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Suppression of high-affinity ligand binding to the major glutathione *S*-transferase from *Galleria mellonella* by physiological concentrations of glutathione

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The major glutathione *S*-transferase from larvae of *Galleria mellonella* binds a number of synthetic triphenylmethane dyes with dissociation constants of the order of 10^{-6} M or less. The organ distribution of the enzyme activity does not parallel the uptake of such dyes by the insect's organs *in vivo*. The affinity of the protein for such dyes is decreased by about an order of magnitude by the presence of glutathione in normal physiological concentration. This appears to be the cause of this protein's lack of efficacy as a 'ligandin' *in vivo*. The dyes appear to be acting as ineffective substrate analogues, binding at the catalytic site and impeding, in a reciprocal fashion, the binding of glutathione. Fluorescence-quenching titration and kinetic experiments together indicate the existence of a single ligand-binding and catalytic site per dimeric enzyme molecule.

INTRODUCTION

The GSH *S*-transferases (EC 2.5.1.18) are a group of enzymes with the major role of detoxifying electrophilic molecules by conjugation with the thiol group of GSH. The enzymes are widely distributed and have wide substrate specificities (Jakoby, 1978). In vertebrates, these proteins also have the ability to bind non-substrate ligands, generally organic anions. This may be a passive detoxication mechanism, or may relate to hepatic uptake of such ligands (Wolkoff *et al.*, 1979). The ligands are seen in some instances to inhibit the catalytic activity (Clark *et al.*, 1967; Vander Jagt *et al.*, 1982; Hayes & Chalmers, 1983; Boyer *et al.*, 1984), but in other cases, notably that of binding of bilirubin to rat liver 'ligandin', a high-affinity binding site, distinct from the catalytic site, appears to be involved (Bhargava *et al.*, 1978).

In insects, interest in the GSH *S*-transferases has focused on their conjugation of a wide range of pesticides and on the possible consequent development of resistance to insecticides (Oppenoorth *et al.*, 1979; Motoyama *et al.*, 1980). No systematic approach to examining their ligand-binding properties has yet been made. There is reason to believe that the insect GSH *S*-transferases should have such properties. Enzyme preparations from *Costelytra zealandica* were strongly inhibited by anionic triphenylmethane dyes (Clark *et al.*, 1967). Such a dye, BSP, acts as an effective affinity ligand in the isolation of a GSH *S*-transferase from *Galleria mellonella* (Clark *et al.*, 1977) and another, BPB, associates with zones of GSH *S*-transferase during electrophoresis of enzyme preparations from *Musca domestica* (Motoyama & Dauterman, 1977).

The principal glutathione transferase from *Galleria mellonella* is one of the better characterized of the insect GSH *S*-transferases (Clark *et al.*, 1977; Chang *et al.*, 1981) from the point of view of physical and catalytic properties. The present study was initiated to ascertain what ligand-binding properties characterized this protein, to what extent they interacted with the catalytic properties of the protein, and what physiological roles could be ascribed to such properties in the living insect.

MATERIALS AND METHODS

Materials

DEAE-Sepharose, QAE-Sephadex A-25 and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. GSH, ANS and BSP were from Sigma Chemical Co., St. Louis, MO, U.S.A. Amaranth [1-(4-sulpho-1-naphthylazo)-2-naphthol-3,6-disulphonic acid, disodium salt] was obtained as a technical-grade preparation and was recrystallized from aq. 80% (v/v) ethanol. The product was chromatographically homogeneous when examined on 0.25 mm t.l.c. plates of Silica Gel G (Merck) run in butanol/acetic acid/water (4:1:5, by vol.). BSG, sodium dodecyl sulphate, BPB and 1-chloro-2,4-dinitrobenzene were purchased from BDH Laboratories, Poole, Dorset, U.K., and sodium taurocholate (puriss) from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Bilirubin, lithocholic acid, Coomassie Blue R-250 and mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DBSP was a gift from Hynson, Westcott and Dunning, Baltimore, MD, U.S.A. Buffer constituents were from Sigma Chemical Co.

