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ISOLATION OF AN ORGANIC ANION BINDING PROTEIN FROM RAT LIVER PLASMA MEMBRANE FRACTIONS BY AFFINITY CHROMATOGRAPHY

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SUMMARY

As part of a study of hepatic organic anion transport, solubilized liver plasma membrane proteins were subjected to affinity chromatography on bilirubin- and sulfobromophthalein-labeled agarose columns. Both columns retained a Sudan Black and PAS negative protein of molecular weight 60,000 daltons, which cochromatographed with [^{35}S]sulfobromophthalein on Sephadex G-75, and reversibly bound [^{35}S]sulfobromophthalein in vitro with high affinity ($K_a \simeq 10^7\ M^{-1}$) and a valence of 2. Erythrocyte ghost membranes did not contain this protein. Sulfobromophthalein-agarose retained two additional smaller proteins which did not cochromatograph with [^{35}S]sulfobromophthalein. Their significance is unclear. This study supports the hypothesis that liver cell plasma membranes participate in the hepatic transport of organic anions.

INTRODUCTION

The mechanism by which low molecular weight organic anions such as bilirubin and BSP* cross the liver cell plasma membrane remains uncertain. The kinetics of hepatic uptake of these two compounds both in vivo (1) and in the isolated perfused rat liver (2) is suggestive of specific membrane-located carrier mediated transport, as is the recent demonstration of saturable binding sites with a high affinity for BSP on rat liver plasma membrane fractions (3). Moreover, a protein which binds BSP has been extracted from a crude liver plasma membrane preparation (4).

In an attempt to better document the presence of specific bilirubin and BSP-binding proteins in liver plasma membranes, membrane fractions enriched in either sinusoidal or canalicular components were prepared, their proteins solubilized, and the resulting protein mixture subjected to affinity chromatography on agarose columns to which either bilirubin or BSP had been covalently bound. The results of this investigation suggest that specific organic anion binding proteins exist in liver plasma membranes. This, in turn, supports the hypothesis that organic anions cross liver cell plasma membranes by a membrane-associated carrier mediated process.

Abbreviation used: BSP, sulfobromophthalein

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METHODS

Preparation of liver plasma membrane fractions: Previously described modifications (3) of established methods were used to prepare and characterize liver plasma membranes enriched in canalicular (5) and sinusoidal (6) components. Sixty to 120 g. of liver from male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were processed for each experiment. Rat erythrocyte ghosts (7), subjected to the same experimental procedures, served as a control membrane preparation.

Solubilization and detergent removal: Membrane proteins were solubilized by gentle magnetic stirring for 1 hr at 2°C with 1% (w/v) Triton X-100, at a ratio of 1 ml detergent per 1 mg membrane protein. After centrifugation at 100,000 x g for 1 h, excess detergent in the supernatant was removed by chromatography on a 200 ml column of Bio Beads SM-2 (BioRad, New York, NY), at a flow rate of 20 ml/h.

Preparation of affinity chromatography columns: After coupling hexanediamine (Aldrich Chemical Co.) to CNBr activated agarose 4B (Pharmacia), unconjugated bilirubin was covalently linked to the free amino groups, as described by Hierowsky and Broderson (8). The resulting gel contained 11 ± 3 μmol/ml free amino groups and 2.1 ± 0.3 μmol/ml bilirubin (8). After extensive washing in 99% (v/v) and, subsequently, 50% (v/v) dimethylformamide, the bilirubin-agarose was stored in the latter solvent at 2°C in the dark for up to six weeks.

BSP was coupled to agarose 6B by the epichlorhydrin reaction (9). The washed gel was stored in 0.02 M sodium phosphate buffer, pH 7.5, containing 0.2% (w/v) sodium azide, for up to 2 months. The BSP content of the gel, assayed from the E_{580} of an alkaline agarase digest, averaged 3.6 ± 0.2 μmol/ml gel.

Affinity chromatography: Prior to use, 10 ml columns of either bilirubin- or BSP linked agarose were equilibrated for 24 h at 2°C with $NaHCO_3$ 1 mM pH 7.6. Solubilized proteins from 30-50 mg membrane fractions were then passed over the columns at a flow rate of 10 ml/h, and the effluent monitored for protein at 280 nm. The columns were washed with NaCl 0.15 M, buffered with sodium phosphate 0.02 M pH 7.6, until no absorption at 280 nm was detectable. Thereafter, the column was eluted with either 1 M KI, 9 M urea or 50 μM free ligand. All protein-containing washes and eluates were concentrated by ultrafiltration to a protein content of 1 mg/ml, using Nuclepore PSED membranes (Millipore Filter Corp., Bedford, MS) with a nominal molecular weight cut-off of 10,000 daltons.

