The three-dimensional structure of a class-Pi glutathione S-transferase complexed with glutathione: the active-site hydration provides insights into the reaction mechanism

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The structure of mouse liver glutathione S-transferase P1-1 complexed with its substrate glutathione (GSH) has been determined by X-ray diffraction analysis. No conformational changes in the glutathione moiety or in the protein, other than small adjustments of some side chains, are observed when compared with glutathione adduct complexes. Our structure confirms that the role of Tyr-7 is to stabilize the thiolate by hydrogen bonding and to position it in the right orientation. A comparison of the enzyme–GSH structure reported here with

previously described structures reveals rearrangements in a well-defined network of water molecules in the active site. One of these water molecules (W0), identified in the unliganded enzyme (carboxymethylated at Cys-47), is displaced by the binding of GSH, and a further water molecule (W4) is displaced following the binding of the electrophilic substrate and the formation of the glutathione conjugate. The possibility that one of these water molecules participates in the proton abstraction from the glutathione thiol is discussed.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) catalyse the inactivation of a wide range of hydrophobic compounds containing electrophilic centres by conjugation with glutathione (GSH, γ -Glu-Cys-Gly) [1]. The conjugation reaction between GSH and the hydrophobic substrate occurs in a ternary complex with the enzyme. Initial-rate studies show that the addition of GSH and the hydrophobic substrate (e.g. with 1-chloro-2,4-dinitrobenzene and ethacrynic acid) proceeds by way of a random order of addition for the mouse enzyme [2], in agreement with earlier studies on the human class-Pi GST (GST P1-1) [3]. Several workers have presented evidence that the pK_a of the GSH thiol is reduced to approx. 6.5–7 on binding to the enzyme, so that the binary complex with GSH exists as $E \cdot GS^-$. The nucleophilic thiolate anion attacks the electrophilic centre of the hydrophobic substrate, producing GSH adducts with less toxicity and greater water solubility than the free compounds, facilitating their removal from the cells (GSTs have been the subject of many reviews; [4–9]). There are representative crystal structures for several classes of cytosolic mammalian GST, including Pi [10–13], Mu [14,15], Alpha [16,17] and a homologue of the Sigma-class GST, the haematopoietic prostaglandin D-synthase [18]. Recently, the structures of several non-mammalian GSTs have been reported, including the *Lucilia cuprina* and *Arabidopsis thaliana* Theta-class [19,20], the squid Sigma-class [21] and *Schistosoma japonica* Mu-class enzymes [22,23]. All the structures possess a similar GSH-binding site (G subsite), but the hydrophobic substrate-binding site (H subsite) exhibits more variation. Despite these differences, they probably share a similar catalytic mechanism.

A crucial step in the reaction is the mechanism by which the enzyme stabilizes the thiolate anion, GS^- . The closest residue to the thiol group is a tyrosine in the Pi, Alpha and Mu classes (Tyr-7 in GST P1-1). Earlier work on GST suggested that the tyrosine residue could stabilize and orient the thiolate for the nucleophilic

Table 1 Crystal data and refinement statistics for *mGST P1-1*·GSH

Rmsd, root mean square deviation.

Crystal data	
<i>a</i> (Å)	142.68
<i>b</i> (Å)	162.57
<i>c</i> (Å)	63.32
Space group	<i>C</i> 222 ₁
Data collection	
Total number of reflections	171 889
Number of unique reflections	20 740
R_{sym} (%) [*]	8.7
Completeness (%)	82.1
Last shell completeness (resolution) (%)	50.3
	(2.50–2.44 Å)
$I/\sigma(I)$	14.6
$I/\sigma(I)$ (last shell)	3.3
Refinement	
R_{factor} (%) [†]	20.8
R_{free} (%) [‡]	28.7
Resolution (Å)	8.0–2.44
Number of unique reflections > 2 σ (F)	15 756
Total number of atoms	3348
Water molecules	131
Rmsd bonds (Å)	0.010
Rmsd angles (°)	2.02

^{*} $R_{sym} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where hkl are unique reflection indices, $I_i(hkl)$ is the i th measurement intensity of reflection hkl and $\langle I(hkl) \rangle$ is the mean of all measurements of reflection hkl .

