The binding of two different reaction products (p-nitrobenzyl glutathione and the aflatoxin-glutathione conjugate) to mouse glutathione S-transferase A3-3 (mGSTA3-3) has been measured using equilibrium dialysis and a direct fluorescence quenching technique. As expected, p-nitrobenzyl glutathione was found to bind with a stoichiometry of 2.24 ± 0.17 mol/mol of dimeric enzyme. However, the much larger aflatoxin-glutathione conjugate, 8,9-dihydro-8-(S-glutathionyl)-9-hydroxyl-aflatoxin B₁ (AFB-GSH), was found to bind with a stoichiometry of 1.12 ± 0.08 mol/mol of dimeric enzyme. p-Nitrobenzyl glutathione bound mGSTA3-3 with a dissociation constant (Kᵦ) of 59 ± 17 μM while the aflatoxin-glutathione conjugate bound the enzyme with a Kᵦ of 0.86 ± 0.19 μM. Glutathione competitively inhibited binding of AFB-GSH to mGSTA3-3 with a Kᵦ of 1.5 mM, suggesting that AFB-GSH was binding to the enzyme active site. Although AFB-GSH bound to mGSTA3-3 with a stoichiometry of 1 mol/mol of dimeric enzyme, AFB-GSH completely inhibited activity toward 1-chloro-2,4-dinitrobenzene, indicating that AFB-GSH binding to one active site alters affinity for 1-chloro-2,4-dinitrobenzene in the active site of the other subunit. To our knowledge, this is the first report of a glutathione S-transferase reaction product which binds to the enzyme with a stoichiometry of 1 mol/mol of dimer.

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of broad specificity detoxification enzymes which catalyze the conjugation of glutathione to xenobiotics and endogenous substrates with electrophilic functional groups. Conjugation of reactive substrates with glutathione by GST serves primarily to prevent deleterious reactions with nucleophilic centers in cellular macromolecules. As first shown by Benson et al. (1), overexpression of GST often provides protection against the effects of electrophile exposure. In contrast, depletion of glutathione can increase cellular damage caused by GST substrates (2). In vertebrates, the cytosolic GSTs consist of four classes of isoenzymes: alpha, mu, pi, and theta, which all have similar three-dimensional structures (3–6). The active form of GST is a dimer, either a homodimer or a heterodimer, composed of two subunits from the same class (7, 8).

Structural and functional studies have clearly shown that the GST homodimer has two identical binding sites (one per subunit) which act independently rather than cooperatively. Structures have been solved for numerous crystals of GST isoenzymes complexed with different GST products or product analogs in the active site. These crystals do not display any significant structural differences between the two subunits in the dimer, which might suggest differences in activity of the subunits (4, 5, 9, 10). In addition, many of these structures clearly show two products bound per dimer, one in the active site of each subunit (4, 5, 9–11). Ligand binding experiments have shown noncooperative substrate and product binding with a stoichiometry of two ligands per dimer (12). In addition, steady state kinetic experiments with 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene are consistent with two identical, independent active sites (13). Finally, the ability to predict substrate turnover and specificity of heterodimers from homodimer activities provides further evidence that each subunit in the dimer has an independent active site (14). Although the reported ligand binding and kinetic studies of GST generally support a model where GST dimers have two independent active sites, this model has not been extensively tested using different isoenzymes and substrates. In particular, binding studies using large substrates or products have not been reported in the literature.

One relatively large, bulky substrate for certain GSTs is the epoxide of aflatoxin B₁ (AFB). AFB, one of a related group of mycotoxins produced by the common fungal mold *Aspergillus flavus*, is a well documented rat and human carcinogen (15, 16). AFB is activated to the highly reactive aflatoxin 8,9-exo-epoxide (AFBO) by certain cytochromes P450. AFBO then serves as a substrate for some, but not all, GST isoenzymes (16). Mice constitutively express glutathione S-transferase mGSTA3-3 in liver, which possesses high activity toward AFBO, protecting them from aflatoxin B₁-induced hepatocarcinogenicity by conjugating AFBO with glutathione to form 8,9-dihydro-8-(S-glutathionyl)-9-hydroxyl-aflatoxin B₁ (AFB-GSH; Fig. 1) (17). Preliminary studies have suggested that mGSTA3-3 has a very high affinity for AFB-GSH, and thus AFB-GSH may be a useful ligand with which to evaluate the turnover of large compounds by GST. In this study we report that AFB-GSH binds mGSTA3-3 with a stoichiometry of 1 mol/mol of dimeric enzyme.
EXPERIMENTAL PROCEDURES

