Comparison of the affinities of newly identified human bile acid binder and cationic glutathione S-transferase for bile acids

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Abstract The bile acid binding properties of the newly identified bile acid binder (M_r = 36,000) (*FEBS Lett.* 1984. **177:** 31–35) and the major cationic glutathione (GSH) S-transferase (M_r = 50,000) in human liver cytosol were compared. Binding affinities were measured by the competitive displacement by bile acids of 1-anilino-8-naphthalene sulfonate (ANS) bound to the proteins and, in some cases, by direct methods of flow dialysis and equilibrium dialysis. The binding affinities for various bile acids by the human bile acid binder were 2–5 orders of magnitude greater than those by human cationic GSH S-transferase. This suggests an important physiologic role for the former protein in intracellular transfer of bile acids in human liver. — **Takikawa, H., A. Stolz, M. Sugimoto, Y. Sugiyama, and N. Kaplowitz.** Comparison of the affinities of newly identified human bile acid binder and cationic glutathione S-transferase for bile acids. *J. Lipid Res.* 1986. **27:** 652–657.

Supplementary key words 1-anilino-8-naphthalenesulfonate displacement • flow dialysis • equilibrium dialysis • hepatic cytosol binders

The intracellular movement of bile acids in hepatocytes is poorly understood. Bile acid binding by cytosolic proteins has been proposed as having an important role in both intracellular transport of bile acids and protection from potential toxic effects of bile acids. We have previously identified two proteins in rat liver that bind bile acids (1). One is GSH S-transferase or ligandin (M_r = 47,000) and the other is the bile acid binder (M_r = 33,000). We have previously found that both of these rat proteins bind various bile acids with about the same affinity (1). Human liver contains similar proteins, i.e., bile acid binder (M_r = 36,000) (2) and GSH S-transferase (M_r = 50,000) (3). However, little is known about the binding affinities of the human cytosolic proteins for bile acids. In the present study we compared the bile acid binding properties of GSH S-transferase and the bile acid binder purified from human liver.

METHODS

Purification of protein

An operative liver sample (25 g) (apparently normal) containing 1.3 g of cytosol protein was used. The protocol was approved by the institutional Human Subjects Review Committee. Cytosol (33% w/v) was prepared and the GSH S-transferase and bile acid binder fractions were separated on Sephadex G-75 superfine chromatography exactly as we have previously reported (1, 2).

Purification of the major cationic GSH S-transferase was performed according to Mannervik and Jensson's approach for purification of rat liver enzymes (4). Briefly, the purification procedure consisted of affinity chromatography of the Y fraction on S-octylglutathione bound to epoxy-activated Sepharose 6B followed by chromatofocusing on PBE 118 gel. The major cationic transferase was eluted at pH 9 by chromatofocusing and represented more than two-thirds of the transferase activity. Homogeneity was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to the method of Laemmli (5); the cationic transferase was a homodimer (subunit M_r = 25,000). The amount of pure protein obtained was 0.93 mg. Amino composition of proteins was performed as previously described (1, 2).

The bile acid binder was purified according to our previous report (2) using equilibrium dialysis with [^{14}C]lithocholic acid (59 mCi/mmol, New England Nuclear) to identify activity. Briefly, the 30–40 kilodalton fraction

Abbreviations: SDS, sodium dodecyl sulfate; ANS, 1-anilino-8-naphthalenesulfonate; GSH, glutathione.

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from the Sephadex G75 sf column containing peak bile acid binding was pooled and chromatofocused on PBE 94 gel followed by hydroxyapatite chromatography. Purified bile acid binder showed a single band on SDS polyacrylamide gel electrophoresis (M_r = 36,000); 0.47 mg of pure protein was obtained.

Binding studies with purified proteins

1-Anilino-8-naphthalene sulfonate (ANS) fluorescence inhibition technique. To cuvettes containing 2 ml of 0.01 M sodium phosphate buffer, pH 7.4, and purified protein (0.15–0.8 μM), different amounts of ANS (0–50 μM final concentration) were added and fluorescence was determined (Ex 400 nm, Em 480 nm) at room temperature (25–27°C). To elucidate the type of inhibition, ANS binding was determined with the cationic transferase and bile acid binder in the presence and absence of a fixed concentration of bile acid. Results were expressed as a Scatchard plot (6), and the dissociation constants for ANS binding were obtained by nonlinear least squares method using the Michaelis-Menten equation.

