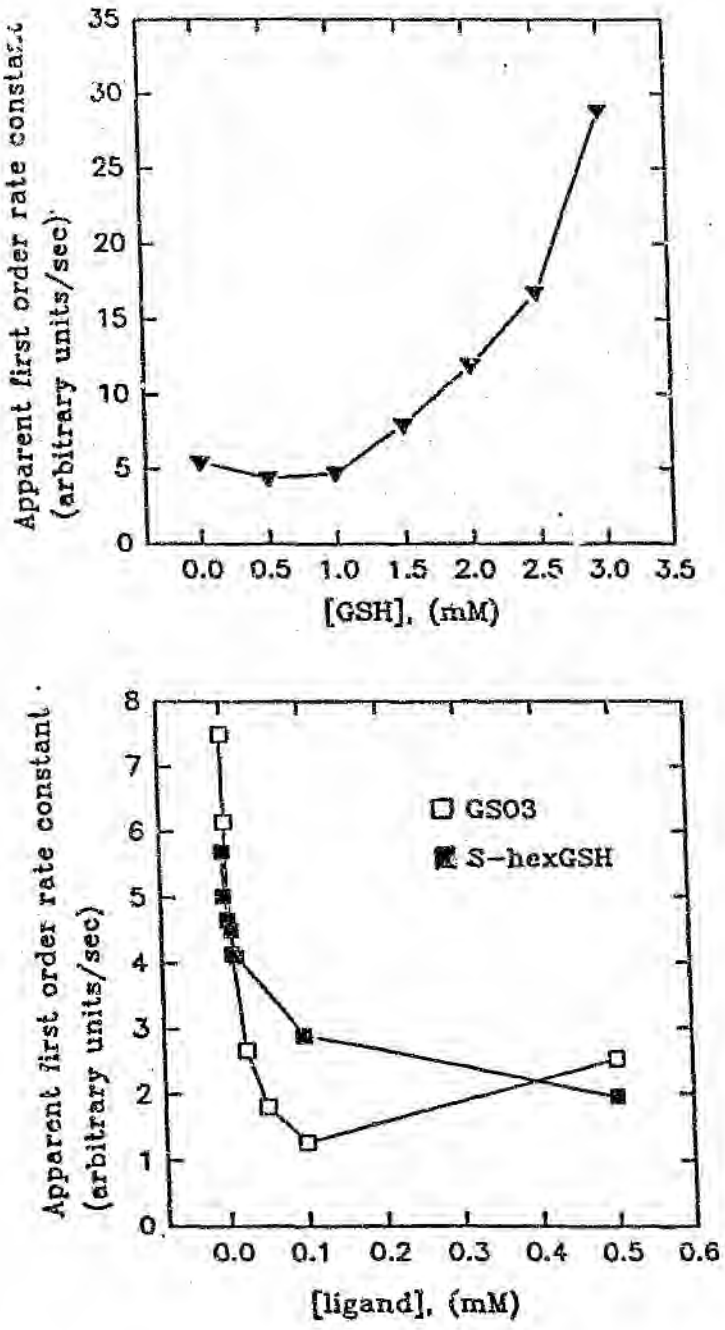


Figure 33. Dependence of the first-order unfolding rate constant of pGSTP1-1 in 8M urea upon the concentration of (▾) Glutathione, (■) S-hexyl glutathione, and (□) glutathione sulphonate.

The dependence of the unfolding first-order rate constant for pGSTP1-1 in 8M urea upon the concentration of glutathione, glutathione sulphonate and S-hexylglutathione is shown in Figure 33. The kinetics data correlates well with the equilibrium data described above in that the presence of glutathione has a destabilising effect on folded pGSTP1-1 and with increasing concentration of ligand in the assay mixture, increases the rate of unfolding (Figure 28A) whereas the two glutathione analogues stabilise folded pGSTP1-1 and increasing concentration of ligand, decrease the rates of unfolding (Figure 28B).

The unfolding data, except in the case for glutathione sulphonate concentrations of greater than 0.1 mM, do not show evidence of the existence of a population of enzyme molecules with different unfolding kinetics. Free pGSTP1-1 and in the presence of ligands, has only one kinetically significant transition state.

Kinetics data for glutathione sulphonate beyond 0.1mM lack the linearity of the log Fmax/(Fmax-Fobs) against time plots, indicating a deviation from first-order unfolding kinetics and violated two-state unfolding mechanism. As a consequence, an increase in the unfolding rate of enzyme at these sulphonate concentrations, could not be interpreted as a destabilising effect on GST structure.

