Inhibition of glutathione *S*-transferase by bile acids

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The effects of bile acids on the detoxification of compounds by glutathione conjugation have been investigated. Bile acids were found to inhibit the total soluble-fraction glutathione *S*-transferase activity from rat liver, as assayed with four different acceptor substrates. Dihydroxy bile acids were more inhibitory than trihydroxy bile acids, and conjugated bile acids were generally less inhibitory than the parent bile acid. At physiological concentrations of bile acid, the glutathione *S*-transferase activity in the soluble fraction was inhibited by nearly 50%. This indicates that the size of the hepatic pool of bile acids can influence the ability of the liver to detoxify electrophilic compounds. The A, B and C isoenzymes of glutathione *S*-transferase were isolated separately. Each was found to be inhibited by bile acids. Kinetic analysis of the inhibition revealed that the bile acids were not competitive inhibitors of either glutathione or acceptor substrate binding. The microsomal glutathione *S*-transferase from guinea-pig liver was also shown to be inhibited by bile acids. This inhibition, however, showed characteristics of a non-specific detergent-type inhibition.

The glutathione *S*-transferase(s) (EC 2.5.1.18) represents the body's most important defence mechanism against metabolically generated electrophilic toxins. These enzymes are present in many tissues and are particularly prevalent in the liver, where they can constitute up to 10% of the extractable protein (Jakoby, 1977). Previous studies of the role of glutathione *S*-transferases in the protection of the liver from hepatotoxins revealed that a decreased glutathione *S*-transferase activity can result in necrosis (Zimmerman, 1978) and most likely also carcinogenesis (Glatt & Oesch, 1977; Summer *et al.*, 1980). It is important, therefore, to understand all of the factors that affect the activity of this family of enzymes *in vivo*.

It was shown previously that bile acids are capable of binding to certain forms of glutathione *S*-transferase with affinity constants in the order of 10^4 – 10^5 M⁻¹ (Tipping *et al.*, 1976; Hayes *et al.*, 1979, 1980). However, there has been no systematic study of the effect of this binding on the enzymic activity of the different forms of glutathione *S*-transferase. In the present paper we show that physiological concentrations of bile acids are inhibitors of glutathione *S*-transferase activity, and pre-

sent a preliminary kinetic analysis of the inhibition of several different forms of the enzyme.

Materials and methods

Materials

1-Chloro-2,4-dinitrobenzene, glutathione sodium glycocholate, sodium taurocholate, chenodeoxycholic acid and sulphobromophthalein were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Cholic acid (Sigma) was dissolved in ethanol, treated with Celite/Norit A, and recrystallized from 70% ethanol. It was then re-dissolved in hot ethanol and dihydroxy bile acid contaminants were removed by precipitation with light petroleum, as described by Hofmann (1973). Deoxycholic acid (Sigma) was dissolved in 40 ml of hot 50% ethanol, and clarified by treatment with Celite/Norit A. The filtrate was stored at –20°C for 2 days. The precipitate was collected, dried and dissolved in water to a concentration of 14% (w/v). The solution was neutralized with NaOH, and the sodium deoxycholate was precipitated by adding 20 vol. of acetone. The precipitated material was collected at –20°C. Glycodeoxycholate (>98% pure; Sigma) and glycocholate (>98% pure; Sigma) were not further purified. *p*-Nitrobenzyl chloride and 1,2-dichloro-4-nitrobenzene were obtained from Aldrich, Mil-

Abbreviation used: GSH, reduced glutathione.

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waukee, WI, U.S.A., and were recrystallized from ethanol/water before use. Sodium taurodeoxycholate was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A.

Methods

The microsomal cell fraction was obtained from guinea-pig liver as described elsewhere (Zakim & Vessey, 1973) and washed once in 0.25 M-sucrose and once in 0.1 M-Tris, pH 8. Soluble-fraction glutathione *S*-transferase activity was prepared from rat liver by passing a 100 000 g supernatant through DEAE-cellulose in a Buchner funnel. This removed most of the red chromophore with only a 2–4% loss of enzyme activity. Glutathione *S*-transferase forms A, B and C were separated by the procedure of Habig *et al.* (1974).

