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CHOLIC ACID BINDING BY ANIONIC GLUTATHIONE-S-TRANSFERASE FROM HUMAN LIVER CYTOSOL

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SUMMARY: An anionic glutathione-S-transferase from human liver cytosol has been purified on the basis of its affinity for the primary bile acid, cholic acid. It represents 18% of the total 1-chloro-2,4-dinitrobenzene conjugating activity in the liver, has an isoelectric point of 4·6 and is made up of two identical subunits of molecular weight 23,000. The enzymes bile acid binding properties are compared with the cationic glutathione-S-transferases.

Maintenance of the enterohepatic circulation of bile acids requires the hepatic uptake of bile acids from blood, their transport across the hepatocyte and secretion into bile. In rat, intrahepatic transport involves cytosol binding proteins. These proteins have been shown to belong to the group of enzymes, the GSH-S-transferases (1,2). There are seven such enzymes in rat liver and while they exhibit overlapping substrate specificities they can be readily separated on the basis of their district binding affinities for both substrate and non-substrate ligands (3). Multiple forms of GSH-S-transferases are also found in human liver cytosol (4). However, unlike the rat enzymes which are all cationic proteins, the GSH-S-transferases in human liver can be fractionated into anionic as well as cationic proteins (5). Because of the possible physiological and clinical significance of this intracellular transport event it was decided to look at the question of the human GSH-S-transferases as bile acid binding proteins.

ABBREVIATIONS

CDNB	1-chloro-2,4-dinitrobenzene
GSH	Glutathione
SDS	Sodium dodecyl sulphate
AH	aminohexylamino

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MATERIALS AND METHODS

Cholic acid-AH-Sepharose 4B was synthesised as described in (6). Human liver cytosol was prepared as previously described (1). The 105,000xg supernatant (~40 ml) was dialysed against 10 mM Tris HCl pH 8·0; 1mM EDTA and passed through a DEAE-Cellulose 52 column (2·5 × 20 cm) pre-equilibrated with the same buffer. The column was washed with the starting buffer until GSH-S-transferase activity ceased to appear in the effluent. Anionic GHS-S-transferase activity which was retained by the column was eluted with a linear salt gradient (0-200 mM). The salt eluted peak of catalytic activity was dialysed against 10 mM phosphate buffer pH 7·4 before being applied to the cholic acid-AH-Sepharose 4B affinity column (1 × 20 cm). Specifically bound protein was competitively eluted with 5 mM cholic acid pH 7·4. The eluent was dialysed against phosphate buffer and applied to an antialbumin column. The enzyme activity was eluted by washing with buffer. The cationic enzymes were further fractionated by application to a CM-Sephadex column pH 6·7 (2·5 × 20 cm). After washing with 70 ml of 10 mM phsophate buffer a linear salt gradient was established (0-100 mM). Two peaks of enzyme activity were resolved. These were dialysed against 10 mM phosphate buffer pH 7·4 before being applied to the affinity column. No catalytic activity was bound by the matrix. All column procedures were carried out at 20°C. SDS discontinuous gel electrophoresis was performed on slab gels by the method of Maizel (8). Analytical isoelectric focusing in polyacrylamide disc gels was performed as described by Wrigley (10) in the ampholine range of 3-7. After focusing, half the gel was fixed in trichloroacetic acid and stained with Coomassie Blue. The other half

Table 1. Purification of glutathione-S-transferases from human liver

Fraction	Total protein (mg)	Total activity (μmole/min)	Specific activity[a] (μmole/min/mg)	Yield (%)
Liver cytosol	580	187	0·32	-
Anionic enzyme				
DEAE-Cellulose	37	37	1·00	(100)
Cholic acid-AH-Sepharose 4B	6·8	31	4·6	84
Antialbumin	2·8	20	7·2	54
Cationic enzyme				
DEAE-Cellulose CM-Sephadex	95	159	1·67	(100)
FI	30	70	2·6	50
FII	12	55	4·6	35
Cholic acid-AH-Sepharose 4B	0	0	0	-

[a] Activity measured @ 20°C and pH 6·5 using 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH as substrates.

was cut into 0·25 cm sections, eluted with distilled H_2O overnight, and the pH measured. GSH-S-transferase activity was measured under standard assay conditions (7). The kinetics of the inhibition of GSH-S-transferase activity by bile acids was determined by the method of Lineweaver and Burk (11), and Dixon (12). Amino acid analysis was performed by hydrolysis of protein samples (~300 μg) with constant boiling HCl in evacuated ampoules for 24 and 72 hr. Analysis was carried out in a Dionex Kit AA analyser.

