Proton release on binding of glutathione to Alpha, Mu and Delta class glutathione transferases

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Potentiometric, spectroscopic and stopped-flow experiments have been performed to dissect the binding mechanism of GSH to selected glutathione S-transferases (GSTs), A1-1, M2-2 and *Lucilia cuprina* GST, belonging to Alpha, Mu and Delta classes respectively. Both Alpha and Mu isoenzymes quantitatively release the thiol proton of the substrate when the binary complex is formed. Proton extrusion, quenching of intrinsic fluorescence and thiolate formation, diagnostic of different steps along the binding pathway, have been monitored by stopped-flow analysis. Kinetic data are consistent with a multi-step binding mechanism: the substrate is initially bound to form an un-ionized pre-complex [$k_1 \geq (2-5) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$], which is slowly converted into the final Michaelis complex ($k_2 = 1100-1200 \text{ s}^{-1}$). Ionization of GSH, fluorescence quenching and proton extrusion are fast events that occur either synchronously or rapidly after the

final complex formation. The Delta isoenzyme shows an interesting difference: proton extrusion is almost stoichiometric with thiolate formed at the active site only up to pH 7.0. Above this pH, at least one protein residue acts as internal base to neutralize the thiol proton. These results suggest that the Alpha and Mu enzymes retain not only a similar catalytic outcome and overall three-dimensional structure but also share a similar kinetic mechanism for GSH binding. The Delta GST, which is closely related to the mammalian Theta class enzymes and is distantly related to Alpha and Mu GSTs in the evolutionary pathway, might display a different activation mechanism for GSH.

Key words: fluorescence, stopped-flow, substrate activation.

INTRODUCTION

The glutathione S-transferase family (GSTs, EC 2.5.1.18) is characterized by a number of isoenzymes that catalyse the conjugation of GSH with different electrophilic co-substrates [1]. The mammalian cytosolic GSTs belong to at least seven classes (Alpha, Kappa, Mu, Pi, Sigma, Theta and Zeta) [2–6] and the phylogenetic relationships between them have been determined from structurally based amino acid sequence alignments. These results suggest a separation of the soluble GSTs into two major subfamilies characterized by either a serine residue (Theta and insect Delta classes; the *Lucilia cuprina* GST was previously termed a Theta or Theta-like GST and has recently been reclassified as a member of the insect Delta class [6]) or a tyrosine residue (Alpha, Mu, Pi and Sigma classes) as the key residue in GSH activation [6]. Despite the structural differences between the classes, a common property of all GSTs might be to lower the pK_a of the thiol group of bound GSH from 9.0 to between 6.0 and 6.9, thus enhancing the rate of the nucleophilic attack of GSH towards the electrophilic co-substrates. This deprotonation causes a 200–300-fold rate acceleration at physiological pH; it therefore represents a crucial step in the enzymic catalysis. Many efforts have been made to clarify, at the molecular level, the origin and mechanism of this forced deprotonation. For example, the three-dimensional structures of Alpha, Mu, Pi class GSTs

show the thiol group of GSH to be within hydrogen-bonding distance of the hydroxy group of a structurally conserved Tyr residue at the active site (Tyr-9, Tyr-6 and Tyr-7 in the human Alpha, Mu and Pi GSTs respectively) [7–9]. On the basis of an unusually low pK_a of this residue (8.0–8.5 in the Alpha and Pi GSTs), it has been proposed that the Tyr hydroxylate might abstract the thiol proton or, at least, destabilize the S–H bond through an $[O-H-S]^-$ species that confers a partial thiolate character on the sulphur atom of the substrate [10–13]. This possibility is unlikely in view of steady-state kinetic results that show that, in the Alpha and Pi isoenzymes, the active species in turnover is probably the undissociated tyrosine [14,15]. In addition, in the Theta and Delta class GSTs, a Ser residue efficiently replaces the Tyr residue [16,17]. In the latter case, the bound GSH has a pK_a of 6.6 but the involvement of the hydroxylate group as a proton acceptor is even more improbable at physiological pH, owing to the low acidic character of the serine residue. It is now thought more likely that the interaction of the hydroxy group (Ser or Tyr) with the thiolate sulphur of GSH is important only for the correct orientation and stabilization of the thiolate in the active site. An alternative mechanism of assisted deprotonation has been proposed on the basis of the transfer of the thiol proton from the thiol group to the carboxylate group of GSH itself [18]. These proposed models, named 'base-assisted deprotonations', imply that the thiol proton is not

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase.

