Influence of the H-site residue 108 on human glutathione transferase P1-1 ligand binding: Structure-thermodynamic relationships and thermal stability

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Abstract: The effect of the Y108V mutation of human glutathione S-transferase P1-1 (hGST P1-1) on the binding of the diuretic drug ethacrynic acid (EA) and its glutathione conjugate (EASG) was investigated by calorimetric, spectrofluorimetric, and crystallographic studies. The mutation Tyr 108 → Val resulted in a 3D-structure very similar to the wild type (wt) enzyme, where both the hydrophobic ligand binding site (H-site) and glutathione binding site (G-site) are unchanged except for the mutation itself. However, due to a slight increase in the hydrophobicity of the H-site, as a consequence of the mutation, an increase in the entropy was observed. The Y108V mutation does not affect the affinity of EASG for the enzyme, which has a higher affinity ($K_d \sim 0.5 \mu\text{M}$) when compared with those of the parent compounds, $K_d^{\text{EA}} \sim 13 \mu\text{M}$, $K_d^{\text{GSH}} \sim 25 \mu\text{M}$. The EA moiety of the conjugate binds in the H-site of Y108V mutant in a fashion completely different to those observed in the crystal structures of the EA or EASG wt complex structures. We further demonstrate that the ΔC_p values of binding can also be correlated with the potential stacking interactions between

Additional Supporting Information may be found in the online version of this article.

Abbreviations: DMSO, dimethylsulfoxide; DSC, differential scanning calorimetry; DTT, dithiothreitol; EA, ethacrynic acid; EACys, ethacrynic-cysteine conjugate; EAME, ethacrynic-mercaptoethanol conjugate; EASG, ethacrynic-glutathione conjugate; GST, glutathione transferase; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; hGST P1-1, human glutathione transferase P1-1; ITC, isothermal titration calorimetry; MES, 2-morpholinoethanesulfonic acid; MPD, 2-methyl-2,4-pentanediol; wt, wild type.

Indalecio Quesada-Soriano and Lorien J. Parker contributed equally to this work.

The coordinates for the structures of the Y108V apo, EA and EASG complex are deposited with the Protein Databank (<http://rcsg.org/pdb/>) with the entry codes 3HJM, 3HKR, and 3HJO, respectively.

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ligand and residues located in the binding sites as predicted from crystal structures. Moreover, the mutation does not significantly affect the global stability of the enzyme. Our results demonstrate that calorimetric measurements maybe useful in determining the preference of binding (the binding mode) for a drug to a specific site of the enzyme, even in the absence of structural information.

Keywords: glutathione S-transferase; ethacrynic acid; calorimetry; binding; X-ray crystallography; EA-conjugates; thermal stability

Introduction

Glutathione transferases (EC 2.5.1.18) (GSTs) are a family of enzymes involved in the cellular detoxication of endogenous and exogenous electrophilic compounds, including carcinogens, by catalyzing their conjugation with the thiol group of reduced glutathione (GSH, γ -Glu-Cys-Gly).^{1,2} Cytosolic mammalian GSTs are classified into at least seven distinct classes: alpha, mu, pi, theta, sigma, zeta, and omega.³ Three-dimensional structures of representatives of all known classes of mammalian cytosolic GSTs have been determined by X-ray crystallography. Despite low sequence similarity between the soluble GST classes, typically less than 30% sequence identity, all cytosolic GSTs have a similar protein fold with the N-terminal domain adopting a thioredoxin fold. There are at least two ligand-binding sites per subunit: the GSH-binding site (G-site), which is highly specific for GSH, and the hydrophobic substrate binding site (H-site), which can bind a variety of different electrophiles.

Overexpression of GST P1-1 has been noted in several human tumors and is thought to contribute to the development of resistance to commonly used anti-cancer drugs including chlorambucil, mephalan, and adriamycin.⁴⁻⁷ Consequently, GST P1-1 expression has been considered as a diagnostic indicator of chemical carcinogenesis.^{8,9} Overexpression of GST P1-1 in tumors was the basis for development of GST P1-1-activated prodrugs for use in cancer chemotherapy.¹⁰ The diuretic drug ethacrynic acid (EA, [2,3-dichloro-4-(2-methylenebutanoyl)-phenoxy] acetic acid) is an inhibitor of GST P1-1. The use of this inhibitor as a means of overcoming acquired multidrug resistance has been thoroughly explored, reaching Phase II clinical trials. Unfortunately, due to unwanted toxic side effects, EA was withdrawn from further trials. EA can also act as substrate of GST, forming a conjugate with GSH via Michael addition (EASG), both spontaneously and by GST-driven catalysis, by pi, mu and alpha class GSTs.¹¹⁻¹⁴ The α,β -unsaturated ketone moiety of EA is the target for conjugation by GSH.¹⁴ The reaction catalyzed by the pi class enzyme is the most efficient, often considered a characteristic feature of this isoenzyme.¹ The GSH conjugate of EA also acts as an inhibitor of GSTs.

Crystallographic studies of alpha¹⁵ and pi¹⁶ enzymes indicate that EA binds in the H-site of the enzyme in a similar position to that found for the hexyl moiety of the S-hexylSG complex crystal structure.¹⁷ On the other hand, two different binding modes with the

GSH conjugate of EA (EASG) were discovered.^{16,18} These two structures (binding modes) present some differences, mainly in the accommodation of the EA moiety in the H-site but in both cases the EA arene ring forms a π -stacking interaction with Tyr 108.

Investigations into the structural basis of differential substrate specificity, among the different classes of GSTs, particularly with EA as substrate, are extensive.^{15,16,18-21} The alpha and pi class isoforms appear to be the most recently evolved classes. A comparison of their H-sites, based mainly on 3-D superposition of their crystal structures, shows some differences.^{15,16,18} An important difference, which greatly affects the substrate specificity, is the Tyr 108 residue in the pi enzyme which has a counterpart Val 111 in the class alpha. The hydroxyl group of this Tyr 108 residue seems to contribute to the catalytic mechanism as a general acid in the Michael addition of GSH to EA.^{16,18-20} We have shown previously a shift in substrate specificity of the pi class enzyme towards that of the alpha class iso-enzyme by generating a single point mutation at this residue (Tyr 108 $\rightarrow$ Val) in the H-site of GST P1-1.²⁰ The essential role of this tyrosine residue in catalysis was also examined using the Y108F mutant.¹⁹ The necessity of this residue for catalysis is further highlighted through the conserved nature of this active site residue in the mu class enzyme (Tyr 115).²²⁻²⁵ This residue is completely absent in alpha class which may explain the low specific activity of human alpha class enzyme for EA.^{1,26} The residual activity toward EA both of hGST A1-1 and GST P1-1/Y108F and GST P1-1/Y108V mutants was discussed previously.^{15,19,20} Another important difference between pi and alpha class isoenzymes is the addition of a C-terminal helix (residues 208-222) in the alpha isoenzyme, which can be described as a dynamic helix (helix 9) wobbling about a hinge, both in the apo-enzyme and in the presence of GSH analogs. Recently, Micaloni *et al.*²¹ redesigned the H-site of GST P1-1 by adding this flexible helix from GST A1-1 enzyme. The addition of this new helix into the open H-site of GST P1-1 produced a significant change in the enzymatic properties of the chimeric enzyme, closely representing those exhibited by the alpha class enzyme. The mutation to a valine at position 108, however, had a more pronounced affect on EA binding than by the addition of C-terminal helix, reinforcing the importance of Tyr 108 in the catalytic process of the pi class enzyme.