3.2.4. Discussion

Glutathione and its S-analogues are sequestered at the G-site via a conserved hydrogen-bonding network (see Figure- 14). The hydrogen

bonding between the γ-glutamyl moiety of glutathione on one subunit and the side chain of Asp96 from the neighbouring subunit (a topologically conserved feature at the subunit interface of class alpha/mu/pi/sigma/ *Schisostoma japonicum* structures) could have a stabilising effect at the dimer interface.

Glutathione affinity for the enzyme is low and does not provide significant stabilising effect on pGSTP1-1 molecule. The structural basis for the observed destabilising effects of glutathione, may not just be steric but may also involve the activation of the thiol group of reduced glutathione at the G-site (see also figure 9(b)). The interaction of glutathione with Tyr7 is not essential for the binding, but it is essential for catalysis. Hydroxyl group of Tyr7 appears to act as a hydrogen bond donor to promote glutathione thiolate formation by decreasing the pKa of the glutathione's thiol group (see paragraph 1.2.2.3.). Therefore, a possible explanation for the observed destabilising effect glutathione exerts on the conformation of pGSTP1-1 when compared with glutathione sulphonate, is that some binding energy is lost to stabilisation against urea as a result of it being required for the activation of the thiol group of glutathione.

The stabilised anionic side chain of glutathione sulphonate (which mimics the thiolate form of glutathione; Kd = 4μM) interacts more tightly with Tyr7 than does the thiol moiety of reduced glutathione (Kd = 200μM). The hydroxyl group of Tyr7 forms a hydrogen bond with the sulphonate moiety of glutathione sulphonate. This interaction could explain the greater affinity of glutathione S-transferases for glutathione analogues in which the cysteine has been replaced by moieties having

electronegative side chains, such as the sulphonate (Graminski et al., 1989; Adang et al., 1990).

For S-hexylglutathione nonspecific apolar contacts with the hexyl moiety at the adjacent H-site provide additional binding energy resulting in tighter binding. Danielson et al. (1987) observed that glutathione transferases displayed increased binding energy in response to increased hydrophobicity of the substrate. For some of the enzymes the increase in the binding strength afforded by increased chain length of the substrate even counteracts the steric limitations at the active site. The hexyl arm in the human pi class enzyme interact with Phe8, Val110, Val35 and Tyr106, but the location of the hexyl group is different in the two subunits of the dimer, suggesting higher conformational freedom allowed in the H-site (Reinemer et al., 1992).

Stabilising effect of glutathione sulphonate and S-hexyl glutathione can be a consequence of both ligands binding more tightly to the fully folded conformation than to the fully unfolded or any other state of the protein. It is possible as well, that during unfolding in the presence of the glutathione analogues, certain intermediate conformational states of pGSTP1-1 become stabilised. Difference in the binding affinities of glutathione to the pre- and posttransitional states of the macromolecule could have shifted the equilibrium between these states causing destabilisating effect to be observed, both in equilibrium and kinetic studies.

Uncomplexed pGSTP1-1 exists in either a completely folded or unfolded state at equilibrium and its experimentally determined m-value compares very well with the expected value (see paragraph 3.1.2.).

According to the m-value data, the binding of glutathione appears not to affect significantly the way the protein responds to urea-induced unfolding, whereas its sulphonate and S-hexyl analogues seem to alter significantly the protein's unfolding pathway, thus giving rise to a multi-state unfolding process (Myers et al., 1995; Carra and Privalov; 1995).

Ligand-induced stabilisation/destabilisation effects could impact significantly on protein turnover *in vivo* since unfolded proteins are highly susceptible to proteolytic degradation (Pace, 1990). The effects observed here may have an important role in determining the life times of glutathione S-transferases *in vivo*.

3.3.3.CONCLUSIONS

The conformational stability and unfolding of porcine class pi glutathione S-transferase has been examined under equilibrium conditions by monitoring a variety of structural and functional parameters (Dirr and Reinemer, 1991). A highly cooperative two-state mechanism of unfolding for the class pi isoenzyme was suggested with only folded dimer and unfolded monomers significantly present in equilibrium. The folded monomer was suggested to be thermodynamically unstable.

Glutathione S-transferases bind the physiological tripeptide thiol glutathione (present up to 10mM in cells) and its S-analogues in a highly specific manner at the polar G-site. The effects of G-site ligand binding on the conformational dynamics of these enzymes were investigated by equilibrium unfolding and first-order unfolding kinetics studies with pGSTP1-1. The data presented indicate a glutathione-induced destabilisation and a glutathione analogue-stabilisation of the pGSTP1-1 conformation against urea.

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Author: Erhardt Julija.
Name of thesis: Class pi glutathione S-transferase- unfolding and conformational stability in the absence and presence of G-site ligands.

PUBLISHER:
University of the Witwatersrand, Johannesburg
©2015

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