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released quantitatively in the surrounding solution in the absence of turnover. Conversely, it has been recently demonstrated that, in the human Pi class isoenzyme, the thiol proton leaves the active site quantitatively when the binary complex is formed [19]. The kinetic behaviour of diagnostic events occurring during the GSTP1-1-GSH complex formation suggested that the binding process cannot be represented by a single bimolecular step but rather by a multi-step mechanism in which a structural transition of a weakly bound pre-complex (with a protonated GSH) into the final Michaelis complex (with an ionized GSH) is probably rate-limiting in the binding process.

There are therefore a number of different currently proposed mechanisms for activation, ionization and the fate of the thiol proton. The aim of the study reported here has been to ascertain whether a common class-independent model can be described. In particular, we have examined whether the quantitative release of the thiol proton, the absence of a proton transfer to a 'stable' base residue in the active site and a multi-step binding mechanism are common properties of all GSTs. Spectroscopic, potentiometric and stopped-flow experiments have been performed on selected GSTs (A1-1, M2-2 and *L. cuprina* GSTs) belonging to the Alpha, Mu and Delta classes.

EXPERIMENTAL

Enzymes

The human A1-1, M2-2 and *L. cuprina* GSTs were expressed in *Escherichia coli* and purified as described previously [20–22]; GST activity was assayed spectrophotometrically at 340 nm with the standard co-substrate 1-chloro-2,4-dinitrobenzene (CDNB), as described by Habig et al. [23]. Protein concentration was obtained from the protein absorbance at 280 nm, assuming $\epsilon_{1 \text{ mg/ml}}$ values of 0.73, 1.48 and 1.41 for A1-1, M2-2 and Delta GSTs respectively. Enzyme molarity is referred to a single subunit. Molecular masses of 25.5, 25.6 and 23.9 kDa per GST subunit were used for A1-1, M2-2 and Delta GSTs respectively.

Spectroscopic experiments

Difference spectra of GSH bound to GST A1-1 and M2-2 were obtained with a Kontron double-beam Uvikon 940 spectrophotometer equipped with a cuvette holder thermostatically controlled at 25 °C. In a typical experiment, 2 mM GSH (final concentration) was added to GST (approx. 12 μM active sites, final concentration) in a suitable buffer (0.1 M). The amount of thiolate was calculated by the peak-to-trough amplitude between 240 and 300 nm on the basis of an ϵ_{240} of 5000 $\text{M}^{-1}\cdot\text{cm}^{-1}$. Spectral contributions of free enzyme and free GSH (dissolved in a BSA solution showing an A_{240} similar to that of the GST sample) were subtracted. The pH dependence of the GSH ionization was obtained with the following buffers: sodium acetate buffers between pH 5.0 and 5.5, and potassium phosphate buffers between pH 6.0 and 7.5.

Potentiometric experiments

Thiol proton extrusion was detected at 25 °C essentially as reported previously [19]. In a typical experiment, performed under a N_2 atmosphere, a GSH solution (4 mM in 0.1 M NaCl) was titrated to a fixed pH with 0.1 M NaOH; 1 ml of this solution was mixed with 1 ml of GST (4 mg/ml for Mu and Alpha GST, and 3 mg/ml for the Delta isoenzyme, all in 0.1 M NaCl) at exactly the same pH. Quantification of the released thiol proton was determined by the suitable addition of 10 mM NaOH to regain the initial pH.

Scheme 1

pK_a values were obtained from spectroscopic and potentiometric experiments by fitting the data to:

$$y = y_{\text{lim}} / (1 + 10^{\text{pK}_a - \text{pH}}) \quad (1)$$

Data from potentiometric experiments with the Delta enzyme were fitted to:

$$y = \frac{y_{\text{lim}} (10^{-\text{pK}_{a1}} 10^{-\text{pH}})}{[(10^{-\text{pK}_{a1}} 10^{-\text{pK}_{a2}}) + (10^{-\text{pK}_{a1}} 10^{-\text{pH}}) + 10^{-2\text{pH}}]} \quad (2)$$

allowing determination of the pK_{a1} and pK_{a2} values.

