Studies of the relationship between the catalytic activity and binding of non-substrate ligands by the glutathione *S*-transferases

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The dimeric enzyme glutathione *S*-transferase B is composed of two dissimilar subunits, referred to as Ya and Yc. Transferase B (YaYc) and two other transferases that are homodimers of the individual Ya and Yc subunits were purified from rat liver. Inhibition of these three enzymes by Indocyanine Green, biliverdin and several bile acids was investigated at different values of pH (range 6.0–8.0). Indocyanine Green, biliverdin and chenodeoxycholate were found to be effective inhibitors of transferases YaYc and YcYc at low (pH 6.0) but not high (pH 8.0) values of pH. Between these extremes of pH intermediate degrees of inhibition were observed. Cholate and taurochenodeoxycholate, however, were ineffective inhibitors of transferase YcYc at all values of pH. The observed differences in bile acids appeared to be due, in part, to differences in their state of ionization. In contrast with the above results, transferase YaYa was inhibited by at least 80% by the non-substrate ligands at all values of pH. These effects of pH on the three transferases could not be accounted for by pH-induced changes in the enzyme's affinity for the inhibitor. Thus those glutathione *S*-transferases that contain the Yc subunit are able to act simultaneously as both enzymes and binding proteins. In addition to enzyme structure, the state of ionization of the non-substrate ligands may also influence whether the transferases can perform both functions simultaneously.

The glutathione *S*-transferases (EC 2.5.1.18) are a family of enzymes that catalyse the conjugation of GSH with numerous electrophiles, many of which are toxic (Jakoby, 1978; Kaplowitz, 1980). Hence they are important enzymes of detoxification. In addition, these same proteins bind a variety of substances that are not substrates, such as bilirubin, bile acids, haematin and Indocyanine Green (Levi *et al.*, 1969; Litwack *et al.*, 1971; Ketley *et al.*, 1975; Hayes *et al.*, 1979). The affinity of the transferases for these non-substrate ligands also may have physiological significance in that the glutathione *S*-transferases may act as storage (Wolkoff *et al.*, 1979) and/or intracellular transport

(Tipping & Ketterer, 1978) proteins of molecules that have poor or limited solubility in water. The possible dual function of the transferases, i.e. catalytic and binding/carrier, presents an interesting problem, especially since the non-substrate ligands act as inhibitors of enzymic activity (Ketley *et al.*, 1975; Bhargava *et al.*, 1978; Vessey & Zakim, 1981; Vander Jagt *et al.*, 1982). In other words, it is not possible to decide at present whether the binding/carrier function or the catalytic function of the glutathione *S*-transferases is their primary activity and whether these functions can be performed simultaneously under conditions present *in vivo*.

In the current study we have investigated the inhibition of three forms of rat liver glutathione *S*-transferase by several non-substrate ligands. We show that there is an interrelationship between the binding/carrier and catalytic functions of these proteins that is susceptible to modulation by changes in the pH of the assay buffer or the

Abbreviations used: GSH, reduced glutathione; NaDodSO₄, sodium dodecyl sulphate. The different forms of the transferase are identified by their subunit structure; the *M_r* values of the individual subunits are 22000 for Ya, 23500 for Yb and 25000 for Yc subunits (Bass *et al.*, 1977).

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concentration of GSH. Some forms of glutathione *S*-transferase are shown to be capable of acting simultaneously as both enzymes and binding/carrier proteins.

Materials and methods

Materials

1-Chloro-2,4-dinitrobenzene was obtained from BDH Chemicals, Poole, Dorset, U.K., and Indocyanine Green was purchased from Pfaltz-Bauer Co., Stanford, CT, U.S.A. GSH, chenodeoxycholic acid (sodium salt), cholic acid and deoxycholic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Taurochenodeoxycholic acid (95% purity) was purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. Cholic acid and deoxycholic acid were recrystallized as described previously (Vessey & Zakim, 1981). The other bile acids were used without further purification. Biliverdin IX α was generously given by Antony McDonagh (McDonagh & Palma, 1980).

Preparation of enzymes

Glutathione *S*-transferase B (YaYc) and the YaYa and YcYc homodimers were purified as described previously (Boyer *et al.*, 1983). All enzymes were free of contaminating proteins and other transferases, as judged by NaDodSO₄/polyacrylamide-gel electrophoresis and isoelectric focusing (Maizel, 1971; Kenney & Boyer, 1981).

