Electrostatic interactions affecting the active site of class Sigma glutathione S-transferase

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We have shown previously that the solvent-induced equilibrium unfolding mechanism of class Sigma glutathione S-transferase (GST) is strongly affected by ionic strength [Stevens, Hornby, Armstrong and Dirr (1998) *Biochemistry* 37, 15534–15541]. The protein is dimeric and has a hydrophilic subunit interface. Here we show that ionic strength alone has significant effects on the conformation of the protein, in particular at the active site. With the use of NaCl at up to 2 M under equilibrium conditions, the protein lost 60 % of its catalytic activity and the single tryptophan residue per subunit became partly exposed. The effect was independent of protein concentration, eliminating the dissociation of the dimer as a possibility for the conformational changes. This was confirmed by size-exclusion HPLC. There was no significant change in the secondary structure of the protein

according to far-UV CD data. Manual-mixing and stopped-flow kinetics experiments showed a slow single-exponential salt-induced change in protein fluorescence. For equilibrium and kinetics experiments, the addition of an active-site ligand (S-hexylglutathione) completely protected the protein from the ionic-strength-induced conformational changes. This suggests that the change occurs at or near the active site. Possible structural reasons for these novel effects are proposed, such as the flexibility of the α -helix 2 region as well as the hydrophilic subunit interface, highlighting the importance of electrostatic interactions in maintaining the structure of the active site of this GST.

Key words: alpha helix, protein stability, protein unfolding.

INTRODUCTION

It is known that electrostatic interactions make a significant contribution to the stability and function of proteins. Buried salt bridges, although uncommon [1], have been shown to contribute to the stability of proteins [2]. Studies on solvent-exposed salt bridges have shown similar effects [3]. Electrostatic interactions are known to be important at the subunit interfaces of multimeric proteins, as observed in studies with ionic denaturants such as guanidinium chloride in comparison with non-ionic denaturants like urea [4]. It has been shown that, although subunit interfaces of proteins are as tightly packed as protein cores, the morphology of interfaces is not always predominantly hydrophobic and that in approximately two-thirds of homodimers of known structure, a mixture of small hydrophobic patches and polar regions occurs [5]. Electrostatic interactions provide the specificity of protein–protein interactions at subunit interfaces, as shown by several site-directed mutagenesis studies on dimeric proteins such as triosephosphate isomerase [6].

The first studies of the effects of ionic strength on proteins were performed by Hofmeister, in which he described the effects of different salts on the stability and solubility of proteins [7]. Many studies on the effects of ionic strength on proteins have focused on molten globules and denatured states [8–10] to determine the importance of electrostatic interactions in these conformational states.

Glutathione S-transferases (GSTs; EC 2.5.1.18) are dimeric proteins that are involved in cellular detoxification in a wide variety of organisms [11]. There are several species-independent

gene classes that can be separated according to the properties of their subunit interfaces. The Alpha, Mu and Pi classes have hydrophobic subunit interfaces that have a conserved hydrophobic lock-and-key motif ([12], and references therein). The key consists of a phenylalanine residue protruding into a hydrophobic pocket on the other subunit. The interfaces of the class Sigma [12] and Theta [13] GSTs do not feature this interaction and have more hydrophilic interfaces, which are flatter in topology. The cephalopod class Sigma GST studied in this work (shown in Figure 1) has almost twice the number of hydrogen bonds and salt bridges compared with a class Mu GST [12]. There are also a significantly greater number of ordered water molecules involved in solvating the interface than in other GST classes. The location of the lock-and-key motif in the Alpha, Mu and Pi GST classes at the subunit interface is indicated in Figure 1. Homodimeric class Sigma GST (GSTS1-1) has been shown to unfold by a four-state mechanism involving a dimeric intermediate with decreased enzyme activity and a thermodynamically stable non-native monomeric state [14]. These conformational states were shown to be sensitive to ionic strength, and NaCl was used to stabilize the monomeric intermediate. In view of these observations this study was undertaken to characterize the effect of ionic strength on class Sigma GST.