Stopped-flow experiments

Stopped-flow measurements were made at 5 °C on an Applied Photophysics kinetics spectrometer, a stopped-flow instrument, as described previously [19]. The kinetics of the thiol proton extrusion was studied by rapid mixing of GST [50 μM Delta GST and 118 μM A1-1 and M2-2] in 0.1 M NaCl/10 μM Phenol Red indicator (Fluka) (pH 7.0) with an identical volume of GSH or *S*-hexyl-GSH [from 0.2 to 10 mM in 0.1 M NaCl/10 μM Phenol Red indicator (pH 7.0)]. The reactions were followed at 560 nm. The kinetics of thiolate formation and the quenching of intrinsic fluorescence were studied in similar experiments by following the increase in A_{240} and changes in the fluorescence at 340 nm (λ_{ex} 295 nm). Analysis of kinetic data was performed as described previously [19].

Kinetic data analysis

Apparent first-order rate constants (k_{obs}) were calculated from the experimental traces of protein fluorescence quenching and changes in A_{240} and A_{560} obtained after mixing the enzyme with different GSH concentrations, by using the program MATLAB (Mathworks, Natick, MA, U.S.A.). The same experimental data were fitted to Scheme 1 to obtain the microscopic rate constants listed in Table 3 by using the program FACSIMILE (Atomic Energy Authority, Harwell, Didcot, Oxon., U.K.). The program performs the simultaneous fitting of the observed time courses into Scheme 1 by means of numerical integration of the ordinary differential equations. By using numerical integration we also obtained simulated time-dependences of the observed events (fluorescence quenching, changes in A_{240} and A_{560}). From these simulated traces we calculated the apparent first-order rate constants plotted as the continuous lines in Figure 4. The protein fluorescence quenching was attributed to the appearance of $\text{E}^*\text{-GSH}$, $\text{H}^+\text{-E}^*\text{-GS}^-$ and E^*GS^- species; the change in A_{240} was attributed to the appearance of $\text{H}^+\text{-E}^*\text{-GS}^-$ and E^*GS^- species, whereas the change in A_{560} was attributed to the appearance of the E^*GS^- species (Scheme 1).

RESULTS

Detection of GSH ionization in GSTA1-1 and GSTM2-2

Only indirect kinetic and spectroscopic evidence for a forced deprotonation of GSH bound to the human Alpha class GST

Figure 1 Spectroscopic evidence for the ionization of GSH in A1-1 (A) and M2-2 (B)

Spectra of GS^- in the binary complex GSH-GST (2 mM GSH, 12 μ M GST) were obtained at different pH values as described in the Experimental section. Each experimental point is the mean for three determinations; the S.E.M. for each point did not exceed 6%. The continuous line is the best fit of the data to eqn. (1). Insets: difference spectra of the GST-GSH binary complex in 0.1 M potassium phosphate buffer, pH 7.0.

A1-1 is available [14,18]. In addition, the dependence of k_{cat}/K_m^{CDNB} on pH, which should reflect the deprotonation of GSH in the binary complex, reveals a multiplicity of ionizations

that do not allow an unequivocal assignment of a pK_a value for the bound GSH [18]. A direct demonstration of the formation of GSH thiolate at the active site and its dependence on pH has now been obtained by differential UV spectroscopy. As shown in Figure 1(A) (inset), the differential UV spectrum of GSTA1-1 at pH 7.0 and in the presence of saturating amounts of GSH shows an absorption band centred on 240 nm that is diagnostic of the thiolate ion [24]. The pH dependence of this spectral perturbation identifies an apparent pK_a of 6.46 ± 0.17 (Figure 1A).

The rat Mu-class GSTM4-4 and GSTM3-3, in complex with GSH [24,25], were the first models to give direct spectroscopic evidence for a forced deprotonation of the bound substrate. Respective pK_a values of 6.6 and 6.9 were found, which are close to those calculated for the binary complex GST-GSH by kinetic approaches (pK_a 6.6 [26] and 6.2 [25] respectively). No results are available for the human Mu class GSTM2-2 used in this work. For this enzyme, direct evidence for the GSH thiolate formation is shown in Figure 1(B). The pK_a of the bound GSH, calculated on the basis of the pH dependence of the spectral perturbation at 240 nm, is 6.60 ± 0.19 (Figure 1B). pK_a values of 6.60 ± 0.11 and 6.48 ± 0.14 have been calculated previously for the *L. cuprina* GST, on the basis of spectroscopic and kinetic data respectively [16].