Abbreviations used: BCG, Bromocresol Green (3,3',5,5'-tetrabromo-*m*-cresolsulphonphthalein); BPB, Bromophenol Blue (3,3',4,4'-tetrabromophenolsulphonphthalein); BSP, phenol-tetrabromophthaleindisulphonic acid; DBSP, phenol-3,6-dibromophthaleindisulphonic acid; ANS, 8-anilinonaphthalene-1-sulphonic acid.

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Experimental animals

Larvae of the greater wax moth, *Galleria mellonella*, were raised as described previously (Clark *et al.*, 1977).

Preparation of enzyme

Whole larvae were homogenized in distilled water, the homogenate was clarified by acidification and the GSH *S*-transferase was extracted in a single step, of combined anion-exchange and affinity chromatography on GSH–BSP conjugate immobilized on Sepharose 4B, as described previously (Clark *et al.*, 1977; Clark & Dauterman, 1982). GSH (7.5 mM) was used as eluent. Fractions defining the enzyme peak were pooled and stored in 10 ml batches at −20 °C until required. Before use in fluorescence quenching or enzyme kinetic experiments, GSH was removed by passing the sample through a column (50 cm × 2.5 cm) of Sephadex G-25.

Determination of protein concentration

Protein solutions (15 ml) were dialysed twice for 16 h against 2-litre portions of 0.15 M-NaCl solution. Protein concentrations were determined by the method of Lowry *et al.* (1951), calibrated with bovine serum albumin.

Electrophoretic analysis

The homogeneity of protein preparations was assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Fluorescence titrations

Measurements of intrinsic protein fluorescence were made with an Aminco–Bowman spectrophotofluorimeter. Excitation was at 290 nm and emission at 350 nm. The cell holder was maintained at 25 °C, and the standard slit arrangement no. 3, as defined by the manufacturers, was used. Titrations were carried out on 2 ml of solutions of the purified GSH *S*-transferase at at least two known concentrations ranging between 2 and 0.2 μM in 10 mM-sodium phosphate buffer, pH 7.4 (buffer A). The fluorescence of the unquenched protein solution was set to a reading of about 100%. Dye was added from a stock solution of a suitable concentration (0.5–3 mM) in the same buffer to a maximum volume of 25 μl. Any GSH additions were made from a stock solution of 120 mM-GSH in buffer A, re-adjusted to pH 7.4. Titrations were carried out in triplicate.

Enzyme inhibition

Experiments with BSP as inhibitor were carried out at 25 °C and in 0.1 M-sodium phosphate buffer, pH 6.5 (buffer B). Concentrations of GSH were varied between 0.4 and 4 mM at a fixed concentration of 1-chloro-2,4-dinitrobenzene of 1 mM. Alternatively, 1-chloro-2,4-dinitrobenzene concentrations were varied between 0.1 and 1.0 mM at a fixed GSH concentration of 2 mM. Concentrations of BSP used ranged from 0 to 7.63 μM.

Experiments in which BCG was the inhibitor were carried out as above, except that the assay mixture was buffered at pH 7.4 with buffer A.

Rate measurements were made spectrophotometrically at 344 nm (Cohen *et al.*, 1964) in quadruplicate.

Tissue distribution of enzyme

Live larvae were chilled, decapitated and dissected in 0.9% NaCl solution. Portions (5–50 mg) of fat-body, Malpighian tubule and gut were removed. Blotted tissue samples were weighed, a known quantity of buffer B was added and the sample was homogenized in a Polytron homogenizer. Suitable volumes (0.05–0.5 ml) were taken for the assay of enzyme activity at 25 °C in buffer B containing GSH (1 mM) and 1-chloro-2,4-dinitrobenzene (1 mM). Samples of tissue homogenate were taken for the determination of protein content. Determinations of enzyme activity and protein content were made at least in triplicate.

Determination of free thiol content of fat-body

Larvae were dissected as above, and portions of fat-body (30–50 mg) were taken, blotted, weighed and homogenized in 3 ml of buffer A. Protein was precipitated by addition of 0.25 ml of 12% (w/v) trichloroacetic acid and was removed by centrifugation at 2000 *g* for 10 min. The supernatant was neutralized by cautious addition of 2 M-NaOH, and the free thiol content was determined by the method of Ellman (1959).