Buffers

Buffers were made with potassium phosphate (20mM) over the pH range 6.0–8.0. The ionic strength of the buffers was adjusted to a conductivity of either 2300 μ S (equal to 25mM-KCl, low ionic strength) or 8050 μ S (equal to 100mM-KCl, high ionic strength) by adding 1M-KCl. Conductivity was measured with a YSI model 31 conductivity bridge (Yellow Springs, OH, U.S.A.). In preliminary studies less inhibition of enzymic activity by Indocyanine Green was observed with the high-ionic-strength buffer as compared with the low-ionic-strength buffer. The decrease in inhibition may have been due to salt-induced aggregation of the Indocyanine Green (Landsman *et al.*, 1976), and the low-ionic-strength buffer was used when studies were performed with Indocyanine Green. In contrast, the extent of inhibition caused by biliverdin and bile acids was unaffected by the ionic strength of the buffer. The addition of enzyme, GSH, 1-chloro-2,4-dinitrobenzene, Indocyanine Green or biliverdin at the highest concentrations used did not affect significantly the pH or the ionic strength of the assay system.

Enzyme assays and inhibition studies

Enzymic activity was measured spectrophotometrically (Perkin-Elmer 555 recording spectrophotometer) at 25°C or 30°C with 1-chloro-2,4-dinitrobenzene and GSH as substrates. The appearance of *S*-(2,4-dinitrophenyl)glutathione was monitored at 340nm (Habig *et al.*, 1974). All enzyme activities were corrected for non-enzyme rates. Protein concentration was determined as described previously (Lowry *et al.*, 1951), with bovine serum albumin as standard.

Indocyanine Green and biliverdin were prepared fresh daily. Biliverdin was dissolved in 0.01M-NaOH. Both solutions were protected from light. Bile acid solutions were adjusted to pH 7–8 and then added directly to the reaction cuvette.

The enzyme and non-substrate ligand were mixed together with GSH and buffer of the specified pH and ionic strength in a 1ml assay cuvette. The mixture was left to stand for 2–5min at room temperature, during which time the cuvettes were protected from light. The enzyme itself was stable under these conditions. 1-Chloro-2,4-dinitrobenzene then was added to initiate the enzymic reaction, and initial rates of enzymic activity were determined. As a control, experiments were carried out as described above but in the absence of non-substrate ligands.

Binding studies

The binding of Indocyanine Green and biliverdin by the transferases was determined by measuring the quenching of intrinsic protein fluorescence by the added ligand (Ketley *et al.*, 1975). Fluorescence-quenching data were analysed by using eqn. (1):

$$\Delta F = \frac{\Delta F_m [L_f]}{K_D + [L_f]} \quad (1)$$

ΔF and ΔF_m are the observed change in fluorescence and maximal change in fluorescence respectively, and $[L_f]$ is the calculated free concentration of non-substrate ligand assuming a single binding site per enzyme dimer. Dissociation constants were obtained from plots of ΔF^{-1} versus $[L_f]^{-1}$. Measurements were made at 25°C with a Perkin-Elmer MPF-44B fluorescence spectrophotometer. The fluorescent spectra of the proteins were affected little by ionic strength or pH, and, with all three transferases, measurements were made at $\lambda_{exc.} = 280$ or 285nm and $\lambda_{em.} = 330$ nm. Corrections were made for the inner-filter effects of Indocyanine Green and biliverdin (McClure & Edelman, 1967).

Results

Time course of inhibition

Vander Jagt *et al.* (1982) reported previously

that the inhibition of glutathione *S*-transferase forms YaYc and YbYb by bilirubin was enhanced by preincubation of the enzyme and inhibitor. In the current study transferase YaYc was incubated with either Indocyanine Green or biliverdin in the presence or absence of GSH at pH 6.0 and 8.0. The maximum extent of inhibition was achieved within the mixing time irrespective of pH, presence or absence of GSH or the concentration of the latter substrate (0.2 or 5 mM). These latter investigators (Vander Jagt *et al.*, 1982) also reported that the presence of 2.5 mM-GSH during the incubation diminished the inhibition by bilirubin. The intra-hepatic concentration of GSH is known to decrease with starvation or after exposure to certain drugs (Kluwe & Hook, 1981; Pessayre *et al.*, 1979). We were interested, therefore, in how a physiological (4–5 mM) and a very low (0.2 mM) concentration of GSH would affect inhibition by the non-substrate ligands. The low concentration of GSH was selected because it is equal to the apparent K_m of the rat liver transferases (Jakoby, 1978).