Chemicals and Reagents—The mouse GSTA3 cDNA was previously cloned (18). The plasmid pET11d was purchased from Novagen (Madison, WI). Aflatoxin B1, 1-chloro-2,4-dinitrobenzene, and glutathione were purchased from Sigma. Other chemicals used were of analytical grade and were purchased from various commercial sources.

AFBO was synthesized, separated from the endo-epoxide, and reacted with glutathione to form AFBO-GSH, using the procedure developed by Raney et al. (19). Synthesized AFBO-GSH was purified by HPLC using a protocol also described by Raney et al. (19), which allows separation of exo-epoxide conjugate from endo-epoxide conjugate. The synthesized exo-epoxide conjugate did not contain detectable amounts of endo-epoxide conjugate. The purified AFBO-GSH coeluted from the HPLC column with enzymatically generated conjugate. The concentration of purified AFBO-GSH was determined by measuring the absorbance at 365 nm using the extinction coefficient 21,800 \( \text{M}^{-1} \text{cm}^{-1} \) (20).

Glutathione S-transferase Expression and Purification—Escherichia coli BL21 (DE3) were transformed with the pET11d plasmid containing the mGSTA3 cDNA and optimized for high levels of mGSTA3-3 protein expression. mGSTA3-3 was expressed using a procedure modified from Sambrook et al. (21). All purification steps were conducted at 4°C. Bacteria expressing recombinant protein were lysed with a Heat Systems Ultrasonics model W-220 sonicator at power level five three times for 20 s each. Cellular debris was removed by centrifugation at 20,000 \( \times \) g two times for 20 min each. Bacterial supernatant was loaded onto a glutathione agarose affinity column and washed overnight with \( \geq 10 \) column volumes of 50 mM Tris/HCl, pH 7.4; 200 mM NaCl; 0.5 mM dithiothreitol. GST proteins were eluted with 50 mM glutathione in 200 mM Tris, pH 9.0. The proteins were dialyzed against 4 \( \times \) 2 liters of 40 mM Na\(_2\)HPO\(_4\), pH 7.4, 1 mM EDTA, 0.5 mM dithiothreitol over a period of 40 h. All bacterially expressed GSTs yielded a single band that migrated at the predicted size on SDS-polyacrylamide gel electrophoresis gels (data not shown). Protein concentrations were determined with the Bradford colorimetric assay (Bio-Rad), using bovine serum albumin as a standard. Molar concentrations of homodimeric mGSTA3-3 were calculated from protein mass measurements using a molecular mass of 50.5 kDa (17).

Direct Measurement of AFBO-GSH Binding to mGSTA3-3—Apparent dissociation constant, \( K_d \), and binding stoichiometry for AFBO-GSH were determined by measuring quenching of the fluorescence of AFBO-GSH upon binding to mGSTA3-3. AFBO-GSH is fluorescent when free in aqueous solution with a fluorescence maximum at excitation 365 nm, and emission 440 nm. This fluorescence is completely quenched when AFBO-GSH is bound to mGSTA3-3 (see Fig. 2). Thus, fluorescence intensity of a solution containing both the enzyme mGSTA3-3 and the ligand AFBO-GSH is proportional to the concentration of free (unbound) AFBO-GSH. The measurements were performed on a Perkin-Elmer LS50 luminospectrometer in the presence of 1 \( \mu \)M mGSTA3-3 dimer and 0.125–3 \( \mu \)M AFBO-GSH in 0.1 M potassium phosphate buffer, pH 7.2. Measurements were performed at 37 °C, maintained by a Lauda MS circulating water bath. The excitation wavelength was 365 nm and emission was detected at 440 nm. Concentration of free AFBO-GSH was determined by comparison to a standard curve. Quenching of tryptophan fluorescence of mGSTA3-3 upon ligand binding was also monitored by excitation at 280 nm and emission at 330 nm.