Tissue distribution of dyes

Larvae were immersed in artificial haemolymph (Dreaux & Grapin-Poupeau, 1964) containing the ligand to be tested at a concentration of approx. 10 μM, and the preparation was left for 10 min at room temperature. The dye-containing saline was then poured off, and the viscera of the insect were washed with two changes of clean saline. They were then examined for visual evidence of dye distribution. ANS was detected by viewing under u.v. light, and BSP by introduction of sufficient alkali to cause coloration of the dye.

Data fitting

Data from kinetic experiments were fitted to theoretical equations by a least-squares-regression program, KINETICS 301, written for the Apple IIe computer. The program performs an initial fit by using the random search routine of Koeppe & Hamann (1980) followed by a matrix-inversion routine based on that published by Cleland (1967). Coefficients of variation on quadruplicate rate measurements were approximately constant. Data from enzyme kinetic experiments were therefore weighted according to $(1/v)^2$ (Wilkinson, 1961).

Unweighted data from fluorescence-quenching titrations were fitted directly to a theoretical equation by using the same least-squares-regression procedures as above in the program FQUENCH. The equation employed was based on the assumption that dye (total concentration $[D_t]$) bound to a single class of n non-interacting sites on the protein (concentration $[E_t]$) governed by a dissociation constant, K_d. The consequential decrease in the observed fluorescence, corrected for inner-filter effects by the method of Chignell (1977), is denoted ΔF_c. The effective absorption coefficients used in the correction method ranged from 3550 $M^{-1} \cdot cm^{-1}$ for DBSP to 8300 $M^{-1} \cdot cm^{-1}$ for bilirubin. The limiting value of ΔF_c was designated ΔF_m. The regression equation used was thence:

$$\Delta F_c = \frac{\Delta F_m}{2n[E_t]}\{([D_t]+n[E_t]+K_d) - \sqrt{([D_t]+n[E_t]+K_d)^2-4n[D_t][E_t]}\}$$

in which the regression parameters were ΔF_m, n and K_d. Data were obtained at two values of $[E_t]$ differing by factors of 3–5-fold. This reduced the high covariance

Table 1. Characteristics of binding of ligands to the major GSH *S*-transferase from *G. mellonella*

Binding constants were determined by fluorescence titration as described in the Materials and methods section. The weighted means of the regression parameters are shown as means ± S.D. where possible. The numbers in parentheses in the first column show the numbers of different experiments carried out.

Ligand	n	$10^7 \times K_d$ (M^{-1})	ΔF_m
BSP (4)	0.78±0.16	6.2±0.1	53±2
DBSP (3)	1.28±0.25	18.2±2.7	34±5
BPB (2)	0.65±0.10	10.1±1.9	76±4
BCG (3)	1.12±0.20	11.8±2.5	64±1
Amaranth* (1)	0.80 (0.69–0.92)	200 (160–260)	43 (38–49)
Bilirubin* (1)	0.90 (0.82–0.97)	43.3 (37–52)	72 (66–78)

* Data not sufficiently well conditioned for the matrix-inversion routine to converge. Best-fit values are shown together with Radex (Goldberger, 1968) error ranges.

between n and K_d found when only a single value was used and gave increased precision and stability in the regression procedure. In some instances, particularly in cases of weakly binding ligands with a strong inner-filter effect, ill-conditioned data were obtained and the matrix-inversion routine would not converge. In such cases the best fit was obtained by the random search procedure and, instead of calculating standard errors on the regression parameters, the Radex range (Goldberger, 1968) (which is the range of parameter values defined at the limits by a doubling of the minimum sum of squared residuals) was calculated instead. When data are presented in the form of Scatchard plots, the data transformations were made after calculation of the best-fit values of free and bound ligand.

RESULTS

Dye uptake into organs of *G. mellonella* larvae

The uptake of model dyes into various organs of larvae of *G. mellonella* was studied qualitatively with the dyes amaranth, BCG, BPB, BSP and ANS. All of these were found to be concentrated in the Malpighian tubules, leading to pronounced staining. Such a result was expected in the light of previous work (e.g. see Maddrell, 1971). No evidence was seen of comparable uptake into other organs. The fat-bodies remained almost colourless in these experiments.

Organ distribution of enzyme activity

The specific activities of GSH *S*-transferase in fat-body, gut and Malpighian tubule were 0.72±0.30, 0.18±0.04 and 0.095±0.04 μmol/min per mg of protein respectively.