[†] $R_{factor} = \sum_{hkl} \|F_o(hkl) - |F_c(hkl)|\| / \sum_{hkl} |F_o(hkl)|$, where $F_o(hkl)$ and $F_c(hkl)$ are the observed and calculated structure factors, respectively.

[‡] R_{free} is the R value calculated for a subset of 10% of randomly selected reflections not used in the refinement.

Figure 1 Ribbon representation of one monomer of the *mGST P1-1*·GSH structure

The GSH molecule is shown as magenta sticks and the S γ atom as a yellow sphere. The figures are prepared with the program MOLMOL [47].

attack [24,25]. It was also argued that this residue could act as a general base, accepting the proton released by the thiol group [26,27]. However, the ionization of a tyrosine residue would result in a highly reactive group that had to be stabilized in the active-site pocket. Several hypotheses have been formulated on how this stabilization is achieved, including the positive electrostatic potential of the active site enhanced by the helix dipole of α A [28], or the interaction of positive-charged residues, such as Arg-15 in the Alpha class [29]. García-Sáez and co-workers [12] suggested that, should Tyr-7 be in its ionized form, a positively charged residue could be in closer proximity in the free enzyme and be removed when GSH binds. The structure of the unliganded Cys-47-modified enzyme [30] proves that this is not the case (see below). Other observations [9,31,32] also argue against the tyrosinate hypothesis, and other functional groups of the protein [33] or even the carboxyl groups of GSH [34] have been proposed as the proton acceptors. It seems clear from recent work on human GST P1-1 that the proton removed from GSH does not remain bound to the enzyme but is extruded into the solvent in a step that is not rate limiting [35].

The recent unliganded crystal structures of the human GST class Alpha [17] and the *S. japonica* GST [23] show an intact G subsite in the absence of substrate or inhibitor. The unliganded Cys-47-modified class-Pi mouse liver GST (*mGST P1-1*) [30] also shows an unmodified Tyr-7 environment, although some of the GSH-binding residues are disordered. Based on that structure and the preliminary report of the *mGST P1-1* in complex with

GSH [36], we have proposed a mechanism for thiol activation where a water molecule initially accepts the thiol proton. Here we present the fully refined structure of the GSH complex with a detailed analysis of the water structure at the active-site pocket. This structure is compared with the unliganded structure and the binary complex with glutathione S-conjugates, since these represent 'snapshots' of various intermediates in the reaction pathway and provide further insight into the mechanism of thiol activation.

MATERIALS AND METHODS

mGST P1-1 homodimer was purified from LACA mouse liver as described previously [2]. The protein was dialysed against 10 mM Mes buffer at pH 6.5 and concentrated to 15 mg/ml using Centricon microconcentrators. Vapour-diffusion crystallization was performed using hanging drops containing 3 μ l of *mGST P1-1*, 1 μ l of GSH (18 mM in 50 mM Mes, pH 6.6) and 2 μ l of precipitant solution (20% poly(ethylene glycol) 6000, 50 mM Mes buffer, pH 5.5), equilibrated against a reservoir solution of 0.3 M Na/K phosphate buffer. Long bar crystals appeared within 10 days at 20 °C. The crystals were mounted on X-ray capillaries using crystallization drops equilibrated for 2 days as harvesting buffer (the crystals were unstable in harvesting buffer not containing protein). Three-dimensional diffraction data were collected up to 2.44 Å at 20 °C with a Mar Research Image Plate using an Elliot GX21 rotating anode X-ray generator (Table 1). The data were

Figure 2 C^α superposition of both monomers of the *mGST* P1-1·GSH structure

Monomer A is shown in blue, while helices αB and $3_{10}B$ of monomer B are shown in red. The side chains of residues Trp-38 and Leu-42, and the GSH molecule from monomer A are shown as sticks.

processed with the program XDS [37]. The crystals were centric orthorhombic, space group $C222_1$, with cell dimensions $a = 142.68$ Å, $b = 162.57$ Å and $c = 63.32$ Å, and one dimer of *mGST* P1-1 in the asymmetric unit.