Equilibrium Dialysis—The dissociation constant, \( K_d \), and binding stoichiometry for p-nitrobenzyl glutathione and AFBO-GSH were determined by equilibrium dialysis. The two compartments in each cell were separated by \( M \), 12,000–14,000 cut off Spectra/Por dialysis membrane from Spectrum Medical Industries, Inc. (Houston, TX). One compartment was loaded with 300 \( \mu \)l of mGSTA3-3 enzyme in 0.1 M potassium phosphate buffer, pH 7.2. For analysis of p-nitrobenzyl glutathione binding, a protein concentration of 3.0–4.3 mg/ml (61–86 \( \mu \)M dimeric enzyme) was used; for analysis of AFBO-GSH binding, a protein concentration of 0.15–0.21 mg/ml (3.0–4.3 \( \mu \)M dimeric enzyme) was used. The other compartment was loaded with 300 \( \mu \)l of ligand (60–1500 \( \mu \)M p-nitrobenzyl glutathione or 1–16 \( \mu \)M AFBO-GSH) in the same buffer. In each control cell, one compartment was loaded with 300 \( \mu \)l of ligand in 0.1 M potassium phosphate buffer, pH 7.2, and the other compartment was loaded with only buffer. The cells were rotated for 4.5 h at 37 °C for equilibration. The control cells were used to confirm that equilibrium had been achieved and to measure nonspecific binding of the ligand. Ailquots from both compartments of the control cells and from the ligand-only compartment of the enzyme-containing cells were recovered, and ligand concentrations were determined by measuring absorbance at 280 nm (p-nitrobenzyl glutathione) or fluorescence at excitation 385 nm/emission 440 nm (AFBO-GSH).

Substrate Assay—Activity toward CDNB was measured as described by Habig and Jakoby (22).

Data Analysis—Binding parameters, the dissociation constant (\( K_d \)), and binding capacity (\( n \)) for binding of AFBO-GSH to mGSTA3-3 and \( K_d \) for inhibition of AFBO-GSH binding by glutathione were determined by nonlinear regression using the program ENZFITTER (Biosoft, Cambridge, UK).

RESULTS

Direct Measurement of Product Binding—The dissociation constant (\( K_p \)) and binding capacity (\( n \)) for binding of AFBO-GSH to mGSTA3-3 were measured directly by monitoring the quenching of AFBO-GSH fluorescence. The fluorescence of AFBO-GSH in buffer was proportional to AFBO-GSH concentration from 0–3 \( \mu \)M AFBO-GSH (Fig. 2, inset). This fluorescence was quenched by binding of AFBO-GSH to mGSTA3-3 (Fig. 2). AFBO-GSH fluorescence was not efficiently quenched by rGSTA3-3, which is 86% identical to mGSTA3-3 (results not shown). Table I summarizes the parameters for AFBO-GSH binding to mGSTA3-3. The data were fit to a model for noncooperative ligand binding:

\[ y = (n \cdot x)/(K_d + x) \]  
(Eq. 1)
The data were obtained from equilibrium dialysis experiments at 37 °C except where indicated. The parameters were obtained by fitting the data to the model \[ y = \frac{n \cdot x}{K_d + x}, \]
using nonlinear regression with the program ENZFITTER (Biosoft, Cambridge, UK). \( n \) is the binding capacity in moles of ligand/mol of dimeric enzyme, \( K_d \) is the dissociation constant, \( x \) is the free ligand concentration, and \( y \) is the moles of ligand bound per mol of dimeric enzyme. The data were fit by nonlinear regression using the program ENZFITTER (Biosoft, Cambridge, UK). Analysis of ligand binding from quenching of AFB-GSH fluorescence yielded a \( K_d \) of 0.72 ± 0.11 \( \mu M \) and a binding capacity of 1.31 ± 0.088 mol/mol of dimer.

The intrinsic fluorescence of tryptophan in mGSTA3-3 was also monitored (23), and this fluorescence decreased with increasing concentrations of AFB-GSH, indicating that AFB-GSH was binding to mGSTA3-3 in a concentration-dependent manner (data not shown). Data from quenching of tryptophan fluorescence were not used to estimate binding parameters, because ligand binding capacity cannot be determined from changes in spectroscopic properties of the protein which rely on fractional change (24).