GSH concentration in fat-body

The mean concentration of GSH in fat-body, sampled from five different larvae, was found to be 3.3±0.7 mM, assuming that non-protein thiol, determined as described in the Materials and methods section, was accounted for entirely by GSH.

Purification of enzyme

The enzyme obtained was apparently homogeneous. The M_r of the native enzyme, calculated from the previously derived subunit M_r (Chang *et al.*, 1981), was taken to be 51000 for subsequent calculation, and we confirm that it is a dimer of subunits of identical or very similar M_r values.

Binding of ligands to *G. mellonella* GSH *S*-transferase

The dyes tested led to a decrease in the intrinsic fluorescence of the protein. Data from the titration procedure were fitted to the theoretical equation as described in the Materials and methods section. A fit to untransformed data was chosen to avoid the subjective elements in graphical methods of analysis (Halfmann & Nishida, 1972; Mertens & Kägi, 1979) and the bias introduced in fitting to transformed data. In general, a good fit was obtained, coefficients of variation on the regression parameters within any one experiment being of the order of 0.05–0.15. Values of K_d, n and ΔF_m obtained are shown in Table 1. The data from typical experiments, transformed as Scatchard plots, are shown in Fig. 1.

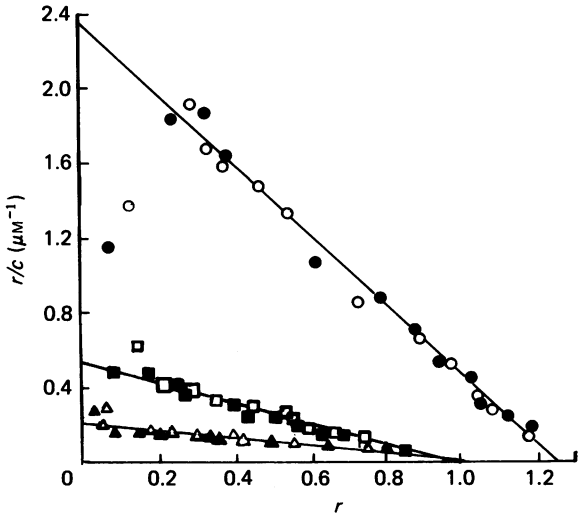


Fig. 1. Fluorescence-quenching titration of triphenylmethane dyes with the major GSH *S*-transferase from *G. mellonella*

Scatchard plots of fluorescence-quenching titrations of bilirubin (▲ and △), BCG (● and ○) and DBSP (■ and □). The titrations were carried at protein concentrations of 0.710 μM (▲, ● and ■) and 0.171 μM (△, ○ and □).

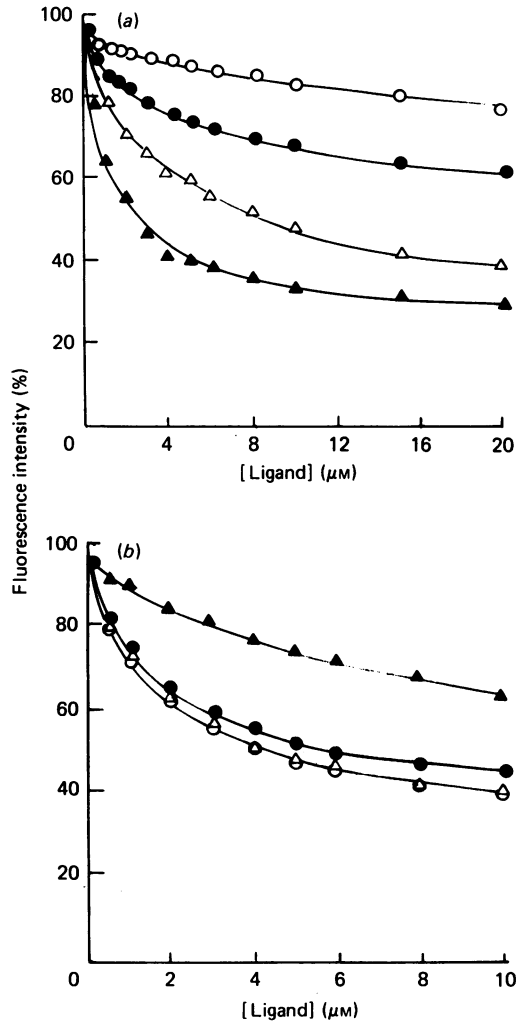


Fig. 2. Effect of thiols on the binding of triphenylmethane dyes to *G. mellonella* GSH *S*-transferase

