Kinetic Characterization of Native and Cysteine 112-Modified Glutathione *S*-Transferase A1-1: Reassessment of Nonsubstrate Ligand Binding†

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Received June 11, 2002; Revised Manuscript Received July 9, 2002

ABSTRACT: We have prepared human glutathione *S*-transferase isoform A1-1 (GST A1-1) which has been chemically modified at cysteine 112. These modifications include formation of mixed disulfides with glutathione ("glutathiolation") and cross-linkage of the GST dimer with bis-maleimides reacting with the equivalent Cys 112 residues of the two monomers. This residue (Cys 112) lies adjacent to the hydrophobic substrate binding site, and its side chain thiol projects into the large, solvent-filled cleft which is widely reported in the literature to be the binding site of nonsubstrate ligands. Both types of modification block this intersubunit cleft region and significantly change its chemical environment. Kinetic experiments with these altered enzymes revealed that neither type of modification affects the catalytic activity of GST A1-1 or the binding of nonsubstrate ligands. The lack of an effect on glutathione conjugation activity is somewhat surprising given the proximity of cysteine 112 to the hydrophobic substrate binding site. More surprising, however, is the observation that modification at cysteine 112 has no effect on the binding of nonsubstrate ligands. Furthermore, two of these ligands, lithocholic acid and estradiol disulfate, unexpectedly exhibited competitive inhibition of the unmodified enzyme, suggesting that they bind in the hydrophobic substrate site rather than some accessory ligand binding site. Together, these results strongly argue against the intersubunit cleft as the nonsubstrate ligand binding site and prompt a reassessment of how these ligands interact with GST A1-1.

The glutathione *S*-transferases (GSTs)[1] are a family of cytosolic proteins with multiple known functions (*1*, *2*). As the name of the family implies, their most well-documented function is the catalytic activity wherein the nucleophilic tripeptide glutathione (GSH, γ-Glu-Cys-Gly) is conjugated to structurally diverse hydrophobic electrophiles. This activity serves to detoxify foreign electrophiles and reactive products of oxidative stress, as well as being part of the biosynthetic pathway of endogenous compounds, including leukotriene A4 and prostaglandin H2. Several different isoforms of these enzymes have been characterized and grouped by sequence similarity into classes A, P, M, T, K, and S; each class contains multiple members identified numerically (e.g., A1, A2, P1, etc.). The enzymes exist as homodimers wherein each subunit contributes a catalytically independent active site containing a glutathione binding subsite ("G-site") and a hydrophobic substrate site ("H-site"). Numerous crystallographic studies have clearly identified these sites, with the observation that the residues contributing to the G-site are well-conserved across GST classes, whereas those of the H-site are highly variable and thus produce the varying hydrophobic substrate specificities of the different isoforms.

A second, and only recently appreciated, GST function is in the modulation of signal transduction via binding to protein kinases involved in cell proliferation and apoptosis (*3*, *4*). Specifically, GST isoforms P1-1 and M1-1 have been reported to modulate the phosphorylation activity of JunK and ASK1, respectively. No structural models have been reported for these complexes, and nothing is known about which domains of the GST are involved in the protein−protein interaction. However, it appears likely that GST−JunK or GST−ASK1 interactions make important contributions to the cellular antioxidant response.

A third function of GSTs is their capacity to reversibly bind, without catalytic turnover, a variety of hydrophobic ligands (*5*). Historically, this binding capacity has been termed the "ligandin" activity. The GST isoform with the most extensively studied ligandin behavior is GST A1-1, the structure of which is shown in Figure 1. This isoform is particularly abundant in the liver, comprising ~3% of all cytosolic protein in human hepatocytes (*6*). Prototypical ligands for this enzyme are aromatic and contain at least one anionic functional group; examples include endogenous compounds such as porphyrins, steroids, and bile acids. These nonsubstrate ligands are inhibitors of the glutathione conjugation activity, and early investigators of the ligandin activity reported that they inhibit noncompetitively, suggesting that they bind at a site distinct from the H-site (*7*−*9*). Furthermore, early reports also indicated that in some cases, complete inhibition could be attained at a stoichiometry of

† This work was supported by National Institutes of Health Training Grant T32 GM07750 (R.P.L.), Grant GM62284 (W.M.A.), and the Department of Medicinal Chemistry at the University of Washington.

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[1] Abbreviations: GST, glutathione *S*-transferase; GSH, reduced glutathione; GSSG, glutathione disulfide; DTT, dithiothreitol; CDNB, chloro-2,4-dinitrobenzene; LCA, lithocholic acid; EDS, β-estradiol 3,-17-disulfate; BMB, 1,4-bis-maleimidobutane; BMOE, bis-maleimidoethane; $BMPEO_3$, 1,8-bis-maleimido triethylene glycol; $BMPEO_4$, 1,11-bis-maleimido tetraethylene glycol.