Molecular replacement was performed with the program AMoRe [38] using the co-ordinates of the *mGST* P1-1 enzyme structure as in the *S*-(*p*-nitrobenzyl)glutathione complex structure (Brookhaven Protein Data Bank pdb code 1GLQ [12]). One dimer of the protein was orientated and located in the asymmetric unit with a correlation coefficient (for Fobs) of 0.78 and an *R*-factor of 0.295. Special care was taken to eliminate all the solvent and inhibitor molecules from the model before starting the structure refinement. A combined X-ray and energy minimization refinement then followed using XPLOR [39]. 10% of the reflections were set aside for the *R*-free calculations [40]. After rigid body and positional refinement starting at the resolution interval 8–3.5 Å and increasing the maximum resolution to 3.0 Å, $2F_o - F_c$ and $F_o - F_c$ difference Fourier syntheses revealed the two GSH molecules (one per monomer) in the active sites, and were modeled in the electron density. Most of the molecule fitted the electron density perfectly except for the residues 35–47 where the electron density was very weak. Positional refinement, increasing the resolution to 2.44 Å, simulated annealing and atomic isotropic B-factor refinement with restraints imposed on atoms directly bonded were then carried out and yielded an *R*-free of

0.287 and an *R*-factor of 0.208 (Table 1). The atomic co-ordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (pdb code 1GSY).

RESULTS AND DISCUSSION

The present study reports the three-dimensional crystal structure of the GST P1-1 homodimer bound to its natural substrate GSH (from now on, complex referred to as *mGST* P1-1·GSH). The global structure of the protein in this complex is similar to that of the native protein bound to various glutathione adducts [12] (Figure 1). It shows a dimeric protein with two independent active sites, each of them including a GSH-binding site or G subsite, and a hydrophobic-binding site or H subsite.

Helices αB and $3_{10}B$ show higher mobility in the absence of a hydrophobic substrate

The C^α superposition of both monomers of the protein in *mGST* P1-1·GSH and several other *mGST* P1-1 GSH adduct complexes show root-mean-square deviations around 0.2 Å. Both monomers present higher deviations in the zone comprising residues 35–47, which includes helix αB and the following helix $3_{10}B$ (maximum deviation 1.5 Å for residue Leu-42) (Figure 2). The $2F_o - F_c$ difference Fourier map shows very weak electronic density

Figure 3 Hydration of the active site in three different structures

(A) Unliganded Cys-47-modified *mGST* P1-1 (pdb code 1BAY [30]); (B) *mGST* P1-1 · GSH; (C) *mGST* P1-1 complexed with S-(*p*-nitrobenzyl)glutathione (pdb code 1GLQ [12]). Water molecules W1 and W2 are present in the three structures. W0 (coloured yellow) occupies in the unliganded Cys-47-modified *mGST* P1-1 structure the location of the S γ atom of GSH. W4 is not present in the structure of *mGST* P1-1 complexed with S-(*p*-nitrobenzyl)glutathione, its position being occupied by the nitrobenzyl moiety.

in this zone that is even broken at some residues, and is related to the high atomic temperature factors, indicating that this zone is flexible. This flexibility is independent of the packing environment and seems to be an intrinsic characteristic of the area.

Some results indicate that the α B–3 $_{10}$ B zone is stabilized upon binding of the substrates, as observed in the structure of the unliganded Cys-47-carboxymethylated *mGST* P1-1 enzyme,

where the absolutely disordered helices α B and 3 $_{10}$ B are partially organized upon binding of S-(*p*-nitrobenzyl)glutathione [30]. We observe also that the binding of a hydrophobic substrate in the form of a GSH adduct [12] increases the definition of the electron density, and decreases the atomic B-factors (although they are still higher than the rest of the protein), in comparison with *mGST* P1-1 · GSH. Despite the flexibility of the α B–3 $_{10}$ B zone, it seems unlikely that it is associated with stabilizing a thiolate anion, since none of the residues of this area in the *mGST* P1-1 · GSH complex exhibit conformational changes that would result in these side chains approaching the thiol group of GSH or the hydroxyl group of Tyr-7. Nor do new contacts appear in this region in the unliganded Cys-47-carboxymethylated enzyme structure. Furthermore, kinetic studies have involved helix α B motions in the modulation of K_m^{GSH} , whereas a similar effect on K_{cat} cannot be determined unequivocally [41].