(*a*) Fluorescence-quenching titrations were carried out with DBSP (● and ○) and BCG (▲ and △) in the absence of GSH (● and ▲) and in the presence of 3 mM-GSH (○ and △). (*b*) Titrations were carried with BSP as the ligand, in the presence of no thiol (○), 2 mM-mercaptoethanol (△), 2 mM-L-cysteine (●) and 2 mM-GSH (▲).

The bile salts taurocholic acid and lithocholic acid caused no quenching of the protein fluorescence when studied at the concentrations used for other test compound, nor were they able to displace from the protein the dyes BCG or amaranth even when present at 100-fold the dye concentration. It was concluded that these bile acids have no significant affinity for the protein.

The values of n obtained were consistently, for all ligands studied, approximately 1. We have no evidence for a second population of binding sites of lower affinity, or of there being significant non-specific binding.

Effects of thiols on ligand binding

In view of previous apparently conflicting observations on the effects of GSH and other thiols on ligand binding by these enzymes (Ketterer *et al.*, 1975; Jakoby *et al.*, 1976; Clark *et al.*, 1977), we decided to examine the effects of thiols on the binding phenomena reported above. It was found that GSH at a concentration of 3 mM had a marked depressant effect on the binding of BSP, BCG, DBSP and BPB. Examples of such results are shown in Fig. 2. Cysteine and mercaptoethanol were tested for these effects. Neither of these thiols had a significant effect on ligand binding.

The effect of GSH was not dependent on order of addition of reagents. Although addition of GSH alone to a solution of the protein caused no significant change in fluorescence, if the intrinsic fluorescence of the protein had been quenched by addition of a ligand, addition of

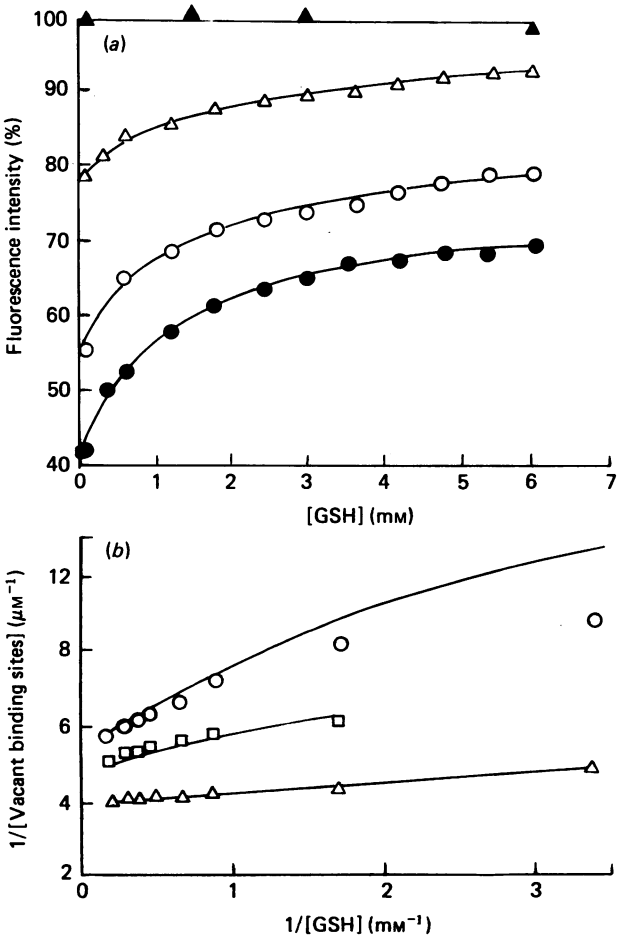
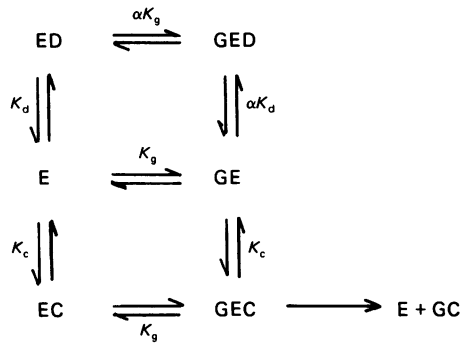