10.1021/bi0262810 CCC: $22.00
Published on Web 08/15/2002

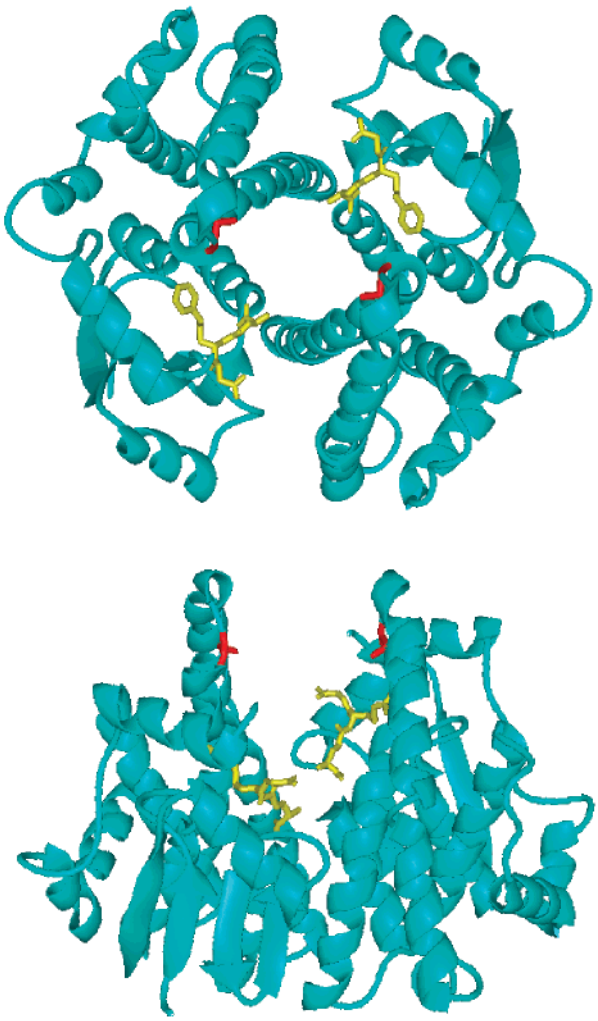

FIGURE 1: Two views of the GST A1-1 dimer. One molecule of *S*-benzylglutathione (yellow) is shown bound at each active site. The side chain of cysteine 112 (red) is shown inserting into the intersubunit cleft.

1 mol of ligand/mol of GST dimer, suggesting the existence of only one binding site per dimer for some nonsubstrate ligands (*10*, *11*). These observations led to speculation that the ligandin site may lie within the large, V-shaped intersubunit cleft near the 2-fold symmetry axis (Figure 1). This hypothesis seemed to be confirmed when the structure of a cytosolic GST from *Schistozoma japonicum* was determined, and the antihelmenthitic drug praziquantel was observed to be bound within the solvent-accessible intersubunit cleft (*12*). Furthermore, this hypothesis has been supported by affinity labeling studies (*13*, *14*) and fluorescence resonance energy transfer studies (*15*) with rat and human GST A1-1, respectively. However, no crystal structures of GST A1-1 in complex with a nonsubstrate ligand have been determined, and the binding modes of nonsubstrate ligands therefore remain uncertain.

Human GST A1-1 contains a single cysteine residue per monomer (Cys 112)[2] which lies within the intersubunit cleft (Figure 1). This residue is in the loop region between helices 4 and 5, adjacent to the hydrophobic substrate binding site. Its side chain thiol inserts into the intersubunit cleft, very near the 2-fold symmetry axis. This residue is therefore ideally located to serve as a reactive handle for probes of the intersubunit cleft region. In the current study, we have exploited two types of probes to explore the importance of the intersubunit cleft region in nonsubstrate ligand binding. First, we have prepared an enzyme in which both cysteine residues of the dimer are involved in mixed disulfides with glutathione. Such "glutathiolation" has recently been observed to modulate the activity of numerous proteins, including other GST isoforms (*16*−*18*). The insertion of two highly charged and hydrophilic glutathione moieties into the intersubunit cleft would certainly be expected to alter the binding affinity of hydrophobic ligands if indeed they bind in this region. Second, we have exploited the proximity of the two equivalent cysteine residues of the dimer by cross-linking them with several different agents, thus imposing a direct blockade of the intersubunit cleft. Here we report the results of kinetic studies with Cys 112 glutathiolated GST A1-1 and the Cys 112 cross-linked enzyme which strongly argue against the intersubunit cleft as the nonsubstrate ligand binding site. Moreover, a rigorous kinetic analysis of classical nonsubstrate ligands suggests that in many cases they may act as simple competitive inhibitors, rather than as noncompetitive inhibitors as previously considered. Together, these results prompt a reassessment of the ligandin activity of GST A1-1.

EXPERIMENTAL PROCEDURES

Reduced glutathione, ethacrynic acid, chloro-2,4-dinitrobenzene, β-estradiol 3,17-disulfate, hematin, and lithocholic acid were purchased from Sigma. The cross-linking agents BMOE, BMB, $BMPEO_3$, and $BMPEO_4$ were purchased from Pierce. The glutathione−ethacrynic acid conjugate was prepared as described previously (*19*).

Recombinant human glutathione *S*-transferase A1-1 was expressed in *Escherichia coli* and purified as described previously (*20*). The enzyme was incubated with 10 mM dithiothreitol to ensure complete reduction of cysteine residues, followed by extensive dialysis to remove the reducing agent.

