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Conformational stability of Cys^{45}-alkylated and hydrogen peroxide-oxidised glutathione *S*-transferase

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Abstract **A highly reactive cysteine residue in class pi glutathione *S*-transferases enhances their susceptibility to chemical alkylation and oxidative stress. Alkylation of the reactive Cys^{45} in the porcine class pi enzyme (pGSTP1-1) with either *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine or iodoacetamide results in a loss of enzyme activity and glutathione-binding function. Similarly, oxidation of pGSTP1-1 with hydrogen peroxide (H_2O_2) also results in a loss of catalytic and gluthathione-binding function, but these effects are reversed by the addition of 5 mM glutathione or dithiothreitol. Analysis by SDS-PAGE of the H_2O_2- oxidised enzyme indicates oxidation-induced formation of disulphide bonds involving Cys^{45}. Equilibrium-unfolding studies with guanidinium chloride indicate that the unfolding of Cys^{45}-alkylated and H_2O_2-oxidised pGSTP1-1 can be described by a two-state model in which the predominant thermodynamically stable species are the folded dimer and unfolded monomer. Unfolding transition curves suggest that the introduction of a large and bulky AEDANS at Cys^{45} does not affect the unfolding pathway for pGSTP1-1. H_2O_2-oxidised pGSTP1-1, on the other hand, appears to follow a different unfolding pathway. This appears not to be a result of the introduction of disulphide bonds since the reduction of these bonds in the oxidised protein with dithiothreitol does not affect the unfolding transition. Furthermore, the conformational stability of the oxidised protein is significantly diminished ($\Delta G(H_2O)$ = 11.6 kcal/mol) when compared with unmodified and AEDANS-alkylated enzyme ($\Delta G(H_2O)$ = 22.5 kcal/mol).**

Key words: Conformational stability; Unfolding; Glutathione S-transferase; Oxidative stress; Hydrogen peroxide

1. Introduction

Cytosolic glutathione *S*-transferase (EC2.5.1.18), a superfamily of dimeric proteins (M_r ~ 50,000), are present in most aerobic organisms protecting them against chemical-induced toxicity and stress [1]. The proteins can be catalogued under one of four species-independent gene classes (alpha, mu, pi and theta) [2,3], for which a number of X-ray crystal structures are known (see [4] for review). The proteins share a similar folding topology with each polypeptide chain arranged into two structurally distinct domains.

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Abbreviations: pGSTP1-1, porcine glutathione S-transferase class pi with two 1-type subunits; IAEDANS, *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; SEC-HPLC, size-exclusion-high performance liquid chromatography; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione; DTT, dithiothreitol.

Class pi glutathione *S*-transferases are homodimers with three highly conserved cysteines per subunit but no disulphide bonds. They have a reactive cysteine situated near the glutathione-binding site (Cys^{45} in pGSTP1-1, and Cys^{47} in hGSTP1-1, rGSTP1-1 and mGSTP1-1; for aligned sequences see [4]) which is highly susceptible to oxidation and covalent modification by biological disulphides and thiol-reagents [5–11]. A structural basis for the reactivity of Cys^{45} has been given elsewhere [4,12]. Modifications of the reactive cysteine are an important source of enzyme inactivation by blocking glutathione binding. This may even function to modulate catalytic activity in vivo [13–15]. Replacement of the oxidation-sensitive cysteine markedly diminishes the class pi enzyme's susceptibility to oxidative stress [8,13].

To investigate further the impact of chemical modifications on the dynamics of the class pi glutathione *S*-transferases, we have examined the unfolding and conformational stability of both Cys^{45}-alkylated and H_2O_2-oxidised pGSTP1-1.