EXPERIMENTAL

Materials

GSTS1-1 was overexpressed in *Escherichia coli* BL21(DE3) transformed with the plasmid GST5/pET in the presence of

Abbreviations used: GST, glutathione S-transferase; GSTS1-1, homodimeric class Sigma glutathione S-transferase; S-hexyl-GSH, S-hexylglutathione; Sj26GST, 26 kDa glutathione S-transferase from *Schistosoma japonicum*.

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Figure 1 Representation of the structure of class Sigma glutathione S-transferase

The structure is shown perpendicular to the crystallographic two-fold axis. Both subunits are shown, as are domains I and II. The active-site ligand, 1-(S-glutathionyl)-2,4-dinitrobenzene and Trp-38 in α -helix 2 are shown as ball-and-stick representations on one subunit. Asp-37, Lys-42 and Pro-51 are shown as ball-and-stick representations on the other subunit [12]. The position of the hydrophobic lock-and-key motif that is present in some other GST classes, but is absent from class Sigma GST, is shown by a star.

50 μ g/ml ampicillin, as described [12]. The protein was purified by *S*-hexylglutathione (*S*-hexyl-GSH) affinity chromatography and the ligand was removed with a Sephadex G-25 column. *S*-hexyl-GSH was prepared as described [15]. The protein was stored in 20 mM sodium phosphate buffer, pH 7, containing 1 mM EDTA and 5 mM dithiothreitol and the concentration was determined by taking the molar absorption coefficient as 31660 M⁻¹·cm⁻¹ at 280 nm [16]. 1-Chloro-2,4-dinitrobenzene was purchased from Sigma; reduced glutathione (GSH) and dithiothreitol were purchased from Boehringer Mannheim. All other reagents were of analytical grade.

NaCl-induced conformational changes at equilibrium

Samples of GSTS1-1, at a final concentration of 1 μ M, were equilibrated in the presence of a range of NaCl concentrations (0–2 M) for at least 3 h at 20 °C in 20 mM sodium phosphate buffer, pH 7, containing 1 mM EDTA and 5 mM dithiothreitol. When the effect of active-site ligands was being examined, the protein was incubated with the ligand for at least 30 min before the addition of NaCl. The conformation of the protein was monitored by using a number of methods. The fluorescence of the single tryptophan residue per subunit (Trp-38) was monitored with a Hitachi 850 fluorescence spectrophotometer at an excitation wavelength of 295 nm. The results are presented as a ratio of the fluorescence intensity (*F*) at 355 nm (the emission maximum for fully denatured protein) to that at 330 nm (the emission maximum for fully folded protein). The enzyme activity was monitored spectrophotometrically by adding aliquots of the NaCl-treated samples to an assay mixture containing 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene in 0.1 M potassium phosphate buffer, pH 6.5, containing 3% (v/v) ethanol [17]. Linear progress traces were collected for 1 min at 340 nm with 0.67 nM GSTS1-1 and were corrected for non-enzymic rates. Reactivation, within this time, owing to renaturation was found

to be less than 10%. CD data were collected with a Jasco J-710 spectropolarimeter with a 1 mm path length in a 10 mM sodium phosphate buffer, pH 7, at a protein concentration of 3 μ M and were averaged over 16 runs. The effect of NaCl was found to be reversible by monitoring the recovery of native fluorescence and enzyme activity after a 1:10 dilution of protein in 2 M NaCl. The recovery of enzyme activity and fluorescence was close to 100% after 6 h.

Kinetics of the NaCl-induced conformational changes

Stopped-flow data were collected by using a stopped-flow SX-18 MV spectrofluorimeter (Applied Photophysics) with symmetrical mixing at a final protein concentration of 1 μ M and a final concentration of NaCl or KCl of 2 M. The excitation and emission pathlengths were 10 nm and 2 nm respectively, with an excitation bandwidth of 2.32 nm. An excitation wavelength of 280 nm was used with a 320 nm emission cut-off filter. Excitation at 280 nm yielded a better signal than that at 295 nm and it was shown at equilibrium that the results were superimposable with either wavelength (results not shown). Manual-mixing data were collected with a Hitachi 850 fluorescence spectrophotometer at an excitation wavelength of 295 nm and an emission wavelength of 355 nm, with both bandpasses at 10 nm. The temperature was maintained at 20 °C in a thermostatically controlled water bath.