Proton release on binding of GSH

A simple approach to determining the fate of the thiol proton derived from the ionization of GSH is based on pH measurements of the enzyme solution before and after the addition of GSH, in the absence of any buffer. Figure 2 shows three representative experiments performed with A1-1, M2-2 and Delta GSTs at approx. pH 7.5, 7.0 and 6.0. For example, after the addition of saturating GSH to GSTA1-1, the pH of the resulting solutions decreased from 7.50, 6.98 and 6.01 to 7.33, 6.79 and 5.95 respectively. Back-titration with 10 mM NaOH allowed a quantitative estimation of the released proton. The pH dependence of the proton extrusion (Figure 3A) was obtained by similar experiments at different pH values (from 6.0 to 7.5). The apparent pK_a is 6.67 ± 0.12 , a value similar to those found by spectrophotometric experiments (Figure 1A). The amount of GSH thiolate formed at each pH (calculated by UV spectroscopy, see Figure 1A) is close to that of the released protons (Figure 3A), suggesting a stoichiometric correspondence.

Figure 2 Potentiometric evidence for the thiol proton extrusion for A1-1, M2-2 and Delta GSTs

Representative experiments performed at three different pH values are shown. pH changes (indicated by the arrows) were observed after mixing 1 ml of 4 mM GSH with 1 ml of a solution containing 4 mg of A1-1 or M2-2, or 3 mg of Delta GST. All solutions contained 0.1 M NaCl and were titrated to the same pH before mixing.

Figure 3 pH dependence of the thiol proton release in A1-1 (A), M2-2 (B) and Delta (C) GST

The concentration of the released thiol proton ($[H^+]_{\text{released}}$) was calculated at each pH value as described in the Experimental section and normalized to the quantity of active sites. Results are means for three different experiments. The S.E.M. for each point did not exceed 8%. Solid lines are the best fits of the data to eqn. (1) (A, B) and to eqn. (2) (C) (see the Experimental section for the definition of both equations). The broken line in (C) represents the pH dependence of GSH ionization, previously reported for the *L. cuprina* GST [16].

Similar results have been obtained for GST M2-2 (Figures 1B, 2 and 3B). In that case, the pH dependence of the proton extrusion showed an apparent pK_a of 7.14 ± 0.15 for the bound GSH.

Different behaviour was found for the *L. cuprina* GST. Potentiometric experiments reported in Figure 2 indicate a significant decrease in proton extrusion above pH 7.0. A quantitative analysis revealed that the amount of proton released into the surrounding solution reached a maximum of approx. 0.4 equivalents of proton per active site at pH 6.97, close to the amount of thiolate found by spectroscopy at the same pH (Figure 3C) [16]. Above pH 7.0 this stoichiometry was lost and, at pH 7.5, only 0.2 proton equivalents per active site were measured, when about 0.75 thiolate equivalents per active site had been formed [16]. The bell-shaped trend of the proton release on pH (Figure 3C) indicated a different mechanism of activation compared with those of the Mu and Alpha isoenzymes and, possibly, at least one protein residue had a role as a stable proton acceptor. By assuming that the pK_a for the deprotonation of

Table 1 pK_a values for the thiol group of GSH obtained by different methods

Isoenzyme	pK_a	
	Spectroscopic	Potentiometric
A1-1	6.46 ± 0.17	6.67 ± 0.12
M2-2	6.60 ± 0.19	7.14 ± 0.15
P1-1	$6.20 \pm 0.12^*$	$6.18 \pm 0.09^*$
Delta	$6.60 \pm 0.11^\dagger$	—

* From [19].

† From [16].

Table 2 Pre-steady-state kinetic parameters derived by stopped-flow experiments at 5 °C

Kinetic constants were obtained by rapid mixing of the enzyme with an identical volume of 2 mM GSH in the absence of any buffer and at 5 °C. Experiments were performed in triplicate. The S.E.M. for each value did not exceed 8%.