Protein assay and polyacrylamide gel electrophoresis: Protein was assayed by a micromodification of the Coomassie Blue method (10), chosen because it is not influenced by detergents such as Triton X-100 (10). Absence of albumin and ligandin in the membrane fractions was documented by double radial immunodiffusion, using antibodies against rat albumin (Cappel Laboratories) and rat liver glutathione transferase B (a kind gift of Dr. William B. Jakoby). Membrane proteins and affinity column eluates were further characterized by SDS polyacrylamide gel electrophoresis after complete reduction with mercaptoethanol and dithiothreitol (11). The proteins were stained for lipid content and PAS-reactive components by the Sudan Black and PAS methods, respectively (12).

Organic anion binding properties of the observed proteins: The properties of a specific 60,000 dalton protein retained by both BSP- and bilirubin-agarose were further examined by cochromatography of the purified proteins with [^{35}S]BSP (Amersham-Searle Corp., Arlington Heights, IL; Sp.A. 112 mCi/mmol) on Sephadex G-75 (Pharmacia). Effluents, in 1 ml fractions, were monitored for protein at 280 nm, and for ^{35}S content by liquid scintillation spectrometry (3). In an attempt to quantitate BSP binding to these proteins, 200 μg aliquots of protein, isolated from bilirubin-agarose, were incubated in 1 mM $NaHCO_3$ buffer, pH 7.6, containing various concentrations of [^{35}S]BSP (10^{-9} to 10^{-5} M). The reaction mixture was ultra-filtered in a 1.5 ml cell using

Nuclepore PSED membranes (Millepore). The dry membranes were disolved in methoxyethanol and counted for ^{35}S radioactivity (3). Appropriate filter blanks, treated in the same way with [^{35}S]BSP in the absence of protein, were subtracted, and the amount of BSP bound at each initial BSP concentration calculated (3). The binding constant of BSP for the protein was estimated graphically from a Scatchard plot of the data.

RESULTS

In 20 experiments, the protein content of whole liver homogenates averaged 176 ± 22 (SD) mg/g liver, of which 0.28 ± 0.07 and 0.68 ± 0.09 mg protein/g liver, respectively, were recovered in the sinusoidal and canalicular-enriched membrane fractions. The sinusoidal fraction was enriched seven-fold compared to the canalicular fraction in glucagon-stimulated adenylate cyclase (0.012 ± 0.001 vs. 0.002 ± 0.001 μmol/h/mg protein), whereas the canalicular fraction was enriched five-fold over the sinusoidal fraction in 5' nucleotidase (93.2 ± 6.4 vs. 17.4 ± 2.7 μmol/h/mg protein) (3). Neither fraction contained either albumin or ligandin detectable by radial immunodiffusion against appropriate antibodies.

Of the total protein in the two membrane fractions, approximately 87% was recovered after solubilization and 75% after detergent removal on Bio Beads SM-2. However, only 0.3 ± 0.1 and 0.2 ± 0.1% of the protein in the sinusoidal and canalicular fractions, respectively, was retained by bilirubin-agarose after extensive washing. For BSP-agarose, the corresponding figures were 0.4 ± 0.2 and 0.6 ± 0.2%. These retained proteins were readily eluted with either free ligand (bilirubin or BSP, 50 μM), 9 M urea, or 1 M KI. They showed no immuno-reactivity by radial immunodiffusion against either anti-ligandin or anti-rat albumin antibodies.

SDS polyacrylamide gel electrophoretic patterns from the original membrane fractions, the solubilized proteins therefrom, and the eluates from the Bio Bead SM-2 columns revealed multiple bands, with no change in protein composition (i.e., loss of bands) during solubilization or Bio Bead SM-2 chromatography. In contrast, the final eluates from the bilirubin-agarose columns contained, after reduction, only a single PAS and Sudan Black negative protein band of estimated molecular weight 60,000 daltons which was present in both sinusoidal and canalicular membrane fractions. In additional experiments, the un-reduced protein eluted from bilirubin-agarose had an estimated molecular weight of 55,000 by gel filtration of Sephadex G-75, and of 58,000 by Triton