The structure of the active site and conformation of GSH

The architecture of the active site is maintained in the absence of a hydrophobic substrate in the H subsite. The interactions of GSH with the residues of the protein that form the G subsite resemble the ones that the GSH moiety of different GSH adducts maintain with the protein [12], which include contacts with Tyr-7, Trp-38, Lys-44, Gln-51, Leu-52, Glu-64, Ser-65 and Asp-98 (from the other monomer). Even though the zone 35–47 is partially disordered, the side chain of Trp-38 is in contact with GSH (interaction between N $^{\epsilon 1}$ of Trp-38 and O 1 of Gly), and seems responsible for maintaining the integrity of helix α B (Figure 2). As in all of the *mGST* P1-1 complexes, the hydroxyl group of Tyr-7 is the only protein group within hydrogen-bond distance to the sulphur atom of GSH.

The GSH conformation is similar to that found for the GSH moiety of other *mGST* P1-1 complexes. The Ψ angle of the cysteine residue is 20.4°, which means that the carbonyl oxygen is pointing in a direction opposite to the hydrophobic pocket. A different conformation was observed in the GSH complexes with class Mu [14,42] and *S. japonica* GST [22], where the carbonyl group of Cys is pointing to the H subsite. The resolution of the present data (2.44 Å) does not allow us to completely rule out such a conformation for the Cys–Gly amide bond of GSH, with a Ψ angle of 155.3°. If indeed GSH could adopt two conformations when bound to *mGST* P1-1, the movement of the cysteinyl carbonyl oxygen could affect the water positions at the active site since, for example, in the $\Psi = 155.3^\circ$ conformation, the carbonyl would be within hydrogen-bond distance of water W4. The $\Psi = 20.4^\circ$ conformation fits better in the electron density, but, since the co-crystals obtained to date are binary complexes of GST with GSH or a product analogue, and not the result of the GSH–hydrophobic substrate conjugation in a ternary complex, we do not know the conformation of the GSH moiety in a real reaction product, making it impossible to ascertain whether, during the catalytic reaction, some GSH conformational changes occur.

Active-site hydration

Several water molecules occupy strategic positions in the active site, satisfying all of the hydrogen-bond requirements of GSH (Figure 3b). In particular, W1, W2 and W4 are found at hydrogen-bond distance from the thiol group of GSH. W1 binds both γ -Glu carbonyl atom and the sulphur atom of GSH; the distances between the water O and the GSH S γ in monomer A and B are 3.6 and 2.8 Å, respectively, in W2, 2.5 and 3.5 Å respectively, and in W4, 3.6 and 3.5 Å respectively. In addition,

Figure 4 Van der Waals surface of one monomer of the *mGST* P1-1 · GSH structure after omitting internal water molecules

A water-filled channel starts at W1 and W2 (hydrogen-bonded to the sulphur atom of GSH) and reaches the solvent at residue Arg-11. The right-hand figure corresponds to a 90° rotation of the left-hand figure and is sectioned vertically to show the interior of the channel.

W3 binds the hydroxyl group of Tyr-108, which has been proposed to participate in the catalytic mechanism in both the Pi [13,43] and Mu [44,45] enzymes. W4 occupies the equivalent position of the C¹ atom of the benzyl ring of S-(*p*-nitrobenzyl)-glutathione when bound to the protein. The net of water molecules seems very stable, as it is also observed in the structure of the unliganded Cys-47-carboxymethylated enzyme, where W1, W2, W3 and W4 occupy the same positions as in the *mGST* P1-1 · GSH complex, even in the absence of any ligand [30] (Figure 3a). In this structure, one more water molecule is observed (W0) situated in a position equivalent to the thiol group of GSH.