Parameter	k_{obs} (s^{-1})		
	A1-1	M2-2	Delta
Proton extrusion	—	735	323
GSH thiolate formation	600	859	—
Fluorescence quenching	648	784	254

GSH was 6.60, as determined by difference spectrometry (Table 1) [16], the best fit of the experimental data reported in Figure 3(C) suggests an apparent pK_a value of 7.18 ± 0.26 for the protein basic residue(s).

Kinetics of the formation of the GST–GSH binary complex

Stopped-flow experiments have been performed to dissect the binding mechanism of GSH to A1-1, M2-2 and Delta GSTs. Different events occurring during GSH binding have been monitored, i.e. quenching of the intrinsic fluorescence of the protein, formation of GSH thiolate (by means of spectrophotometric perturbation at 240 nm) and proton extrusion (followed by visible spectroscopy in the presence of an acid–base indicator). As shown in Table 2, the apparent rate constants of thiolate formation and fluorescence quenching measured for GSTA1-1 are similar; these events might therefore be synchronous. The kinetics of proton release cannot be determined for this isoenzyme because Bromocresol Purple or Phenol Red, used as acid–base indicators, interfere with the GSH binding process. The dependence of the apparent rate constants for fluorescence quenching and thiolate formation on GSH concentration is not linear but hyperbolic, reaching a plateau above 3 mM GSH (Figure 4).

In addition, with GSTM2-2, the kinetics of the proton extrusion process, thiolate formation and quenching of intrinsic fluorescence are very similar; the absolute values are slightly higher than that found for the Alpha isoenzyme (Table 2). The GSH dependence of the observed kinetic parameters is again hyperbolic, reaching a constant value above 1 mM GSH (Figure 4).

For the *L. cuprina* GST, proton extrusion and intrinsic fluorescence quenching appear to be synchronous events but the

Figure 4 Dependence of the observed first-order rate constants on [GSH] for Mu (■), Alpha (□) and Delta (●) GSTs

Each point is the mean of the apparent first-order kinetic constants for proton extrusion, GSH ionization and protein fluorescence quenching, obtained by stopped flow experiments at different GSH concentrations (from 0.1 to 5 mM) and at 5 °C. The S.E.M. for each point did not exceed 8%. The continuous lines show the apparent first-order rate constants obtained from the simulation of the observed time courses with Scheme 1, by using numerical integration of the ordinary differential equation and by employing the microscopic rate constants listed in Table 3. The apparent first-order rate constants calculated for the GST Pi 1-1 [19] are also shown (broken line).

values of the apparent microscopic rate constants are lower than those of the Alpha and Mu GSTs (Table 2). The rate of thiolate formation is not measurable accurately under our experimental conditions. In the stopped-flow experiments we were forced to use a wide slit aperture (bandwidth approx. 20 nm) to provide enough light energy in the 240 nm region; this enzyme shows relevant spectral perturbations below this wavelength. Alternatively, the rate of thiolate formation is scarcely defined because it might occur within the instrument's dead time (less than 1 ms). The dependence of the apparent rate constants for proton extrusion and fluorescence quenching on GSH concentration is shown in Figure 4. As observed for other GST classes, this dependence is again hyperbolic and reaches a plateau at GSH concentrations higher than 4 mM.

DISCUSSION

It has previously been reported that GSH interacts with GSTP1-1 via a multi-step binding process and that, after thiolate formation at the active site, the thiol proton is quantitatively released into solution [19]. The goal of the work reported here was to verify whether a similar mechanism occurred in other isoenzymes, i.e. human A1-1, M2-2 and *L. cuprina* GSTs. A preliminary study on the A1-1 and M2-2 isoenzymes allowed us to quantify the amount of GS⁻ formed at the active site at various pHs and then to calculate apparent pK_a values of 6.45 and 6.60 respectively (Figure 1). In a second step, potentiometric experiments were performed to verify the possible output of the thiol proton in the surrounding solution. This occurred for all the isoenzymes tested (see Figure 2); the quantity of protons for Alpha and Mu GSTs, measured by back-titration at distinct pH values (Figure 3), were almost stoichiometric with the amount of the GSH thiolate formed at the active site (Figure 1). Therefore the proposed role of a conserved Tyr residue (or of the γ -carboxylate group of GSH itself) as possible thiol proton acceptor [10,18], previously excluded for the Pi isoenzyme [19], can now be ruled out even for Alpha and Mu GSTs. In contrast, the Delta isoenzyme behaved differently above pH 7.0, releasing sub-stoichiometric amount of proton at high pH values. The bell-shaped pH dependence of the proton extrusion (Figure 3C) was

