The Binding of Substrates and a Product of the Enzymatic Reaction to Glutathione S-Transf erase A*

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The binding of substrates and a product to glutathione S-transferase A from rat liver was studied by use of equilibrium dialysis and equilibrium partition in a two-phase system. The radioactive substrates glutathione and 3,4-dichloro-1-nitrobenzene, S-(2-chloro-4-nitrophenyl)glutathione, gave hyperbolic binding isotherms with a stoichiometry of 2 mol per mol of enzyme (i.e. 1 molecule per subunit). Glutathione (and glutathione disulfide) had an equilibrium (dissociation) constant for the binding of about 10 \( \mu M \), whereas bromosulfophthalein and the product had equilibrium constants of about 0.5 \( \mu M \). All ligands showed the same binding stoichiometry, and competition experiments involving unlabeled ligands indicated that glutathione and the glutathione derivatives were binding to the same site. Low affinity sites appeared to exist in addition to the specific high affinity sites (one per subunit) for all ligands tested. The binding studies are fully consistent with a steady state random kinetic mechanism for the enzyme.

One of the glutathione S-transferases (1), transferase B, was originally discovered as a binding protein capable of forming tight complexes with various endogenous compounds as well as with xenobiotics (see Ref. 2). It was named ligandin by Litwack et al. (2), and it has been suggested that in the capacity of a binding protein it may serve intracellularly in the transport of compounds having hydrophobic regions (see Ref. 1). Recently, quantitative models of facilitated diffusion were considered for ligandin (3). In contrast to the extensive investigations of GSH S-transferase B (ligandin), the characterization of the other GSH S-transferases in terms of binding properties has been very limited (1). A study of nonsubstrate ligands was made on four GSH S-transferases from rat liver, and it was concluded that all of the transferases possess binding properties similar to those of ligandin (GSH S-transferase B) (4).

In connection with the study of the kinetic properties of the transferases it became essential to have information available about the binding of substrates. Extensive kinetic investigations have been carried out with GSH S-transferase A and the substrates 3,4-dichloro-1-nitrobenzene and GSH (3, 6), but information on binding of the reactants to the enzyme is lacking. In view of the complex rate behavior of the enzyme (5, 6) it was desirable to study the binding of reactants, in order to evaluate the possibility of cooperative binding as a cause of non-Michaelian kinetics. Some preliminary data have previously been reported (6, 7).

MATERIALS AND METHODS

S-(2-Chloro-4-nitrophenyl)glutathione was prepared enzymatically (6), and the \(^{35}S\)-labeled compound was synthesized by the same procedure using \(^{35}S\)GSH (Schwarz/Mann). The specific radioactivities used in the binding studies were about 0.1 Ci/mol for the GSH derivative and about 1 Ci/mol for GSH. \(^{35}S\)GSSG (about 10 Ci/mol) was prepared by oxidation of GSSG (New England Nuclear), and \([^{35}S]1\)-bromosulfophthalein was from The Radiochemical Centre, Amersham. GSH S-transferase A from rat liver, identical with form II of "GSH-S-aryltransferase," was purified and assayed as earlier described (6, 8). The protein concentration was determined with a micro-biuret method (9) using bovine serum albumin as a standard.

The binding of ligands to the enzyme was studied by two methods: equilibrium partition in a two-phase system and equilibrium dialysis. The equilibrium partition method (10) made use of a system formed by mixing 10 parts of 25% (w/w) Dextran T40 (Pharmacia Fine Chemicals), 3.5 parts of 50% (w/w) polyethylene glycol 6000 (Merck), and 2 parts of enzyme (20 to 40 \( \mu M \) in the lower phase) in 50 nm sodium phosphate, pH 7.3. About 30 \( \mu L \) of a stock solution of the labeled ligand were added to 5 ml of the mixture. Aliquots of 200 \( \mu L \) were then thoroughly mixed in centrifuge tubes with 50 \( \mu L \) of water solutions of different concentrations of the ligand in unlabeled form, thereby keeping the amount of radioactive ligand constant while varying the total ligand concentration. The samples were left at 22°C for 30 min and then centrifuged to speed up the separation of the two phases. Aliquots from the two phases were counted in 10 ml of Aquasol (New England Nuclear) using a Beckman LS-100 liquid scintillation spectrometer. About 99% of the enzyme was recovered in the lower phase of the two-phase system. The corrections made for quenching (<10%) were different for the two phases. The distribution of the ligand between the two phases was determined in the absence of enzyme, and the distribution coefficient was used in the calculation of the concentration of free ligand in the binding experiments.