RESULTS

NaCl-induced conformational changes at equilibrium

Figure 2 shows the effect of increasing NaCl concentration on the intrinsic fluorescence (Figure 2A) and the enzyme activity (Figure 2B) of GSTS1-1. The tryptophan residue is located at the active site and makes contact with the substrate glutathione [12], so a change in fluorescence would be expected to be accompanied by a change in enzyme activity. The maximum emission wavelength of the native protein is 330 nm. There is a wavelength shift in the presence of 2 M NaCl to 340 nm, confirming exposure of the tryptophan residues to a more polar environment. Complete unfolding of the protein results in a shift in the maximum emission wavelength to 355 nm and a fluorescence ratio (F_{355}/F_{330}) of 1.6 [14]. The maximum fluorescence ratio of approx. 1.0 is close to that shown for the dimeric intermediate described with unfolding by urea and is accompanied by a 60% loss in enzyme activity at 2 M NaCl. It seems that the conformational change caused by NaCl is not co-operative because the transitions are not the characteristic sigmoidal shape for two-state unfolding transitions. There was no change in the anilidonaphthalene-sulphonate-binding properties of the protein at up to 2 M NaCl, although there was an increase in anilidonaphthalene sulphonate binding above 2 M NaCl; this was confirmed by light scattering to be due to protein aggregation (results not shown).

CD showed that there was no significant change in the secondary structure content of the protein in the presence of NaCl, as indicated by ellipticity values at 222 nm of 12000 m-degrees for protein in 0 M NaCl and 12280 m-degrees for protein in the presence of 1 M NaCl. To determine whether the native dimer dissociated into monomeric states in the presence of NaCl, the dependence on protein concentration was examined. The changes in the conformation of the protein did not seem to be dependent on protein concentration because there was no shift in the trend with a 10-fold difference in protein concentration (results not shown). This was confirmed by size-exclusion HPLC, which showed that the dimeric native state of the protein was maintained at up to 2 M NaCl (results not shown). Dissociation of the dimer could be expected owing to the hydrophilic nature

Figure 2 Effect of NaCl on GSTS1-1

The protein concentration was 1 μ M in 20 mM sodium phosphate buffer, pH 7, containing 1 mM EDTA and 5 mM dithiothreitol at 20 °C. (A) Change in intrinsic fluorescence with increasing NaCl concentration, using an excitation wavelength of 295 nm; (B) loss of enzyme activity, shown as a percentage.

of the subunit interface, which is mainly stabilized by salt bridges and hydrogen bonds [12].

Because NaCl is not commonly observed to denature proteins, it is likely that these effects are specific and localized to a certain region of the protein. Figure 3 shows the effect of NaCl in the presence of active-site ligands. GSH (the physiological substrate) at 10 mM decreases the extent of the effect of NaCl, whereas the product analogue *S*-hexyl-GSH at 1 mM seems to completely prevent the loss of structure and function induced by NaCl. Both the native fluorescence properties and the enzyme activity were unaffected by up to 2 M NaCl in the presence of *S*-hexyl-GSH. The greater effect observed with *S*-hexyl-GSH was expected because it has a much higher affinity than GSH for the protein. The dissociation constant (K_d) for GSH is approx. 600 μ M; that for *S*-hexyl-GSH is 18 μ M. The apolar hexyl moiety of *S*-hexyl-GSH increases the number of interactions with the active site of the protein and so exerts a more significant effect.

Kinetics of the NaCl-induced conformational change

To elucidate further the effect of NaCl on the protein, the kinetics of the process was examined by using stopped-flow and manual-mixing fluorescence techniques. An example of a stopped-flow fluorescence trace over 1000 s with the addition of 2 M NaCl is shown in Figure 4. The slow change in fluorescence fits well to a single-exponential function [$F_t = F_i \exp(-t/\tau) + F_\infty$,

Figure 3 Effect of NaCl on GSTS1-1 in the presence of active-site ligands

The protein concentration was 1 μ M in 20 mM sodium phosphate buffer, pH 7, containing 1 mM EDTA and 5 mM dithiothreitol at 20 °C. The protein was preincubated with GSH or *S*-hexyl-GSH (●, no ligand; ○, 10 mM GSH; ▼, 1 mM *S*-hexyl-GSH) for 30 min before the addition of NaCl. Upper panel: change in intrinsic fluorescence with increasing NaCl concentration, with an excitation wavelength of 295 nm. Lower panel: loss of enzyme activity, shown as a percentage.