In this article, we have explored the characteristics of the Y108V mutant through thermodynamic studies

Figure 1. A: Structure of the EASG-Y108V mutant complex showing a close up view of the active site. The ligand is shown in blue bonds and the protein in green ribbon. Phe 8 is shown in green stick. The blue mesh signifies the $2F_o - F_c$ electron density map contoured at 1σ . B: Stereo superposition of the Y108V EASG (light and dark grey) complex superimposed on the 3GSS structure (light and dark blue). The rotation of the EA moiety and the shift of the Phe 8 are clearly depicted. C: Orthogonal views of the superposition of 3GSS (blue), 11GS (yellow), 2GSS (pink), and the Y108V structures (green) showing the differing orientations of the EA moiety. GSH has been omitted for clarity.

on its interaction with EA and the EASG conjugate using fluorescence spectroscopy, isothermal titration calorimetry (ITC), and differential scanning calorimetry (DSC). We have also investigated the mutant structurally using X-ray crystallography. Furthermore, the ΔC_p values of binding can also be correlated with the potential stacking interactions between ligand and residues located in the H-site as predicted from structural analysis. Such an approach may prove to be a useful tool for determining the preference of binding (the binding mode) for a drug to a specific site of the enzyme.

Results

To elucidate the role of the amino acid residue at position 108 in GST P1-1 through the thermodynamics of binding with EASG and EA, we have utilized the Tyr108Val mutant.²⁰ In class alpha GST the corresponding residue is a valine (Val 111) and so this amino acid was chosen for comparative purposes.

Crystal structures of the Apo and EASG complexes

The crystal structure of the apo Y108V enzyme was essentially identical to that of the wt enzyme with a r.m.s deviation in α -carbon positions on superposition of 0.3 Å and no significant deviations between the models. A water molecule normally found in wt structures that interacts with the hydroxyl group of Tyr 108, is missing in the mutant. Otherwise the microenvironment around residue 108 is very similar in the two structures.

In the structure of the EASG mutant complex, the GSH and EA portions of the conjugate molecule have bound in the G- and H-sites, respectively, as expected.

The EA moiety, however, has bound in a novel mode not previously observed (2GSS, 3GSS, 11GS, and 1GSE).^{15,16,18} The electron density maps are consistent with one of three ligands binding to the active site (one ligand per monomer for each GST molecule in the crystal): the product EASG, the substrates EA plus GSH (broken down EASG), and GSH only. This was not seen in the previously published wt EASG complexes where only the product was observed and this might be due to the mutation of Y108 which is a key residue in Michael addition of GSH to EA. Interestingly, we observed that unconjugated EA binds in exactly the same orientation in the active site as the EA moiety of the GSH conjugated product [Fig. 1(A), main plot].

The structure of the mutant enzyme determined from enzyme cocrystallized with EA alone showed no evidence of EA binding in the H-site at all. We cannot rule out that the crystallization conditions may have affected EA binding since these were different to those of wt-EA complexes¹⁶ and might not be favorable for EA binding. An alternative explanation is that replacement of the Tyr 108 causes a loss of π - π stacking interactions with EA, which cannot be completely compensated by Phe 8, resulting in significantly weaker binding.

Thermodynamics of EASG binding

ITC titrations of the Y108V mutant of GST P1-1 were performed to investigate and establish a comparative and detailed analysis of the role of Tyr 108 in the interaction with the inhibitor EASG. Figure 2 shows exemplary raw data from a single experiment. Notably, no evidence for ligand binding cooperativity was observed [Fig. 2(A); inset]. Titrations of the Y108V

Figure 2. Characterization of EASG binding to Y108V mutant of GST P1-1. A: Representative isothermal titration calorimetry measurements of the binding of 1.95 mM EASG to 47 μ M Y108V mutant of GST P1-1. Titration was performed in 20 mM sodium phosphate, 5 mM NaCl and 0.1 mM EDTA at pH 7.0 and 25.2°C. *Inset plot*: Hill graph. The Hill coefficient value was 1.08 ± 0.01 . B: Binding of EASG to the Y108V mutant of GST P1-1 obtained by fluorescence spectroscopy at pH 7.5 and 25°C. The degree of binding, v , is plotted *versus* $\ln[\text{ligand}]_f$ (with $[\text{ligand}]_f$ equal to free ligand concentration). The dimeric enzyme concentration was 8 μ M and the buffer solution was 20 mM sodium phosphate buffer, pH 7.5, containing 0.1 mM EDTA. The *solid line* gives the best fit to the data for a non-cooperative model. *Inset plot*: Evolution of fluorescence emission spectra corresponding to titration of Y108V mutant with EASG. Their evolution with increasing ligand concentration is indicated by the arrow.

mutant [Fig. 2(B)] and wt enzyme with EASG by fluorescence spectroscopy (25°C) resulted in quenching curves that are consistent with the behavior obtained by calorimetry. Figure 3 summarizes the thermodynamic parameters and the binding constants extracted from the binding isotherms at different temperatures. From the thermodynamic parameters obtained at different temperatures, the heat capacity change ($\Delta C_p = d\Delta H/dT$) for the binding reaction can be calculated. The negative ΔC_p values were considerably larger (approximately twice) for EASG binding to the Y108V mutant ($\Delta C_p = -415 \pm 17 \text{ cal mol}^{-1} \text{ K}^{-1}$) when compared with the wt enzyme ($\Delta C_p = -264 \pm 24 \text{ cal mol}^{-1} \text{ K}^{-1}$).

GSH and S-hexylSG binding

The interaction of GSH and S-hexylSG with the Y108V mutant, in phosphate buffer and pH 7.0 (buffer A), was examined as a function of temperature between 15–35°C (cf. Fig. S4, Supporting Information). The values and signs of the thermodynamic parameters obtained for GSH binding were very similar to those deduced for the wt enzyme²⁷ (Table I). However, in that case, it was determined a higher and negative value of heat capacity change [$\Delta C_p = -0.44 \pm 0.05 \text{ kcal mol}^{-1} \text{ K}^{-1}$] for the wt enzyme with S-hexylSG than that calculated herein for the Y108V mutant [$\Delta C_p = -0.28 \pm 0.06 \text{ kcal mol}^{-1} \text{ K}^{-1}$] (Table I).

EA interaction

The binding of EA to GST P1-1 was investigated by ITC and fluorescence spectroscopy at several temperatures in the range 20–35°C and pH 7.0 (phosphate buffer (Buffer A)). Figure 4(A) shows a representative thermogram corresponding to a titration of wt GST P1-1 with EA, at pH 7.0 and 25°C. Examination of the

Figure 3. Temperature dependence of the thermodynamic parameters for binding of EASG to both the Y108V mutant and the wt enzyme. The parameters for the Y108V and the wt GST P1-1 are shown by *open* and *filled* symbols, respectively. The heat capacity changes, associated with the binding, were determined by linear regression analysis as the slopes of the plots of ΔH *versus* temperature.

Table I. Thermodynamic Parameters of the Interaction of GSH, S-hexylSG, EA, and EASG to wt GST P1-1 and the Y108V Mutant at 25°C and pH 7.0

Ligand	Protein	K_d (μM)	ΔG° (kcal mol $^{-1}$)	ΔH (kcal mol $^{-1}$)	$T\Delta S^\circ$ (kcal mol $^{-1}$)	ΔC_p° (cal mol $^{-1}$ K $^{-1}$)
GSH	WT ^a	91.0 $\pm$ 8.1	-5.53 $\pm$ 0.02	-11.21 $\pm$ 0.12	-5.65 $\pm$ 0.12	-294 $\pm$ 30
	Y108V	27.8 $\pm$ 2.2	-6.20 $\pm$ 0.40	-12.21 $\pm$ 0.48	-6.01 $\pm$ 0.48	-230 $\pm$ 21
S-hexylSG	WT ^a	1.2 $\pm$ 0.3	-8.04 $\pm$ 0.02	-16.13 $\pm$ 0.07	-8.04 $\pm$ 0.07	-442 $\pm$ 49
	Y108V	2.4 $\pm$ 0.2	-7.65 $\pm$ 0.33	-12.59 $\pm$ 0.34	-4.94 $\pm$ 0.31	-284 $\pm$ 61
EA	WT	13.0 $\pm$ 1.5	-6.65 $\pm$ 0.23	-3.41 $\pm$ 0.18	3.24 $\pm$ 0.32	-380 $\pm$ 60
	Y108V	30.3 $\pm$ 2.7	-6.19 $\pm$ 0.17	-0.91 $\pm$ 0.08	5.28 $\pm$ 0.41	-250 $\pm$ 10
EASG	WT	0.5 $\pm$ 0.1	-8.58 $\pm$ 0.26	-14.17 $\pm$ 0.37	-5.59 $\pm$ 0.37	-264 $\pm$ 24
	Y108V	0.3 $\pm$ 0.1	-8.79 $\pm$ 0.37	-13.34 $\pm$ 0.46	-4.55 $\pm$ 0.31	-415 $\pm$ 17