Equilibrium dialysis was carried out by use of MSE Dionorm equipment. One compartment was loaded with 200 \( \mu L \) of enzyme in 50 nm sodium phosphate, pH 7.3, unless otherwise stated, and the other compartment was loaded with 200 \( \mu L \) of ligand(s) in the same buffer. The total amount of radioactivity was constant in a cell, and the concentration of measured ligand was varied by addition of unlabeled ligand. The cells were rotated (12 rpm) for 3 h at 22°C for equilibration. Aliquots (130 \( \mu L \)) from each cell compartment were then analyzed by liquid scintillation spectrometry in 10 ml of Aquasol. The two methods used to study the equilibrium binding gave similar results, but it should be noted that the binding of bromosulfophthalein was studied only by equilibrium dialysis owing to the extremely uneven distribution of this ligand between the phases of the two-phase system. The possibility of unspecific binding to enzyme, membranes, or cells of the radioactive ligands tested was excluded by the finding that the unlabeled ligands were fully competitive versus their corresponding radioactive forms in the binding experiments.

In experiments with GSH dithioerythritol was added to prevent oxidation of GSH during equilibration. Blocking the sulfhydryl groups

* This investigation was supported by grants (to B. Mannervik) from the Swedish Natural Science Research Council and the Magn. Bergvalls Stiftelse. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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of GSH by addition of 50 μl of 10 mM N-ethylmaleimide to the
scintillation vial before mixing with Aquasol was necessary to obtain
stable counts of radioactivity. When bromosulfophthalein was used
50 μl of 0.2 M NaH₂PO₄ was added to each scintillation vial to bleach
the color of the dye by lowering the pH value.

RESULTS

Binding of a Single Ligand—The binding of the product
of the enzymatic reaction, S-(2-chloro-4-nitrophenyl)glutathione to GSH S-transferase A was studied by two methods.
Fig. 1 shows a Scatchard plot of the results of the equilibrium
partition method. By this method as well as by equilibrium
dialysis the graphs were linear, indicating hyperbolic (i.e. noncooperative) binding of the product. The extrapolated
maximal number of product molecules per enzyme molecule
was about 2. Likewise, the substrate GSH gave a linear
Scatchard plot showing a maximal binding of 2 molecules of
GSH per enzyme molecule (Fig. 2). Most of the binding studies
were carried out at pH 7.3, because this pH value was optimal
for the location of enzyme to one of the two phases in the
equilibrium partition method. It was checked by use of equi-
libration dialysis that the product and GSH gave the same
stoichiometry and hyperbolic binding curve at pH 8.0 (Fig. 2),
which was the pH value used in most of the previous kinetic
experiments. The inhibitor glutathione disulfide (8) gave sim-
lar results (data not shown). The second substrate 3,4-di-
chloro-1-nitrobenzene was not available in radioactive form
and could accordingly not be studied by the same methodology.
However, an alternative substrate bromosulfophthalein
was studied by the equilibrium dialysis method and was also
found to give a hyperbolic saturation curve and a binding
stoichiometry of 2. A summary of the binding parameters is
given in Table I.

Competition of Ligands for Binding to the Enzyme—Fig.
3 shows that addition of a fixed concentration of unlabeled

**Table I**

| Ligand                        | n      | Kᵦ (μM) |
|-------------------------------|--------|---------|
| GSH                           | 2.27 ± 0.14 | 7.06 ± 1.47 |
| GSSG                          | 2.10 ± 0.03 | 7.47 ± 0.31 |
| S-(2-Chloro-4-nitrophenyl)glutathione | 1.89 ± 0.03 | 0.34 ± 0.05 |
| Bromosulfophthalein           | 2.01 ± 0.04 | 0.44 ± 0.02 |