Glutathiolation incubations contained 1 μM GST in 100 mM phosphate buffer (pH 8.0). As a glutathiolation agent, 1 mM GSSG was added. Enzyme to be used for the kinetic studies was shaken at 25 °C until no native enzyme could be detected by electrospray mass spectrometry; that is, complete glutathiolation of the GST monomers was achieved. Native enzyme to be used as a control for the kinetic studies was incubated under the same conditions but without GSSG. Monitoring the extent of glutathiolation was achieved by LC−MS, using a 5 cm Poros R2 column (elution by a fast linear gradient from a 5% AcCN/0.05% TFA mixture to a 60% AcCN/0.05% TFA mixture in 7 min) coupled to a Fisons VG Quattro II mass spectrometer fitted with a Z-spray ESI source. Electrospray mass spectrometry data were deconvoluted using the Maximum Entropy utility of the MassLynx software package.

Cross-linkage with various bis-maleimides was achieved by incubation of 10 μM GST in 100 mM phosphate buffer (pH 7.2) with the appropriate cross-linking agent. Stocks of

[2] Residue numbering reflects the presence of the transcription initiator codon for an N-terminal methionine residue. Although mass spectrometry indicates that this residue is not present in our protein preparations, we retain the numbering convention to maintain consistency with other literature.

the cross-linking agent were prepared in DMSO and added in aliquots of 0.5 equiv. After each addition, the vial was shaken for 30 min at 25 °C and the extent of the reaction determined by LC−MS as described above. Additional cross-linker was aliquoted until the intensity of the unmodified enzyme peak in the mass spectrum represented less than 5% of the intensity of the cross-linked dimer. Typically, this required the addition of 2−3 equiv of cross-linker (total incubation time of 2−3 h). Native enzyme to be used as a control was incubated under the same conditions but without any cross-linking agent.

The activity of glutathiolated and native GST with chloro-2,4-dinitrobenzene (CDNB) as the electrophilic substrate was determined at 10 nM GST with 3 mM glutathione and variable CDNB concentrations or 2 mM CDNB and variable GSH concentrations. Rates were determined by measuring the absorption at 340 nm (λ_{max} of the glutathione−dinitrobenzene conjugate) for 1 min on a Beckman DU 7400 spectrophotometer. Data were fit to the Michaelis−Menten equation using Graphpad Prism 3.0 to determine K_m and V_{max}. The activity of the cross-linked enzymes was determined at a single substrate concentration (3 mM GSH or 750 μM CDNB) and compared to that of the unmodified control.

For inhibition assays with glutathiolated and native enzyme, the GST concentration was 10 nM and CDNB concentrations of 250 μM, 500 μM, 1 mM, and 2 mM were paired with variable inhibitor concentrations as follows: 0, 100 nM, 200 nM, 400 nM, 800 nM, 1.6 μM, and 3.2 μM for hematin, 0, 2.5, 5, 10, and 20 μM for lithocholic acid, 0, 25, 50, 100, 400, and 800 μM for estradiol disulfate, and 0, 500 nM, 2 μM, 4 μM, and 8 μM for the glutathione−ethacrynic acid conjugate. Data were analyzed by global nonlinear regression using Microcal Origin.

For inhibition assays with the cross-linked enzymes, the GSH concentration was 3 mM and the CDNB concentration was 750 μM. IC_{50}s were determined by varying the concentration of hematin.

RESULTS

Preparation of Modified Enzymes. Figure 1 illustrates the location of the two equivalent cysteine 112 residues within the overall architecture of the GST A1-1 dimer. This residue lies in the loop region between helix 4 and helix 5, and its side chain thiol protrudes into the large, V-shaped intersubunit cleft. As seen in the bottom panel of Figure 1, this cleft is roughly V-shaped and approximately 27 Å in depth. The side chain of cysteine 112 resides ~10 Å from the top of the cleft, while the span separating the cysteinyl thiols of the two monomers varies from 14.6 to 18.3 Å in the four published crystal structures (*21*, *22*). This difference is largely due to the varying orientations of the side chain, as the distance between the α-carbons of the two residues varies by less than 1 Å. We have prepared an enzyme in which both of the cysteine residues are coupled to glutathione via disulfide bonds ("glutathiolated"). By reference to the two glutathione moieties of the bound *S*-benzylglutathione in Figure 1, the amount of space they would occupy within the cleft can be appreciated at a glance. We have also cross-linked these cysteine residues using four bis-maleimides, the lengths of which vary from 8 to 18 Å (Figure 2). While two of these agents appear to be shorter than the span between

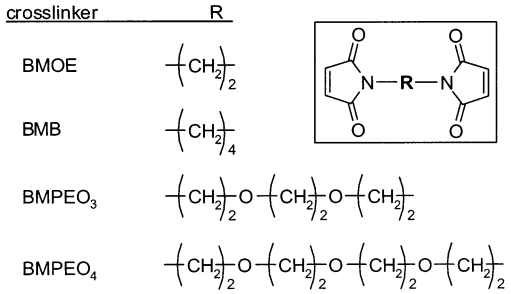

FIGURE 2: General structure of bis-maleimides used to cross-link GST A1-1. Lengths of the individual cross-linking agents vary and are specified in Table 2.