Fig. 3. Effect of GSH on the binding of BCG to *G. mellonella* GSH *S*-transferase

(*a*) Fluorescence-quenching titrations were carried out as described in the Materials and methods section. The meter reading of fluorescence intensity of the solutions of the native protein was set to 100%. BCG was added to final concentrations 0 μM (▲), 0.25 μM (△), 1 μM (○) and 2.5 μM (●). GSH was then added to the indicated concentrations, and fluorescence readings were taken. (*b*) Data from the above titrations were taken and transformed into a double-reciprocal plot. The concentration of binding sites not occupied by BCG was calculated. The reciprocals of these concentration terms were plotted against 1/[GSH]. Symbols: △, 0.25 μM-BCG; □, 1 μM-BCG; ○, 2.5 μM-BCG. The continuous lines are derived from theoretical expressions giving the best fit to the untransformed data shown in (*a*).



Scheme 1. Model for ligand and substrate binding to *G. mellonella* GSH *S*-transferase

GSH could cause an increase in the fluorescence of the system. The quenching by the ligands could, however, be only partially reversed by GSH. This is illustrated by the data shown in Fig. 3. In an experiment in which the quenching effect of BCG was countered by successive additions of GSH, it was found that the protein fluorescence tended to increase towards a horizontal asymptote, the value of which depended on the amount of BCG present (Fig. 3*a*). For clarity of presentation fluorescence readings were converted into a calculated value for the concentration of unoccupied ligand-binding sites. The corresponding concentrations of free BCG were also calculated. The reciprocals of these derived data were plotted against 1/[GSH] and are displayed in Fig. 3(*b*). The transformed data describe non-linear curves, not intersecting at the vertical axis. The data conformed to eqn. (3), generated by the model in Scheme 1 with substrate concentration [C] = 0. It was assumed that free protein and the protein–GSH complex had the same intrinsic fluorescence. It was further assumed that protein–dye and dye–protein–glutathione complexes had the same reduced intrinsic fluorescence. The total concentration of unliganded protein is given by:

$$[E_{free}]+[EG] = [E_t] \Big/ \left(1+\frac{[D_{free}]}{K_d}\cdot\frac{(\alpha K_g+[G])}{(\alpha K_g+\alpha[G])}\right) \quad (3)$$

Fitting the data in Fig. 3 to this equation generated values of 0.48 ± 0.12 μM for K_d, 0.61 ± 0.06 mM for K_g and 9.9 ± 2.2 for α. Equations constrained to fit to a competitive model ($\alpha = \infty$) or to a pure non-competitive model ($\alpha = 1$) both generated an inferior fit to the experimental data.

Enzyme inhibition

The kinetics of this enzyme have been shown to conform to a random Bi Bi model (Chang *et al.*, 1981). Substrate concentration ranges were chosen to give an apparent rapid-equilibrium system, yielding linear double-reciprocal plots. Under the assay conditions employed (Habig *et al.*, 1974), BSP was found to be a potent inhibitor (see Fig. 4). The mode of inhibition was found to be competitive with the aromatic substrate 1-chloro-2,4-dinitrobenzene (concentration [C]) with a $K_{d(app.)}$ value of 0.48 ± 0.06 μM with values of V (0.110 ± 0.01 μmol/min) and $K_{c(app.)} = 0.386\pm0.07$ mM in the equation:

$$v = V \Big/ \left[1+\frac{K_{c(app.)}}{[C]}\left(1+\frac{[D]}{K_{d(app.)}}\right)\right] \quad (4)$$

The inhibition data with respect to GSH as the varied-concentration substrate gave the best fit to a mixed mode of inhibition (Dixon & Webb, 1964) conforming to the rate equation:

$$v = \frac{V}{1+\frac{K_{g(app.)}}{[GSH]}\left(\frac{\alpha[D]+\alpha K_{d(app.)}}{[D]+\alpha K_{d(app.)}}\right)} \quad (5)$$

