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Purification and reconstitution of the bile acid transport system from hepatocyte sinusoidal plasma membranes

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The taurocholic acid transport system from hepatocyte sinusoidal plasma membranes has been studied using proteoliposome reconstitution procedures. Membrane proteins were initially solubilized in Triton X-100. Following detergent removal, the resultant proteins were incorporated into lipid vesicles prepared from soybean phospholipids (asolectin) using sonication and freeze-thaw procedures. The resultant proteoliposomes demonstrated Na^+-dependent transport of taurocholic acid which could be inhibited by bile acids. Greatly reduced amounts of taurocholic acid were associated with the phospholipid or membrane proteins alone prior to proteoliposome formation. Membrane proteins were fractionated on an anionic glycocholate-Sepharose 4B affinity column which was prepared by coupling (3α,7α,12α-trihydroxy-5β-cholan-24-oyl)-$N^α$-lysine to activated CH-Sepharose 4B via the ε-amino group of lysine resulting in the retention of a free carboxyl group. The adsorbed proteins enriched in components in the 54 kDa zone, which were originally identified by photoaffinity labeling to be components of the bile acid transport system, were also incorporated into liposomes. This vesicle system showed almost a 4-fold increase in Na^+-dependent taurocholic acid uptake when compared to proteoliposomes formed from total membrane protein, as well as sensitivity to inhibition by bile acids. These results demonstrate that the bile acid carrier system can be reconstituted in proteoliposomes and that utilizing proteins in the 54 kDa zone leads to a significant enhancement in the transport capacity of the reconstituted system, consistent with the role of 54 kDa protein(s) as component(s) of the bile acid carrier system.

Introduction

Bile acids such as taurocholic acid, play an important role in (a) the formation of bile which is involved in digestive and excretory processes, and (b) in the regulation of cholesterol biosynthesis [1]. These compounds are transported across the hepatocyte sinusoidal plasma membrane by sodium-dependent and sodium-independent carrier mediated processes [2–7]. The kinetic properties of this system have been extensively characterized in purified plasma membrane vesicles from the hepatocyte sinusoidal surface domain [8–11]. Studies have also characterized the binding of bile acids to specific plasma membrane sites [12]. The initial identification of the membrane carrier for these substrates has been accomplished using the techniques of photoaffinity labeling with a photo-reactive diazirine derivative of taurocholic acid [13–15]. These studies indicated that a 54

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kDa intrinsic membrane protein was the bile acid carrier or one of its subunits. In order to establish the functional properties of putative carriers, as well as to monitor their purification, studies have been concerned with the reconstitution of several transport systems in different stages of purity, using a variety of liposome preparations [16]. The production of selectively permeable liposomes for amino acids [17], inorganic anions such as phosphate and sulfate [18,19], glucose [20,21], nucleosides [22], and the organic anion, sulfobromophthalein [23] have been reported. The procedure of affinity chromatography has been used extensively for the identification and purification of a large number of soluble as well as membrane associated proteins [24]. In this study we describe the purification of the bile acid carrier protein by affinity chromatography on an anionic glycocholate-Sepharose 4B column, the reconstitution of the taurocholic acid transport system in soybean phospholipid (asolectin) liposomes and the effects of a sodium gradient, protein concentration and carrier purity on proteoliposomal transport capacity.

Experimental procedures

Preparation of plasma membranes. Livers from male Sprague-Dawley rats were homogenized and sinusoidal surface plasma membranes were isolated using a sucrose density gradient procedure as previously described [25]. The purity of this preparation was assessed by measuring the appropriate enzyme markers [14]. Membranes were stored at −80°C in 5 mM Tris-HCl (pH 8.0).

Membrane solubilization. Sinusoidal plasma membranes (1–4 mg) were mixed with 1 ml of 0.6% Triton X-100, 136 mM KCl, 5 mM Tris-HCl (pH 7.4), and the suspension stirred at 4°C for 2 h. The solubilized proteins were separated from insoluble material by centrifugation at 250000 × *g* for 30 min. The resultant supernatant was treated with 0.8 g of Bio-Beads SM-2 for 1 h to remove the detergent.