^a Ref. 27.

calorimetric traces revealed some important features when compared with a thermogram corresponding to a typical binding process. Thus, the calorimetric responses after each injection of EA (peaks in the thermogram) show two exothermic phases clearly differentiated by their response times. The response time for the first phase, ~ 2 min (comparable with the response time in the dilution experiment) is smaller than that obtained for the second phase (~ 12 min). It is also worth noting that each injection includes ligand dilution, which always occurs in any binding experiment and is evaluated in a separate experiment.

The possibility of an irreversible modification with loss of activity has also been examined after preincubation of the enzyme with EA in similar conditions to those used during a calorimetric experiment. Only when treated with a large excess of β -mercaptoethanol was the initial enzymatic activity restored (cf. Fig. S5, Supporting Information). The possible targets for the chemical reaction with EA might be a free $-SH$ group present either in the buffer, in the enzyme or both. The reaction with some buffer component was discarded because no reducing agents with free thiols (as β -mercaptoethanol or DTT) were included. However, GST P1-1 has four Cys residues with Cys 47 the most reactive^{28–33} and thus probably the target for covalent reaction with EA, via a Michael addition, to form a thioether linkage. The calorimetric titrations with C47S mutant and EA, in similar experimental conditions that those studied for wt enzyme, do not show any apparent kinetic process and the baseline recovery after each EA injection is typical for a single binding process [Fig. 4(B)]. It can therefore be concluded that the C47S mutation removes the covalent modification potential of EA towards this enzyme. Thus, a mechanism with two consecutive steps can be postulated for the interaction of wt enzyme (P) with EA (Scheme 1).

Scheme 1.

Figure 4. Calorimetric thermograms. A: Exemplary calorimetric thermogram for the titration of 23 μM wt GST P1-1 with 5 μL injections (1 μL first injection) of 2.1 mM EA in 20 mM sodium phosphate, 5 mM NaCl and 0.1 mM EDTA at pH 7.0 and 25°C. The first peak corresponds to a preinjection of 1 μL . Inset plot: Comparison of a peak from a calorimetric thermogram for the titration of wt enzyme with EA (which reflects the slow kinetic process caused by covalent modification) (solid line) and a representative calorimetric trace of a typical binding peak in the absence of kinetic process (dashed line). B: Calorimetric titration of 37 μM C47S-hGSTP1-1 mutant with 4 mM EA. A zoom plot of the same peaks is shown in the inset. The chemical process (irreversible covalent modification of the enzyme with EA) is not apparent in the raw calorimetric thermogram with this mutant.

In the reversible first step (fast binding process), EA binds to the H-site and in the irreversible second step Cys 47 reacts (slow chemical process), via Michael addition, with the α,β -unsaturated ketone of EA.

A theoretical deconvolution procedure, similar to that described elsewhere,²⁹ was applied to the calorimetric traces with EA, to obtain the kinetic and thermodynamic parameters for this association, according to the model postulated in Scheme 1 (Supporting Information). A similar behavior to that described for wt enzyme, was also observed for the binding of EA to the Y108V mutant. After the mathematical deconvolution, the resulting binding thermograms were analyzed according to a non-cooperative model for two binding sites for EA (one site per monomer). The peaks corresponding to the chemical process (heat generated by the conjugation reaction of EA with the enzyme (wt and Y108V mutant)), were analyzed and the kinetic constant at each temperature was calculated as described in the Supporting Information. Activation energy values (calculated by Arrhenius equation) for the chemical conjugation of EA with Cys 47 were ~ 2 and ~ 5 kcal mol⁻¹ for wt enzyme and Y108V mutant, respectively.

Although the C47S mutation allowed us to study the non-covalent binding of EA to enzyme, our interest was further focused on the investigation of such binding interaction (both with wt enzyme and with Y108V mutant) using alternative methods without the need to make additional mutations both on the wt enzyme and on the Y108V mutant. Thus, further experiments, using bromopyruvate as thiol-reactive alkylating agent,³⁴ were performed in order to prevent the covalent modification of the enzyme by EA. We observed that after treatment with this reagent, the modified enzyme (see Materials and Methods for details) was unable to bind EA (cf. Fig. S7, Supporting Information).

On the other hand, it was of interest to examine if the presence of S-methylSG in G-site might produce a protective effect on Cys 47. Such a G-site-based inhibitor was expected to lock down helix $\alpha 2$, by binding to the G-site, and in doing so bury Cys 47. However, the calorimetric thermograms show the occurrence of a chemical reaction similar to that found in the absence of this inhibitor. Therefore, this procedure does not prevent the chemical reaction with EA. The next strategy employed to prevent covalent modification of Cys 47 by EA lead us to modify the EA molecule so as to block the reactive α,β -unsaturated ketone. Thus, we synthesized the EA derivatives, EAME and EACys, obtained by conjugation reaction, via Michael addition, between EA and β -mepcaptoethanol, and EA and L-cysteine, respectively (cf. Materials and Methods). In both cases, the size of the EA conjugate moiety could be sufficiently small to avoid filling the G-site, and consequently their binding to wt enzyme and Y108V mutant would be expected to be very similar to that

for EA (unconjugated form). In both cases, the binding of these derivatives to either wt enzyme or Y108V mutant did not produce the kinetic process observed in the binding of EA.

DSC studies

The thermal stability of the Y108V mutant of GST P1-1 was studied by DSC (Buffer B) and compared to the wt enzyme. The thermal denaturation of mutant was always irreversible (in the absence and the presence of EASG), as no transition could be detected in reheating runs. Thermal unfolding of the mutant was very similar to that of wt²⁹ Figure 5 (inset plot). Although the transition temperatures, T_m , should be closely similar for both enzymes, it is slightly higher for the Y108V mutant. In contrast with the results obtained for the wt enzyme,²⁹ the thermogram shape obtained for the Y108V mutant showed only one transition, instead of the two apparent transitions detected in the traces of the wt enzyme. Profiles of excess heat capacity *versus* temperature were obtained for scanning rates of 0.2–1.5 K min⁻¹. The position of the maximum on the C_p curve is shifted toward higher temperatures as the scanning rate increases [Fig. 5(A), main plot]. Such effect verifies the irreversible character of the thermal denaturation of the protein^{35–38} and, therefore, a kinetic control for this process. Standard numerical integration of the transition curves, and determination of T_m values [Eq. (4)], yields the parameters presented in Table II which reveals that, at a heating rate of 1.5 K min⁻¹ (maximum value for the VP-DSC instrument), the transition temperature (T_m) seems to reach a maximum value and that a further increase in the heating rate should not change this value significantly. Furthermore, the van't Hoff enthalpy approaches the true calorimetric enthalpy with the increase of the heating rate, thereby yielding a cooperativity ratio $\Delta H^{cal}/\Delta H^{VH}$ equal to 1. Considering together the results for the thermal unfolding of wt enzyme²⁹ and those obtained here for this mutant, the general model described by Lumry-Eyring³⁸ is sufficient to describe the thermal unfolding pathway for this mutant at all the scan rates studied.

Scheme 2.