2. Materials and methods

2.1. Materials

Class pi glutathione *S*-transferase was purified from porcine lung and assayed as described [5]. The protein concentration of the purified dimeric pGSTP1-1 was determined using an absorbance coefficient of 49,600 $M^{-1}cm^{-1}$ at 280 nm calculated according to [16]. IAEDANS, iodoacetamide and guanidinium chloride (Aristar) were from Sigma, Fluka and BDH, respectively. DTT, DTNB and GSH were obtained from Boehringer Mannheim. All other chemicals were of analytical grade.

2.2. Alkylation and oxidation of pGSTP1-1

IAEDANS is a fluorescent sulphydryl reagent that reacts readily with thiol groups in proteins yielding photostable covalent derivatives [17]. Cys^{45} in pGSTP1-1 was alkylated with a 5-fold molar excess of IAEDANS for 4 h at room temperature in 0.1 M Tris-HCl, 1 mM EDTA, pH 7.5. Unreacted IAEDANS was removed by filtration through Sephadex G-25 and the AEDANS-modified pGSTP1-1 separated from unmodified enzyme by affinity chromatography on *S*-hexylglutathione Sepharose [5]. The stoichiometry of labelling was determined spectrophotometrically using a coefficient of 6000 $M^{-1}cm^{-1}$ for AEDANS at 340 nm [17]. Alkylation with iodoacetamide (10-fold molar excess) was performed in 0.2 M Tris-HCl, pH 8.0. Gel filtration and affinity chromatography were performed as described above. H_2O_2- induced oxidation of pGSTP1-1 was performed with 1 mM H_2O_2 for 30 min in 0.2 M Tris-HCl, pH 8.0. Gel filtration to remove H_2O_2 and affinity chromatography to separate oxidised from unoxidised protein were performed as described above. The number of free cysteines in untreated, alkylated and oxidised pGSTP1-1 was determined with DTNB as described elsewhere [5]. SEC-HPLC under non-denaturing conditions was used to obtain M_r values [18]. Discontinuous SDS-PAGE [19] in 15% (m/v) polyacrylamide separating gels under reducing and non-reducing conditions was employed to determine the presence of disulphide bonds.

2.3. Equilibrium unfolding and conformational stability analysis

All unfolding studies with native, alkylated and oxidised pGSTP1-1

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(0.1–10 μM) were performed in 20 mM Mes-NaOH, 0.1 M NaCl, 1 mM EDTA, 0.02% NaN_3, 0–4 M guanidinium chloride, pH 6.5, at room temperature as described elsewhere [20]. The unfolding process was monitored by measuring the steady-state fluorescence of tryptophan (295 nm excitation, 300–400 nm emission) and of AEDANS (340 nm excitation, 500 nm emission). Reversibility of unfolding was determined by diluting a solution of unfolded pGSTP1-1 (10 μM unmodified or modified protein containing 2 M urea) 10-fold with buffer without guanidinium chloride and then measuring fluorescence. The residual denaturant (0.2 M) did not affect the measurements of folded pGSTP1-1. All measurements were made after equilibrium was attained. Analysis of the experimental data as well as the determination of the conformation stability for the different forms of pGSTP1-1 were as described in [20]. Curve-fitting was done with the SigmaPlot programme.

3. Results and discussion

3.1. Properties of alkylated and oxidised pGSTP1-1

Alkylation with either IAEDANS or iodoacetamide as well as H_2O_2-induced oxidation of pGSTP1-1 leads to a loss in enzyme activity as a consequence of the modified enzyme's inability to bind GSH at its G-site. This property does allow for the convenient separation of modified protein from native protein by affinity chromatography on *S*-hexylglutathione Sepharose. According to SEC-HPLC (data not shown), the gross dimeric conformation of the modified proteins was similar to that for the native pGSTP1-1 (see [18] for examples of elution profiles). pGSTP1-1 has four cysteines in each subunit (Cys^{14}, Cys^{45}, Cys^{99} and Cys^{169}) but no disulphide bonds in the native protein [4,5]. Alkylation of pGSTP1-1 with thiol-specific reagents occurs at Cys^{45}; a structural basis for the reactivity of Cys^{45} and the inability of the alkylated enzyme to bind glutathione at its G-site has been suggested elsewhere [4,12]. Treatment of the H_2O_2-oxidised pGSTP1-1 with 1 mM GSH resulted in a 70–80% reactivation. Similar treatment of the oxidised human orthologue yielded only 40% reactivation [7]. However, simultaneous treatment of the latter with both GSH and thioltransferase resulted in almost complete reactivation.