where F_t is the total fluorescence amplitude at time t , F_i is the amplitude at zero time, τ is the time constant and F_∞ is the amplitude at infinite time], yielding a rate constant of $(1.40 \pm 0.13) \times 10^{-3} \text{ s}^{-1}$. Approx. 20% of the fluorescence change occurred within the dead time of the instrument (2 ms [18]). Manual-mixing data were collected under the same conditions and the results are shown as triangles superimposed on the stopped-flow data in Figure 4, showing that the two techniques produced kinetic traces with similar rates. Manual mixing was also used to confirm the signal at the endpoint of the reaction because results could be collected for a longer period than with stopped flow, which was limited to 1000 s. Confirmation of this value validates the single-exponential fit of the stopped-flow data, which could not be collected to the endpoint of the reaction.

To determine whether the effect of NaCl was salt-specific, similar experiments were performed with other salts such as KCl, as shown in the middle trace in Figure 4 (upper panel). A single-exponential reaction was observed with KCl, although the reaction was slower, with a rate constant of $(1.02 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$. Similar experiments were performed under equilibrium

Figure 4 Stopped-flow and manual-mixing kinetics of the effect of NaCl (in the presence and the absence of *S*-hexyl-GSH) and KCl on GSTS1-1

Protein (2 μ M) was mixed with 2 M NaCl or KCl at 20 °C and the fluorescence was monitored with time [excitation wavelength 280 nm, emission cutoff filter 320 nm (stopped flow); excitation wavelength 295 nm, emission wavelength 355 nm (manual mixing)]. For the results collected in the presence of *S*-hexyl-GSH, GSTS1-1 was preincubated with 1 mM *S*-hexyl-GSH before being mixed with NaCl. The fluorescence signal of native protein is indicated by the lower arrow in the upper panel; the upper arrow indicates the signal on completion of the reaction. The results were fitted to a single-exponential function and the residual values for the stopped-flow data for NaCl and KCl are shown in the lower panels. The units for the results are arbitrary fluorescence units. The stopped-flow data points are indicated as dots and the manual-mixing data for NaCl are shown as open triangles.

conditions with $(\text{NH}_4)_2\text{SO}_4$ and KCl; both salts caused conformational changes but neither to the extent caused by NaCl (results not shown). It therefore seems that other salts cause conformational changes in the protein, resulting in tryptophan exposure and loss of catalytic function. These results suggest that the ionic-strength effect is non-specific in terms of the salt used.

Similar stopped-flow experiments were performed in the presence of 1 mM *S*-hexyl-GSH; a sample is shown as the lower traces in Figure 4. As shown for the equilibrium studies above, the protein was completely protected and no change in fluorescence signal was observed over 1000 s. There was a decrease in the fluorescence signal in the presence of the ligand because binding of the ligand quenched the protein fluorescence, as expected.

DISCUSSION

According to the Hofmeister series of cations and anions, NaCl occurs at what is considered to be the midpoint of stabilizing and destabilizing effects. Salts such as $(\text{NH}_4)_2\text{SO}_4$ commonly stabilize proteins; salts such as guanidinium chloride are known to destabilize proteins, according to the rank order described by Hofmeister (see [19]). It is therefore unexpected for NaCl to have general destabilizing effects on proteins, suggesting that the effects observed in this study are related to specific localized effects.

The results shown indicate that GSTS1-1 undergoes a protein-concentration-independent NaCl-induced conformational change that results in partial exposure of the tryptophan residue in α -helix 2 and a loss of enzyme activity of up to 60%. Far-UV

CD spectra of protein in the presence and absence of 1 M NaCl are similar, indicating that the conformational change is small in terms of secondary structure. The fact that the binding of *S*-hexyl-GSH prevents these ionic-strength effects suggests similar conformational changes to those observed with urea [14]. The first transition in the unfolding mechanism of urea has been shown to involve local unfolding in the active-site region of the protein, a conformational change that was substantially prevented in the presence of active-site ligands and was shown to be influenced by ionic strength. Studies have shown that α 2 helices in GSTs have high conformational flexibilities [20]. There is crystallographic evidence that this region is flexible in the class Sigma GST, as shown by above-average temperature factors [12]. Additional crystallographic evidence has been provided that this region in class Pi GSTs is highly flexible in the absence of active-site ligands [21,22]. The single-exponential increase in the fluorescence of GSTS1-1 with time suggests that the conformational change is a single event, such as the local unfolding of α -helix 2.