I would be a dissociate intermediate state and partially unfolded in equilibrium (K) with native dimer (N_2), and **D** an irreversible denatured state that cannot fold back into the native state. However, from our results (Table II), at the maximum heating rate, this general model can be simplified to a single and reversible non-two-state model: $N_2 \rightleftharpoons 2D$.

Figure 5. Heat capacity measurements. A: Variation with heating rate of the C_p transition curves of Y108V mutant of hGST P1-1. Thermal transitions were carried out in 10 mM HEPES-NaOH at pH 7.5, and calorimetric traces were baseline corrected. The numbers near the peaks refer to the heating rates in K/min. The experiments were performed at a protein concentration of 12 μ M. (inset plot) Comparison of representative calorimetric traces (heat capacity scans) of Y108V mutant (dashed line) and wt enzyme (solid line) at a scan rate of 1.5 K/min. B: Excess heat capacity versus temperature for the thermal denaturation of Y108V-GST P1-1 at different EASG concentrations, in the range of 0–2.51 mM. Protein concentration was 12 μ M, and the heating rate was 1.5 K/min. Single and symmetrical C_p curves obtained at low ligand concentrations (14, 27, and 54 μ M EASG) are shown as inset plot.

Heat capacity scans of Y108V mutant at different EASG concentrations. Figure 5(B) shows some C_p curves of Y108V at different EASG concentrations. A biphasic behavior was detected with increasing EASG concentrations. For these bimodal thermograms, the lower and higher denaturation temperatures observed were denoted as $T_{m,1}$ and $T_{m,2}$, respectively. A progressive increase in T_m s with ligand concentration is expected for denaturation processes

coupled to ligand binding on the native protein, even at levels beyond those where all binding sites are essentially fully occupied.^{39–42} This tendency was observed when the concentration of EASG was increased at constant protein concentration [Fig. 5(B)]. As the affinity of EASG to the native protein (folded state) is high, for the sake of simplicity, we assume that ligand binding to the unfolded state is negligible. Fitting of the DSC thermograms for the thermal denaturation of the Y108V mutant and wt enzyme in the presence of different EASG concentrations was carried out using the following equation

$$C_p^{\text{exc}} = \frac{\Delta H_1^{\text{cal}} \cdot \Delta H_1^{\text{VH}}}{RT^2} \cdot \frac{K_1}{(1 + K_1)^2} + \frac{\Delta H_2^{\text{cal}} \cdot \Delta H_2^{\text{VH}}}{RT^2} \cdot \frac{K_2}{(1 + K_2)^2} \quad (1)$$

where the equilibrium constants are given by

$$K_i = \exp \left[-\frac{\Delta H_i^{\text{VH}}}{R} \left(\frac{1}{T} - \frac{1}{T_{m,i}} \right) \right] \quad (2)$$

with i equal to 1 or 2. Equation 1 includes terms corresponding to two non-two-state transitions described phenomenologically in terms of van't Hoff enthalpy values (ΔH_i^{VH}), which may differ from the corresponding calorimetric enthalpies (ΔH_i^{cal}). From our data it can be deduced that, the first transition can be formally treated as two states ($\Delta H^{\text{cal}}/\Delta H^{\text{VH}}$ ratio closed to 1), whereas the second transition, where the cooperativity ratio calculated was always lower than the unity, can not (cf. Fig. S8A and Table T3, Supporting Information). The reason for the low value of $\Delta H^{\text{cal}}/\Delta H^{\text{VH}}$ for the second transition might be the result of the irreversibility of global thermal unfolding mechanism.

We have applied the general statistical-mechanical analysis of conformational transitions in proteins described in the literature.^{42–45} This provides a basis for obtaining an estimate of the average number of ligands bound per mole of native protein, $\bar{X}_N$, from the heat capacity data by equation,

$$\frac{\partial(1/T_m)}{\partial \ln[L]} = -\frac{R \bar{X}_N}{\Delta H} \quad (3)$$

Hence, we will use characteristic temperature ($T_{m,1}$ and $T_{m,2}$) and ΔH^{cal} (area below curve) values determined by the fitting of calorimetric traces to Eqs. (1) and (2). At low EASG concentrations [see Fig. 5(B) (inset plot)], the second term from Eq. (1) was ignored in the fitting and the T_m value for this transition was assigned to the characteristic temperature, $T_{m,1}$ (cf. Fig. S8B, Supporting Information). The inset in Figure 6 shows plots of $1/T_{m,1}$ and $1/T_{m,2}$ versus $\ln[\text{EASG}]$ for the higher EASG concentrations used and plotted in the main plot of Figure 6. According to Eq. (3), the numbers of ligand molecules released ($\bar{X}_N$) in the

Table II. Geometric Data Comparison Deduced From EASG Y108V, 3GSS, and 11GS Structures

Structure PDB code	Plane angles		Centroid distance		Closest contact	
3GSS ^a	EA – F8	51.9°	EA – F8	8.2 Å	EA(C4) – F8(CE2)	6.7 Å
	EA – Y108	1.7°	EA – Y108	3.80 Å	EA(C4) – Y108(CZ)	3.6 Å
11GS ^b	EA – F8	45°	EA – F8	6.0 Å	EA(C5) – F8(CD2)	4.7 Å
	EA – Y108	7.5°	EA – Y108	4.4 Å	EA(C5) – Y108(CE1)	3.7 Å
3HJO	EA – F8	12.7°	EA – F8	4.5 Å	EA(C2) – F8(CG)	3.9 Å

Single letter amino acid abbreviations are used for clarity.

^a Ref. 16.

^b Ref. 18.

corresponding transitions might be obtained from the slopes of these plots. The slopes for these plots are practically equal within experimental error (Fig. 6). A similar procedure to that described for the Y108V mutant was done for wt enzyme using the T_m values ($T_{m,1}$ and $T_{m,2}$) deduced from calorimetric traces (cf. Fig. S9, Supporting Information). Finally, an estimate of the pK_s value for the ligand binding equilibrium can be deduced by plotting the inverse transition temperature ($1/T_m$) versus the log [ligand].^{43,44} As described, the pK_s value can be estimated from the abscissa value where the initial $1/T_m$ value deviates from the horizontal line (cf. Fig. S10, Supporting Information).

Discussion

Binding of EA conjugates

The binding of EASG conjugate to both wt GST P1-1 and Y108V mutant is non-cooperative with a stoichiometry 1:1 (one molecule of EASG per subunit) (Fig. 2). The interaction with EASG was favorable enthalpically (exothermic) and unfavorable entropically for both enzymes at all studied temperatures (Fig. 3). The affinity is high and similar for both enzymes over the temperature range studied. The order of K_d ($K_d^{EASG} \sim 0.5 \mu M$) values for both enzymes reflects the inhibitor potency for this ligand compared with their parent compounds ($K_d^{EA} \sim 13 \mu M$; $K_d^{GSH} \sim 25 \mu M$). These results support the findings deduced from structural studies for the complexes with wt enzyme.^{16,18} Moreover, the order of affinity (expressed as K_d) was similar to that determined, by ITC at pH 7.0 and 25°C, for alpha class GST (GST A1-1) (K_d^{EASG} (GST A1-1) = $0.4 \mu M$ ⁴⁶). However, while EASG binding to wt GST A1-1 was both enthalpically and entropically driven, we find that the binding to wt GST P1-1 was solely enthalpically driven (Fig. 3). It has been argued that the flexible C-terminal $\alpha 9$ helix of GST A1-1 is required for entropically driven ligand binding in this isoenzyme.⁴⁶ We expected that ligand binding to GST P1-1 (where the C-terminal mobile helix seen in GST A1-1 is not present) would not be entropically driven and this is in agreement with our experimental results.