The inhibition constants (K_i) for various bile acids were determined by varying bile acid concentrations at a constant ANS concentration (15 μM). The glucuronidated and sulfated bile acids were synthesized as previously reported (7, 8) and the other bile acids were recrystallized substrates that were commercially available. K_i values were calculated by nonlinear least squares method as previously described (9).

Flow dialysis and equilibrium dialysis. Binding of lithocholic and glycochenodeoxycholic acid by the human proteins was measured by flow dialysis or equilibrium dialysis to confirm the results of the ANS technique. Flow dialysis was performed at room temperature according to the method of Colowick and Womack (10). A Spectrapor 2 membrane (Spectrum Medical Industries, Los Angeles, CA) was placed between the upper and lower chambers. [^{14}C]Lithocholic acid (59 mCi/mmol) or [^{3}H]glycochenodeoxycholic acid (2.3 Ci/mmol) (New England Nuclear) was dissolved in 0.5 ml of 0.01 M sodium phosphate buffer, pH 7.4, and placed in the upper chamber. The lower chamber was eluted with the same buffer (30 ml/hr); the outflow was collected every 2 min and radioactivity of each fraction was counted. Equilibrium for diffusion of bile acids was achieved within 6 min and transfer rates of labeled bile acids from the upper to the lower chamber did not change with the addition of unlabeled bile acids to the upper chamber in the absence of protein. Next, 0.5 ml of protein solution in the same buffer and labeled bile acid were placed in the upper chamber. The lower chamber was eluted under the same conditions and the outflow was collected every 2 min. Unlabeled bile acid was added stepwise into the upper chamber every 8 min and the radioactivity in each fraction was determined. After the experiment using the protein solution, the transfer rate of labeled bile acid in the absence of protein was repeated to confirm that the transfer rate was the same as the initial control experiment, indicating no change in the permeability of the membrane. Free fraction (f) at each bile acid concentration was calculated as follows:

$$f = \frac{\text{transfer ratio (protein)}}{\text{transfer ratio (buffer)}}$$

where transfer ratio is the radioactivity filtered per 2 min divided by the radioactivity in the upper chamber in experiments with buffer or protein. The free and bound concentrations of bile acids were calculated from f values.

Equilibrium dialysis was performed at 4°C as previously reported (1, 9). A tracer amount of labeled bile acid with various concentrations of unlabeled bile acid was added to the protein compartment. After equilibrium was reached (48 hr), radioactivity on both sides of the Spectrapor 2 membrane was determined. Results determined by both methods were expressed as Scatchard plots (6) and the number of binding sites (n) and K_d values were calculated by nonlinear least squares using the following equation:

$$C_b = \frac{n(p) \cdot C_f}{K_d + C_f}$$

where C_b and C_f are bound and free concentrations, respectively, of bile acid and p is protein concentration. In some cases, equilibrium dialysis and flow dialysis were performed using only the tracer amount of labeled bile acids. Using a very low concentration of bile acid ($K_d >> C_f$), the above equation can be simplified to

$$C_b = \frac{n(p) \cdot C_f}{K_d},$$

and K_d can be estimated (1).

Binding of lithocholic acid in gel filtration

To 6 ml of human cytosol, 1.5 μCi of [^{14}C]lithocholic was added; after 1 hr of incubation at 4°C, the sample was applied to a Sephadex G75 sf column (120 × 2.5 cm) and eluted with 0.01 M sodium phosphate buffer, pH 7.4, at a flow rate of 12 ml/hr. Individual fractions (2.5 ml) were assayed for A_{280}, GSH S-transferase activity (1) and radioactivity.