consistent with the involvement of a protein residue in a 'stable' capture of the released proton with an apparent pK_a of 7.2. In this context, we note that in the Delta isoenzyme, Ser-9 is within hydrogen-bonding distance of the sulphur atom of the bound GSH. This residue replaces the structurally conserved tyrosine residue found in the Alpha, Mu and Pi GSTs [27]. Owing to the lower propensity of the serine hydroxy group to dissociate (in comparison with a Tyr residue), its involvement in the observed proton capture is unlikely. Two His residues are close to the bound GSH: His-38 and His-50 are within hydrogen-bonding distance of the carboxylate group of the Gly moiety of GSH [27]. The negative charge of the carboxylate would stabilize the protonated imidazole ring(s) and even increase their pK_a values. Therefore these histidine residues become reasonable candidates for the observed proton neutralization; the apparent pK_a of 7.2 for the unknown basic residue is compatible with this assumption. In contrast, A1-1, P1-1 and M2-2 GSTs, which release the thiol proton quantitatively even above pH 7.0, have their nearest histidine residues at 13 Å (or more) from GSH [7–9]. Tyr-113, in the active site of the Delta enzyme, could have a role in the deprotonation of GSH. This residue is related via a hydrogen-bonding network both to the thiol of GSH and to Tyr-105 [27]; however, a direct involvement of Tyr-113 can be ruled out on the basis of the unchanged apparent pK_a of GSH bound to the Tyr-113 → Phe mutant enzyme [16]. In conclusion, the residues involved in proton neutralization in the Delta enzyme seem to be restricted to His-38 and/or His-50.

The GSH binding mechanism was studied by stopped-flow experiments. Apparent rate constants for proton extrusion, quenching of the intrinsic fluorescence and GSH ionization are very similar in the Mu GST (Table 2). They are also close to those previously reported for the Pi isoenzyme [19]. Similarly, quenching of the intrinsic fluorescence and thiolate formation seem to be synchronous events in the Alpha A1-1 isoenzyme, although the kinetics for proton extrusion could not be determined owing to an unexpected interference by the acid–base indicator. It is possible that, in A1-1 and M2-2, the synchronism of these different events might be only apparent and caused by the occurrence of a slower preceding step. This possibility is supported by the hyperbolic behaviour of the apparent rate constants against GSH concentration (Figure 4). In fact, these kinetic data can be fitted to the minimal two-step binding mechanism shown in Scheme 1 [19]. This describes a fast interaction of GSH to give a pre-complex, E–GSH, followed by a rate-limiting step (possibly a structural transition of the binary complex) to give the final Michaelis complex E*–GSH. Protein quenching, deprotonation of GSH and proton extrusion occur after the rate-determining step of the binding and might be very fast. The existence in GST P1-1 of a slow interconversion between a weakly bound precomplex and a tightly bound Michaelis complex has recently been supported by two-dimensional NMR [28] and dynamic fluorescence data [29]. It is clear that the two-step mechanism shown in Scheme 1 is a reasonable model that agrees with our results; however, other, more complicated pathways, could be designed as well. The fitting procedure, performed on the basis of Scheme 1, allowed us to calculate the microscopic rate constants listed in Table 3, which are close to those previously obtained for GSTP1-1 [19].

The available results for the Delta isoenzyme raise some questions of interpretation. Although proton extrusion and fluorescence quenching display similar kinetic constants (although lower than those measured for all other GSTs) and their dependence on GSH concentration is hyperbolic, as was also observed for the Alpha and Mu GSTs (Table 2 and Figure 4), thiolate formation cannot be measured accurately. This might

Table 3 Microscopic constants of the GSH binding process at 5 °C

All microscopic rate constants were as defined in Scheme 1 and were calculated by fitting the stopped-flow results as described in the Experimental section. The S.E.M. for each value did not exceed 10%. $K_d = (k_{-1}/k_1)(k_{-2}/k_2)$ is the overall apparent dissociation constant for the complex E*–GSH to give free GSH and free enzyme.

