Binding of glutathione and an inhibitor to microsomal glutathione transferase

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Microsomal glutathione transferase is an abundant liver protein that can be activated by thiol reagents. It is not known whether the activation is associated with changed binding properties of the enzyme. Therefore the binding of GSH and an inhibitor to rat liver microsomal glutathione transferase was studied by use of equilibrium dialysis and equilibrium partition in a two-phase system. The radioactive substrate glutathione and an inhibitor (glutathione sulphonate) give hyperbolic binding isotherms with a stoichiometry of 1 mol per mol of enzyme (i.e. 1 molecule per

homotrimer). Glutathione had an equilibrium binding constant of 18 μM . Competition experiments involving glutathione sulphonate showed that it could effectively displace GSH. These and kinetic studies showed that the K_d and K_i for glutathione sulphonate are close to 10 μM . No change in these parameters was obtained after *N*-ethylmaleimide activation of the enzyme. Thus activation does not result from changes in binding affinity to GSH.

INTRODUCTION

Glutathione transferases (glutathione transferase, EC 2.5.1.18; see [1–3] for reviews) are a group of enzymes involved in the detoxication of numerous carcinogenic, mutagenic, toxic and pharmacologically active compounds [4]. A membrane-bound member of this family has been isolated [5] and named microsomal glutathione transferase. This homotrimeric enzyme has a unique amino acid sequence and immunological properties in comparison with its cytosolic counterparts, and appears to have no closely related isoenzyme [6,7]. A distant relationship to leukotriene C_4 synthase and the similar 5-lipoxygenase-activating protein is, however, indicated [8,9]. These proteins are involved in the production of mediators of smooth muscle constriction implicated in the pathogenesis of asthma [10]. Interestingly the microsomal glutathione transferase displays an affinity for the leukotriene C_4 synthase protein [11] and is also a high-capacity ‘low-affinity’ binder of leukotriene C_4 [12]. In the study on binding of leukotriene C_4 to microsomal glutathione transferase it was noted that the enzyme bound one molecule per trimer [12]. Other than that, no binding studies have been performed with this enzyme. Microsomal glutathione S-transferase (GST) is activated by thiol reagents, and it is not known whether this influences the binding properties. Therefore we have studied the binding of glutathione and an inhibitor, glutathione sulphonate (GSO_3^-), to unactivated and activated forms of microsomal GST.

MATERIALS AND METHODS

Chemicals and enzyme

^{35}S -labelled glutathione was from NEN research products (Dreieich, Germany), hydroxyapatite (HA) was from Bio-Rad (Richmond, CA, U.S.A.), Sephadex G25 and CM-Sepharose were from Pharmacia (Uppsala, Sweden) and Triton X-100, *N*-ethylmaleimide (NEM) and GSH were from Sigma (St. Louis, MO, U.S.A.).

All other chemicals were of highest purity and were purchased from common commercial sources.

Unless otherwise stated all experiments were carried out in a standard buffer containing 10 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 % (v/v) Triton X-100, 20 % (v/v) glycerol (buffer A). Microsomal GST was purified from male Sprague–Dawley liver as described previously [5]. The protein concentration was determined by the procedure of Peterson [13]. The purity of the microsomal GST was assessed by SDS/PAGE on 12.5 % (w/v) gels [14]. A single band of M_r 17000 was obtained after Coomassie Blue staining. To remove GSH in the enzyme preparation before binding studies, microsomal GST was dialysed against buffer A in the cold room for 3 days with two or more daily buffer changes. The residual amount of GSH was lower than 0.5 μM , as determined by the method of Grassetti and Murray [15].

Activation of the microsomal GST by NEM was performed as described earlier [5]. In short, the enzyme was incubated with 5 mM NEM (final concentration) on ice for up to 30 min and at maximal activation the reaction was stopped by adding 5 mM GSH (adjusted to pH 7.0 by KOH). Excess GSH and GSH–NEM adduct was removed by dialysis as described above.

Binding experiments

The binding of ligands to the enzyme was studied by two methods: equilibrium partition in a two-phase system and equilibrium dialysis.