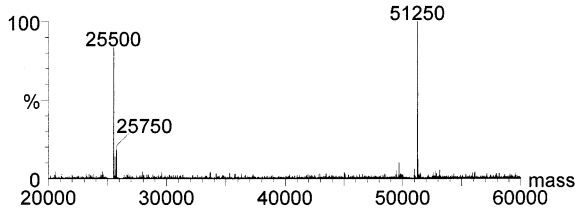

FIGURE 3: Deconvoluted electrospray mass spectrum of GST A1-1 partially cross-linked with the bis-maleimide BMB. At this early time point in the cross-linkage reaction, the unmodified monomer is still present (M_R of 25 500) along with the cross-linked dimer (M_R of 51 250). The reaction intermediate in which only one monomer has reacted with the cross-linker (M_R of 25 750) is observed only with the short cross-linkers BMB and BMOE.

the cysteinyl thiols, the crystallographic *B* values indicate that cysteine 112 resides within a very dynamic region of the protein. We therefore employed these short cross-linking agents to probe the stability of the intersubunit cleft, as observed in crystal structures.

The progress of the chemical modifications made at cysteine 112 was followed by electrospray mass spectrometry. Maleimides react very selectively with cysteine residues at pH 7.2, and the formation of glutathione mixed disulfides is of course specific to cysteine residues. As this protein contains only a single cysteine residue per monomer, peptide mapping was not necessary to localize the modifications to cysteine 112. Since the LC−MS process dissociates the dimer into its equivalent subunits, the mass spectrum of the wild-type enzyme consists of a single peak at an M_R of 25 500. Enzyme which has been cross-linked with bis-maleimides results in the appearance of a peak at 51 000 + M_R of the cross-linking agent. This is illustrated in Figure 3 with the cross-linking agent BMB, which has a molecular weight of 250. Also visible in this spectrum are the unmodified monomer (M_R of 25 500) and the monomer which has reacted with one end of the cross-linker, but which has not yet been coupled to the other subunit (M_R of 25 750). Interestingly, this intermediate species in the cross-linkage reaction (25 500 + M_R of the cross-linker) is observed only with the relatively short cross-linking agents BMB and BMOE, which are shorter than the span between the cysteine residues observed in the crystal structures. This is consistent with a scheme in which the second reaction step is too fast to observe when the geometry of the cross-linker is ideal. Even with the shortest cross-linker, however, nearly complete cross-linkage is observed in a 3 h incubation. Incubation with excess GSSG leads to formation of glutathiolated enzyme (M_R of 25 805), while incubation with DTT restores native enzyme (M_R of 25 500, data not shown). The kinetics of this

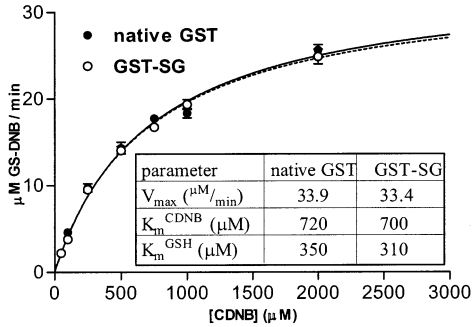

FIGURE 4: Overlaid Michaelis−Menten plots of CDNB turnover by native (●) and glutathiolated [GST-SG (○)] GST A1-1. The kinetic parameters derived from these plots are tabulated in the inset. Also included in the table is the K_m for glutathione of each enzyme form, derived from the corresponding experiment with variable GSH concentrations.

reaction are quite slow, requiring hours at 25 °C to achieve complete glutathiolation as judged by mass spectrometry.

Kinetics of Native and Glutathiolated GST A1-1. The glutathione conjugation activity of native and glutathiolated GST A1-1 was determined using variable concentrations of the diagnostic substrate chloro-2,4-dinitrobenzene (CDNB). Michaelis−Menten plots generated from these kinetic data are superimposable for the two forms (Figure 4), indicating that glutathiolation has no significant effect on glutathione conjugation activity. The kinetic parameters V_{max} and K_m were determined by nonlinear regression of the Michaelis−Menten plots, and are tabulated within Figure 4. Using a fixed concentration of CDNB, a second set of assays was performed with variable GSH concentrations, which demonstrated that the K_m for GSH was also unchanged by glutathiolation (Figure 4, inset).

To assess the effect of glutathiolation on the binding of nonsubstrate ligands in the ligandin site, three such endogenous ligands were assayed for their inhibition of the enzyme activity: lithocholic acid (LCA, a bile acid), estradiol 3,17-disulfate (EDS), and hematin. The inhibition data were analyzed by global nonlinear regression, which is a more robust analysis method than the linear regressions which have previously been used to characterize inhibition of GST by nonsubstrate ligands. Nonlinear regression is superior to linear regression as it does not require linear transformations which introduce distortions into the data set. Further, the global fitting method seeks to find the best fit to all of the data simultaneously rather than treating the data set from each concentration condition independently, which effectively decreases the weight of any single data point on the overall fit. Our data were fit to the rate equation

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]\left(1 + \frac{[I]}{\alpha K_i}\right)}$$

which is derived from the general inhibition expression shown in Scheme 1.