Glutathione *S*-transferase activity was measured essentially as described by Habig *et al.* (1974). The standard assay for measuring sulphobromophthalein conjugation contained: 100 mM-phosphate buffer, pH 7.5; 5 mM-GSH; 0.06 mM-sulphobromophthalein and protein in a 1 ml volume. The reaction was followed spectrophotometrically at 330 nm. The standard assay for conjugation of 1,2-dichloro-4-nitrobenzene contained 100 mM-phosphate buffer, pH 7.5, 5 mM-GSH, 0.5 mM-1,2-dichloro-4-nitrobenzene and protein in 1 ml. The reaction was followed at 345 nm. The standard assay for conjugation of 1-chloro-2,4-dinitrobenzene contained 100 mM-phosphate buffer, pH 6.5, 3 mM-GSH, 0.25 mM-1,2-dichloro-4-nitrobenzene and protein. The reaction was followed at 340 nm. The standard assay for the conjugation of *p*-nitrobenzyl chloride contained 100 mM-phosphate buffer, pH 7.6, 5 mM-GSH, 0.5 mM-*p*-nitrobenzyl chloride and protein. The reaction was followed at 310 nm. Protein was determined by the method of Gornall *et al.* (1949). All bile acid solutions were adjusted to pH 7–8; additions were made directly to the reaction cuvette.

Results

Soluble glutathione *S*-transferase

A crude preparation of glutathione *S*-transferase, containing all of the different forms of the enzyme (see under 'Methods'), was tested for sensitivity to bile acids. When sulphobromophthalein was used as a substrate, all bile acids tested were inhibitory at concentrations well below their critical micelle concentrations (Table 1). One possible mode of bile acid-dependent inhibition of glutathione *S*-transferase is formation of complexes between the substrate and bile acid. This possibility can be tested because the substrates used in these studies all react with glutathione non-enzymically. These non-enzymic rates of conjugation were found to be unaffected by the concentrations of bile acid used in the present study. It was considered most likely, therefore, that the bile acids exerted their effect directly on the enzyme.

To determine whether inhibition was due to a non-specific surfactant effect of the bile acid, we varied the concentration of protein in the assay from 60 µg to 800 µg. The concentration of cholate required for 50% inhibition was 0.4 mM for all concentrations of protein up to 250 µg of protein/ml. Thus the inhibition of glutathione *S*-transferase is independent of the ratio of bile acid to protein and, therefore, must result from the stoichiometric binding of bile acids to specific sites on the enzyme. The apparent K_i for cholate increased at protein concentrations higher than 0.25 mg/ml, suggesting that non-specific binding of bile acid to protein decreases the concentrations of free bile acid.

The inhibitory effect of bile acids on glutathione *S*-transferase also was tested with 1,2-dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride as substrates. These data are shown in Table 1. Bile acids inhibited the conjugation of all substrates. The rates of conjugation of

Table 1. The concentration of different bile acids that cause 50% inhibition of glutathione *S*-transferase activity towards various substrates

Initial rates of conjugation of chlorodinitrobenzene, sulphobromophthalein, dichloronitrobenzene and *p*-nitrobenzyl chloride were determined for the DEAE-cellulose eluate preparation of enzyme, using the standard assays (see under 'Methods') and in the presence of various concentrations of the indicated bile acids. The values indicated in the Table are the approximate concentrations of bile acid required for 50% inhibition.

Bile acid	Concentration for 50% inhibition (mM)			
	Chlorodinitrobenzene	Sulphobromophthalein	Dichloronitrobenzene	<i>p</i> -Nitrobenzyl chloride
Cholate	0.5	0.45	2.7	0.35
Glycocholate	0.9	0.4	6.0	—
Taurocholate	1.2	0.4	—	0.90
Deoxycholate	0.1	0.15	0.25	0.17
Taurodeoxycholate	0.3	—	—	0.22
Chenodeoxycholate	0.05	0.1	—	0.09

1-chloro-2,4-dinitrobenzene, sulphobromophthalein and *p*-nitrobenzyl chloride showed similar sensitivities towards bile acids, whereas the rate of conjugation of 1,2-dichloro-4-nitrobenzene, compared with the other substrates, was less sensitive to inhibition by trihydroxy bile acids. In all cases, the dihydroxy bile acids were considerably more inhibitory than the trihydroxy bile acids. Also the glycine and taurine conjugates of the bile acids were, in most cases, less inhibitory than the parent bile acid. When the data for these inhibitions are plotted by the method of Dixon (1953), i.e. $1/v$ versus concentration of inhibitor, the lines are often non-linear. Generally, they bend upward (parabolic), although in a few cases they bend downward (hyperbolic) or are linear.