Equilibrium-dialysis experiments were carried out using dialysis cells constructed in our workshops. These consisted of two hollowed-out discs that were clamped together with a dialysis membrane in between. The two chambers so formed could each hold up to 1 ml of solution. Membranes (Spectra-pore No. 1, cut-off 6000–8000 kDa) were boiled in 1 % (w/v) Na_2CO_3 and rinsed thoroughly with distilled water before use. On each side of the membrane, 0.2 ml of buffer A solution was poured into the chambers: on one side 25–600 μM microsomal GST was present,

Abbreviations used: GST, glutathione S-transferase; GSO_3^- , glutathione sulphonate; HA, hydroxyapatite; NEM, *N*-ethylmaleimide; CDNB, 1-chloro-2,4-dinitrobenzene.

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and on the other side the ligand concentration was varied from cell to cell.

For equilibration the cells were agitated (120 rev./min) overnight in the cold room. Control experiments revealed that equilibrium was reached within 4 h. Concentrations of bound + free and free ligand were determined by withdrawing 150 μ l samples of solution from either side of the membrane. The radioactivity was counted in 10 ml of Aquasol (New England Nuclear) using a Beckman LS-100 liquid-scintillation spectrometer. The GSH contents of some samples were also determined in phosphate-buffered saline by derivatization of the sample *in situ* with the thiol-reactive fluorigen monobromobimane. The resultant GSH-bimane adducts were separated by reversed-phase HPLC and quantified by fluorimetry, as in [16].

The equilibrium partition method made use of a system formed by mixing 33 mg of HA with 200 μ l of buffer A containing microsomal GST at a concentration of ≤ 0.86 mg/ml and varying GSH concentrations (7.5–90 μ M). Samples were mixed, left on ice for 25 min and then centrifuged at 5000 *g* in an Eppendorf centrifuge for 3 min. The pellet contains enzyme (99% as determined by enzyme activity measurements) with bound GSH, whereas the supernatant reflects the free GSH concentration. By determining the radioactivity of the supernatant compared with the added amount, free and bound GSH could be estimated. The binding of GSH was dependent on the presence of enzyme. Competition experiments involving GSO_3^- were performed to estimate the K_d of this compound and to verify the experimental procedure. Microsomal GST (50 μ M) and GSH (7.5 μ M) were mixed and various concentrations of GSO_3^- (7.5–500 μ M) were added. From the competition data bound and free GSO_3^- were calculated as in [17].

In all binding experiments dithioerythritol (0.1 M) was added to prevent oxidation of GSH. The microsomal GST was stable throughout the equilibrium dialysis and HA binding period, as assessed by activity measurements. Binding properties of the activated enzyme, however, could only be investigated by the equilibrium partition method, which is much faster. We also checked whether, at the high protein concentrations used, the Donnan effect influenced the distribution of the ligand. By varying the ionic strength of the medium (up to 0.2 M KCl) or including glutamic acid (same charge as GSH) at 0.2 mM or 1 mM, ionic effects could be excluded to within experimental error. Each of the experimental results shown or referred to below is the mean of at least three determinations. Binding constants and capacity were derived by non-linear regression in the program Ultrafit (Biosoft Ltd., London, U.K.).

Measurement of inhibition kinetics

Enzyme activities with 1-chloro-2,4-dinitrobenzene (CDNB, 0.5 mM) were measured as described previously [18,19].

The effect of GSO_3^- on enzyme activity was determined by monitoring the rate with varying GSH (0.25–10 mM) and CDNB as the second substrate at fixed concentrations of GSO_3^- (5–40 μ M). Triplicate determinations were performed and the experiments were repeated at least once. The type of inhibition was determined by examining double reciprocal plots (Figure 3). Apparent K_m' values at different inhibitor concentrations were derived by the program Ultrafit. The inhibition constant K_i was derived from the fit of the linear dependence of K_m' on inhibitor concentration according to eqn. (1) by the program Ultrafit.