* Obtained by the method of equilibrium partition.
GSH inhibited the binding of labeled \( S-(2\text{-chloro-4-nitrophenyl})\)glutathione. The effect was competitive, indicating that the two ligands bind to the same site. Other ligands which were also shown to decrease the binding of the product were \( S\text{-hexyl-} \) and \( S\text{-octylglutathione} \) and bromosulfophthalein. All effects appeared to be competitive. Competition experiments involving labeled GSH and unlabeled GSH derivatives gave similar results. The second substrate, 3,4-dichloro-1-nitrobenzene in concentrations up to 1 mM (near the limit of solubility) had no measurable effect on the binding of product. (The effect on GSH binding cannot be studied owing to the enzyme-catalyzed reaction between the two substrates.) The binding curves of the radioactive ligands were hyperbolic in the concentration range considered, whereas the effect of the unlabeled competitors appeared to be nonhyperbolic. Figs. 4 and 5 show the results of displacement of a fixed concentration of the radioactive product using variable concentrations of unlabeled GSH and bromosulfophthalein, respectively. However, it should be noted that the concentrations used of the competitors are considerably higher than those of the same compounds in labeled form used in the binding studies (cf. Fig. 2). In fact, the concentration range covered with radioactive GSH in Fig. 2 corresponds to the four points closest to the \( x \) axis in Fig. 4, and these points can be approximated by a straight line. The binding constants \( (K_d) \) for GSH and bromosulfophthalein were calculated from those data obtained at low concentrations of the competitor (cf. Ref. 13). The values of constants thus obtained from the competition experiments were in good agreement with the values calculated from directly measured binding of labeled competitor (cf. Fig. 2). On the other hand, it was found that the lines in the Scatchard plots curve to the right near the \( x \) axis (cf. Fig. 1) when the concentration of the radioactive ligand was increased. These findings reveal low affinity binding in excess of the stoichiometry of 2 molecules of ligand per enzyme molecule.

**FIG. 4. Effect of variable concentrations of glutathione on the binding of \( S-(2\text{-chloro-4-nitrophenyl})\)glutathione.** The measurements were made at 22°C by equilibrium dialysis using 24 \( \mu \)M total concentration of \( ^3\text{H}\)-labeled product, 21 \( \mu \)M enzyme, and 50 mM sodium phosphate buffer (pH 7.3).

**FIG. 5. Effect of variable concentration of bromosulfophthalein (BSP) on the binding of \( S-(2\text{-chloro-4-nitrophenyl})\)glutathione.** The measurements were made at 22°C by equilibrium dialysis using 24 \( \mu \)M total concentration of \( ^3\text{H}\)-labeled product, 21 \( \mu \)M enzyme, and 50 mM sodium phosphate buffer (pH 7.3).

**DISCUSSION**

The results reported in the present paper show that GSH, GSSG, bromosulfophthalein (an electrophilic substrate), as well as a product, \( S-(2\text{-chloro-4-nitrophenyl})\)glutathione, bind noncooperatively to GSH S-transferase A with a stoichiometry of 2 molecules per enzyme molecule (1 molecule per subunit) when their concentrations are kept low. At high ligand concentrations a much weaker nonspecific binding appears to occur. Similar results were obtained by use of equilibrium dialysis in a study of the binding to ligandin (GSH S-transferase B) of ligands such as some steroids, bromosulfophthalein, and the conjugate N-methyl-4-aminoazobenzene-glutathione (13). However, a notable difference between the results of the two studies appears in the stoichiometry, which for GSH S-transferase B was reported as 1 molecule of ligand per enzyme molecule (13), whereas our data indicate that 2 molecules are bound per GSH S-transferase A molecule. An unequivocal explanation for the difference cannot be given, but the finding that the two subunits may be dissimilar in GSH S-transferase B (14, 15) could mean that in this protein only one subunit binds the ligands tested. The subunits of GSH S-transferase A, on the other hand, appear to be identical in their binding properties. The mutual competition of the ligands (Figs. 3 to 5) indicate that they bind to the same or to overlapping sites on the GSH S-transferase A molecule.
The finding that bromosulfophthalein displaces S-(2-chloro-4-nitrophenyl)glutathione from the enzyme (Fig. 5) indicates that bromosulfophthalein binds to (at least a part of) the same site as the GSH derivative. The effect is not completely reciprocal, because S-(2-chloro-4-nitrophenyl)glutathione could only partly inhibit the binding of bromosulfophthalein (data not shown). Therefore, it appears probable that bromosulfophthalein can bind to different positions on the enzyme, some of which are inaccessible to the glutathione derivative. It has been suggested to explain inhibition experiments (7, 16) that the active center of the enzyme consists of a binding site for the peptide part of GSH and GSH derivatives (G site) as well as a hydrophobic site (H site) for the binding of the second hydrophobic substrate. The latter site would also bind hydrophobic S-substitutents of GSH derivatives. The H site must be able to bind the variety of large and small substrate molecules, and the lack of effect of 3,4-dichloro-1-nitrobenzene on the binding of product could mean that both the S-substituent of the product and the relatively small substrate molecule could be accommodated simultaneously.