Constant	A1-1	M2-2	P1-1*	Delta
k_1 (M ⁻¹ s ⁻¹)	$\geq 2 \times 10^6$	$\geq 5 \times 10^6$	$\geq 10^6$	$\geq 0.5 \times 10^6$
k_{-1} (s ⁻¹)	≥ 1500	≥ 2500	≥ 1250	≥ 1000
k_{-1}/k_1 (mM)	0.75	0.5	1.25	2.0
k_2 (s ⁻¹)	1250	1100	1000	1250
k_{-2} (s ⁻¹)	50	100	37	25
k_{-2}/k_2	0.04	0.09	0.037	0.02
K_d (μM)	30	45	46	40

* From [19].

Figure 5 Hydration of the active site in the crystal structure of two different GST isoenzymes

(A) GST A1-1 in the apo form (PDB code 1GSD [7]); (B) GST A1-1 in complex with ethacrynic acid–GSH conjugate (PDB code 1GSE [7]); (C) GST from *Schistosoma japonicum* in complex with drug praziquantel (PDB code 1GTA [31]); (D) GST from *Schistosoma japonicum* in complex with GSH (PDB code 1GNE [32]). Water molecules W1 and W2 are present in both apo forms (A, C); in (B) their positions are occupied by the S^γ atom of the GSH moiety and by the ethacrynic acid moiety respectively; in (D) W1 is not present and its position is occupied by the S^γ atom of GSH.

be due simply to an intrinsic instrumental limit and thus the GSH binding to this specific enzyme could always be described by the mechanism depicted in Scheme 1. Alternatively, thiolate formation could be a very fast event that occurs mainly within the instrument's dead time. In the latter case, a different modality of interaction of GSH with this specific isoenzyme and possibly other serine-containing GSTs must be considered and Scheme 1 does not describe it.

Nevertheless, the fact that at least Alpha, Pi and Mu GSTs display a very similar multi-step mechanism for the binding of the substrate and yield a similar fate for the thiol proton is an important finding. It is reasonable, for these enzymes, which have almost certainly diverged from a common ancestral protein [6], that the evolutionary development of each should have preserved not only a similar molecular architecture and catalytic outcome but also a similar kinetic mechanism for GSH binding. Furthermore, the finding that in Alpha, Pi and Mu class enzymes the proton extrusion process is always fast and always occurs after the formation of the final Michaelis complex might be an indication that a similar mechanism for proton output exists for these enzymes. This suggestion is compatible with crystallographic data on GSTs. In the crystal structure of the Pi class GST, one water molecule is lost when GSH binds to the active site, and a second water molecule is released when the GSH conjugate is bound [30]. Thus one water molecule could assist proton release from GSH and the proton might be extruded bound to this specific water molecule. Figure 5 shows that a similar displacement of one water molecule from the active site occurs during the binding process in Alpha and Mu GSTs. We cannot verify the involvement of water molecules in the extrusion process for the Delta class (owing to the lack of a crystal structure for the apo form of the enzyme) but the available results suggest that the modality of proton output is preserved at least in the Alpha, Mu and Pi enzymes.

This work is a part of special projects supported by the Italian Ministry of University and of Scientific and Technological Research, the Italian National Research Council and the Italian Ministry of Health.