$$K_m'/K_m = 1 + [\text{GSO}_3^-]/K_i \quad (1)$$

RESULTS

GSH binds to unactivated and NEM-activated microsomal GST with equal affinity (Figure 1). The K_d , ≈ 20 μ M, is similar to many other glutathione transferases [20,21]. The binding stoichiometry revealed that one GSH molecule is bound by each homotrimeric enzyme. The results of the binding experiments are summarized in Table 1. In order to control for the Donnan effect, the equilibrium dialysis experiments were performed at different ionic strengths with essentially identical results (results not shown). It was also shown that glutamic acid did not compete for binding. When the activated enzyme was examined it turned out to be unstable during the course of equilibrium dialysis. A new binding assay, relying on two-phase partition of the enzyme with bound ligand and free ligand, was developed using HA. This assay was tested also with unactivated enzyme to verify its validity in comparison with equilibrium dialysis. Since the values were in agreement and this assay is considerably less time

Figure 1 Binding of GSH to activated (●) and unactivated (○) microsomal GST (16.7 μ M trimer) determined by equilibrium dialysis

Details are described in the Materials and methods section.

Table 1 Binding of GSH and GSO_3^- to microsomal GST

Experimental details are described in the Materials and methods section. The K_d (GSH) and capacity values were determined by equilibrium dialysis and HA partition. Corresponding values for the activated enzyme were obtained from HA partition only. Results are means $\pm$ S.D. of values from (*n*) separate experiments. The K_d (GSO_3^-) value was determined by competition in HA partition. The K_i values were determined from the CDNB assay as described. n.d. = not determined.

	Dissociation/inhibition constant (μ M)	
	Unactivated enzyme	Activated enzyme
K_d (GSH) (μ M)	18 $\pm$ 14 (6)	22 $\pm$ 5 (4)
K_d (GSO_3^-) (μ M)	8.6 $\pm$ 5 (6)	n.d.
K_i (GSO_3^-) (μ M)	9.5 $\pm$ 2.3 (4)	8.8 $\pm$ 1.9 (5)
Capacity ratio		
Capacity GSH/trimer	0.33 $\pm$ 0.12 (6)	0.36 $\pm$ 0.02 (4)

Figure 2 Competition for GSH (7.5 μM) binding to the microsomal GST (16.7 μM trimer) by varying GSO_3^- in the two-phase partition method

Inset: Binding of GSO_3^- to microsomal GST determined from the competition data. Details are described in the Materials and methods section.

Figure 3 Competitive inhibition of unactivated (top) and activated (bottom) microsomal GST by GSO_3^-

Left: Plot of the reciprocal of initial velocity versus the reciprocal of GSH concentration in the presence of 0.5 mM CDNB and (a) (○) 0 μM , (●) 5 μM , (■) 10 μM , (□) 20 μM GSO_3^- , (b) (○) 0 μM , (●) 10 μM , (■) 20 μM , (□) 30 μM , (△) 40 μM GSO_3^- respectively. Right: Determination of the value of K_i for GSO_3^- . The plotted values of K_m and K_m' were determined from non-linear regression fits to the data in the Figure on the left. The value of K_i was determined from a linear least-squares fit of the data to eqn. (1).

consuming it was possible to obtain accurate binding data also with the less-stable activated enzyme. Radioactive [^{35}S]GSH was used in most studies but the data were also verified by using

unlabelled GSH (determining the amounts by fluorescent derivatization with monobromobimane followed by HPLC-based separation and quantification as in [16]). By this method we could exclude the possibility that radiodecomposition of the [^{35}S]GSH influenced the data.

Competition experiments were performed to determine the binding affinity of the inhibitor GSO_3^- (Figure 2). The binding constant ($K_d \approx 10 \mu\text{M}$) for GSO_3^- was also verified independently by kinetic experiments that showed competitive inhibition ($K_i \approx 10 \mu\text{M}$) (Figure 3). Again, there was no difference between activated and unactivated enzyme. The data are summarized in Table 1.

DISCUSSION

Binding experiments performed by equilibrium dialysis can be complicated by the Donnan effect. When, as in this case, enzyme and ligand are of opposite charge, false-positive data can be generated. In order to control for artifacts, several controls were made. Changes in ionic strength, inclusion of L-glutamic acid and the use of a two-phase partition assay had no influence on the results; we can conclude that the binding data are valid.