Jakobson et al. (12) have previously shown that glutathione and small substrates inhibit binding of the small reaction product S-(2-chloro-4-nitrophenyl)glutathione to GST in a competitive manner. To verify that AFB-GSH was binding to the active site of the enzyme, the ability of glutathione and CDNB to compete with AFB-GSH for binding to mGSTA3-3 was measured. As shown in Fig. 3, glutathione inhibits binding of AFB-GSH to mGSTA3-3 in a competitive manner, with a \( K_d \) of 1.5 mM. Inhibition of AFB-GSH binding by CDNB also appeared to be competitive (data not shown). To further explore the surprising result suggesting that binding capacity was saturated at a stoichiometry of 1 mol of AFB-GSH/mol of dimer, ligand binding was remeasured by equilibrium dialysis.

**Equilibrium Dialysis—**An equilibrium dialysis apparatus was used to measure binding of two ligands, AFB-GSH and p-nitrobenzyl glutathione, to mGSTA3-3. Binding curves obtained for both ligands were hyperbolic, consistent with noncooperative binding (Fig. 4). The residuals appeared to be normally distributed, indicating that the model used was appropriate to describe the observed binding. As shown in Table I, the binding stoichiometry for AFB-GSH was 1.12 ± 0.078 mol/mol of dimer, while binding stoichiometry for p-nitrobenzyl glutathione was 2.24 ± 0.17.

**Inhibition of Activity toward CDNB by AFB-GSH—**Given our finding that 1 molecule of AFB-GSH binds per molecule of mGSTA3-3 homodimer, we measured the ability of AFB-GSH to inhibit activity toward CDNB. Based on previous studies, CDNB conjugation occurs in the active site of each subunit in a noncooperative manner (14). Fig. 5 shows the inhibition of activities toward CDNB by AFB-GSH under standard assay conditions for both mGSTA3-3, an enzyme with high activity toward AFBO, and rGSTA3-3, an enzyme with low activity toward AFBO.

Although mGSTA3-3 and rGSTA3-3 have similar CDNB activities (9.9 and 15.7 \( \mu M/mg/min \) respectively), they have very different activity toward AFBO (265 nmol/mg/min and <0.2 nmol/mg/min). AFB-GSH inhibited activity toward CDNB in mGSTA3-3 in a concentration dependent manner; however, it did not inhibit activity toward CDNB in rGSTA3-3 (Fig. 5).

**DISCUSSION**

The results of this study show that p-nitrobenzyl glutathione binds to mGSTA3-3 with the expected stoichiometry of 2 mol/mol of dimer, while the much larger glutathione conjugate

| Ligand | \( n \) | \( K_d \) |
|--------|--------|--------|
| AFB-GSH | 1.31 ± 0.088 | 0.72 ± 0.11 * |
| AFB-GSH | 1.12 ± 0.078 | 0.86 ± 0.19 |
| p-nitrobenzyl glutathione | 2.24 ± 0.17 | 59 ± 17 |

* Data obtained by direct measurement of AFB-GSH fluorescence quenching as described under “Experimental Procedures.”
 * Parameter values ± standard error.
AFB-GSH binds with a stoichiometry of 1 mol/mol of dimer. This is the first study to show different binding stoichiometries with two different glutathione conjugates. Although Schramm et al. used a spin-labeled product analog ([S]-[[2,2,5,5-tetramethyl-1-oxo-3-pyrrolidinyl]cambamoyl]methyl[glutathione]) to study ligand binding and found a binding stoichiometry of one per dimer in rGSTA1-1, they did not attempt to measure the binding stoichiometry of any alternative ligands (25). Schramm et al. (25) suggested that their results were indicative of one active site per dimer or of generalized half-sites reactivity for GSTs, conclusions which have not been supported by subsequent research (4, 5, 14). In this study, we have verified the previous findings that small glutathione conjugates bind to GST with a stoichiometry of two per dimer (12). However, we have found a large glutathione conjugate which binds to mGSTA3-3 with a stoichiometry of one per dimer.