RESULTS AND DISCUSSION

This study examines the binding properties for bile acids of human hepatic cationic GSH S-transferase and bile acid binder and contrasts these results to our previous findings with similar proteins purified from rat liver. **Table 1** contrasts the molecular weight, subunit size,

TABLE 1. Comparison of the properties of human and rat bile acid binding proteins

	Human		Rat	
	Cationic GSH S-Transferase	Bile Acid[a] Binder	GSH S-Transferase B	Bile Acid[b] Binder II
Molecular weight	50,000	36,000	47,000[c]	33,000
Subunit molecular weight	25,000	36,000	22,000[c] 25,000	33,000
Eluted pH on chromatofocusing	9.0	7.3	9.5[c]	5.5
Amino acid composition				
Aspartic acid	38.3	35.3	37[d]	32.7
Threonine	7.8	10.4	11	13.2
Serine	23.6	19.6	18	12.9
Glutamic acid	53.0	35.4	46	31.7
Proline	28.8	19.0	20	10.5
Glycine	22.7	20.0	21	14.9
Alanine	32.0	26.0	31	15.6
Valine	20.0	26.4	25	14.9
Methionine	9.3	3.4	8	4.6
Isoleucine	29.3	15.4	18	13.6
Leucine	61.3	35.2	50	27.1
Tyrosine	9.0	11.6	13	9.5
Phenylalanine	19.8	12.9	17	13.2
Histidine	5.8	8.9	6	6.2
Lysine	45.4	25.7	36	17.8
Arginine	24.3	14.4	22	11.9

[a,b] The values are from our previous reports (1, 2).
[c] Mannervik and Jensson (4).
[d] Habig, Pabst, and Jacoby (11).

apparent isoelectric point (pH at which the protein was eluted in chromatofocusing), and amino acid composition of these proteins. Although similarities exist in the properties of human and rat proteins, they clearly differ with respect to chemical composition. In addition, antisera raised against either of the rat proteins do not cross-react with human proteins (A. Stolz and N. Kaplowitz, unpublished observations).

Initially, we determined whether bile acids could competitively inhibit the binding of ANS. Lithocholic acid competitively inhibited the binding of ANS by the human cationic GSH S-transferase and bile acid binder as shown in **Fig. 1, a and b**, respectively. In our previous report (2), the concentrations of lithocholic acid (up to 2 μM) were too low to show inhibition of ANS binding by human GSH S-transferase because of its low affinity for bile

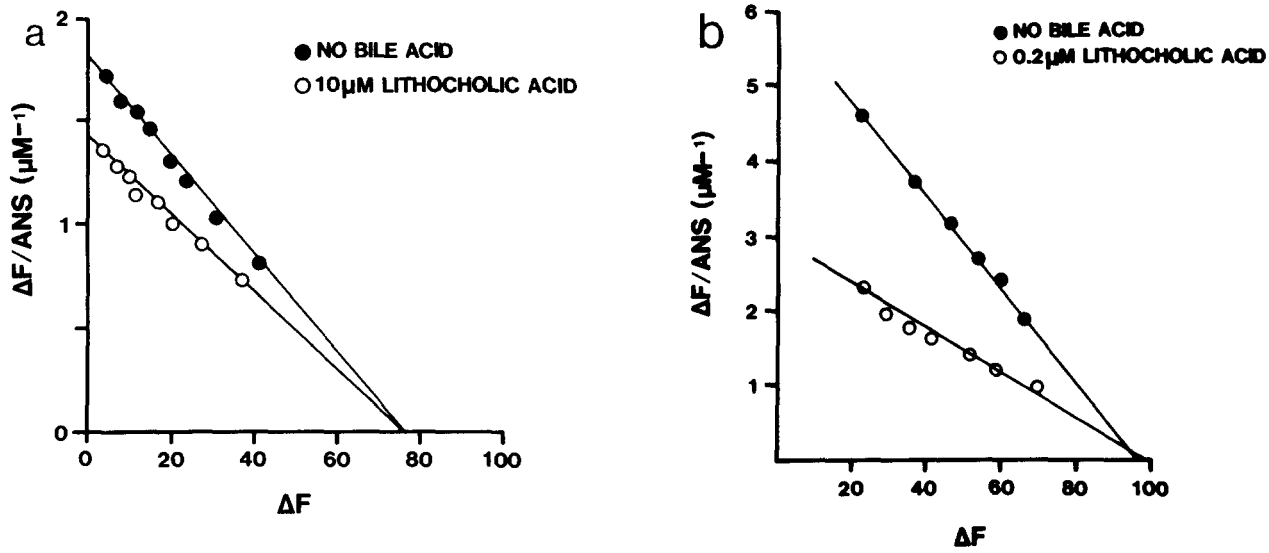

Fig. 1. Scatchard plots of the binding of ANS by cationic GSH S-transferase (a) and bile acid binder (b) from human liver in the absence and presence of lithocholic acid. The concentration of ANS was varied from 5 to 50 μM while concentrations of lithocholic acid and protein (0.8 μM cationic transferase or 0.15 μM bile acid binder) were held constant. The binding of ANS was determined fluorometrically (Ex 400 nm, Em 480 nm) in 0.01 M sodium phosphate buffer, pH 7.4, at room temperature.