There are several likely structural reasons for the sensitivity of GSTS1-1 to ionic strength. At the termini of α -helix 2 (located at the active site, as shown in Figure 1) are charged residues, with Asp-37 appearing at the N-terminus and Lys-42 at the C-terminus. Charged residues in these positions are known to stabilize α -helices owing to favourable interactions with the helix dipole, which is positive at the N-terminus and negative at the C-terminus [23,24]. It is also known that ionic strength affects the stabilization of α -helices by charged residues by shielding the electrostatic effects [25,26]. It might be that the cause of the effects of ionic strength observed with GSTS1-1 (which are not salt-specific) are related to interference of the ions with the helix dipole-stabilizing charged residues at the helix termini. The α 2-helices of some other GSTs have similar motifs [such as the 26 kDa glutathione *S*-transferase from *Schistosoma japonicum* (Sj26GST), which has Glu-36 and Lys-43 at the N- and C-termini of α -helix 2 respectively] and these interactions might contribute to stabilizing these flexible helices in GSTs in general. The fact that this helix in GSTS1-1 is short, as well as the absence of the conserved lock-and-key interaction in the Sigma class GST, might account structurally for the low stability and high sensitivity to ionic strength of its α -helix 2. This region of the protein has also been shown to have a low propensity for forming a stable helix, according to a prediction algorithm (AGADIR [27]), suggesting that interactions with the rest of the protein, as well as active-site ligands, are required to stabilize this helix. In addition, there is a *cis*-proline residue that is conserved in all GSTs at the end of the loop region containing the hydrophobic lock-and-key phenylalanine residue in the Alpha, Mu and Pi classes. This *cis*-proline residue in the class Sigma GST (Pro-51) might contribute to the flexibility of the α -helix 2 region. Pro-51 is located at the end of the loop connecting α -helix 2 to the core of domain I. Isomerization of the peptide bond preceding Pro-51 might confer additional conformational flexibility on α -helix 2 and the loop at the subunit interface. The role of the conserved *cis*-proline residue in maintaining the active sites of other GSTs has been confirmed by mutagenesis studies [28,29]. Ionic strength could also affect the salt bridges at the hydrophilic subunit interface of GSTS1-1 by charge shielding, further contributing to conformational instability.

To establish the significance of the hydrophobic lock-and-key interaction in maintaining the conformation of the active-site region in other GSTs, the effect of NaCl on Sj26GST was examined. Under equilibrium conditions and examining manual-mixing fluorescence kinetics, it was found that Sj26GST was insensitive to ionic strength (results not shown). The protein

maintained its catalytic activity up to 2 M NaCl; no fluorescence change was observed. Sj26GST has a tryptophan residue (Trp-40) in a similar topological position to that of Trp-38 of GSTS1-1 [30]. The α -helix 2 of Sj26GST also has potential helix-dipole-stabilizing charged residues at each end. However, it does have a more hydrophobic subunit interface and might be expected to be less sensitive to changes in ionic strength.

In conclusion, this work highlights the significance of electrostatic interactions in maintaining the active-site region of class Sigma GST. The sensitivity to ionic strength seems to be a consequence of the hydrophilic subunit interface and other structural features. The lack of the hydrophobic interaction that is conserved in some other GST classes seems to make the protein more susceptible to disruption of the electrostatic interactions that stabilize the active site. Evolution seems to have favoured the hydrophobic subunit interface in the development of the GST superfamily [12], with the more recent GST classes (Alpha, Mu and Pi) featuring the hydrophobic lock-and-key interaction. The change in the nature of the interface might be related to the low stability of the catalytic region in the absence of this interaction.

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