Effect of pH on inhibition of glutathione S-transferase YaYc by Indocyanine Green, biliverdin and cheno-deoxycholic acid

Fig. 1 shows the pattern of inhibition of glutathione *S*-transferase YaYc by Indocyanine Green at various values of pH. The extent of inhibition was dependent on the pH of the assay buffer, with less inhibition being observed at the higher values of pH. For example, in the presence of 10 μ M-Indocyanine Green transferase YaYc was without enzymic activity at pH 6.0 whereas at pH 8.0 it was inhibited by only 37%. At all values of pH, increasing amounts of Indocyanine Green were added until inhibition was complete or until the extent of inhibition no longer changed as a function of the concentration of added non-substrate ligand. At low values of pH, complete inhibition of transferase YaYc was observed, whereas inhibition was partial at high values of pH (Fig. 1). Increasing the concentration of 1-chloro-2,4-dinitrobenzene from 0.1 mM to 0.9 mM did not affect the inhibition caused by 50 μ M-Indocyanine Green at any value of pH. Increasing the concentration of GSH from 0.2 mM to 5 mM, however, was associated with a decrease in inhibition by Indocyanine Green, especially at the low values of pH (Table 1).

The results obtained with biliverdin and cheno-deoxycholic acid were similar to those described above for experiments with Indocyanine Green, in that the higher the pH of the buffer, the less inhibition observed for a given concentration of non-substrate ligand. Increasing the concentration of GSH from 0.2 mM to 5 mM did diminish the extent of inhibition observed; however, the effect

Fig. 1. Effect of pH on the inhibition of glutathione *S*-transferase YaYc by Indocyanine Green

Assay mixtures contained 20 mM-potassium phosphate buffer (2300 μ S), 0.1 mM-1-chloro-2,4-dinitrobenzene, 0.2 mM-GSH and 1.5 μ g of transferase YaYc/ml. The pH of the buffer was 8.0 (■), 7.5 (●), 7.0 (△), 6.5 (○) or 6.0 (□). Enzymic activities were determined at each pH value in the presence and in the absence of Indocyanine Green. Relative activity is defined as activity in the presence of inhibitor divided by activity in its absence.

Table 1. Effect of GSH concentration on inhibition of transferase YaYc by Indocyanine Green

Transferase YaYc (final concn. 1.5 μ g/ml), GSH and Indocyanine Green (final concn. 50 μ M) were mixed together and left to stand for 5 min at room temperature in 20 mM-potassium phosphate buffer at the specified pH. Enzymic activity was initiated by adding 1-chloro-2,4-dinitrobenzene. Enzymic activity measured in the presence of Indocyanine Green was compared with enzymic activity measured in its absence.

pH	Concn. of GSH (mM)	Inhibition (%)
6.0	0.2	100
6.0	5.0	76
7.0	0.2	66
7.0	5.0	40
8.0	0.2	47
8.0	5.0	30

was less dramatic than that seen with Indocyanine Green.

The reversibility of the pH effect on inhibition was determined by incubating the enzyme in the presence of Indocyanine Green or biliverdin at pH 6.0 and then raising the pH to 8.0 with dilute

NaOH. Increasing the pH of the assay lead to an increase in enzymic activity that was similar to that observed if the entire incubation had been performed at pH8.0 (Table 2).

Studies of transferases YaYa and YcYc

The effect of pH on the inhibition of the glutathione *S*-transferases YaYa and YcYc by Indocyanine Green, biliverdin and chenodeoxycholic acid was determined. Initial studies were performed at pH6.0 and 8.0 and various concentrations of Indocyanine Green (Fig. 2). Transferase YcYc was inhibited completely by 10 μ M-Indocyanine Green at pH6.0, whereas at pH8.0

inhibition was limited to 14% despite the use of saturating concentrations of Indocyanine Green (50 μ M). On the other hand, transferase YaYa was inhibited by 80–90% at all values of pH, even at the lowest concentrations (3 μ M) of Indocyanine Green

Table 2. *Reversibility of inhibition of transferase YaYc by Indocyanine Green and biliverdin*

The enzyme (final concn. 7.3–11.1 μ g/ml), non-substrate ligand (25 μ M) and GSH (5mM) were mixed together in phosphate buffer at the prescribed pH for 2½ min. The final pH was then obtained, if necessary, by adding diluted NaOH. Each assay was performed in duplicate.

Initial pH	Final pH	Non-substrate ligand	Enzymic activity (μ mol/min per mg)
6.0	6.0	Indocyanine Green	0.45
6.0	8.0	Indocyanine Green	1.94
8.0	8.0	Indocyanine Green	2.11
6.0	6.0	Biliverdin	0.54
6.0	8.0	Biliverdin	2.28
8.0	8.0	Biliverdin	2.00

Fig. 2. *Effect of pH on the inhibition of transferases YaYa and YcYc by Indocyanine Green*

The assay conditions were as described in Fig. 1 legend except that the assay mixtures contained (1.5 μ g/ml) either transferase YaYa assayed at pH8.0 (○) or pH6.0 (●) or transferase YcYc assayed at pH8.0 (Δ) or pH6.0 (▲).