TABLE 2. Dissociation constants (μM) for bile acid binding by cationic GSH S-transferase and bile acid binder from human and rat liver determined by the ANS technique

Bile Acid	Human		Rat	
	Cationic GSH S-Transferase	Bile Acid Binder	GSH S-Transferase B[a]	Bile Acid Binder II[a]
Lithocholic acid	20 (4.9[b], 9.0[c])	0.03 (0.12[b])	0.3	0.6
Taurolithocholic acid	26	0.03	11	8
Chenodeoxycholic acid	120	0.08	25	1.5
Glycochenodeoxycholic acid	76 (60[d])	0.07	13	6
Taurochenodeoxycholic acid	120	0.09	19	25
Chenodeoxycholic acid-3-sulfate	66	0.03	ND	ND
Chenodeoxycholic acid-3,7-disulfate	ND	0.05	ND	ND
Chenodeoxycholic acid-3-glucuronide	4300	0.08	ND	ND
Deoxycholic acid	51	0.5	16	40
Ursodeoxycholic acid	120	0.15	ND	ND
Cholic acid	900	1.5	90	350
Taurocholic acid	1900	0.5	120	550

K_i values were calculated from the results of Figs. 2 and 3 by nonlinear least squares method as previously reported (9); ND, not determined. All experiments using the ANS technique were repeated two or more times and the mean values are listed.

[a] The values are from our previous report (1).

[b] Equilibrium dialysis using purified transferase (single point).

[c] Flow dialysis using purified transferase.

[d] Flow dialysis using purified transferase (single point).

acids. In the present studies, 10 μM lithocholic acid was used; this concentration revealed competitive inhibition. Chenodeoxycholic acid also competitively inhibited the binding of ANS (not shown). Having established that bile acids compete for the ANS binding site, the ANS–protein mixture was then titrated with increasing concentrations of bile acids to yield displacement curves. From these data, K_i values were calculated (**Table 2**). **Fig. 2** shows the inhibition of the fluorescence of ANS bound by the human cationic transferase by various bile acids. Among the unconjugated bile acids, concentrations required to displace ANS were lowest with lithocholic acid and highest with cholic acid. This indicates that the order of affinities is mono->di->trihydroxy bile acids. Among the various conjugates of chenodeoxycholic acid, the affinity for the 3-sulfate was greater and that for the 3-glucuronide was lower than for chenodeoxycholic acid. Glycine or taurine conjugation had no influence on the binding of mono-, di-, or trihydroxy bile acids compared with the unconjugated bile acids.

With the human bile acid binder, all bile acids studied inhibited the fluorescence of ANS at much lower concentrations than required to inhibit ANS binding by the cationic transferase (**Fig. 3**). The preferential binding of bile acids by the binder compared to cationic GSH S-transferase was reflected in the exclusive elution of radioactive lithocholic acid in association with the 30–35 kilodalton fractions distinct from GSH S-transferase activity