This rate equation is noncommittal with respect to inhibition mechanism; rather, the regression routine is permitted to vary the factor α to achieve the best fit. If the best fit is obtained by expanding α to infinity, the iterations stop and the user can fix its value at some suitably large number; the equation then reduces to the rate equation for a competitive

Scheme 1

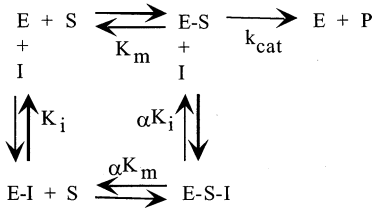

Table 1: Comparative Kinetic Parameters of GST A1-1 Inhibitors[a]

inhibitor	native GST	GST-SG
GS−EA		
K_i (μM)	1.2 (0.1)	0.94 (0.04)
α	∞	∞
EDS		
K_i (μM)	105 (9)	130 (10)
α	∞	∞
LCA		
K_i (μM)	7.3 (0.5)	5.5 (0.5)
α	∞	∞
hematin		
K_i (μM)	0.20 (0.01)	0.24 (0.010)
α	3.4	5.0

[a] Values for K_i and α were determined by global nonlinear regression for the native and glutathiolated (GST-SG) enzyme; α represents a measure of the inhibition mechanism (see the text; Results).

Table 2: Kinetic Parameters of Cross-Linked GST A1-1 (Results)

cross-linker	length (Å)	% activity	hematin IC_{50} (nM)
none (control)	−	100	200
BMOE	8.0	89	280
BMB	10.9	103	250
$BMPEO_3$	14.7	86	260
$BMPEO_4$	17.8	99	280

inhibitor. Convergence on an α value of 1 produces the rate equation for a noncompetitive inhibitor. Values between 0 and 1 or between 1 and infinity imply mixed inhibition with positive or negative cooperativity, respectively, in the binding of the substrate and inhibitor. Fitting data to this equation therefore provides a quantitative measure of the mechanism along the continuum from pure competitive to pure noncompetitive inhibition. The kinetic parameters resulting from this analysis are shown in Table 1, along with results for the classical competitive GST inhibitor glutathione−ethacrynic acid conjugate (GS−EA). Again, no significant differences were seen between the native and glutathiolated enzymes. Unexpectedly, the parameter α, which provides a quantitative measure of the inhibition mechanism ($\alpha = 1$ for noncompetitive inhibitors, $\alpha = \infty$ for competitive inhibitors) expanded to infinity for LCA and EDS, suggesting that they, like GS−EA, are in fact competitive inhibitors with respect to CDNB. Only hematin produced an α indicative of an inhibitor possessing substantial noncompetitive character.

Kinetics of Cross-Linked GST A1-1. For each of the cross-linked enzymes, CDNB conjugation activity at a single substrate concentration was determined relative to that of the unmodified control (Table 2). Again, it is evident that modification at cysteine 112 has little effect on the catalytic turnover of CDNB. Because the previous inhibition studies with nonsubstrate ligands revealed that only hematin was in fact a noncompetitive inhibitor, this was the only compound assayed for its inhibition of the cross-linked enzymes. The

hematin concentration required for 50% inhibition at a single substrate concentration (IC_{50}) was determined for each cross-linked enzyme and unmodified control, and the results are included in Table 2. As with glutathiolation, cross-linkage of these two residues across the intersubunit cleft has little effect upon the inhibition of GST A1-1 by hematin.

DISCUSSION

Cysteine 112 is not an active site residue in GST A1-1; however, several nearby residues on helix 4 are directly involved in substrate binding. The adjacent residue, Val 111, comprises the "top" of the hydrophobic substrate binding site (H-site). When substrate is bound, the highly dynamic C-terminal helix closes over the active site and contacts Val 111 and Pro 110. On the next turn of helix 4, three consecutive residues with aliphatic side chains (Leu 108, Leu 107, and Ile 106) comprise the side of the H-site, providing direct hydrophobic contacts with the electrophilic substrate. Because of the proximity and connectivity of these residues to Cys 112, it is reasonable to expect that perturbations of the protein structure caused by modification of this residue may have implications for substrate binding and catalysis. Moreover, the orientation of the cysteinyl side chain into the intersubunit cleft suggests that adducts of this residue would insert into the space postulated to be the binding site of nonsubstrate ligands.

On the basis of recent reports in the literature that glutathiolation of cysteine residues modulates the activity of numerous proteins, including other GST isoforms (*16*−*18*), we determined whether glutathiolation of cysteine 112 has any effect on the function of GST A1-1. The results of our kinetic studies with completely glutathiolated GST A1-1 indicate that glutathiolation has no effect on the catalytic turnover of this isoform (Figure 3). It thus appears that the addition of the large and highly hydrophilic glutathione moiety to Cys 112 has no major effect on the conformation of the nearby hydrophobic residues which contribute to electrophilic substrate binding. The lack of structural perturbation upon glutathiolation has been confirmed by crystallography of the partially glutathiolated protein (*23*). In these structures, the subunits which have been glutathiolated retain their native conformation, while only the cysteinyl side chain of the attached glutathione is crystallographically observed, indicating the glutathione moiety is unstructured and highly dynamic.