REFERENCES

- Armstrong, R. N. (1997) Chem. Res. Toxicol. **10**, 2–18
- Mannervik, B., Alin, P., Guthenberg, C., Jennson, H., Tahir, M. K. and Jornvall, H. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 7202–7206
- Meyer, D. J., Coles, B., Pemble, S. E., Gilmore, K. S., Fraser, G. M. and Ketterer, B. (1991) Biochem. J. **274**, 404–414
- Buetler, T. M. and Eaton, D. L. (1992) Environ. Carcinog. Ecotoxicol. Rev. **10**, 181–203
- Pemble, S. E., Wardle, A. F. and Taylor, J. B. (1996) Biochem. J. **319**, 749–754
- Board, P. G., Baker, R. T., Chelvanayagam, G. and Jermin, L. S. (1997) Biochem. J. **328**, 929–935
- Cameron, A. D., Sinning, I., L'Hermite, G., Olin, B., Board, P. G., Mannervik, B. and Jones, T. A. (1995) Structure **3**, 717–727
- Raghunathan, S., Chandross, R. J., Kretsinger, R. H., Allison, T. J., Penington, C. J. and Rule, G. S. (1994) J. Mol. Biol. **238**, 815–832
- Oakley, A. J., Lo Bello, M., Battistoni, A., Ricci, G., Rossjohn, J., Villar, H. O. and Parker, M. W. (1997) J. Mol. Biol. **274**, 84–100
- Atkins, W. M., Wang, R. W., Bird, A. W., Newton, D. J. and Lu, A. Y. H. (1993) J. Biol. Chem. **268**, 19188–19191
- Dietze, E. C., Wang, R. W., Lu, A. Y. H. and Atkins, W. M. (1996) Biochemistry **35**, 6745–6753
- Meyer, D. J., Xia, C., Coles, B., Chen, H., Reinemer, P., Huber, R. and Ketterer, B. (1993) Biochem. J. **293**, 351–356
- Karshikoff, A., Reinemer, P., Huber, R. and Ladenstein, R. (1993) Eur. J. Biochem. **215**, 663–670
- Bjornestedt, R., Stenberg, G., Widersten, M., Board, P. G., Sinning, I., Jones, T. A. and Mannervik, B. (1995) J. Mol. Biol. **247**, 765–773
- Kolm, R. H., Sroga, G. E. and Mannervik, B. (1992) Biochem. J. **285**, 537–540
- Caccuri, A. M., Antonini, G., Nicotra, M., Battistoni, A., Lo Bello, M., Board, P. G., Parker, M. W. and Ricci, G. (1997) J. Biol. Chem. **272**, 29681–29686
- Tan, K. L., Chelvanayagam, G., Parker, M. W. and Board, P. G. (1996) Biochem. J. **319**, 315–321
- Widersten, M., Bjornestedt, R. and Mannervik, B. (1996) Biochemistry **35**, 7731–7742
- Caccuri, A. M., Lo Bello, M., Nuccetelli, M., Nicotra, M., Rossi, P., Antonini, G., Federici, G. and Ricci, G. (1998) Biochemistry **37**, 3028–3034

-
- 20 Board, P. G. and Pierce, K. (1987) *Biochem. J.* **248**, 937–941
- 21 Ross, V. L. and Board, P. G. (1993) *Biochem. J.* **294**, 373–380
- 22 Board, P. G., Coggan, M., Wilce, M. C. J. and Parker, M. W. (1995) *Biochem. J.* **311**, 247–250
- 23 Habig, W. H. and Jakoby, W. B. (1981) *Methods Enzymol.* **77**, 398–405
- 24 Graminski, G. F., Kubo, Y. and Armstrong, R. N. (1989) *Biochemistry* **28**, 3562–3568
- 25 Liu, S., Zhang, P., Ji, X., Johnson, W. W., Gilliland, G. L. and Armstrong, R. N. (1992) *J. Biol. Chem.* **267**, 4296–4299
- 26 Chen, W.-J., Graminski, G. F. and Armstrong, R. N. (1988) *Biochemistry* **27**, 647–654
- 27 Wilce, M. C. J., Board, P. G., Feil, S. C. and Parker, M. W. (1995) *EMBO J.* **14**, 2133–2143
- 28 Nicotra, M., Paci, M., Sette, M., Oakley, A. J., Parker, M. W., Lo Bello, M., Caccuri, A. M., Federici, G. and Ricci, G. (1998) *Biochemistry* **37**, 3020–3027
- 29 Stella, L., Caccuri, A. M., Rosato, N., Nicotra, M., Lo Bello, M., De Matteis, F., Mazzetti, A. P., Federici, G. and Ricci, G. (1998) *J. Biol. Chem.* **273**, 23267–23273
- 30 Parraga, A., Garcia-Saez, I., Walsh, S. B., Mantle, T. J. and Coll, M. (1998) *Biochem. J.* **333**, 811–816
- 31 McTigue, M. A., Williams, D. R. and Tainer, J. A. (1995) *J. Mol. Biol.* **246**, 21–27
- 32 Lim, K., Ho, J. X., Keeling, K., Gilliland, G. L., Ji, X., Rüker, F. and Carter, D. C. (1994) *Protein Sci.* **3**, 2233–2244
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Received 26 May 1999/29 July 1999; accepted 8 September 1999