Identification and purification of a 36 kDa bile acid binder in human hepatic cytosol

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We recently purified two closely related 33 kDa proteins from rat hepatic cytosol, designated bile acid binder I and II, which selectively bind bile acids with comparable affinity as glutathione *S*-transferase B. This work has now been extended to human liver in which we have identified a similar cytosolic binding activity in the 30–40 kDa fraction from gel filtration. Subsequent chromatofocusing and hydroxyapatite chromatography resulted in the isolation of a homogenous monomeric protein of 36 kDa. The binding affinity of this protein for lithocholate using the displacement of 1-anilino-8-naphthalenesulfonate (ANS) was 0.1 μM, whereas human hepatic glutathione *S*-transferases purified from glutathione affinity chromatography demonstrated no competitive displacement of ANS.

GSH S-transferase *Ligandin* *Bile acid binder* *Protein purification* *Human liver*

1. INTRODUCTION

The liver efficiently extracts 70–90% of the bile acid pool present during a single pass through the liver [1]. Although recent progress has been made in elucidating the mechanism of hepatic bile acid uptake [2] and canalicular excretion [3], little is known about the process of intracellular translocation of bile acids. As a first step in understanding intracellular bile acid transport, we sought to identify cytoplasmic proteins which specifically bind bile acids. Previously, it has been widely held that the glutathione *S*-transferases were the predominate cytoplasmic bile acid binding proteins [4–6]. In the rat, we recently isolated a newly recognized pair of similar 33 kDa bile acid binding proteins (bile acid binders I and II), initially recognized as lithocholate binding activity in the 30–40 kDa fractions from gel filtration [7,8]. These proteins bind most lipophilic bile acids with comparable or greater affinity than glutathione *S*-transferase B. Since little is known about human hepatic bile acid binding proteins, it was of interest to determine if a similar binding activity exists in the 30–40 kDa fractions from gel filtration of human hepatic cytosol and to purify the protein responsible for this activity.

2. MATERIALS AND METHODS

2.1. *Preparation of cytosol*

Normal human liver was obtained during surgical lobectomy for an isolated metastasis from colon carcinoma. Tissue was homogenized (33%, w/v) in 0.01 M sodium phosphate, pH 7.4 (Buffer A) with 0.25 M sucrose. The supernatant (cytosol) was harvested after centrifugation at 100000 × *g* for 60 min.

2.2. *Lithocholate binding activity*

Lithocholate binding was determined by equilibrium dialysis of individual column fractions against 2 l buffer A with 25 nmol [^{14}C]lithocholate (3 μCi) (59 Ci/mmol; Amersham) at 4°C for 48 h using Spectrapor Membrane (12–14 kDa exclusion) (Spectrum Medical Industries, Los Angeles, CA). The ratio of bound over free lithocholate for individual fractions was calculated by the dif-

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ference of total dpm minus free dpm divided by free dpm [7].

2.3. *Gel filtration of hepatic cytosol*

Human hepatic cytosol (8 ml) was chromatographed on a 2.5 × 120 cm Sephadex G-75 superfine (Pharmacia) column eluted with buffer A at 4°C and fractions were assayed for A_{280}, glutathione (GSH) *S*-transferase activity by monitoring the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) [9] and lithocholate binding activity.

2.4. *Purification of lithocholate binding protein*

Human hepatic cytosol (150 ml) was fractionated on a 5 × 100 cm Sephadex G-75 sf column eluted with buffer A. The 30–40 kDa fractions containing lithocholate binding activity were pooled, concentrated by ultrafiltration and applied to a 1 × 50 cm column of Polybuffer Exchanger 94 (Pharmacia) equilibrated with 0.025 M imidazole, pH 7.4, and eluted with a 1:8 dilution of polybuffer 74 (adjusted to pH 4 with HCl) at a flow rate of 24 ml/h. Individual fractions (3.5 ml) were assayed for A_{280}, GSH *S*-transferase activity, lithocholate binding and pH. Fractions containing lithocholate binding activity from chromatofocusing were pooled, adjusted to pH 6.7 with dilute phosphoric acid and applied to a 0.9 × 27 cm hydroxyapatite column (HA-Ultrogel, LKB) equilibrated with 0.01 M potassium phosphate, pH 6.7 (buffer B). Following elution with 40 ml buffer B, the column was eluted with a linear gradient of 0.025 M potassium phosphate and 0.25 M potassium phosphate pH 6.7 (130 ml each). Fractions were assayed for A_{280}, lithocholate binding activity and conductivity.