In addition to the well defined active site, which consists of the glutathione binding site and the hydrophobic binding site (26), GSTs also contain at least one non-substrate binding site. Many non-substrate ligands have been shown to bind GST with a stoichiometry of one per dimer, or two per dimer with a single high affinity site and a single low affinity site (27). The exact location of the non-substrate binding site has not been identified. However, a recent crystal structure of GST from Schistosoma japonica shows the anti-schistosomal drug praziquantel bound to a site in the dimer interface, identifying this cleft as a binding site for at least some non-substrates (28). A binding stoichiometry of one mole ligand per mole dimeric enzyme could be interpreted as evidence that AFB-GSH is binding to the non-substrate binding site. However, we found that AFB-GSH completely inhibits activity toward CDNB in mGSTA3-3, but fails to inhibit activity toward CDNB in rGST A3-3 (which is 86% identical to mGSTA3-3 but has no detectable activity toward AFBO). In addition, we found that the binding of AFB-GSH to mGSTA3-3 is competitive with glutathione and CDNB. These data indicate that AFB-GSH is most likely binding to the catalytic site of one subunit.

Sinning et al. (5) reported that the hydrophobic binding site in alpha class hGSTA1-1 is relatively small, capable of holding molecules with ten or fewer carbons. Larger substrates bound to this site would likely extend into the cleft of the dimer interface. Aflatoxin B1 is a difuranocoumarin compound with 22 heavy atoms (see Fig. 1), thus it is likely that a large portion of the molecule extends into the cleft formed by the dimer interface. It is possible that the glutathione moiety of AFB-GSH is bound to the G-site of one subunit, and the aflatoxin moiety extends into the non-substrate binding site identified by McGtigue et al. (28).

The binding of AFB-GSH to an alpha-class isoenzyme was modeled using coordinates from the crystal structure of human GSTA1-1 (74% identical to mGSTA3-3) complexed with S-benzyl glutathione (5). The glutathione moiety of S-benzyl glutathione was used to anchor the glutathione moiety of AFB-GSH in the active site of each subunit. The aflatoxin moiety was rotated about the sulfur bond without regard to steric conflicts with the enzyme. With the glutathione moieties anchored in the G-sites, no binding conformation resulted in any steric conflict between the aflatoxin moiety from one subunit and any part of AFB-GSH bound to the other subunit. The two ligands were separated by at least 7 Å. Although a single AFB-GSH does not appear to physically interact with both enzyme active sites, the binding of AFB-GSH to one subunit may block access to the other subunit active site or may induce a conformational change which lowers the affinity for substrates at the other subunit active site.

Our findings do not support the generally accepted model of GST function, an enzyme with two independent active sites, one on each subunit. Although there have not been previous reports of GST reaction products which bind to GST with a stoichiometry of one per dimer, the available alpha-class crystal structures (5, 29) and ligand binding studies (12) have utilized glutathione conjugates that are much smaller than AFB-GSH. We propose that the presence of AFB-GSH in one subunit active site either physically blocks access to the active site on the other subunit or induces a conformational change which prevents binding to the other subunit. There are a number of other large substrates which are metabolized by various GST isoenzymes to form glutathione conjugates; for example benzo(a)pyrene-7,8-diol-9,10-oxide, 7-hydroxymethylbenz[a]anthracene, and cholesterol-5,6-oxide (see Hayes and Pulford (30) for review). Additional study is needed to determine whether our result is specific to AFB-GSH and mGSTA3-3 or is generally applicable to large glutathione conjugates binding to GST.

Acknowledgments—We thank Eric Dietze for computer modeling of AFB-GSH in the active site of alpha class glutathione S-transferase. We also thank Dr. Theo Bammler for helpful discussions and critical reading of the manuscript.

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FIG. 5. Inhibition of 1-chloro-2,4-dinitrobenzene activity by AFB-GSH. Activity was measured as described by Habig and Jakoby (22) in 0.1 M KPO4 buffer, pH 6.5, with 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene. The activity of mGSTA3-3 (●) and rat glutathione S-transferase A3-3 (○) are shown in the presence of increasing concentrations of AFB-GSH. The CDNB activity of mGSTA3-3 and rGST A3-3 in the absence of AFB-GSH was 9.9 μmol/mg/min and 15.7 μmol/mg/min, respectively. The curve shown for inhibition of mGSTA3-3 activity was fit to the data by nonlinear regression using the equation $y = 1 - (x/K_d + x)$; where $K_d$ is the apparent binding constant of AFB-GSH to mGSTA3-3 under assay conditions.
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