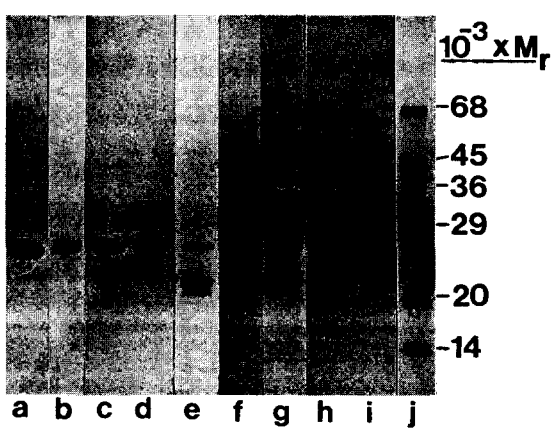

Fig. 1. SDS-polyacrylamide gel electrophoresis of unmodified and modified pGSTP1-1 under non-reducing (without β-mercaptoethanol) and reducing (with β-mercaptoethanol) conditions. Lanes a and b, unmodified pGSTP1-1 under non-reducing conditions, respectively; Lanes c and d, iodoacetamide-alkylated pGSTP1-1 under non-reducing and reducing conditions, respectively; Lanes e and f, H_2O_2-oxidised pGSTP1-1 under non-reducing and reducing conditions, respectively; Lane g, H_2O_2-oxidised pGSTP1-1 treated with 5 mM GSH before electrophoresis under non-reducing conditions; Lanes h and i, iodoacetamide-alkylated pGSTP1-1 exposed to H_2O_2 and electrophoresed under non-reducing and reducing conditions, respectively; Lane j, standard M_r markers.