2.5. *Purification of human GSH* S-*transferases*

Fractions from preparative gel filtration containing GSH *S*-transferase activity were pooled and purified by GSH affinity chromatography using *S*-octylglutathione coupled to epoxy activated Sepharose 6B (Pharmacia) [10]. The product was a single band (25 kDa) on SDS–polyacrylamide gel electrophoresis (PAGE) and represents a mixture of human transferases.

2.6. *SDS–PAGE*

Discontinuous SDS–PAGE was performed according to [11]. 10 μl of Sigma VII-L molecular mass standards were used for calibration of the gel, which was stained with Coomassie brilliant blue.

2.7. *Amino acid composition*

Purified protein (80 μg) was hydrolyzed with 2 ml of 12 N HCl in sealed flamed flasks at 110°C for 24 h and analyzed on a Beckman 6300 HPLC amino acid analyzer using Beckman buffers A, B, and D.

2.8. *Binding affinity*

Binding affinity of both the purified human bile acid binder and the glutathione *S*-transferase was determined by the competitive displacement of the fluorescent probe, 1-anilino-8-naphthalenesulfonate (ANS). The fluorescence of bound ANS was determined at excitation 400 nm and emission 480 nm. To determine the affinity of both proteins for ANS, each protein was studied at 0.5 μM along with increasing concentrations from 0 to 40 μM ANS. For inhibition studies, ANS concentrations were varied in the absence or presence of lithocholate (1 μM) for bile acid binder and (2 μM) for the GSH *S*-transferases. Data were analyzed by the method of [7].

3. RESULTS AND DISCUSSION

The elution profile of lithocholate binding activity in gel filtration of human hepatic cytosol is shown in fig.1. Lithocholate binding activity eluted as two peaks: a minor peak corresponding to the GSH *S*-transferase (45–50 kDa) and a major peak corresponding to 30–40 kDa. The elution of this major peak of lithocholate binding activity with human cytosol corresponds almost exactly to the elution of the recently identified lithocholate binding activity in rat cytosol. In contrast to the results with human cytosol, the lithocholate binding activity (as determined by equilibrium dialysis) in the rat corresponding to the GSH *S*-transferases containing fractions greatly exceeded that of the 30–40 kDa fractions (not shown). Since we have previously shown that the rat GSH *S*-transferases and the newly identified bile acid binders bind lithocholate with about the same affinity, the greater binding activity of the rat transferase frac-

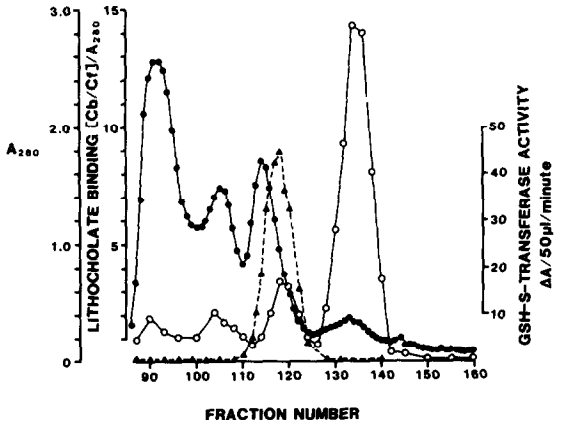

Fig.1. Gel chromatography (Sephadex G-75 sf) of human hepatic cytosol (8 ml). Fractions were assayed for A_{280} (●—●), GSH *S*-transferase activity (▲---▲), and lithocholate binding (○—○).

tion in gel filtration is probably due to its greater cytoplasmic content. The contrary result with human cytosol (fig.1) suggests either a higher binding affinity and/or greater binding capacity for lithocholate by the newly identified human bile acid binder.