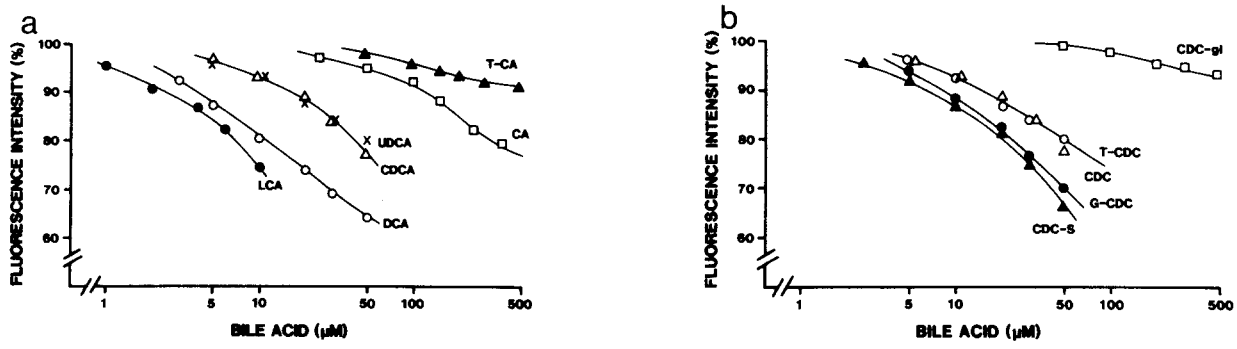

Fig. 2. Inhibition of the fluorescence of ANS (15 μM) bound to cationic GSH S-transferase (0.8 μM) by various bile acids (a) and various conjugates of chenodeoxycholic acid (CDCA) (b). The fluorescence was determined at 480 nm during excitation at 400 nm in 0.01 M sodium phosphate buffer, pH 7.4, at room temperature. Bile acids examined were lithocholic acid (LCA), CDCA, deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), cholic acid (CA), and taurocholic acid (T-CA). Conjugates of chenodeoxycholic acid (CDC) used were its tauro-(T-) and glyco-(G-) conjugates and its 3-sulfate (-S) and 3-glucuronide (-gl).

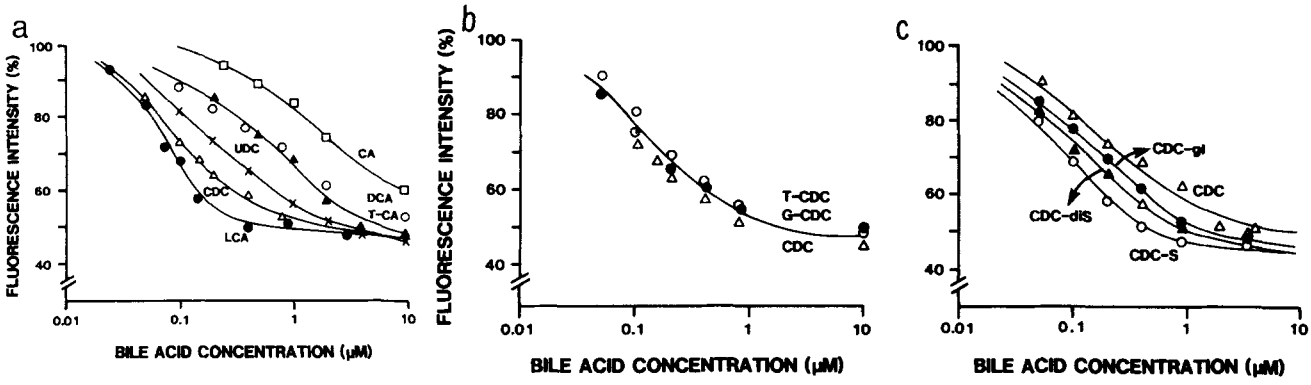

Fig. 3. Inhibition of the fluorescence of ANS (15 μM) bound to human bile acid binder (0.1 μM) by various bile acids (a) and various conjugates of chenodeoxycholic acid (b, c). The experimental condition and bile acids used were the same as Fig. 2 except for the addition of chenodeoxycholic acid-disulfate (CDC-diS).

in gel filtration of human cytosol (not shown). The influence of the number of hydroxyl groups of the bile acids on the inhibition of ANS binding was similar to that seen with the cationic transferase (Fig. 3a), while the state of conjugation of the bile acids had little effect on binding affinity (Fig. 3b). The excellent resolution of the technique is evident in the clear separation of displacement curves in Fig. 3 for lithocholic acid (K_d 0.03 μM), chenodeoxycholic acid (K_d 0.08 μM), and ursodeoxycholic acid (K_d 0.15 μM). These results were highly reproducible (<5% variation). However, smaller relative differences in K_d as between chenodeoxycholate (K_d 0.08 μM) and glycochenodeoxycholate (K_d 0.07 μM) are probably beyond the limit of resolution of the ANS technique. The important point, however, is that the affinity for bile acids is extremely high and most mono- and dihydroxy bile acids are bound with an affinity of the same order of magnitude.