RESULTS

Summary of the results of purification of both anionic and cationic forms of the GSH-S-transferases is presented in Table 1. The two major forms are separated on DEAE Cellulose (Fig. 1). The anionic form which represented 18% of total CDNB conjugating activity was further purified by its specific affinity for the cholic acid-AH-Sepharose 4B affinity matrix. Human serum albumin also bound to the matrix. This was removed by passage through an antialbumin column. The cationic GSH-S-transferases were separated on CM Sephadex into two fractions (designated I and II). It is not known which of the human GSH-S-transferases (named α,β,γ,δ,ε) they represent (4), but neither of these fractions bound to the affinity matrix. Because of the similar kinetic properties between the cationic enzymes (4) the binding studies were performed on a mixture of both these fractions.

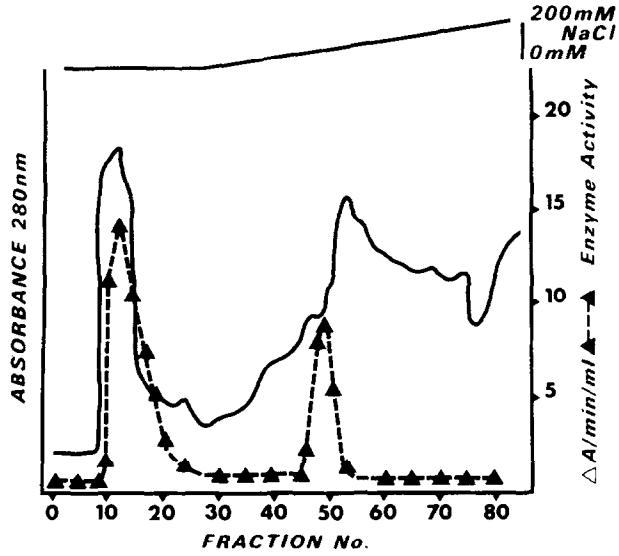

Fig. 1 Separation of glutathione-S-transferases by chromatography on a DEAE-Cellulose column eluted with a linear salt gradient (0-200 mM). Two peaks of enzyme activity were measured. The first peak contained the cationic enzymes and the second peak the anionic enzyme.

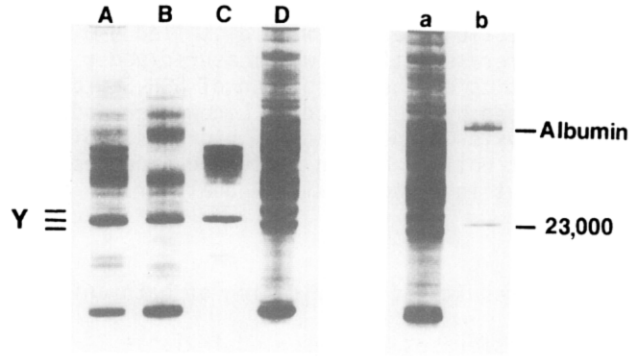

Fig. 2 SDS discontinuous slab polyacrylamide electrophoresis of: A, Whole human liver cytosol; B, Crude anionic enzyme eluted from the DEAE-Cellulose column; C, Crude cationic enzyme eluted from the DEAE-Cellulose column; D, Female rat liver cytosol; a, female rat liver cytosol; b, affinity chromatography purified anionic GSH-S-transferase. The three subunits of rat liver cytosol Y fraction are indicated.

SDS discontinuous electrophoresis confirmed the homogeneity of the anionic enzyme and indicated that it was composed of two identical subunits of molecular weight 23,000. Unlike the Y fraction of rat liver cytosol which resolved into three bands, the Y fraction of human liver cytosol ran as a single band. Both the cationic and anionic enzymes are made up of subunits of molecular weight 23,000.

Isoelectric focusing of the anionic GSH-S-transferase in the pH range 3-7 resulted in a single band of pI 4·6 $\pm$ 0·05 (Fig. 2).

The amino acid composition of the anionic GSH-S-transferase is given in Table 3. It is significantly different from those of the cationic GSH-S-transferases (α-ε) of human liver reported by Kamisaka *et al.* (4). However, it is almost identical to the anionic GSH-S-transferase reported by Awasthi *et al.* (5) except that its glycine content is considerably lower and it contains methionine.

Both the anionic and cationic enzymes were examined for inhibition of their catalytic activity by bile acids. As would be predicted from the results of the affinity chromatography step, cholic acid inhibited only the anionic enzyme. On the other hand, both the anionic and cationic enzymes were inhibited by chenodeoxycholate. The K_i in both instances were identical.

Table 2. Inhibition by bile acids of hepatic glutathione-S-transferases

Bile acid	Glutathione-S-transferase K_i ($\times 10^{-3}$M)[a]	
	Anionic	Cationic
Cholic acid	0·17	No inhibition
Chenodeoxycholic acid	0·12	0·12
Sulfolithocholic acid	0·03	0·006
	K_m CDNB	K_m CDNB
	$0{\cdot}4 \times 10^{-3}$M	$1{\cdot}0 \times 10^{-3}$M

[a] Each individual value represents the mean of triplicate determinations.