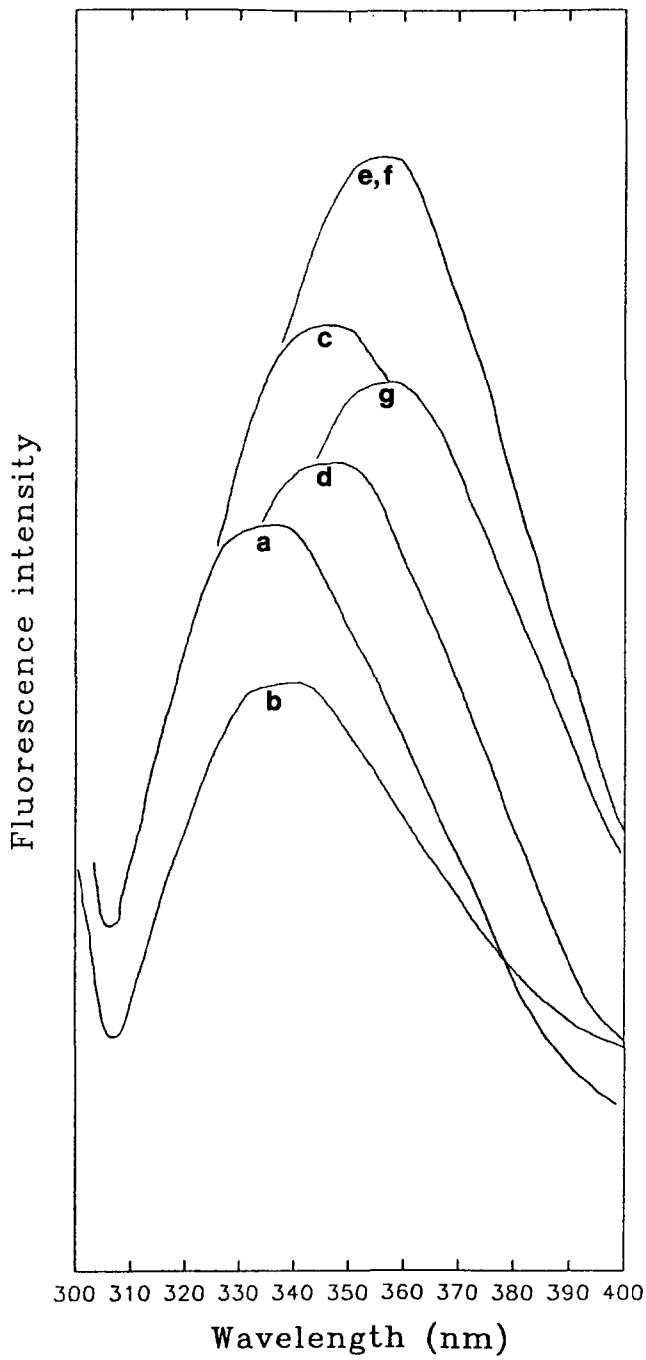

Fig. 2. Fluorescence emission spectra of pGSTP1-1 excited at 295 nm. Spectrum a, folded pGSTP1-1; Spectrum b, folded AEDANS-alkylated pGSTP1-1; Spectrum c, folded H_2O_2-oxidised pGSTP1-1; Spectrum d, folded H_2O_2-oxidised pGSTP1-1 after treatment with 5 mM DTT; Spectrum e and f, unfolded pGSTP1-1 and unfolded H_2O_2-oxidised pGSTP1-1, respectively; Spectrum g, unfolded AEDANS-alkylated pGSTP1-1.

An SDS-PAGE analysis of pGSTP1-1 is illustrated in Fig. 1. Unmodified enzyme is shown in lanes a and b – the pGSTP1 subunit displaying a molecular mass of 25.1 kDa in the absence and presence of β-mercaptoethanol. The electrophoretic mobility of the pGSTP1 polypeptide is not affected by alkylation with

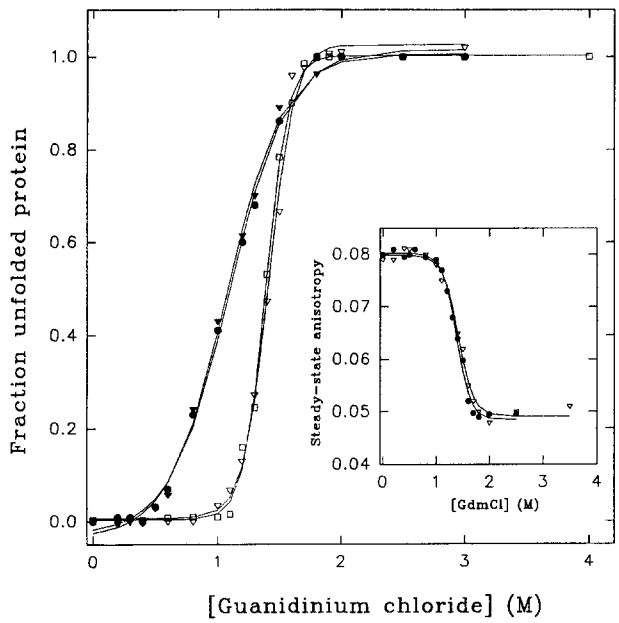

Fig. 3. Guanidinium chloride-unfolding curves for unmodified pGSTP1-1 (▽), AEDANS-alkylated pGSTP1-1 (□), H_2O_2-oxidised pGSTP1-1 (▼) and H_2O_2-oxidised pGSTP1-1 after treatment with 5 mM DTT (●). Insert: steady-state tryptophan (●; excitation 295 nm, emission 355 nm) and AEDANS (▽; excitation 340 nm, emission 500 nm) anisotropy of AEDANS-alkylated pGSTP1-1.