Frequently, negative ΔC_p values are usually attributed to the burial of apolar groups from water. However, in many systems it is also thought to be associated with hydrophobic stacking interactions,

presumably resulting from the dehydration of highly ordered water molecules surrounding hydrophobic surfaces.^{47–50} Thus, the different values of observed heat capacity change upon binding to the Y108V mutant and wt might be caused by a rearrangement in the π – π stacking interactions between the ring of the EA moiety of the conjugate and the aromatic side chain of Phe 8. In the EASG mutant complex structure presented here, the EA-Tyr 108 stacking interaction present in the 3GSS¹⁶ and 11GS¹⁸ wt structures is lost, and the aromatic ring of EA now solely stacks against the ring of the Phe 8 residue. However, the GSH and EA portions of the conjugate molecule have bound in the G- and H-sites, respectively, as expected [Fig. 1(B)]. The statistics, arising from analysis of both previously published structures (Table II), indicate that Phe 8 in 3GSS has a very weak T-shaped interaction with the EA aromatic ring of the EASG conjugate. In the 11GS structure, the EA-Phe 8 interaction is much

Figure 6. DSC measurements. A: Effect of EASG concentration on the transition temperatures for the two transitions observed in the DSC thermograms for the thermal denaturation of the Y108V mutant of hGST P1-1. The symbols are the values of $T_{m,1}$ and $T_{m,2}$ derived from the experimental thermograms. B: (inset) Plots of $1/T_{m,1}$ and $1/T_{m,2}$ versus logarithm of EASG concentration [see Eq. (3)] for the higher ligand concentrations used (in the range of 0.49–2.51 mM EASG). The numbers of ligand molecules released ($\bar{X}_N$), obtained from the slopes of these plots, were $\bar{X}_N^{T_{m,1}} = 1.2 \pm 0.1$ and $\bar{X}_N^{T_{m,2}} = 1.1 \pm 0.2$ for lower and higher transition, respectively.

more energetically relevant, with a substantial reduction in the centroid distance, increasing the ring-stacking effect. In the Y108V complex structure reported here the EA moiety has bound in a novel mode [Fig. 1(C)] not previously observed (2GSS, 3GSS, 11GS and 1GSE).^{15,16,18} In the binding mode seen in the wt structure PDB code 11GS, the EA binds so it is sandwiched between the aromatic rings of Tyr 108 and Phe 8, where it forms parallel π - π stacking interactions primarily with Tyr 108. In the mutant structure reported here the EA arene moiety stacks up more closely against the aromatic ring of Phe 8 (centroid distance: 4.5 Å, Table II) with a concomitant rotation of the ring by about 45°, presumably compensating for the loss of interaction with the Tyr 108 aromatic ring [Fig. 1(B)]. Thus, in the absence of the aromatic ring of residue 108, the EA moiety of the conjugate has swung around to give an almost optimal parallel-displaced interaction with Phe 8, restricted of course by the local environment. We have used this structural information to explain the experimental negative ΔC_p values obtained for EASG interaction with wt ($\Delta C_p \cong -260 \text{ cal mol}^{-1} \text{ K}^{-1}$) and the Y108V mutant ($\Delta C_p \cong -410 \text{ cal mol}^{-1} \text{ K}^{-1}$). This analysis is based on the assumption that the overall conformation of the Y108V mutant complexed with EASG is relatively unchanged from that of the wt protein and that the main effect is the π -stacking between the EA and flanking aromatic amino acids. Other effects, such as a greater predilection for valine to bury itself from solvent than that for tyrosine, may be responsible for the difference in ΔC_p values obtained for the wt and mutant proteins. Thermodynamic results described herein might be used to make an assessment on the predilection of the Y108V mutant to adopt a particular 3D-structure, when complexed with EASG, similar to that observed in 11GS (where there is a high reduction in the centroid distance between EA moiety and Phe 8; Table II). In the absence of Tyr 108, the EA ring can stack optimally with Phe 8 to produce a π - π interaction between the Phe 8 and EA aromatic rings that is more energetically favorable and optimal than those described for the 3GSS binding mode. This increase in the strength of π - π interactions in the Y108V mutant might explain the higher and negative ΔC_p value for the interaction with this mutant than that obtained for the wt enzyme. Therefore, our results demonstrate that ITC measurements can provide a thermodynamic fingerprint of drug-protein interactions, which can be related to the molecular mode of binding in multimeric enzymes.^{51,52} Calorimetric data can therefore be a useful method to complement X-ray crystallographic studies. However, even in the absence of structural information, thermodynamics can give a guideline to improve drug potency. Reports of comparisons of drug binding energies with relevant structural studies are still rather scarce in the literature. Thus, studies such as ours are of great general interest.

The thermodynamic parameters for the interaction of GSH with Y108V were very similar to those deduced from wt.²⁷ However, the thermodynamic parameters determined for S-hexylSG-binding to this mutant show some differences to those obtained for this inhibitor's interaction with wt enzyme: the entropy change values for the Y108V mutant are less unfavorable than those for the wt enzyme, while the enthalpy changes are more favorable for the binding to wt enzyme. (cf. Fig. S4, Supporting Information). The main and only difference between the two 3D-structures is the substitution of a residue at the position 108 (Tyr→Val) of H-site with loss of a contacting water molecule. The Y108V mutation increases the hydrophobicity of this site which might explain the difference in the thermodynamic parameter values between both enzymes (Y108V mutant and wt enzyme) with S-hexylSG (Table I). As there are less ordered water molecules in the more hydrophobic H-site of the Y108V mutant, binding of ligand should be accompanied by a higher entropy change (ie, less negative) and a less negative enthalpy change, which is in agreement with our results. Therefore, the mutation Y108V does not seem to greatly affect the ligand interaction at the G-site.

EA interaction

The EA interaction with wt and the Y108V mutant is associated with a chemical reaction in which a cysteine residue must be near to the H-site and exposed to solvent. Cys 47 has been demonstrated to be the most reactive cysteine toward electrophilic compounds of the four cysteine residues found in wt.^{28,30,31} Cys 47 is located near the C-terminal end of the $\alpha 2$ loop (which lines the G-site) and sits in a hydrophobic pocket defined by residues Trp 38, Leu 43, Leu 52, Tyr 63, and the aliphatic portion of Lys 54.¹⁷ Although Cys 47 is not involved directly in either substrate binding or catalysis^{17,32}, chemical modification of its side-chain inactivates the enzyme^{53,54}, possibly by blocking a structural transition during catalysis⁵⁵ or by inducing an unfavorable conformational change for GSH binding.^{28,31,53,56} In addition, in the absence of bound GSH, Cys 47 is exposed to the solvent and available for reactions.⁵⁷ It has also been proposed that the high reactivity of Cys 47 (its sulfhydryl group has a low pK_a of 4.2) is due to formation of an ion pair between the thiolate of Cys 47 and the protonated ϵ -amino group of Lys 54.^{34,58} The C47S mutation removes this chemical reaction with EA. On the other hand, the strategy to use S-methylSG had a dual objective: (1) To fill the G-site trying to avoid the accessibility of EA to Cys 47, and (2) to establish a similar scenario to the productive binding of EA to the wt enzyme. These objectives would be achieved by GSH binding in the G-site. As the methyl moiety of S-methylSG is too small to fill the H-site it was expected to promote productive binding of EA in similar fashion to GSH.^{2,15,16} In this case,

however, the thiol group of S-methylSG is blocked thus preventing conjugation with EA. This situation would mimic the *in vivo* situation where the intracellular concentrations of GSH (1–10 mM) ensures the majority of enzyme molecules have GSH bound, and thus, promote the productive binding mode of EA. However, we found that the binding of S-methylSG did not prevent the chemical reaction of EA with the enzyme. Therefore, these results indicate either that the accessibility of Cys 47 of $\alpha 2$ loop is maintained even when S-methylSG is bound in the G-site or that another residue can now be modified by EA.