Although in agreement with data on the binding of leukotriene C_4 , we were somewhat surprised when we obtained evidence for only one GSH binding site per homotrimer. The architecture of a single binding site in a homotrimer can only be speculated upon but two possibilities come to mind. Binding to one subunit prevents binding to the others (by a conformational change as in the F1-ATPase [22]) or three overlapping and hence mutually exclusive binding sites are present on the three subunits. Structural data obtained so far [23] do not give any information on the active site, but it is tempting to speculate that a GSH binding site, common to most GSH-utilizing enzymes [24,25], is present also in microsomal GST. Such a site could conform to both binding possibilities. Work in progress will hopefully define the structure in complex with GSH. The stoichiometry obtained here is also compatible with earlier studies on Meisenheimer complex formation [26,27]. Absorption coefficients of the 1-(S-glutathionyl)-2,4,6-trinitrocyclohexadienate Meisenheimer complex, stabilized by the enzyme, were calculated assuming one binding site per subunit and came out much lower than expected ($5\text{--}15 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). If, however, one active site per trimer is assumed, the absorption coefficient agrees much more closely with that expected ($26 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) from data on the free complex in solution [28] and that stabilized by other GSTs [29]. It must be borne in mind, however, that anomalous absorption coefficients have been obtained with some forms of cytosolic GSTs [30], so that this absorption coefficient cannot be used to infer stoichiometry.

The binding data obtained here cannot exclude the existence of additional, low-affinity binding sites. Enzyme concentrations as high as $650 \mu\text{M}$ set a lower limit on the K_d of putative additional sites at approximately that concentration. The data on the Meisenheimer complex stabilization, where high concentrations of GSH and 1,3,5-trinitrobenzene are reached, at least do not favour additional binding sites.

Activation of microsomal GST is a highly interesting phenomenon that can be effected by thiol reagents and proteolysis, and is accompanied by a conformational change [31]. Activation can also be observed *in vivo* [32], under conditions that produce oxidative stress or where reactive intermediates are formed [33,34]. It is thus an attractive hypothesis that reactive electrophiles that modify the enzyme's thiol group (single cysteine at position 49) also increase the rate of their own conjugation in some cases. The kinetic consequences of activation have been

described previously [19,26,35]. Here we can further add that activation is not accompanied by a change in binding affinity to GSH (or GSO_3^-). Neither is the binding stoichiometry affected. It is also of interest to compare the formation constant of a Meisenheimer complex, $15 \times 10^4 \text{ M}^{-1}$ (which is also unaffected by activation [26]), and the association constant for the enzyme and GSH, $6 \times 10^4 \text{ M}^{-1}$. Since these values are similar it is suggested that stabilization of the Meisenheimer complex is largely a result of GSH binding and that a modest extra binding energy is contributed by the 2,4,6-trinitrocyclohexadienate moiety. This suggestion is in keeping with the broad second-substrate specificity of the enzyme.

An inhibitor of microsomal GST (GSO_3^- [36]) was used to verify the binding assay and to obtain an independent, kinetic, estimate of binding. Competitive inhibition is shown by the pattern of intersecting lines in Lineweaver–Burk plots (Figure 3). Identical binding properties of activated and unactivated enzyme indicates that the conformational change that takes place upon activation does not affect this binding site. It is also shown that the binding affinities of GSH and GSO_3^- are similar.

Microsomal GST has earlier been identified as the high-capacity binding protein for leukotriene C_4 found in most tissues [12]. The estimated dissociation constant for leukotriene C_4 ($K_d = 6.3 \text{ nM}$) indicates much stronger binding than for GSH [12]. However, it was also shown that GSH at physiological concentrations could be able to displace leukotriene C_4 . Thus, depending on the relative concentrations of the competing ligands, a scenario where microsomal GST acts as a regulatable store of leukotriene C_4 can be depicted. Experiments are underway to elucidate the possible physiological significance of leukotriene storage on microsomal GST.

In conclusion, the GSH binding affinity and stoichiometry of microsomal GST have been characterized. It is highly significant that only one binding site per trimer can be detected.

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