iodoacetamide (lanes c and d). H_2O_2-oxidised protein run under non-reducing conditions yields major bands at 25.1 kDa, 22.4 kDa and 20.9 kDa and minor bands at 50.1 kDa and 39.8 kDa (lane e). All of these protein bands except for the one at 25.1 kDa disappear when the oxidised protein is run under reducing conditions (lane f) or when the oxidised protein is pretreated with 5 mM GSH or dithiothreitol prior to electrophoresis under non-reducing conditions (lane g). The data suggest that H_2O_2-induced oxidation of pGSTP1-1 results in the formation of intrasubunit and to a much lesser extent intersubunit disulphide bonds. Similar results were obtained for copper-induced oxidation [9] of the enzyme (data not shown). The involvement of Cys^{45} in the formation of these disulphide bonds was demonstrated by shutting down its side chain iodoacetamide then oxidising the alkylated pGSTP1-1 with H_2O_2 followed by SDS-PAGE under non-reducing (Fig. 1, lane h) and reducing (Fig. 1, lane i) conditions. Under these conditions, only one protein band is observed its size corresponding with that for the unmodified pGSTP1-1.

Based on data obtained from DTNB-reactivity assays, folded H_2O_2-oxidised pGSTP1-1 has no exposed free cysteines whereas the denatured protein has two free cysteines per subunit. This indicates the involvement of Cys^{45} in disulphide linkage with another cysteine residue. Reduction of the H_2O_2-oxidised pGSTP1-1 with either DTT or GSH yields one and four free cysteines per subunit for the folded and denatured protein, respectively. At this stage, it is not certain which of the other cysteine residues in pGSTP1-1 are involved with Cys^{45}. Oxidative stress-induced disulphide bond formation in other class pi enzymes has also been reported [7,8,10,22]. It has been suggested that an intrasubunit disulphide bond between Cys^{47} and Cys^{101} (topologically equivalent to Cys^{45} and Cys^{99} in pGSTP1-1) forms in oxidised hGSTP1-1 [9] and rGSTP1-1 [7].

pGSTP1-1 contains two tryptophan residues (Trp^{28} and Trp^{38}) per subunit that can be used as fluorescent reporter groups for studying protein conformation [12]. Tryptophan emission spectra for different forms of pGSTP1-1 are shown in Fig. 2. In its native state, unmodified pGSTP1-1 displays an emission maximum at 335 nm (spectrum a) consistent with partially buried tryptophans [12,20]. AEDANS-alkylated enzyme displays a similar emission maximum wavelength but a lower fluorescence intensity (spectrum b). The latter is due to a primary inner-filter effect by AEDANS and to a transfer of some excitation energy from tryptophan to AEDANS (due to a spectral overlap of protein's emission spectrum with AEDANS absorbance spectrum). H_2O_2-oxidised pGSTP1-1 has an emission maximum red-shifted to 343 nm and a fluorescence intensity higher than the native protein (spectrum c). The introduction of intrasubunit disulphide bonds, therefore, appears to induce a conformational change that results in a weaker quenching microenvironment for tryptophan that becomes more exposed to solvent. It seems quite reasonable to expect the local environment of Trp^{38} to become modified since it is located close to Cys^{45} and is in a protein region displaying structural mobility [4,12]. Treatment of the oxidised protein with 5 mM DTT did not return the emission properties (wavelength and intensity, curve d) to those observed for the native protein indicating that the conformational changes induced in the H_2O_2-oxidised protein were not fully reversed by reductive cleavage of the disulphide bonds. Although the structural basis for this is not known it is supported by the finding above that the oxidised protein is not fully reactivated by reductive treatment.