Therefore, all results reported here indicate that EA inhibits GST P1-1 both competitively toward CDNB and through a covalent modification, of the sulphydryl group of Cys 47 of $\alpha 2$ loop of the enzyme. A mechanism, with two consecutive steps, was described for the binding reaction with EA (Scheme 1). Moreover, the activation energy value for the chemical reaction of EA with the Cys 47 of the Y108V mutant is $\sim$ twofold higher than that for the wt enzyme. This point mutation may affect the chemical reaction of Cys 47 with EA by a mechanism related to the high mobility of the $\alpha 2$ loop and a possible tendency for it to move into the H-site upon EA binding. Thermodynamic parameters determined for the binding process at each temperature, are shown in Figure 7. EA binding to wt and Y108V is both enthalpically and entropically favored, although, in the case of the wt enzyme, the entropy change is negative at temperatures above 30°C. In addition, the affinity of EA to the wt enzyme is higher than that for the Y108V mutant due to a more negative enthalpy change and a more positive entropy change for the wt enzyme. On the other hand, the structural data presented herein does not show any evidence that EA binds to this mutant. There are several reasons that might explain why: (i) The lower affinity of EA for the Y108V mutant, taken together with its poor solubility, suggests much higher concentration of the substrate and longer soak times might be required to observe binding. The lower affinity for EA results from a strong reduction of π - π stacking interactions that usually stabilize binding of EA in wt. A lower affinity of EA to the Y108V mutant was also detected by kinetic studies²⁰; (ii) The crystallization conditions used here are different to those of wt-EA complexes¹⁶ and might not be favorable for EA binding. It should be noted that the high levels of DTT used in the crystallization trials would have prevented covalent modification of the enzyme at Cys 47.

An interesting result can be deduced particularly from the signs and values of the entropy changes. These values contrast with those obtained for the binding of EASG and S-hexylSG to both enzymes (wt enzyme and the Y108V mutant) (Table I). In those cases, the EA and S-hexyl moieties of the inhibitors fill the H-site of the enzyme, whereas the GSH moiety fills the G-site.^{16,18} Clearly, the resulting thermodynamic

Figure 7. Temperature dependence of the thermodynamic parameters for binding of EA to both the Y108V mutant and the wt enzyme of GST P1-1. The parameters for the Y108V and the wt GST P1-1 are shown by open and filled symbols, respectively. The heat capacity changes, associated with the binding, were determined by linear regression analysis as the slopes of the plots of ΔH versus temperature (Table I).

parameters will be the net balance of the interaction with both sites. However, the binding of EA (to the wt enzyme or the Y108V mutant), occurs at the hydrophobic H-site, leaving the G-site unoccupied. This may explain the difference in observed signs and values for the entropy and enthalpy changes with EA binding in comparison to the binding of the GSH based, or GSH conjugated, ligands. This explanation can also be extended to the binding of EAME and EACys to this enzyme. Moreover, the Tyr→Val mutation increases the hydrophobicity of the H-site so the enthalpy changes were less exothermic (less favorable) and the entropy changes more positive for the Y108V mutant over the wt enzyme. Therefore, the EA interaction with these enzymes will be governed by the higher hydrophobicity and thus favorable entropy changes in the mutant when compared with the wt enzyme (Table I).

Taking into account all of these results, we conclude that the binding of EA (but not the EA conjugates) is the only one which is entropically driven whilst the differing thermodynamic properties of the conjugates, mainly in the signs of entropy changes, were significantly affected by the conjugated moieties.

Furthermore, while crystallography studies were unable to characterize EA binding, the use of a thermodynamic approach allowed us to describe both the covalent association and the non-covalent binding properties of this diuretic drug.

DSC experiments

The replacement of tyrosine 108 by valine produces a negligible structural perturbation which seems to be

important from a solely kinetic point of view, a result that is confirmed by the crystal structure of this mutant (see earlier). Furthermore, the thermodynamic parameters for the binding of the ligands to this mutant were very similar to those obtained for wt enzyme. Thus it was expected that the thermal unfolding behavior of the mutant would be closely similar to that of the wt enzyme²⁹ and this was confirmed by the comparison of heat capacity scans of wt and the Y108V mutant shown in Figure 5(A) (inset plot). An analogous result was deduced from thermal stability studies (non-calorimetric) on the Y108F mutant.¹⁹ In that case the amino acid at 108 position (Tyr) was changed to a Phe, resulting in a structure identical to that of the wt enzyme¹⁹, as happened with the Y108V mutant studied herein. The Lumry-Eyring general model³⁸ can be used to describe the thermal unfolding pathway for this mutant (Scheme 2). However, at high heating rates a cooperative two-state model might also be applicable (the DSC scan is symmetric [Fig. 5(A)] and the cooperativity ratio was approximately unity (Table III)). For wt enzyme, a consecutive and irreversible two step model was proposed.²⁹ Therefore, the general thermal unfolding mechanism for this mutant was similar to that described by Aceto *et al.*⁵⁹ for the unfolding/refolding of wt GST P1-1 using chemical denaturants. Thus, this mutation (Tyr108→Val) only alters, albeit slightly, the GST P1-1 thermal denaturation pathway.

The Y108V mutant is stabilized by EASG binding, as judged by the shift of the thermograms when the ligand/protein molar ratio ($[L]_{\text{total}}/[P]_{\text{total}}$) is increased [Fig. 5(B)]. Bimodal thermograms are observed, as expected, due to the presence of different protein populations represented by the ligand-free and ligand-bound protein species.^{60–64} Furthermore, from Eq. (3), we obtained $\bar{X}_N^{T_{m,1}} = 1.2 \pm 0.1$ and $\bar{X}_N^{T_{m,2}} = 1.1 \pm 0.2$ for lower and higher transition, respectively. Thus, an independent dissociation mechanism of two inhibitor molecules (per dimer of protein) can be expected for the thermal unfolding of Y108V/EASG complex (Scheme 3).

Scheme 3.

This independent dissociation was corroborated from ITC results described previously. The first and second step corresponds to the dissociation and unfolding of the protein respectively. Finally, we also noted that the third step is just a convenient, phenomenological representation of the irreversible denaturation for this enzyme. However, for the wt-EASG complex, two molecules of inhibitor are released in the first transition ($\bar{X}_N^{T_{m,1}} = 2.1 \pm 0.2$). In the second, smaller transition, the higher-temperature transition

Table III. Variation of Apparent Thermodynamic Transition Parameters of Y108V With Heating Rate

Scan rate (K min ⁻¹)	T_m (°C)	ΔH^{cal} (kcal mol ⁻¹)	ΔH^{VH} (kcal mol ⁻¹)	$\Delta H^{\text{cal}}/\Delta H^{\text{VH}}$
0.2	51.43	176.98	252.8	0.70
0.5	52.65	170.30	232.9	0.73
0.83	53.45	165.30	199.2	0.83
1.16	54.05	162.30	180.3	0.91
1.5	54.62	160.21	158.3	1.01

($T_{m,2}$) is not associated with a ligand release on unfolding ($\bar{X}_N^{T_{m,2}} = 0.05 \pm 0.3$) (Scheme 4). This result is also consistent with our assignment of the low-temperature transition to the unfolding and dissociation of the dimeric structure in the absence of ligand.²⁹ Therefore, the thermal denaturation of wt GST P1-1 in the presence of EASG can be depicted as:

Scheme 4.

Furthermore, the K values estimated from DSC studies were very similar ($pK_1 \approx 5.7$, $pK_2 \approx 5.6$) which corroborates the non-cooperativity and independence of the sites deduced also by ITC in this work.