The best-fit values for the regression parameters were $V = 0.109\pm0.01$ μmol/min, $K_{g(app.)} = 0.55\pm0.14$ mM; $K_{d(app.)} = 0.659\pm0.14$ mM and $\alpha = 14\pm8.5$. Although the value of α is poorly defined, its inclusion caused a definite improvement in fit over the cases in which it had values of 1 or ∞. Overall the mechanism appears to be that described as competitive-mixed (Clark, 1971), depicted in Scheme 1. Similar results were obtained at pH 7.4, with BCG as inhibitor. The inhibition with respect to 1-chloro-2,4-dinitrobenzene was again found to be competitive, with values of V, $K_{c(app.)}$ and $K_{d(app.)}$ of 1.07 ± 0.11 μmol/min, 0.308 ± 0.061 mM and 1.51 ± 0.28 μM respectively. Again a mixed mode of inhibition best explained the results obtained when GSH

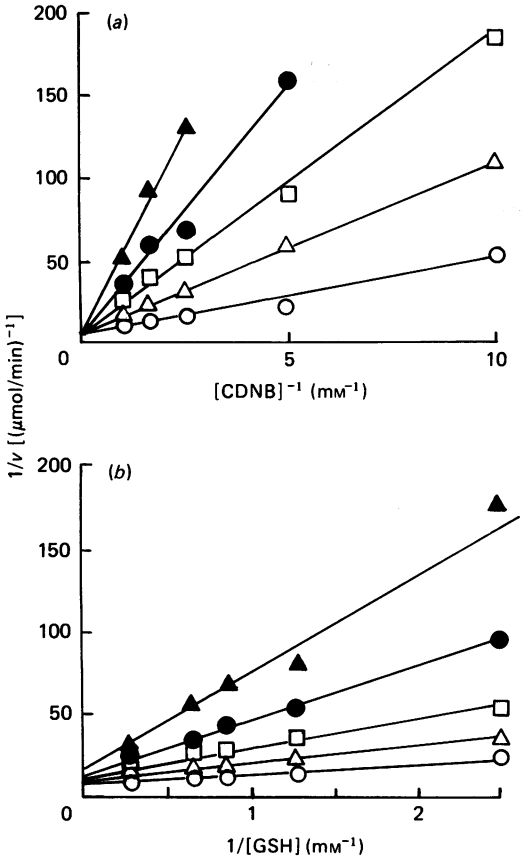


Fig. 4. Inhibition by BSP of the catalytic activity of *G. mellonella* GSH *S*-transferase

(*a*) Initial velocities were measured at 25 °C at pH 6.5 at a constant GSH concentration of 2 mM. Concentrations of 1-chloro-2,4-dinitrobenzene were varied as indicated, and BSP concentrations were 0 μM (○), 0.77 μM (△), 1.54 μM (□), 3.8 μM (●) and 7.63 μM (▲). (*b*) Initial velocities were measured as in (*a*), at a constant 1-chloro-2,4-dinitrobenzene concentration of 1 mM. GSH and BSP concentrations were varied. Symbols are as for (*a*).

concentration was varied; values for the regression parameters were $V = 0.99 \pm 0.065$ μmol/min, $K_{g(app.)} = 0.684 \pm 0.11$ mM, $K_{d(app.)} = 1.9 \pm 0.05$ μM and $\alpha = 2.93 \pm 1.79$.

DISCUSSION

As indicated in the Introduction, this work was approached on the assumption that the major GSH *S*-transferase isolated from *G. mellonella* did have ligandin activity. Studies in vertebrates have concentrated on the binding of bilirubin (Kamisaka *et al.*, 1975; Bhargava *et al.*, 1978; Vander Jagt *et al.*, 1982) and bile acids (Hayes & Chalmers, 1983) to hepatic ligandins as being of particular physiological importance. Since insects are not known to possess bile acids (Downer, 1978) and, generally lacking haem-containing blood respiratory proteins, should not as a rule produce large quantities of bilirubin, it seemed likely that any physiological role for an insect ligandin should differ in the identity of those ligands normally bound from those mentioned above.