Since the ANS technique is an indirect competitive displacement method, it was important to verify the results using methods that directly determine binding. **Fig. 4** shows a Scatchard plot of binding of lithocholic acid by the human cationic transferase determined by flow dialysis. The cationic GSH S-transferase had one binding site for lithocholic acid and the dissociation constant obtained (9.0 μM) was in reasonable agreement with the value determined by the ANS technique (20 μM). The presence of GSH (2 mM) had no effect on the binding of lithocholic acid by the transferase.

In addition, where indicated in Table 2, equilibrium dialysis was employed to spot-check the results of the ANS technique. In general, the results were in good agreement. Insufficient purified material was available to determine whether endogenous bile acids remained associated with the final purified protein fraction. To address this, however, we examined the fate of [^{14}C]lithocholic acid added to the crude protein fractions and we found complete separation of the label from the proteins in chromatofocusing (not shown). Thus, it is unlikely that endogenous bile acids could remain with the purified protein.

We considered the possibility that GSH S-transferase might have lost high affinity binding during purification. Therefore, we performed equilibrium dialysis with the crude GSH S-transferase activity peak from gel filtration of cytosol using 2-3 mg/ml of protein and 0.1 to 20 μM [^{14}C]lithocholic acid. No high affinity binding (i.e., K_d < 5 μM) greater than with purified protein could be detected. Therefore, it is unlikely that high affinity binding was lost during purification.

The human bile acid binder exhibits affinity for bile acids 3-5 orders of magnitude greater than does the major human GSH S-transferase. This difference contrasts with

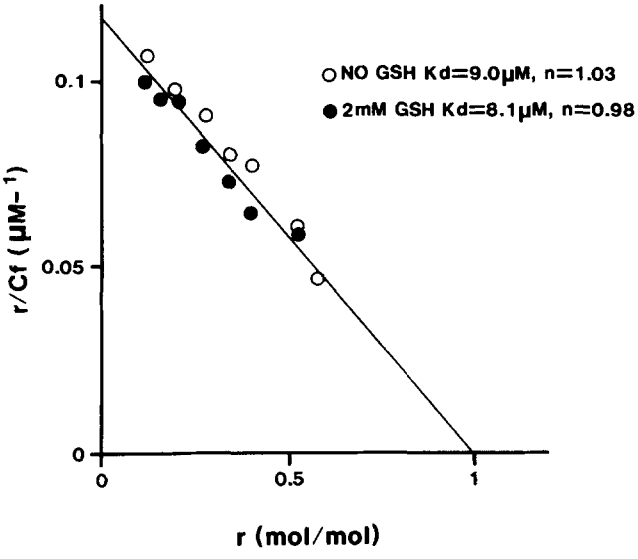

Fig. 4. Scatchard plot of the binding of lithocholic acid by human cationic GSH S-transferase determined by flow dialysis in the absence and presence of 2 mM GSH. Protein concentration was 16.5 μM and the concentration of lithocholic acid was varied (3.3-23.3 μM) by titration of unlabeled lithocholic acid into the upper chamber containing [^{14}C]lithocholic acid. The lower chamber was eluted with 0.01 M sodium phosphate buffer, pH 7.4 (30 ml/hr) at room temperature and eluate was collected in 2-ml aliquots and radioactivity was measured.

results of studies in the rat. Rat bile acid binder and rat ligandin exhibit comparable affinities for bile acids. The human bile acid binder, however, has markedly greater affinity for bile acids compared to either rat protein (Table 2). On the other hand, human ligandin has much lower affinity for bile acids than rat ligandin. Thus, the human bile acid binder even exhibits relatively high affinities ($K_d \sim 1 \times 10^{-6}$ M) for di- and trihydroxy bile acids which are bound relatively poorly by the cytosolic proteins in rat liver. In view of the remarkably high affinity of the human bile acid binder for all bile acids, an important physiologic role for this protein in intracellular transfer and detoxication of bile acids seems likely. This hypothesis assumes that, analogous to the rat, these proteins are predominantly localized in parenchymal liver cells in man. This will require future validation.

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