The A, B and C isoenzymes of glutathione *S*-transferase were separated and the kinetics of inhibition of each form studied separately. All kinetic studies were conducted at a concentration of protein within the range over which 50% inhibition values were independent of protein concentration. A Dixon plot of the inhibition of glutathione *S*-transferase A by cholate with sulphobromophthalein as substrate was non-linear; 50% inhibition was obtained at 0.7 mM-cholate. Kinetic studies of the inhibition by cholic acid of glutathione *S*-transferase form A with sulphobromophthalein as substrate are shown in Fig. 1. A mixed-type inhibition pattern was obtained with respect to both GSH and sulphobromophthalein. Thus cholic acid was not a competitive inhibitor of the binding of either substrate. The inhibition of glutathione *S*-transferase A by cholic acid with 1-chloro-2,4-dinitrobenzene as substrate also was mixed. However, with 1,2-dichloro-4-nitrobenzene as substrate an anomalous plot was obtained; cholate enhanced the apparent rate of reaction at low concentrations of 1,2-dichloro-4-nitrobenzene and inhibited at high concentrations of substrate.

With glutathione *S*-transferase form C, 50% inhibition was obtained at 0.08 mM-chenodeoxycholate and 0.2 mM-cholate. The pattern of inhibition by cholate is once again mixed with regard to both GSH and sulphobromophthalein. This conclusion is supported by the finding that cholic acid inhibited glutathione *S*-transferase form C at true $V_{\max}$; that is, activity extrapolated to saturation with both substrates. Glutathione *S*-transferase C assayed with *p*-nitrobenzyl chloride as substrate was also inhibited by cholate and deoxycholate. The inhibition pattern was mixed versus variable [GSH] for both cholate and deoxycholate. However, when the concentration of *p*-nitrobenzyl chloride was varied, the inhibition pattern was parallel for deoxycholate. In the presence of cholate, the plot for variable *p*-nitrobenzyl chloride intersected the control plot in the first quadrant, i.e. at low concentrations of

Fig. 1. Double-reciprocal plot of $1/v$ of sulphobromophthalein conjugation versus $1/[GSH]$ (a) or $1/[\text{sulphobromophthalein}]$ (b) for rates determined in the presence or absence of cholate

Initial rates of glutathione *S*-transferase A catalysed conjugation of sulphobromophthalein were determined in the presence (□) or absence (○) of cholate and are expressed as $\Delta A_{330}/\text{min}$.

p-nitrobenzyl chloride, a slight activation is caused by cholic acid.

Glutathione *S*-transferase form B was also tested for inhibition. The enzyme was assayed with 1-chloro-2,4-dinitrobenzene as substrate. Cholate, chenodeoxycholate and deoxycholate were all inhibitory; 50% inhibition was obtained at 0.07 mM-chenodeoxycholate, 0.12 mM-deoxycholate and 0.6 mM-cholate. The Dixon plots were hyperbolic for all three bile acids.

Fig. 2. Effect of cholate on microsomal glutathione *S*-transferase

Guinea-pig liver microsomes were assayed for glutathione *S*-transferase activity using the standard chlorodinitrobenzene assay and variable concentrations of cholate. Rates are expressed as $\Delta A_{340}/\text{min}$.

Microsomal glutathione *S*-transferase

A distinct form of glutathione *S*-transferase exists in the microsomal fraction of the liver cell (Friedberg *et al.*, 1979; Morgenstern *et al.*, 1980). Using 1-chloro-2,4-dinitrobenzene as substrate, we studied the effect of variable concentrations of cholic acid on the activity of the microsomal enzyme from guinea-pig liver. Fig. 2 shows the results of such a titration. The effect is quite complex. There is a 10–20% inhibition at 0.1 mM-cholic acid followed by a biphasic activation/inactivation at higher concentrations. If the concentration of microsomal protein in the assay is increased, then more cholate is required to give the same degree of inhibition. The conclusion drawn from these experiments is that the inhibition by cholate may be partially related to detergent effects of the bile acids. Since these effects require a relatively high ratio of detergent to microsomal protein, they are not expected to be significant *in vivo*, except perhaps under cholestatic conditions.