In order to isolate the lithocholate binding protein, a large pool of human hepatic cytosol (150 ml) was fractionated on a preparative Sephadex G-75 sf column which gave the same separation as shown in fig.1 (not shown). The fractions with peak lithocholate binding activity were pooled, concentrated and separated by chromatofocusing (fig.2). The majority of the lithocholate binding activity eluted in a single peak at pH 7.32. Nearly identical results were obtained with another human liver (not shown). Other minor lithocholate binding peaks eluted at both higher and lower pH and were not further characterized. The bulk of GSH *S*-transferase activity eluted prior to the pH gradient and represents the cationic enzymes. The major lithocholate binding peak did not exhibit GSH *S*-transferase activity. Fractions 44–52 from chromatofocusing were pooled, adjusted to pH 6.7 and further purified by hydroxyapatite chromatography. The lithocholate binding activity co-eluted with the major protein peak (fig.3).

Fig.4 shows the SDS–PAGE of the sequential steps in the purification of the lithocholate binder. The lithocholate binding activity peak from hydroxyapatite was a single band indicating that it is a monomeric protein of 36 kDa. The amino acid composition of the purified human lithocholate binder is shown in table 1. Aside from the higher valine and isoleucine content, the human protein shows remarkable similarity to the rat binder [7].

Lithocholate binding affinity of both the purified human GSH *S*-transferases and the bile acid binder were determined by the ANS displace-

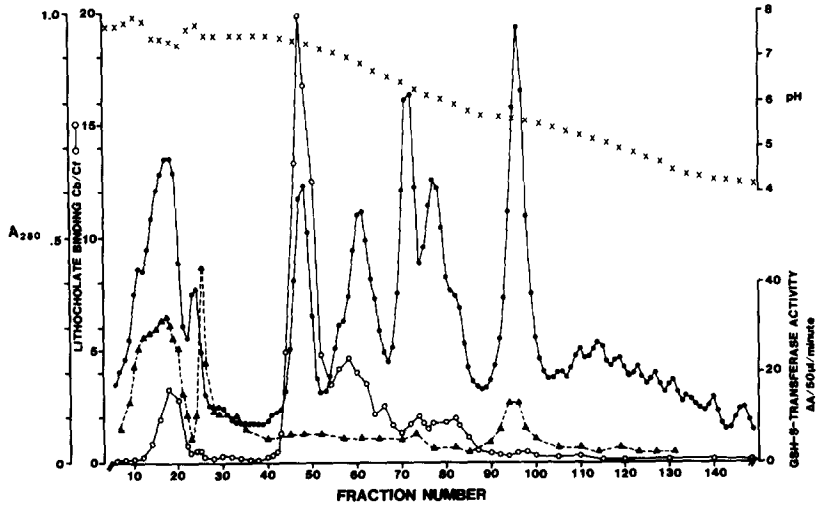

Fig.2. Chromatofocusing of the lithocholate binding activity from 30 to 40 kDa from preparative gel filtration. Fractions were assayed for A_{280} (●—●), GSH *S*-transferase activity (▲---▲), lithocholate binding (○—○), and pH (×).