While sulfolithocholate was the most effective inhibitor of both GSH-S-transferases the K_i for the cationic enzyme was five times greater than the anionic enzyme (Table 2). Conjugates of both primary bile acids were not as effective in inhibiting catalytic activity as their unconjugated forms. Bile acid inhibition in all instances was partially-competitive. Bilirubin strongly inhibited the catalytic activity of the cationic enzymes (K_i; 10 μM) but had no effect on the activity of the anionic GSH-S-transferase at concentrations as high as 200 μM.

DISCUSSION

The isolation of the anionic GSH-S-transferase from human liver is based on its specific affinity for the cholic acid-AH-Sepharose 4B matrix. The affinity matrix has previously been used in the isolation of GSH-S-transferase A and C from rat liver cytosol (1,9).

The abundance, pI and amino acid composition of the homogeneous anionic human transferase suggests that it corresponds to GSH-S-transferase ω described recently by Awasthi and colleagues (5). Unlike the rat enzymes which are made up of dimeric combinations of any of three subunits no heterogeneity of the

Table 3. Amino acid composition of human liver anionic GSH-S-transferase

	Amino acid composition (No. of residues/molecule of mol. wt. 46,000)
Aspartic acid	44.6 ± 3.4
Threonine	19.2
Serine	26.8
Glutamic acid	52.8 ± 2.3
Proline	30.3 ± 3.9
Glycine	31.6 ± 2.5
Alanine	34.1 ± 2.3
Valine	29.1
Methionine	7.8 ± 1.3
Isoleucine	21.9
Leucine	43.4 ± 2.6
Tyrosine	14.2 ± 1.3
Phenylalanine	19.2 ± 3.5
Lysine	27.3 ± 4.1
Histidine	10.2 ± 1.3
Arginine	15.2 ± 3.7

Values are the mean of 8 analyses ± SD. Samples were hydrolysed for 24 and 72 h.

subunits for human GSH-S-transferases is apparent. Both the cationic and anionic GSH-S-transferases are made up of subunits of mol. wt. 23,000. However, the amino acid composition of the anionic GSH-S-transferase is considerably different from the published data for the cationic enzymes, which suggests that it is the product of a distinct gene.

The narrow substrate specificity of GSH-S-transferase ω had led the authors who first isolated the enzyme to pose the question of its exact functional role. This study suggests that one of the major physiological roles of the enzyme is the binding and transport of the most abundent primary

bile acid, cholic acid. Interestingly, while the cationic enzymes did not bind cholic acid they bound chenodeoxycholate with an identical affinity to the anionic enzyme and sulfolithocholate with an affinity five times that for the anionic enzyme. Bilirubin was not bound by the anionic GSH-S-transferase. Thus a functional differentiation exists between the anionic and cationic GSH-S-transferases. A similar differentiation is seen with the rat GSH-S-transferase enzymes in which cholic acid is specifically bound by transferases A and C (1,9) while lithocholate is most strongly bound by ligandin and transferase B (2). The exact physiological implications of this binding heterogeneity are still to be determined.

The binding data for the bile acids give an idea of the nature of the binding site. The decreasing inhibition with the more hydrophilic bile acids suggests it has an important hydrophobic component and since the inhibition is partially-competitive, the binding side is probably separate from the substrate binding site but sufficiently close to effect substrate affinity.

In conclusion, the results show a significant difference in the amino acid composition and ligand binding properties between the anionic and cationic GSH-S-transferases and suggest an important role for the anionic enzyme in the intrahepatic transport of cholic acid.

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REFERENCES

1. Pattinson, N.R. (1981) Biochim. Biophys. Acta 667, 70-76.
2. Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1979) Biochem. J. 181, 699-708.
3. Jakoby, W.B. (1978) Adv. Enzymol. 46, 383-414.
4. Kamisaka, K., Habig, W.H., Ketley, J.N., Arias, I.M. and Jakoby, W.B. (1975) Eur. J. Biochem. 60, 153-161.
5. Awasthi, Y.C., Dao, D.D. and Saneto, R.P. (1980) Biochem. J. 191, 1-10.
6. Pattinson, N., Collins, D. and Campbell, B. (1980) J. Chrom. 187, 409-412.
7. Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) J. Biol. Chem. 249, 7130-7139.

8. Maizel, J.V. (1971) In "Methods in Virology" (R. Maramorosch and H. Kroprowsky, eds.), Vol. 5, pp 179-246, Academic Press, New York.
9. Pattinson, N.R. (1981) Anal. Biochem. (In Press)
10. Wrigley, C.W. (1972) Methods Enzymol. 22, 559-564.
11. Lineweaver, H. and Burk, P. (1934) J. Am. Chem. Soc. 56, 658-666.
12. Dixon, M. (1953) Biochem. J. 55, 170-171.