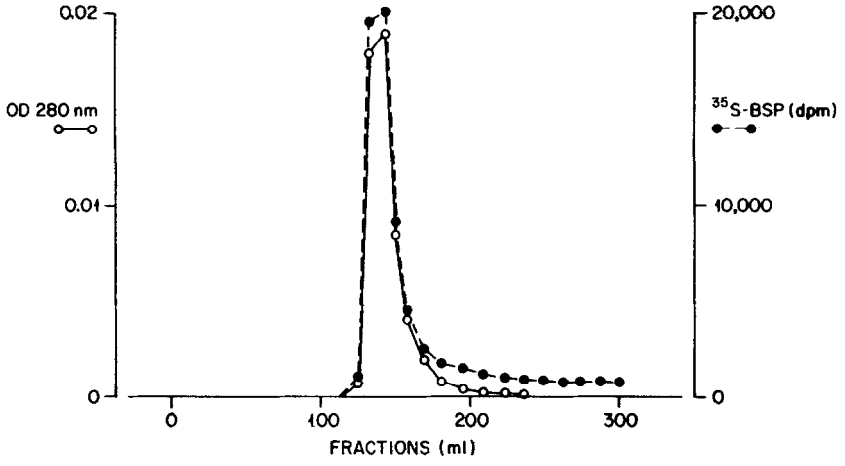

Figure 1: Cochromatography of the 60,000 dalton binding protein with [^{35}S]BSP on a 300 ml Sephadex G-75 column. Flow rate was 21 ml/hr. For the experiment shown, the protein was obtained from a canalicular-enriched liver plasma membrane fraction by affinity chromatography on bilirubin-agarose.

X-100 polyacrylamide gel electrophoresis (12). It cochromatographed on Sephadex G-75 with [^{35}S]BSP (Figure 1), and, in binding studies, bound BSP with an estimated association constant of 10^7 M^{-1} and a maximal binding capacity of 2 moles BSP/mole protein (Table 1).

Table 1

Results of in vitro binding studies. 1 ml aliquots of various concentrations of [^{35}S]BSP were incubated with 200 µg of the 60,000 dalton protein isolated by affinity chromatography on bilirubin-agarose. The column "Percent of Predicted" was obtained by calculating the theoretical concentration of [^{35}S]BSP bound at each concentration of free BSP assuming an association constant of 10^7 M^{-1} and a maximal binding capacity of 2. These estimates were obtained graphically from a Scatchard plot of the observed values.

Free [^{35}S]BSP Concentration (M)	DPM Bound	Bound [^{35}S]BSP Concentration (moles BSP/mole protein)	Percent of Predicted
10^{-5}	771	1.55	94
10^{-6}	693	1.40	92
3×10^{-7}	522	1.05	84
10^{-7}	398	0.80	96
3×10^{-8}	214	0.43	112
10^{-8}	92	0.19	122
10^{-9}	10	0.03	139

SDS polyacrylamide gel electrophoresis of the final BSP-agarose eluates also revealed a PAS and Sudan Black negative protein with a molecular weight of 60,000, which cochromatographed on Sephadex G-75 with [^{35}S]BSP, and exhibited identical electrophoretic properties under various conditions with the protein retained by bilirubin-agarose. Small quantities of two additional proteins of molecular weights 40,000 and 46,000, which did not cochromatograph on Sephadex G-75 with [^{35}S]BSP, were also observed but have not yet been further characterized.

In contrast to the results with liver plasma membrane fractions, none of the solubilized proteins from erythrocyte ghosts was retained on either bilirubin or BSP-agarose. As a further control, none of the proteins from canalicular membrane fractions was retained by either native agarose or agarose-hexanediamine.

DISCUSSION

These studies demonstrate that liver plasma membrane fractions enriched in either sinusoids or canaliculi contain an organic anion binding protein consisting of a single polypeptide chain with an approximate molecular weight of 60,000 daltons. It is likely that the proteins eluted from the bilirubin- and BSP-agarose columns are the same because of their similar chromatographic behavior, absence from both of stainable lipid or polysaccaride components, and the high affinity for BSP of the protein originally identified by its affinity for bilirubin-agarose.

The affinity of these proteins for their respective ligands has many properties expected of pysiologically significant binding proteins. Thus, (a) binding was reversible with low concentrations of free ligand, whereas otherwise drastic conditions were required to elute the proteins from their affinity column binding sites; (b) their binding to BSP was reversible, with a high affinity but a low valence or capacity; (c) binding of the proteins to affinity columns did not involve non-specific interaction with either the carbohydrate or free amino group components of the columns. Moreover, the dual affinity of the proteins for both BSP and bilirubin, and their absence from membranes not involved in organic anion transport, parallel properties of the in vivo organic anion transport mechanism.

It is noteworthy that the affinity of BSP for the 60,000 dalton protein is similar to that recently reported for a low-capacity, high affinity binding site on intact liver plasma membranes (3), and exceeds its affinity for both rat albumin (13) and ligandin (14). The latter is not the case for a BSP binding protein of molecular weight 170,000 daltons recently isolated by Tiribelli and colleagues from a crude liver plasma membrane preparation (4). The reported association constant of BSP for this protein, 2.5×10^5 M^{-1}, is less than for either albumin or ligandin. However, it is possible that the protein described above is a monomer or subunit of the one reported by Tiribelli et al. (4), since the isolation procedures employed in that study did not involve detergents, or reduction prior to polyacrylamide gel electrophoresis.

The studies reported above indicate the presence in liver plasma membranes of a protein, the properties of which suggest a role in hepatic transport of organic anions. The significance of the protein in vivo remains to be established.

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