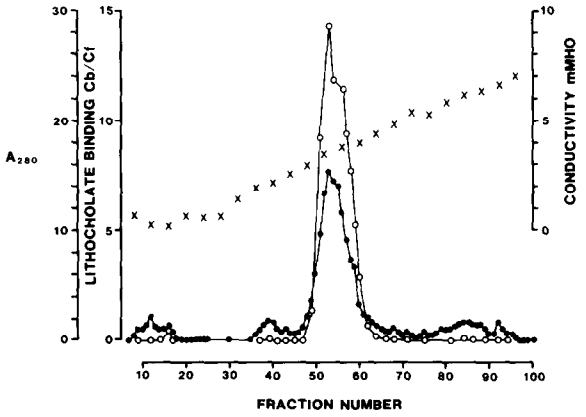

Fig.3. Hydroxyapatite chromatography of major lithocholate binding activity from chromatofocusing. Fractions were assayed for A_{280} (●—●), lithocholate binding (○—○) and conductivity (×).

ment method. The K_d for ANS binding was 13 μM for the bile acid binder and 16 μM for GSH *S*-transferase. Lithocholate competitively inhibited

Table 1
Amino acid composition of human bile acid binder

Amino acid	Human binder
Aspartic acid	35.3
Threonine[a]	10.4
Serine[a]	19.6
Glutamic acid	35.4
Proline[b]	19.0
Glycine	20.0
Alanine	26.0
Valine[c]	26.4
Methionine[b]	3.4
Isoleucine[c]	15.4
Leucine[c]	35.2
Tyrosine[b]	11.6
Phenylalanine	12.9
Histidine	8.9
Lysine	25.7
Arginine	14.4

[a] Extrapolated to zero time from 24- and 72-h incubation
[b] 24-h incubation result
[c] 72-h incubation result

Results are expressed as amino acid analysis of mol amino acid/mol protein (mean of 4 determinations; two incubated for 24 h and two for 72 h)

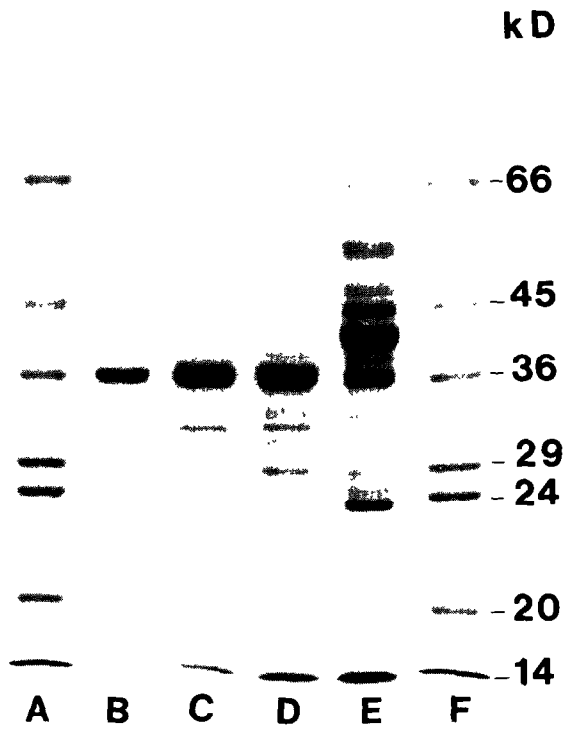

Fig.4. Lanes A and F, molecular mass standards; lane B, 6–8 μg protein from the pooled fractions with lithocholate binding activity from hydroxyapatite chromatography; lane C, 15 μg proteins from the pooled major lithocholate binding peak from chromatofocusing; lane D, 20 μg protein from pooled 30–40 kDa fractions from gel filtration; lane E, 30 μg hepatic cytosolic protein.

the ANS binding bile acid binder (not shown). The K_i for lithocholate was 0.1 μM with the bile acid binder. No inhibition of ANS binding to GSH *S*-transferase was detected. These data, coupled with the greater lithocholate binding activity of the 30–40 kDa fractions from gel filtration of hepatic cytosol (fig.1), suggest that the human bile acid binder which we have identified binds lithocholate with markedly greater affinity than human GSH *S*-transferase. Further work will be required to assess the relative binding of various bile acids using several techniques to determine the binding affinities and specificities of these proteins.

In summary, we have identified and purified a unique lithocholate binder in human hepatic cytosol which is similar to the forms we have

previously found in the rat. The precise role of this binder in intracellular bile acid transport or storage remains to be determined.

ACKNOWLEDGEMENTS

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