We first examined the possibility that this protein was a determinant in the excretion of organic ions by the major excretory organs, the Malpighian tubules. Such a role has been proposed for vertebrate ligandins in liver (Reyes *et al.*, 1969). It is now apparent that this insect enzyme has no such role. Dyes of differing chemical structure were concentrated in the Malpighian tubules and were then excreted into the hind gut, whereas there was no detectable uptake into the fat-body. In contrast, GSH *S*-transferase activity, measured with 1-chloro-2,4-dinitrobenzene, was present in fat-body at 10 times the specific activity and over 100 times the total activity seen in the Malpighian tubules. Preferential uptake of dyes into the Malpighian tubules could not be rationalized in simple terms of distribution of the major GSH *S*-transferase.

In spite of this finding, studies performed *in vitro* confirmed that this protein did indeed have a ligandin character. It bound triphenylmethane dyes strongly (Table 1). The dye BSP was found to bind most strongly, with a dissociation constant equal to that reported for its binding to rat liver ligandin (Kamisaka *et al.*, 1975). Bilirubin bound with a 10-fold lesser affinity, and amaranth less tightly still.

Taurocholic acid and lithocholic acid displayed no ability to interact with the protein, and it thus appears that this enzyme can have no special physiological role as a carrier of bile acids or bilirubin in *G. mellonella*.

This work was also initiated with a view to studying the effect of GSH on ligand binding by this transferase. GSH can be used to elute these enzymes specifically from an affinity matrix having BSP as the immobilized ligand (Clark *et al.*, 1977), and also appears to compete with triphenylmethane dyes for binding to other insect enzymes (Clark *et al.*, 1967). In the present work, the dyes showing the strongest binding (BSP, DPSP, BCG and BPB) were all shown to be displaced from the protein by GSH at a concentration (3 mM) approaching that found in *G. mellonella* fat-body (Fig. 2). In a more detailed study with BCG, it was demonstrated that a state of partial competition appeared to exist between GSH and the dye. The binding of one species to the protein decreased the affinity of the other by about 10-fold, in a fashion consistent with the mechanism shown in Scheme 1.

Kinetic experiments also supported the suggested mechanism. The mechanism of inhibition by BSP and BCG appears to be competitive-mixed (Clark, 1971), and this is compatible with the partially competitive relationship found between BCG and GSH under equilibrium conditions.

The apparent Michaelis constants for GSH obtained in these experiments agree well with the value of K_g obtained by the titration shown in Fig. 3. The inhibition constants for both BSP and BCG agree closely with the dissociation constants shown in Table 1 and that obtained from data in Fig. 3. In sum, both equilibrium and kinetic experiments support the competitive-mixed mechanism in which ligand and GSH each decrease, but do not abolish, the affinity of the other for the enzyme. This situation may most simply be explained by proposing that the ligands examined here act as ineffective substrate analogues. Having a hydrophobic character, they bind to a hydrophobic site normally occupied by the electrophilic substrate, but are so large that they may impede access of GSH to its binding site. Conversely, GSH, if already bound, impedes access by the ligand to the other site. Such a model explains the elution by GSH of the enzyme from an immobilized BSP affinity support (Clark *et al.*, 1977), and may explain the apparently competitive relationship between GSH and triphenylmethane dyes reported for GSH *S*-transferases from *Costelytra zealandica* (Clark *et al.*, 1967).

This model also provides a mechanistic explanation for the lack of functional ligandin activity shown by this protein *in vivo*. Physiological concentrations of GSH depress the affinity of the protein for organic anions by about 10-fold. Although the protein binds anionic dyes strongly *in vitro*, it seems unlikely that it normally plays a role in transporting such compounds *in vivo*. Since a severe toxic challenge may deplete tissue concentrations of GSH markedly (Sugiyama & Kaplowitz, 1984), it seems possible that this pseudo-ligandin activity might constitute an emergency passive detoxication mechanism able to bind toxic organic anions only under such conditions.

It seems likely that these considerations may apply to GSH transferases not only of insect origin but also those of mammalian origin. Previous published work (e.g. Carne *et al.*, 1979; Vander Jagt *et al.*, 1982; Boyer *et al.*, 1984) shows that GSH may depress the binding of ligands to some rat liver GSH *S*-transferases. This suggests that ligand binding, contrary to implicit assumptions in the literature, may be of only minor physiological significance for GSH *S*-transferases lacking the high-affinity bilirubin-binding site.

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