Fig. 3. *Effects of GSH concentration and pH on inhibition of transferases YaYa, YaYc and YcYc by 50 μ M-Indocyanine Green*
The assay conditions are as described in Fig. 1 legend except that the concentration of GSH in (a) was 0.2 mM and in (b) was 5 mM. Each assay mixture contained 1.0–1.8 μ g of transferase YaYa (○), transferase YaYc (◇) or transferase YcYc (Δ)/ml.

Fig. 4. Effects of GSH concentration and pH on inhibition of transferases YaYa, YaYc and YcYc by 50 μM-biliverdin. Assay conditions and symbols were as described in Fig. 3 legend.

Fig. 5. Inhibition of transferase YcYc at pH 6.0 by different bile acids

Assay mixtures contained 20mM-potassium phosphate buffer, pH 6.0 (8050 μS), 0.25mM-1-chloro-2,4-dinitrobenzene, 4mM-GSH and 1.8 μg of transferase YcYc/ml. The individual bile acids were chenodeoxycholate (□), deoxycholate (●), cholate (○) and taurochenodeoxycholate (Δ).

Transferase YcYc behaved in a manner similar to transferase YaYc, whereas glutathione *S*-transferase YaYa retained little enzymic activity in the presence of 50 μM-Indocyanine Green or 50 μM-biliverdin irrespective of pH or the concentration of GSH (Figs. 3 and 4).

Chenodeoxycholic acid also was found to be an effective inhibitor of transferase YaYa at all values of pH, but with transferase YcYc it was a progressively poorer inhibitor as the pH was increased to 8. Three additional bile acids were also studied (deoxycholate, cholate and taurochenodeoxycholate); they caused little inhibition of transferase YcYc at pH 8.0, whereas at pH 6.0 the extent of inhibition was different with each bile acid and followed the order chenodeoxycholate > deoxycholate > cholate > taurochenodeoxycholate (Fig. 5).

Effect of pH on binding of Indocyanine Green and biliverdin by the glutathione *S*-transferases

The binding of Indocyanine Green and biliverdin by the three transferases was determined by measuring the ligand-induced quenching of protein fluorescence as described in the Materials and methods section. The change in relative fluorescence of the transferases after the addition of a given concentration of non-substrate ligands was greater at pH 6.0 compared with that at 8.0 (Fig. 6). This difference was reflected in the somewhat higher K_D values (1.3–3.0-fold) obtained at pH 8.0 than at pH 6.0 (Table 3). The differences in affinity did not account, however, for the effects of pH on inhibition, as the use of saturating concentrations

(Fig. 2). Similarly, biliverdin inhibited transferase YaYa at all values of pH, but only inhibited transferase YcYc at low values of pH. The pH titration curves for the inhibition of all three transferases by either 50 μM-Indocyanine Green or 50 μM-biliverdin are shown in Figs. 3 and 4.

Table 3. Effect of pH on binding of Indocyanine Green and biliverdin by glutathione *S*-transferases

The K_D values were determined by measuring the quenching of protein fluorescence by Indocyanine Green and biliverdin. The measurements were performed in 20 mM-potassium phosphate buffer (2300 μ S) containing 0.2 mM-GSH and 0.1–0.47 μ M-protein.

pH	Ligand Form of transferase ...	K_D (μ M)		
		YaYa	YaYc (B)	YcYc
6	Indocyanine Green	0.18	0.66	2.09
8	Indocyanine Green	0.54	0.83	4.38
6	Biliverdin	0.43	0.81	2.04
8	Biliverdin	0.85	1.21	3.41

of inhibitor did not remove the differential in inhibition between pH6.0 and pH8.0 (Figs. 3 and 4).

Discussion

We have studied the inhibition of glutathione *S*-transferases YaYa, YaYc and YcYc by the non-substrate ligands Indocyanine Green, biliverdin and bile acids. The extent of inhibition of transferases YaYc and YcYc was shown to be dependent on the pH of the assay buffer. Saturating concentrations of these non-substrate ligands caused a complete loss of activity at pH6.0, whereas the extent of inhibition was decreased successively as the buffer pH was increased (Figs. 1 and 3–5). Transferase YaYc is a hybrid, formed *in vivo*, of the subunits that form the homodimers YaYa and YcYc (Kitahara & Sato, 1981; Hayes *et al.*, 1981; Boyer *et al.*, 1983). A pH effect on inhibition was not seen with transferase YaYa (Figs. 3 and 4). Hence the pH effect on the inhibition of transferase YaYc by non-substrate ligands can be ascribed to the presence of the Yc subunit.