3.2. *Equilibrium unfolding and conformational stability*

Solvent-induced unfolding of unmodified, AEDANS-al-

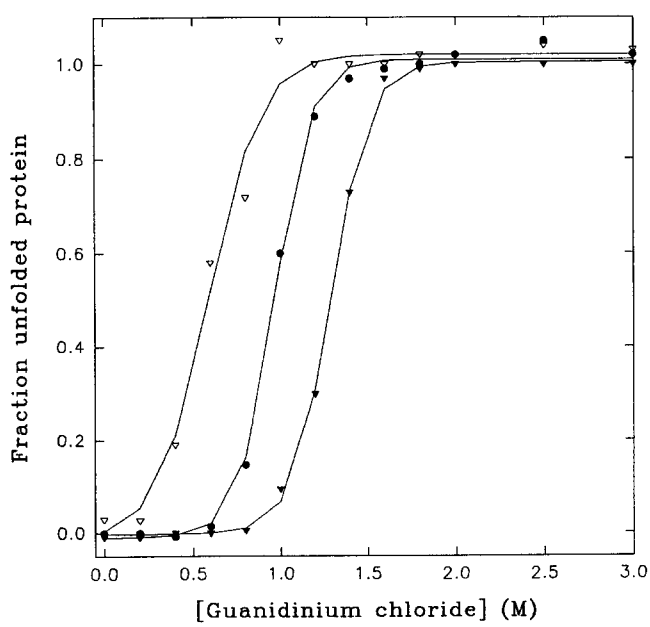

Fig. 4. Protein concentration dependence of guanidinium chloride-induced unfolding of H_2O_2-oxidised pGSTP1-1 at 0.1 μM (▽), 1 μM (●), and 10 μM (▼).

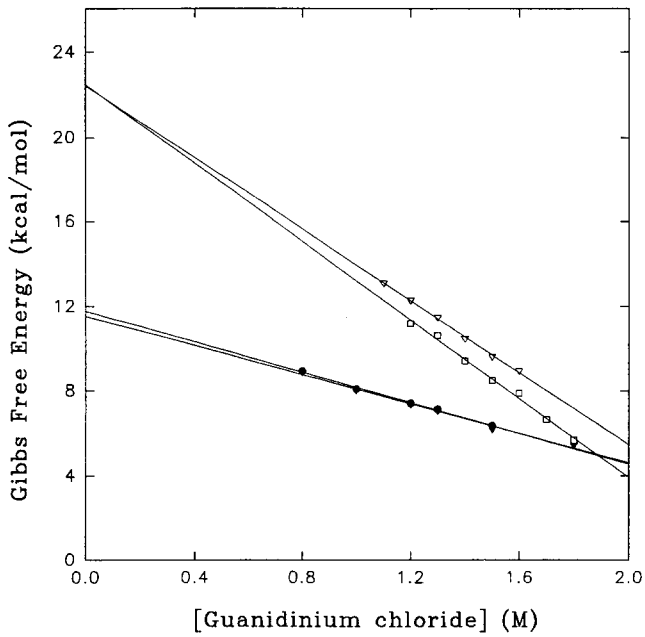

Fig. 5. Gibbs free energy changes of unfolding of unmodified pGSTP1-1 (▽), AEDANS-alkylated pGSTP1-1 (□), H_2O_2-oxidised pGSTP1-1 (●) and H_2O_2-oxidised pGSTP1-1 treated with 5 mM DTT (▼) as a function of guanidinium chloride concentration. Initial data was from the unfolding transitions shown in Fig. 3. Linear extrapolation to the ordinate yields for the Gibbs free energy changes in the absence of denaturant ($\Delta G_u(H_2O)$).

kylated and oxidised pGSTP1-1 produces a red-shift in the emission maximum wavelength to 355 nm and an increase in fluorescence intensity (Fig. 2). These spectral features were used to monitor structural changes occurring during the unfolding process. The lower fluorescence intensity observed for unfolded AEDANS-alkylated pGSTP1-1 (spectrum g, Fig. 2) is a result of the inner filter effects mentioned in section 3.1. Reversibility of unfolding of all forms of pGSTP1-1 (as measured by the recovery of fluorescence) was about 90%.