Upon observing the somewhat surprising result that glutathione conjugation activity is unaffected by this modification, we assessed its impact on the binding of nonsubstrate ligands. Glutathiolation of the two equivalent Cys 112 residues effectively puts a cap on the intersubunit cleft, hindering the movement of bulky solutes from the top of the cleft. Modeling suggests that there may be enough space beneath the glutathiolated residues for relatively small ligands (e.g., steroids) to bind, but not for larger ligands such as porphyrins. Moreover, the highly charged nature of these glutathione moieties (totaling four free carboxylates and two amines) makes the chemical environment of the cleft region considerably more hydrophilic, and would certainly alter the affinity of hydrophobic ligands for this site. For these reasons, we postulated that glutathiolation of Cys 112 may destroy the ligandin binding site of GST A1-1. Such an effect would represent a "molecular switch" between the ligandin and glutathione conjugation activities, since ligand binding inhibits the enzyme but glutathiolation does not. This scheme would be consistent with the role of GST in the detoxification of lipid hydroperoxides and other reactive species generated under oxidative stress, since these conditions would be more likely to result in glutathiolated protein. However, the global nonlinear analysis of our inhibition data with three nonsubstrate ligands demonstrates that their binding is not significantly altered by glutathiolation of Cys 112 (Table 1). Furthermore, this analysis indicates that two of the nonsubstrate ligands, the steroid derivative EDS and the bile acid LCA, inhibit the native enzyme competitively, suggesting that they bind in the H-site. To illustrate the ability of this analytical method to determine the inhibition mechanism, Figure 5 presents the LCA inhibition data and compares the quality of the nonlinear fits when forced to adhere to the competitive versus noncompetitive models (fixed at ∞ vs 1, respectively). The competitive model clearly produces a better fit of the data, as evidenced by the 4-fold greater value of χ^2 (sum of squares deviation of the best fit curves from the experimental data) for the noncompetitive fit. Notice that at an intermediate CDNB concentration of 1 mM (near the K_m) the competitive and noncompetitive fits yield similar curves. However, at substrate concentrations above this, the noncompetitive model consistently predicts reaction velocities which are lower than those actually observed, while at lower substrate concentrations, this model predicts velocities which are too high. Thus, Figure 5 graphically illustrates the importance of performing inhibition assays over a wide substrate concentration range (above and below the K_m) to enable discrimination of the inhibition mechanism.

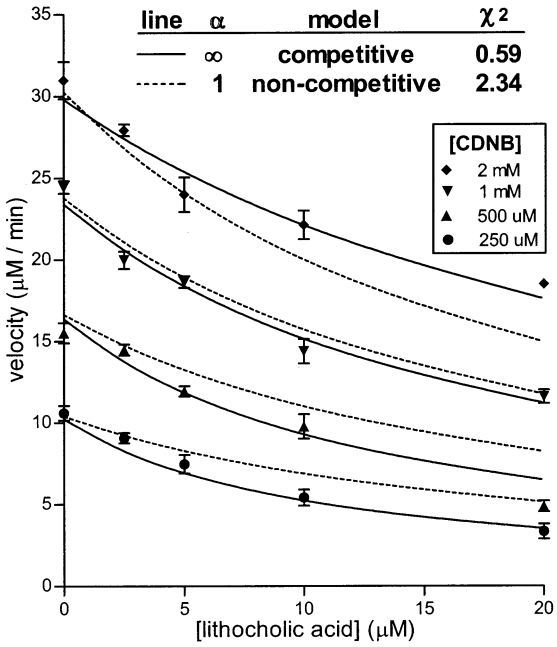

FIGURE 5: Comparative fitting of the LCA inhibition data when forced to fit the competitive [$\alpha = \infty$ (—)] and noncompetitive [$\alpha = 1$ (- - -)] inhibition equations, graphically illustrating the superiority of the competitive model to this system. This difference is quantified by χ^2 (sum of squares deviation of the best fit curves from the experimental data), which is 4-fold greater for the noncompetitive model. When α is allowed to vary as a fitting parameter, the algorithm indeed achieves the best fit by expanding this factor to infinity.

To further probe for binding in the intersubunit cleft, we employed the bis-maleimide cross-linking agents shown in Figure 2 to perturb the local structure and physically block entry of potential ligands. The lengths of these agents vary by a factor of greater than 2, covering a length range from 8 to 18 Å. The length of the shortest of these agents, BMOE, is approximately half the distance between the cysteinyl thiols observed in the available crystal structures (14.6−18.3 Å). Despite this, the two monomers of GST A1-1 are readily cross-linked by even BMOE, with only slightly longer incubation times required to achieve complete cross-linkage with the short cross-linkers BMB and BMOE. This effect is illustrated by mass spectrometry of an intermediate time point of the GST−BMB incubation (Figure 2), wherein three molecular species are clearly observable: monomer (M_R of 25 500), monomer−cross-linker (M_R of 25 750), and monomer−cross-linker−monomer (M_R of 51 250). The rate of the initial reaction (monomer → monomer−cross-linker), which can be monitored by the disappearance of unmodified monomer, is similar for all of the cross-linkers that were tested. The longer cross-linkers $BMPEO_3$ and $BMPEO_4$ then react quickly with the second monomer to form the cross-linked dimer; the intermediate monomer−cross-linker species is not observed when these incubations are carried out at 25 °C. This intermediate species accumulates to a small extent with the shorter cross-linkers, as the second reaction (monomer−cross-linker → monomer−cross-linker−monomer) is dependent upon the dynamics of the protein, requiring partial closing of the intersubunit cleft. That this reaction occurs at only a slightly slower rate with the shortest cross-linker is a testament to the mobility of this region of the protein.