The mechanism by which the pH of the assay buffer modified the inhibition of the transferases YaYc and YcYc by these non-substrate ligands is incompletely understood. The lack of inhibition at the higher values of pH does not appear to result from a failure of the enzyme to bind the non-substrate ligand, as a quenching of protein fluorescence by biliverdin and Indocyanine Green was observed at both pH6.0 and pH8.0 (Fig. 6). Strange *et al.* (1977) and Vander Jagt *et al.* (1982) also noted that lithocholate and bilirubin respectively bound to but did not inhibit transferase YaYc (assay pH7.4). The data indicate that glutathione *S*-transferases YaYc and YcYc at high values of pH form enzyme–inhibitor–substrate

Fig. 6. Effect of pH on the quenching of the protein fluorescence of the transferase YcYc by biliverdin. The cuvette contained 20 mM-potassium phosphate buffer (2300 μ S) at either pH6.0 (○) or pH8.0 (■), 0.2 mM-GSH and 0.2 μ M-transferase YcYc. The relative fluorescence (corrected for the inner-filter effects of biliverdin) is shown plotted versus the concentration of added biliverdin. In the inset, the double-reciprocal plot of ΔF versus the free concentration of biliverdin is shown.

complexes that remain catalytically active. Thus these proteins can function simultaneously as both enzymes and binding proteins.

The effects of pH on inhibition of transferases YaYc and YcYc could be mediated via changes in the non-substrate ligands. Bile acids are known to lose their charge and self-associate at low values of pH (Small, 1971), and this could influence their inhibitory properties. The concentrations of bile acids used in the current study, however, were low (<2 mM), and little aggregation was expected. All the bile acids that we used were fully ionized at pH8.0, and all were poor inhibitors of transferase YcYc at this pH. However, at pH6.0 the bile acids that were partially protonated, chenodeoxycholate (pK5.9) and deoxycholate (pK5.3), were more effective inhibitors of transferase YcYc than were the more ionized bile acids cholate (pK5.0) and

taurochenodeoxycholate ($pK_{1.8}$) (Fig. 5). Hence the state of ionization of the bile acid affects its ability to act as an inhibitor of transferase YcYc. Transferase YaYc demonstrated behaviour similar to that of transferase YcYc. In contrast, transferase YaYa was inhibited by chenodeoxycholate equally at both pH 6.0 and pH 8.0. Hence the state of ionization of bile acids is an important but not sole determinant of their ability to act as inhibitors.

In addition to affecting the non-substrate ligand, the buffer pH could influence the conformation of the enzyme-inhibitor complex. Vander Jagt *et al.* (1982), in explaining the effect of pH on the inhibition of transferase YaYc by bilirubin, concluded that different enzyme-inhibitor conformers formed at different values of pH. The conformer that formed at pH 6.5 was catalytically inactive, whereas the conformer that formed at pH 7.4 retained significant enzymic activity. Our results are similar to those reported by Vander Jagt *et al.* (1982), and the pH-dependent variation in inhibition (Figs. 3 and 4) is thought to reflect a change in the ratio of active to inactive conformers. In contrast with the study by Vander Jagt *et al.* (1982), however, we found that the inactive conformer could be converted into the active conformer by increasing the buffer pH (Table 2).

In the current study we have investigated only the three predominant forms of glutathione S-transferase present in rat cytosol, making it difficult to determine completely the significance *in vivo* of our observations. The results do suggest that intracellular pH and GSH concentration may be important determinants of how well the transferases function as enzymes in pathological states. For example, during cholestasis there are high intrahepatic concentrations of bile acids (approx. 0.6 mM) (Greim *et al.*, 1972) and bilirubin (0.03–0.3 mM in serum). Transferase YaYa would be inhibited extensively under these conditions. Since the normal intracellular pH is about 7.0 (Arieff *et al.*, 1980), transferases YaYc and YcYc would be expected to retain significant enzymic activity despite these high concentrations of inhibitors. However, a small decrease in intracellular pH, especially at low concentrations of GSH, may be associated with a significant loss of enzymic activity (Figs. 3 and 4), rendering the liver susceptible to injury by toxic electrophiles. Hence the accumulation of non-substrate ligands within the hepatocyte may act in concert with other factors in causing hepatic injury.

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