The main reason for this specific mutation was to establish a comparison with the properties of the alpha class GST. However, there is currently no information available regarding the thermal unfolding pathway for that enzyme. Consequently, no acceptable comparison with the behavior expected for the thermal unfolding of the alpha class can be extracted from our DSC studies. However, there are reported studies, addressing the chemical unfolding pathway using chemical denaturants, for the alpha class isoenzyme.^{65,66} Unfolding/refolding experiments (fluorimetric studies), using urea as denaturant agent, yield similar curves with both enzymes (Y108V mutant and wt enzyme) showing that both stability and unfolding pathways (data not shown) for the two are similar. Hence, the thermal and chemical stability of the Y108V mutant are very similar to wt enzyme. A three-state pathway was proposed, similar to that described for the wt enzyme of pi class in the absence of ligands.⁵⁹ The only difference between the chemical unfolding mechanisms, for alpha and pi class GSTs, was the dissociation step for the native dimer. However, the structural differences between alpha and pi class GSTs extend further than just the change in amino acid at position 108, since the alpha class enzyme possesses an additional C-terminal helix. These, and others structural differences between both isoenzymes, might explain the different behavior in their unfolding pathways. What's more, even for GSTs within the same class, different mechanisms are possible. This fact, might explain in part,

why the thermal unfolding mechanism for the Y108V mutant is different to that for the wt enzyme, both in the absence and in the presence of ligand, although the global stability is similar.

Materials and Methods

Materials

GSH, bromopyruvate, S-methylSG, S-hexylSG, and EA were purchased from Sigma. All other chemicals were of analytical grade of the highest purity available. All solutions were made with distilled and deionized (Milli Q) water. All solutions were degassed and filtered through a 0.45 μm Millipore filter before use.

Synthesis of EA conjugates

Conjugation of EA with GSH, β -mercaptoethanol (ME) and Cys was accomplished by modifying the methods described by Dietze *et al.*⁶⁷ and Pettigrew *et al.*⁶⁸. To a suspension of EA and the corresponding nucleophile in a mixture of EtOH and H₂O was added a saturated solution of NaHCO₃ and the mixture was stirred at room temperature. Except for the synthesis of EAME, an excess of EA was used to ensure complete consumption of the nucleophile and hence simplify the purification step. After the reaction was over, the solvent was removed by evaporation under vacuum and the crude compound was purified by column chromatography and lyophilized. Both EASG and EAME were obtained as mixtures of two diastereomers (named A and B for NMR characterization purposes, cf. Supporting Information).

Protein expression and purification

Wt and Y108V mutant enzymes were produced as described previously.^{20,69} C47S mutant was constructed and produced as described elsewhere.²⁸ Wt and GST mutant enzymes were purified by affinity chromatography on immobilized GSH.⁷⁰ After affinity purification, the wt and the mutant enzymes (Y108V and C47S) were homogeneous as judged by SDS-PAGE.⁷¹ Protein concentration was determined by the method of Lowry *et al.*⁷² GST activity was checked spectrophotometrically at 340 nm according to Habig and Jakoby (1981).⁷³ Assay conditions were similar to that described previously.²⁰ Solutions of wt and mutants were prepared by dialysis of the enzyme against several changes of buffer solution at 4°C. Protein concentrations were determined from absorbance measurements at 280 nm using an extinction coefficient of 1.3 mg⁻¹ mL cm⁻¹ for the dimer. The experimental conditions for the treatments with bromopyruvate (haloacid that reacts quickly with thiols in a quasi-stoichiometric amount) were similar to those described by Lo Bello *et al.*³⁴ The usefulness of bromopyruvate as an alkylating agent of protein -SH groups, over DTNB or iodoacetic acid, has been reported previously.³⁴ Briefly, this compound was chosen because

this is a thiol reactive reagent of small size. Therefore, we assumed that any effect on the enzymatic activity would be due only to the chemical reactivity and not to any steric hindrance.

Fluorescence spectroscopy

Intrinsic fluorescence of GST P1-1 was measured with a Perkin Elmer LS50B spectrofluorometer equipped with a thermostatted sample holder set at 25°C. Excitation was at 278 $\pm$ 3 nm, and the emission was at 342 $\pm$ 3 nm. A number of samples containing $\cong$ 7–10 μM of GST (wt and mutants) in 2 mL of 20 mM sodium phosphate buffer (containing 5 mM NaCl, 0.1 mM EDTA) at pH 7.0, were added to a 4.0 mL quartz fluorescence cell and the fluorescence intensity measured. A suitable amount of the ligand (EA, EASG, etc.), dissolved in the same buffer was then added to each sample and the fluorescence intensity measured after mixing. The measurements were corrected for dilution and inner filter effects. The procedure and data analysis used were similar to those described elsewhere.²⁷

Equilibrium unfolding/refolding experiments were performed as described previously⁵⁹ at 25°C in 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 1 mM DTT.

Isothermal titration calorimetry (ITC)

Calorimetric experiments were conducted in a MCS⁷⁴ or an ultrasensitivity VP-ITC instrument (MicroCal, Northampton, MA). ITC measurements were routinely performed in 20 mM sodium phosphate, 5 mM NaCl, 0.1 mM EDTA at pH 7 (Buffer A). 1% (v/v) DMSO was also included in the buffer solution in the titrations of EA with the Y108V mutant. The presence of DMSO in the buffer solution increased the solubilization of a higher amount of EA, which was needed due to a lower affinity of EA for Y108V mutant compared to wt enzyme. The presence of this amount of DMSO in the buffer did not modify the enzymatic activity. In control experiments, under identical experimental conditions, the ligand was injected into buffer. Raw data were collected, the ligand heats of dilution were corrected for and the data was integrated using the Microcal Origin software supplied with the instrument. The experimental design and data analysis was similar to that described elsewhere.²⁹

Differential scanning calorimetry (DSC)

Measurements were performed on a VP-DSC instrument (MicroCal, Northampton, MA) with cell volumes of 0.5 mL at the indicated scan rates. Prior to the DSC experiments, all samples were exhaustively dialyzed against 10 mM HEPES-NaOH at pH 7.5 (Buffer B). Calorimetric cells were kept under an excess pressure of 30 psi to prevent degassing during the scan. The DSC traces in the presence of EASG inhibitor were recorded at least three times at each EASG concentration. The heating rate was routinely 1.5 K/min except as

otherwise indicated. As thermal transitions were always found to be completely irreversible, the calorimetric traces were baseline-corrected by subtracting the sample reheating scans. The chemical baseline was subtracted according to the procedure of Takahashi and Sturtevant.⁷⁵

The integration of the transition enthalpies, ΔH^{cal} , was carried out numerically and the van't Hoff enthalpies, ΔH^{VH} , were calculated according to:

$$\Delta H^{\text{VH}}(T_m) = 4RT_m^2 \cdot \frac{C_p(T_m)}{\Delta H^{\text{cal}}(T_m)} \quad (4)$$

where T_m represents the temperature at maximal C_p of the unfolding reaction, and $C_p(T_m)$ and $\Delta H^{\text{cal}}(T_m)$ are the molar excess heat capacity and transition enthalpy, respectively, at T_m .

Crystallization of apo Y108V enzyme

The standard crystallization conditions for wt enzyme did not yield any crystals of the mutant. Thus, an 8 × 96 well sparse matrix screen (the C3 screen) was set up in sitting drop plates (SD-2, Innovadyne) at the Collaborative Crystallization Centre (www.csiro.au/c3), with drops consisting of 200 nL protein, as described earlier, and 200 nL reservoir. The plates were incubated at 20°C and were imaged 13 times over the course of 2 months. An initial hit was obtained in only one condition containing 200 mM calcium acetate, 100 mM MES, pH 6.0, 20% (w/v) PEG 8K and 5 mM DTT. This condition was used to generate an optimized screen in-house using a scaled up 24 well plate without any success. Subsequently, a 96-well grid around this condition (200 – 400 mM calcium acetate, 16–24% (w/v) PEG 8K and 100 mM MES pH 5.4–6.4) was set up in the same sitting drop plate and incubated and inspected in the same way as the initial screening experiments. Many of these conditions resulted in suitable crystals, after approximately a week and data were collected off those from a drop which contained 270 mM calcium acetate, 20% (w/v) PEG 8K and 100 mM MES pH 5.4. Crystals suitable for diffraction analysis were harvested directly from the sitting droplets into cryoprotectant (two minutes in the well solution containing 5% (v/v) MPD, then dipped briefly in well solution containing 10% (v/v) MPD). The crystals were then snap frozen at 100 K in the cryostream.