Fig. 3 shows highly cooperative unfolding curves for pGSTP1-1 obtained under equilibrium conditions. The curves for native and alkylated enzyme are coincident and so are the unfolding curves for alkylated pGSTP1-1 obtained from tryptophan and AEDANS anisotropy measurements (Fig. 3, insert). The decreasing anisotropy values suggest increasing mobility of both fluorophores upon unfolding. The coincident data, therefore, indicate that the introduction of a large bulky moiety at position 45 does not affect significantly the unfolding pathway of pGSTP1-1. The transfer of excitation energy from tryptophan to AEDANS was markedly reduced as indicated by a 3.2-fold reduction in the fluorescence intensity of AEDANS accompanied by a red-shift in its emission wavelength from 475 nm to 505 nm (data not shown); the red-shift suggesting a more polar environment for the covalently-bound AEDANS fluorophore in the unfolded protein. The non-coincident unfolding curve with its shallower slope for H_2O_2-oxidised pGSTP1-1, however, suggests an unfolding pathway different from that for the unmodified and Cys^{45}-alkylated proteins. Treatment of the oxidised protein with DTT to reduce the disulphide bonds had no effect and yielded an unfolding curve coincident with that for the untreated oxidised protein (Fig. 3). Previous equilibrium studies [20,23] have indicated a concerted two-state unfolding model for unmodified pGSTP1-1 involving only folded dimer and unfolded monomer as the predominant thermodynamically stable species. Protein concentration-dependent studies also suggest two-state models for alkylated and oxidised enzyme, in that, and in accordance with the law of mass action, the stability of the dimer is increased by increased concentration of protein (data for oxidised pGSTP1-1 is shown in Fig. 4). Intrinsically unstable monomeric species not significantly populated at equilibrium have also been reported for a number of other dimeric proteins (see [24] for a review).

Estimated values for the conformational stability of unmodified, AEDANS-alkylated and H_2O_2-oxidised pGSTP1-1 in the absence of denaturant ($\Delta G_u(H_2O)$) are 22.5 kcal/mol, 22.5 kcal/mol and 11.6 kcal/mol, respectively (Fig. 5). The value obtained for unmodified protein compares very well with previously determined values [20]. The structural basis for the destabilisation of the H_2O_2-oxidised pGSTP1-1 (and its DTT-reduced form) is not clear at present. It seems reasonable, however, to suggest that it is related to structural changes occurring in the vicinity of Cys^{45} but that it does not seem to involve the formation of disulphide bonds. The helical region containing helix α2 and Cys^{45} is flexible and its local conformational state determines glutathione binding (and, thus, enzyme activity) and susceptibility to proteolysis (see [4] and references therein). Although these as yet undefined structural changes impact on the stability of the protein they do not significantly affect catalytic function since about 80% of the enzyme activity was recovered after reductive treatment of the oxidised enzyme with DTT.

Recently, it has been suggested that an ion-pair interaction exists between Cys^{47} and Lys^{54} in human GSTP1-1 [26,27,28]. The ion-pair is suggested to contribute towards the formation of the correct conformation at the G-site and in reducing the mobility of the flexible helix α2 [26,27,28]. As both the cysteine and lysine residues are highly conserved in the class pi enzymes [4], similar ion-pair interactions might also occur in the other class pi enzymes. However, the available crystal structures for the porcine [12,21] and mouse [29] class pi enzymes indicate a distance of greater than 6 Å separating the two residues compared with a distance of 4.9 Å for the human enzyme [30]. Furthermore, it appears that the putative ion pair does not impact on the conformational stability or unfolding pathway of GSTP1-1 since similar data were observed for both the native untreated enzyme (ion pair present) and its Cys^{45}-alkylated form (ion pair absent).

Investigations into the proteolytic susceptibility and turnover of alkylated and oxidised GSTP1-1 is underway since increased turnover is a biological response to protein damage [25].

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