Upon verification of complete cross-linkage of the enzyme by mass spectrometry, activity assays were conducted at a single substrate concentration and compared to that with the unmodified control. As seen in Table 2, cross-linkage had a minimal effect on glutathione conjugation activity. It is remarkable that even cross-linkage with BMOE, which holds the intersubunit cleft about halfway closed relative to its crystallographic conformation, has little effect on catalytic activity. To assess the effect of cross-linkage on nonsubstrate ligand binding, the IC_{50} of hematin with the cross-linked enzymes (in addition to the unmodified control) was determined. Only hematin was assayed as an inhibitor since LCA and EDS both exhibited competitive inhibition with the native enzyme (Table 1), indicating that they bind in the H-site. These IC_{50} (Table 2) data indicate that, as with glutathiolation, cross-linkage of the enzyme at Cys 112 does not affect the inhibition by hematin.

Much of the interest of enzymologists in the nonsubstrate ligand binding of GST A1-1 lies in the ability of these compounds to elicit noncompetitive inhibition. We therefore employed inhibition kinetics (rather than direct binding measurements) to probe the effect of chemical modifications of cysteine 112. Our results suggest that LCA and EDS are simple competitive inhibitors, while hematin seems to fit the classical criterion of L-site ligands, noncompetitive inhibition. However, the inhibition which it exhibits is not achieved by binding at the intersubunit cleft; that is, the "L-site" must lie elsewhere on the enzyme. As we have not assessed direct binding to the enzyme, it remains possible that hematin (or the other ligands) could bind at the intersubunit cleft of the native enzyme without a catalytic consequence.

The fact that large, structurally diverse hydrophobic compounds bind to GST A1-1 is unsurprising given that its catalytic function is to conjugate glutathione to xenobiotics. Indeed, the primary difference between a substrate for the enzyme and a nonsubstrate ligand may simply be the presence or absence of an electrophilic center suitable for reaction with glutathione. Given the lack of substrate specificity which this enzyme exhibits (and which is necessary for fulfilling its role as a xenobiotic detoxification enzyme), one might reasonably expect that a variety of hydrophobic but unreactive compounds would bind in the active site and act as competitive inhibitors. Due to the inherent lack of specificity of this enzyme, the binding modes of substrates and nonsubstrate ligands alike are probably very dynamic, with small free energy differences between multiple bound conformations. However, the reports that certain hydrophobic compounds (particularly endogenous anions such as porphyrins and bile acids) act as noncompetitive inhibitors led to the assumption that they bind at a discrete site which was dubbed the "nonsubstrate ligand" site, the ligandin site, or the L-site. In the crystal structure of a cytosolic GST from *S. japonicum*, the antihelmenthitic drug praziquantel was observed to be bound within the solvent-accessible intersubunit cleft region, which became a candidate for the ligandin site (*12*). However, it is notable that praziquantel has been reported not to be an inhibitor of this enzyme (*15*, *24*), suggesting that binding within the cleft as observed in the crystal structure does not interfere with catalytic activity. The *S. japonicum* enzyme contains a prominent tyrosine residue (Tyr 104) within the cleft which makes hydrophobic contacts with praziquantel along the entire length of its side chain. The authors of this paper, however, note that mammalian GSTs of known structure lack a tyrosine residue at this position. The equivalent residue in human GST A1-1 is Glu 104, which is involved in a salt bridge with Arg 15. In fact, there are no aromatic residues within the intersubunit cleft of GST A1-1, and the environment there is not particularly hydrophobic. Interestingly, no crystal structures of GST A1-1 have emerged in the presence of nonsubstrate ligands despite the fact that this isoform is most closely associated with the ligandin function.

In a very recent thermodynamic study (*25*), other workers demonstrated that 8-anilino-1-naphthalenesulfonate (ANS) binds to GST A1-1 with a stoichiometry of 2 per dimer. Moreover, they showed conclusively that hydrophobic interactions with phenyalanine 222 contribute significantly to ANS binding. This residue, which lies at the end of the C-terminal helix, closes over the H-site when substrate is bound. For example, in the crystal structure of the GST A1-1−ethacrynic acid complex (*21*), this residue contributes aromatic interactions with the bound substrate. Although the authors of this study concluded that ANS binds at the edges of the intersubunit cleft, their results are perhaps more consistent with ANS binding independently at the two H-sites of the GST A1-1 dimer. One difficulty in comparing the results of this thermodynamic study with those of other workers using different methods (*13*−*15*) is that different ligands were employed in each case. Our kinetic results with the native, unmodified enzyme indicate that nonsubstrate ligands have different inhibition mechanisms (Table 1), and there is no compelling reason to believe that they all bind at the same site. Questions about the binding site, binding

stoichiometry, and inhibition mechanism must be considered on a case by case basis for each nonsubstrate ligand; that is, it cannot be assumed, as it often appears to be, that all hydrophobic anions interact with GST in the same manner.