Structure determination of apo Y108V enzyme

The X-ray diffraction data were collected using a R-Axis IV++ area detector with CuK α X-rays generated by a Rigaku MicroMax-007H rotating anode X-ray generator. Diffraction data were processed with MOSFLM⁷⁶ and scaled with SCALA.⁷⁷ The diffraction data were consistent with the crystals belonging to a new space group for GST P1-1, $P2_1$, necessitating structure determination by molecular replacement. The space group was confirmed with POINTLESS⁷⁸

and molecular replacement calculations were performed with PHASER⁷⁹ using a monomer of the wt enzyme crystal structure (PDB code: 3CSI⁸⁰) as the search model. Two dimers were located in the asymmetric unit of the new unit cell. The model was then refined by rounds of restrained refinement in REFMAC⁸¹ (initial R_{cryst} and R_{free} were 33.4% and 34.3%, respectively) and model building in COOT.⁸² The mutation from a tyrosine to a valine at position 108 was clear in the electron density maps. Six calcium ions were identified in the structure and their positions confirmed by calculation of an anomalous difference map. The R_{cryst} and R_{free} for the final model were 19.2 and 25.2%, respectively for all data to 2.0 Å resolution. A summary of the refinement statistics is given in Table IV. The model was analyzed with the program PROCHECK⁸³ which showed that its stereochemical quality was similar or better than expected for structures refined at similar resolutions.

Crystallization of the EA Y108V enzyme complex

The protein was preincubated with 5 mM EA and 5 mM DTT for 24 h prior to crystallization. The protein-EA solution was screened against the same optimized grid as described above. Many conditions yielded crystals with data collected from crystals which contained 267 mM calcium acetate, 20% (w/v) PEG 8K and 100 mM MES pH 6. Crystals grew at 20°C and reached a suitable size in approximately a week.

Structure determination of the EA Y108V enzyme complex

The X-ray diffraction data were collected at the Australian Synchrotron using protein crystallography beam line PX1 on an ADSC Quantum 210r Detector. Crystals were cryoprotected and frozen as described for the apo enzyme. Diffraction data were processed and scaled with XDS⁸⁴. Unlike the apo wt structure, this complex crystallized in the C2 space group, the more common space group for GST pi crystallized from PEG and MES. A round of rigid body refinement in REFMAC⁸¹ using a wt GST pi crystal structure (PDB code; 5GSS¹⁶) was performed yielding an R_{cryst} of 40% (and R_{free} of 46%). Again the mutation was evident in the electron density maps with no density for the aromatic ring of the replaced tyrosine. Water and MES molecules were added using COOT,⁸² interspersed by rounds of positional and isotropically restrained B-factor refinement using REFMAC.⁸¹ Even after several rounds of refinement no definitive density for the EA compound was observed. Four calcium atoms were built into the structure. The R_{cryst} and R_{free} for the final model were 17.6 and 21.8%, respectively for all data to 1.8 Å resolution. A summary of the refinement statistics is given in Table IV. The model was analyzed with the program PROCHECK⁸³ which showed that its stereochemical quality was similar or better than expected for structures refined at similar resolutions.

Table IV. Summary of Data Collection and Structure Refinement

Data collection	Apo	EASG	EA
Temperature (K)	100	100	100
Space group	$P2_1$	C2	C2
Cell dimensions			
<i>a</i> (Å)	69.0	75.4	77.1
<i>b</i> (Å)	90.4	89.4	90.3
<i>c</i> (Å)	76.0	69.2	68.9
β (°)	97.5	90.1	98.2
GST monomers in asymmetric unit	4	2	2
Maximum resolution (Å)	2.1	1.95	1.8
No. of crystals	1	1	1
No. of observations	302349	164488	331545
No. of unique reflections	53439	29135	46084
Data completeness (%)	78.8 (92.1) ^a	87.2 (87.2)	94.9 (92.6)
R_{merge}^b	10.9 (40.5)	8.3 (38.0)	5.9 (20.4)
I/σ	6.3 (1.8)	8.6 (1.9)	22.4 (7.6)
Multiplicity	5.6	5.6	7.3
Refinement	Apo	EASG	EA
Non-hydrogen atoms			
Protein	6505	3282	3260
EASG	—	78	—
EA	—	—	—
MES	48	—	24
SO ₄	2	1	0
PO ₄	3	—	—
CO ₃	—	—	3
Ca ²⁺	6	6	4
Cl [−]	—	—	—
Solvent (H ₂ O)	445	376	382
Resolution (Å)	2.1	1.95	1.8
R_{cryst}^c (%)	19.2	16.4	17.6
R_{free}^d (%)	25.2	21.5	21.8
Reflections used in R_{cryst} calculations:			
Number	56270	28178	40882
Completeness (%)	94.8	82.2	99.7
rmsd's from ideal geometry:			
Bonds (Å)	0.019	0.014	0.013
Angles (°)	1.7	1.4	1.3
Wilson B-factor	22.7	18.2	24.4
Mean <i>B</i> (protein) (Å ²)			
Main-chain	19.5	12.5	16.5
Side-chain	20.4	13.5	17.9
Mean <i>B</i> (solvent) (Å ²)	22.4	19.5	25.2
Mean <i>B</i> (GSH) (Å ²)	—	13.8	—
Mean <i>B</i> (EA) (Å ²)	—	26.7	—
Residues in most favored regions of Ramachandran plot (%)	92.2	93.9	93.6
Residues in disallowed regions of Ramachandran plot (%)	0	0	0

^a The values in parentheses are for the highest resolution bin.

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \langle I \rangle$, where I_i is the intensity for the i th measurement of an equivalent reflection with indices h, k, l .

^c $R_{\text{cryst}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.

^d R_{free} was calculated with 5% of the diffraction data that were selected randomly and not used throughout refinement.

Crystallization of the EASG Y108V enzyme complex

The protein was preincubated with 5 mM EASG and 5 mM DTT for 24 h prior to crystallization. The protein-EASG solution was screened against the same optimized grid as described earlier. Many conditions yielded crystals with data being collected from crystals grown from

conditions which consisted of 133 mM calcium acetate, 18% (w/v) PEG 8K and 100 mM MES pH 5.4.

Structure determination of the EASG Y108V enzyme complex

Data were collected in-house as outlined for the apo structure above. Diffraction data were processed with

MOSFLM⁷⁶ and scaled with SCALA.⁷⁷ This complex crystallized in the usual C2 space group. A round of rigid body refinement was performed with REFMAC⁸¹ using a wt GST P1-1 crystal structure (PDB code: 5GSS¹⁶) which yielded an R_{cryst} of 44% (and R_{free} of 48%). The mutation from a tyrosine to a valine was evident in the electron density maps. Water and MES molecules were added using COOT,⁸² interspersed by rounds of positional and isotropically restrained B-factor refinement using REFMAC.⁸¹ After several rounds of refinement, the density for the EA and GSH molecules were evident in a $F_o - F_c$ difference map and were subsequently built into the model. The GSH has full occupancy in both subunits whereas EA appears to be present in only 50% of the enzyme molecules. This suggested that the inhibitor and substrate were no longer conjugated but bound separately or that only 50% of the conjugate was bound. This is further supported by the absence of any continuous electron density between GSH and the EA molecule except at very low contour levels. The B-factors for surrounding residues after refinement were similar to those of the bound ligands, with the average B-factor for GSH and EA being 16 and 24 Å², respectively. There were also six Ca²⁺ ions and four MES molecules identified. The R_{cryst} and R_{free} for the final model were 16.4 and 21.5%, respectively for all data to 1.95 Å resolution. A summary of the refinement statistics is given in Table IV. The model was analyzed with the program PROCHECK⁸³ which showed that its stereochemical quality was similar or better than expected for structures refined at similar resolutions.

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