The evidence for the binding of the 2-chloro-4-nitrophenyl group of the product to the enzyme comes from the about 20-fold higher strength of binding of S-(2-chloro-4-nitrophenyl)glutathione in comparison with GSH or GSSG (see Table I). Another explanation of the lack of effect of 3,4-dichloro-1-nitrobenzene might be that this compound has a very low affinity for the enzyme. The assumption of more than one binding site for bromosulfophthalein per subunit is consistent with the complex shape of Fig. 5. Results indicating more binding sites for bromosulfophthalein than for other ligands have previously been reported for GSH S-transferase B (13). The binding of GSH and GSSG occurred with very similar dissociation constants (Table I). This finding lends support to the conclusion that they bind to the same site as previously indicated by their competition in kinetic studies (7, 16). The similarity in binding strength also indicates that GSSG is bound only with one tripeptide moiety to the enzyme. The value for the dissociation constant of GSH (about 10 μM) implies that more than 95% of the two high affinity sites for GSH are saturated in the cytosol of liver cells, because the concentration of GSH is about 10 μM and the concentration of GSH S-transferase A is about 20 μM (1% of the soluble proteins) (1) (cf. Ref. 17).

The results of the binding experiments obtained in the region of higher reactant concentrations indicate that for all of the ligands studied more than two molecules can be bound per enzyme molecule. The measurements were normally restricted to the range of low concentrations, but in Fig. 1 this feature is evident. In some cases it was attempted to resolve the binding curve into two hyperbolas. However, no clearcut stoichiometry could be determined for the binding in excess of 2 molecules of ligand per molecule, and it, therefore, appears as if the additional binding is unspecific. Also in the case of GSH S-transferase B have "primary" and "secondary" binding sites been found, even if the stoichiometry of binding was different, as noted above (13). The competition experiments show that GSH and bromosulfophthalein displace the product of the enzymatic reaction S-(2-chloro-4-nitrophenyl)glutathione (Figs. 3 to 5) from the enzyme. The interaction of the ligands is of the generalized competitive type (18), indicating more binding sites for bromosulfophthalein than for the product. The competition experiments presented in Fig. 6. The scheme shows a dimer, which has two noncooperative binding sites, i.e., the microscopic rate constants for the binding of a ligand to a subunit are always the same irrespective of the occupancy of the other subunit. If only one ligand is present three enzyme forms vanish, and the binding equation becomes a simple hyperbola as found experimentally (Figs. 1 and 2). If two ligands are present simultaneously the binding equation would appear to be a 2:2 function in the labeled ligand (P in Fig. 6) and a 1:2 function in the competitor concentration. However, just as in the case of a single ligand, numerator and denominator have a common
factor, and the expression degenerates to contain only first degree terms if no cooperativity exists. The binding equation of Fig. 6 was fitted by nonlinear regression methods to the data sets of Figs. 3 and 4 both by using the dissociation constants determined from binding curves with a single ligand ($K_1 = 0.5 \mu M$, $K_2 = 7 \mu M$, see Table I) and by using the dissociation constants as parameters in the regression. Both approaches gave good fits. The data were also analyzed under the assumption that the equilibrium constant for the binding of glutathione to the EP complex ($K_3$) was different from that for binding of glutathione to a subunit of the unliganded protein ($K_2$). Thus, three parameters were used in the curve fitting, but the inclusion of an additional parameter did not improve the fits sufficiently to allow rejection of the simpler model. The third constant ($K_3$) was somewhat less than $K_2$, which would have implied heterotropic cooperativity between the two ligands provided that the difference were significant. However, in view of the lack of cooperative effects of either ligand when studied separately, and considering the experimental error, such a heterotropic interaction cannot be supported by existing evidence. Thus, just as the binding studies involving a single ligand, the competition experiments fully support the conclusion that the two binding sites are independent.

In summary, the results show that the binding characteristics of glutathione S-transferase A are fully consistent with the steady state random kinetic mechanism which has previously been founded on initial rate and inhibition data (6, 16).

Acknowledgments—We thank Mr. Claes Guthenberg for valuable collaboration in the preparation of the enzyme, and Dr. Tamás Bartfai for constructive suggestions and discussions.

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The binding of substrates and a product of the enzymatic reaction to glutathione S-transferase A.
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*J. Biol. Chem.* 1979, 254:7085-7089.

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