A variety of organic anions, including dyes and acidic drugs, have been characterized as inhibitors of different GST isoforms. Two such examples are cibacron blue and bromosulfophthalein, both of which are nonsubstrate ligands which have been reported to be noncompetitive inhibitors of GST P1-1. In a recent crystallographic study, however, these ligands were clearly localized to the hydrophobic substrate binding site; the observation of noncompetitive inhibition appears to be due to the large size of this site which can be simultaneously occupied by substrate and inhibitor (*26*). GST A1-1 has a similarly large hydrophobic substrate site, and the fact that nearly all investigators use the relatively small substrate CDNB makes it distinctly possible that dual occupancy occurs in this isoform as well. Noncompetitive inhibition by ligands bound in the H-site may be elicited by several mechanisms. For example, it may change the bound conformation of CDNB such that the electrophilic center is improperly oriented for conjugation, or prevent the proper alignment of the catalytic tyrosine residue with GSH, or prevent product release by holding the C-terminal helix in a "closed" position. Moreover, glutathione conjugation of larger substrates which occupy more of the H-site may be inhibited competitively by compounds which exhibit noncompetitive inhibition of CDNB turnover. We note that researchers in the cytochrome P450 field have recently observed dual occupancy in the active sites of these xenobiotic metabolizing enzymes, producing inhibition profiles which vary when different substrates are employed (*27*, *28*). Indeed, it may be a general phenomenon that in the presence of multiple potential ligands and substrates, enzymes with large, nonspecific active sites produce atypical kinetics in which classical enzymological considerations may not apply.

The H-site has been recognized as the hydrophobic region adjacent to the thiol function of bound glutathione, and is comprised largely of aliphatic residues from helix 4 (Leu 107, Leu 108, Pro 110, and Val 111), Met 208, and several residues of the highly dynamic C-terminal helix which begins at Glu 210. There is a constriction between Met 208 and Pro 110, but on the other side of that constriction, the protein architecture expands again into a broad hydrophobic cavity. This region is bounded by helices 4, 5, and 7 and the loop region preceding the C-terminal helix. As shown in Figure 6, hydrophobic residues from each of these structural elements line the sides of this cavity. In the crystal structure of the apoenzyme, as well as in complex with ethacrynic acid and its glutathione conjugate, this cavity is open to bulk solvent and contains multiple crystallographically observed water molecules (*21*, *22*). Lysine 117 from helix 5 lies at the top of this hydrophobic region, and its side chain is not involved in any salt bridges within the enzyme. An unpaired positively charged residue adjacent to a large, solvent-accessible hydrophobic pocket immediately suggests a potential binding site for organic anions which typify the nonsubstrate ligands of this enzyme. We believe that the hydrophobic nature of this site with its proximity to the side chain of Lys 117 makes this a more plausible binding site for organic anions such as hematin than the relatively hydrophilic intersubunit cleft. It may be noteworthy that the

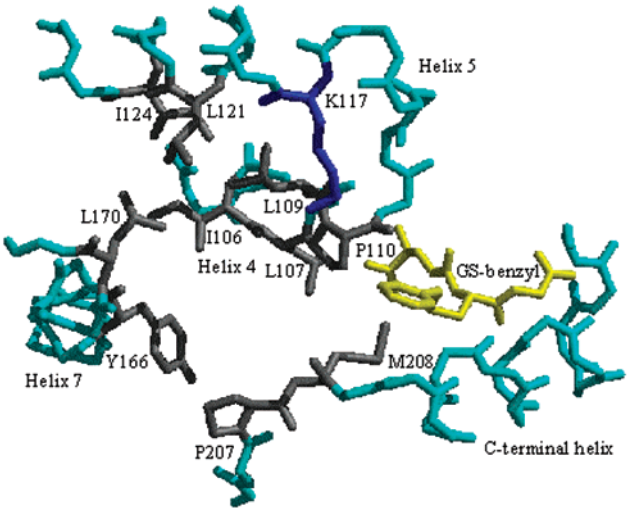

FIGURE 6: Inspection of hydrophobic residues (gray) in the cavity adjacent to the H-site; *S*-benzylglutathione (yellow) is included for reference. These residues are located on helices 4, 5, and 7 and the loop region preceding the C-terminal helix. Lysine 117 (blue) is located on helix 5, and its cationic side chain lies at the top of the cavity. Note the constriction between Met 208 and Pro 110 which partially separates this cavity from the H-site.

loop region between helices 4 and 5 (in which Lys 117 resides) is reported to be involved in crystallographic interactions (*22*), suggesting that the involvement of these residues in protein−ligand interactions may alter the crystallization properties of the enzyme. Indeed, we have tried to obtain diffractable crystals in the presence of hematin with no success and have little doubt that others have tried as well, yet no crystal structures of this high-affinity enzyme−ligand complex have been published to date. This newly described site is separated from the established H-site by a narrow gap between Met 208 and Pro 110. The Cα−Cα distance between these residues varies from 7.4 to 8.4 Å in the available crystal structures, which may restrict direct passage of bound ligands or substrates from one site to the other. However, both of these residues occur in dynamic regions of the protein, with the *B* factors of the α-carbons of Met 208 and Pro 110 being 50 and 47 Å^2, respectively, in the apoenzyme with a global average *B* factor of 26 Å^2 (*22*). Given this mobility, it is difficult to assert whether this second hydrophobic pocket should be viewed as a discrete potential binding site or rather as simply an extension of the H-site.

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BI0262810