Discussion

Bile acids were found to be effective inhibitors of glutathione *S*-transferase activity. A partially purified preparation of enzyme from the soluble fraction of rat livers, which contained almost all of the cellular glutathione *S*-transferase activity, was significantly inhibited by physiological concentrations of bile acid when 1-chloro-2,4-dinitrobenzene, sulphobromophthalein or *p*-nitrobenzyl chloride were used as substrates. The normal bile acid concentration in the soluble fraction of rat liver is 0.2–0.3 mM; the concentration of dihydroxy bile acid (chenodeoxycholic acid, hyodeoxycholic acid and deoxycholic acid) is 65 μM (Greim *et al.*, 1972). At

these concentrations of dihydroxy bile acid, glutathione *S*-transferase activity with 1-chlorodinitrobenzene, sulphobromophthalein and *p*-nitrobenzyl chloride as substrates is inhibited nearly 50% (Table 1). The trihydroxy bile acid (cholate) was less inhibitory than the dihydroxy bile acids, but could be expected to contribute to overall inhibition *in vivo* with certain substrates. The glycine and taurine conjugates of the various bile acids were, in many cases, less inhibitory than the parent bile acid. This suggests that conjugation, in a sense, detoxifies bile acids. With regard to the data in Table 1, it should be noted that they were obtained at near-saturating concentrations of substrates. The sensitivity of glutathione *S*-transferase to inhibition by bile acids is even higher at lower concentrations of most substrates. Thus it seems reasonable to conclude that the glutathione *S*-transferase activity *in vivo* is restricted by the hepatic concentration of bile acid. The extent of this restriction would be expected to vary with the size of the intrahepatic pool of bile acid. During cholestasis, when the concentration of bile acid in the liver can rise 2–4-fold (Greim *et al.*, 1972), the inhibition of glutathione *S*-transferase could be sufficiently great to enhance the potential for toxicity of many electrophilic compounds.

Kinetic analysis of the DEAE-cellulose eluate and of purified glutathione *S*-transferases A and C revealed that the bile acids were not competitive inhibitors of the binding of either glutathione or any of the electrophilic substrates. Dixon plots of the inhibitions were rarely linear. The dihydroxy bile acids always yielded non-linear Dixon plots, suggesting that either there is more than one mode of binding inhibitors or, alternatively, that the preparation contains multiple enzyme forms with differing sensitivities to inhibition. In fact, we have found that forms A, B and C are all heterogeneous, as judged by isoelectric focusing in polyacrylamide gels over the pH range 7–10.

The binding of bile acids by different forms of glutathione *S*-transferase has been reported previously. Tipping *et al.* (1976) used equilibrium dialysis to study the binding of bile acids to a preparation of glutathione *S*-transferase referred to as ligandin, and subsequently shown to contain at least three different forms of glutathione *S*-transferase (Carne *et al.*, 1979). These authors determined binding constants for the bile acids from competitive binding studies with [^3H]oestrone sulphate. The dissociation constants obtained were 0.1 mM for cholate, and 0.05 mM for deoxycholate, taurodeoxycholate and chenodeoxycholate. The values are considerably lower than the values of 0.6 mM for cholate and 0.12 mM for deoxycholate for 50% inhibition obtained in our experiments. Further, our inhibition studies did not show competitive inhibition kinetics as did the work of Tipping *et al.* (1976).

We would suggest that either the binding that these authors were measuring was neither at the inhibitory site nor the active site of the enzyme, or that binding of bile acids may be altered in the presence of both substrates.

Hayes *et al.* (1980) eluted preparations of rat liver glutathione *S*-transferase from a gel-filtration column equilibrated with $10\mu\text{M}$ - $[\text{}^3\text{H}]$ cholate, and uncovered four fractions that eluted with bound cholate. In their study, glutathione *S*-transferases A and C were the only forms that did not bind cholate. In our studies, forms A and C were inhibited by cholate and, in fact, were more sensitive to inhibition than form B. This apparent discrepancy may once again indicate distinct non-inhibitory high-affinity binding sites for bile acid. Hayes *et al.* (1980) used only $10\mu\text{M}$ - $[\text{}^3\text{H}]$ cholate in their binding experiments and were detecting less than 35 d.p.m. of bound cholate. If the binding to inhibitory sites, which is reported in the present paper, has a lower affinity (which is definitely the case for the B form of the enzyme; see above), then the procedure of Hayes *et al.* (1980) would be too insensitive to measure binding.

In conclusion, we have found that physiological concentrations of bile acids inhibit hepatic glutathione *S*-transferase activity by nearly 50%. Dihydroxy bile acids are more inhibitory than cholic acid and conjugated bile acids are often less inhibitory than the parent bile acid. Inhibition is not due to direct binding at the active site of the enzyme.

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