The *mGST* P1-1 · GSH structure presents a low hydrated Tyr-7, without any positively charged residues found in its vicinity. In the unliganded Cys-47-carboxymethylated enzyme, the G subsite is intact in this area, with Tyr-7 occupying the same position as in the complexed enzyme, and a single water molecule hydrogen-bonded to the hydroxyl group, at an O–O distance of 2.7 Å. These results, in agreement with theoretical calculations [36], suggest that the Tyr-7 is not ionized either in the free enzyme or in the GSH complex at physiological pH. The closest water molecule to the S^γ atom in the complex of *mGST* P1-1 with S-hexylglutathione is at 3.2–3.4 Å, and at 3.7 Å in the complex with S-(*p*-nitrobenzyl)glutathione [12] (Figure 3c), showing that W1 and W2 have moved away from the sulphur atom. Here they are most probably neutral species. In those two complexes, W4 is no longer present, as this water molecule is displaced during the binding of the hydrophobic substrate.

Water reorganization and GSH activation

From the structures solved to date, including that of the GSH complex reported here, we now have three crystal structures that can be regarded as snapshots of the reaction (Figure 3). Step 1 is based on the unliganded Cys-47-carboxymethylated enzyme [30] where the position of the S^γ atom of glutathione is occupied by a water molecule (W0 [30]) (Figure 3a). When the GSH molecule enters the G subsite it displaces water molecule W0, leaving W1, W2 and W4 bound to and stabilizing the thiolate anion GS[−] (present structure) (Figure 3b). The thiolate anion is also stabilized by accepting a hydrogen bond from the hydroxyl of

Tyr-7. Liu and co-workers [46] have proposed, for the Alpha-class enzyme, that interactions of the aromatic ring of tyrosine with nearby residues decreases the proton affinity of its hydroxyl group, contributing to the stability of the thiolate of glutathione. A similar effect might occur in the Pi class. The binding of the second substrate in the hydrophobic pocket (or H subsite) displaces W4 and the activated thiol group acts as a nucleophilic reagent attacking the aromatic ring of the electrophilic substrate. The structure of the S-(*p*-nitrobenzyl)glutathione complex gives the final snapshot of the product bound to the enzyme (Figure 3c). Water W4 is not present, its position occupied by the ring of the nitrobenzyl moiety.

It is still unclear how the GSH proton is abstracted, although the present structure shows that the resultant thiolate anion is stabilized by water molecules W1, W2 and W4 and the hydroxyl of Tyr-7. In the absence of other candidates and in agreement with theoretical studies [36] we suggest that one of the water molecules may function in assisting proton release from the GSH thiol. The release of the proton (in the form of H₃O⁺) may be achieved in a number of ways: W0 is ejected as a hydrated proton; W2 releases the proton to the network of water molecules bound to the enzyme, i.e. through the channel filled by four water molecules in a row, described by García-Sáez and co-workers [12], which passes between helices αA and αF and reaches the solvent at Arg-11 (Figure 4), or to a non-identified side-chain residue from the enzyme. An alternative hypothesis (first suggested for the Alpha-class enzyme [34]) is that W1 passes the proton to the α-carboxylate of the γ-Glu residue of the GSH. Water W1 is located on the γ-Glu side of the GSH but we do not observe other water molecules that could act as an intermediate, since W1 is not bound directly to the carboxylate group of GSH. Water molecules equivalent to W0 and W2 appear also in the human apo-enzyme Alpha class [17], whereas a solvent molecule close to the equivalent position of W3 is present in the human Mu class in complex with GSH [15]. A water channel is also observed in the Alpha-class enzyme, although its orientation is slightly different from the one described for *mGST* P1-1 · GSH.

From the *mGST* P1-1 · GSH crystal structure and the previously determined complexes it is not possible to discern the secondary proton acceptor, but all the evidence points to one of

the 'conserved' water molecules (W0, W1, W2 or W4) being the primary acceptor. The formation of a formal $\text{GS}^{\cdot-} \cdots \text{H}_3\text{O}^+$ ion pair must be a transient intermediate and further work is clearly required to discriminate between these hypotheses.

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