Synthesis of glycocholic acid-substituted Sepharose beads. To a solution of cholic acid (100 mg; 0.24 mmol) in 1 ml of dioxane was added 87 μl of tri-*n*-butylamine and the mixture stirred for 30 min at 12°C. Ethyl chloroformate (23 μl; 0.24 mmol) was then added and stirred for an additional 15 min. N^{ϵ}-*t*-Boc-Lysine (120 mg; 0.48 mmol) in 0.5 ml of 1 M NaOH was then added and the reaction stirred for 30 min at 12°C and then at room temperature for 2 h. The reaction was neutralized with 1 M HCl and the solvent removed. The residue was taken up in 500 μl water and acidified with 1 M HCl to pH 2.0. The resultant white precipitate was filtered, and washed with water. The precipitate was dissolved in 1 ml of methanol, filtered and the solvent evaporated. This product was thoroughly dried under vacuum over phosphorus pentoxide to afford 118 mg of the *t*-Boc-lysine derivative. This material was then dissolved in 3 ml of trifluoroacetic acid and maintained at 24°C for 2 h with stirring. The solvent was removed in vacuo and the resulting residue taken up in 1 ml of methanol followed by 0.5 ml chloroform and chromatographed on a 1 × 30 cm silica gel column eluting with 150 ml of $CHCl_3$/MeOH (1 : 2, v/v). The solvent was evaporated, the residue taken up in 5 ml of water and lyophilized to afford 80 mg of 3α,7α,12α-trihydroxy-5β-cholan-24-oyl)-N^{α}-lysine (lysylcholic acid). The resulting product gave one spot on an 0.2 mm silica gel TLC plate which was developed with $CHCl_3$/MeOH (2 : 1, v/v). Elemental analysis was carried out on the HCl salt.

$C_{30}H_{52}N_2O_6Cl$
Calculated: C 63.0; H 9.10; N 4.90.
Found: C 62.7; H 8.86; N 4.73.

This cholic acid derivative (50 mg) was reacted with 1 g of activated CH-Sepharose 4B in 5 ml of 100 mM $NaHCO_3$, 0.5 M NaCl (pH 9.0). The beads were kept in suspension by gentle rotation for 1 h at 4°C. The supernatant was subsequently removed and the beads reacted with 5 ml of 0.1 M Tris, 0.5 M NaCl (pH 8.0) for 1 h. The beads were then washed three times with 0.1 M acetate, 0.5 M NaCl (pH 4.0) followed by 0.1 M Tris, 0.5 M NaCl (pH 8.0) and stored in this buffer at 4°C.

Glycocholate-Sepharose 4B affinity chromatography of solubilized sinusoidal plasma membranes. Solubilized membrane in 0.1% Triton-X-100, 1 M NaCl, 10 mM Tris-HCl (pH 8.0) was applied to the derivatized Sepharose-4B column (4 ml), eluting at 6 ml/h with 10 ml of this buffer to remove non-adsorbed material (GCS-I). The material retained on the column (GCS-II) was then eluted

with 10 ml of 3% Triton X-100, 1 M NaCl, 10 mM Tris-HCl (pH 8.0). The fractions containing protein were pooled (3.5 ml) and were treated for 1 h with 4.5 g Bio-Beads SM-2 to remove the detergent. This procedure was repeated with fresh Bio-Beads for an additional hour. The protein was then stored at −80°C in 137 mM KCl, 10 mM Tris-HCl (pH 7.4) or in phosphate-buffered saline (pH 7.4).

Proteoliposome reconstitution. Soybean phospholipids (asolectin) were extracted for 72 h with acetone [26], filtered and dried under vacuum. 50 mg of this material was suspended in 1.0 ml of 136 mM KCl, 5 mM Tris-HCl (pH 7.4), flushed with nitrogen and sonicated in a sealed tube for 20 min at room temperature in a Branson bath type sonicator [20]. To the resulting liposome suspension was added an equal volume of Triton X-100 solubilized membrane protein or protein derived from the glycocholate-Sepharose 4B affinity column, in 135 mM KCl, 5 mM Tris-HCl (pH 7.4) to give a final protein concentration of 50–150 μg/ml of reconstitution buffer. This mixture was rapidly frozen in an acetone-solid CO_2 bath, thawed at room temperature and resonicated for 15 s in a procedure similar to that previously reported by Kasahara and Hinkle [20]. This proteoliposome preparation was used directly in the transport studies.

Transport measurements. Proteoliposomes (16 μl) were added to 48 μl of a 136 mM NaCl or KCl, 5 mM Tris-HCl buffer, (pH 7.4) containing 2.2 μCi of [^{3}H]taurocholic acid and sufficient nonradioactive taurocholic acid to give a concentration of 5 μM. After incubation at 24°C for the indicated times, a 57 μl aliquot was transferred to a tube at 0°C where no additional uptake will occur. The amount of bile acid associated with the liposome was determined by immediate chromatography of a 50 μl aliquot on a 3 ml G-75 Sephadex column maintained at 2°C. The column was rapidly eluted with 2 ml of 136 mM KCl, 5 mM Tris-HCl (pH 7.4) to yield the proteoliposomal material free of taurocholic acid which remained on the column. The eluate was counted in 15 ml of a scintillation fluid containing 3.3 g/l of butyl PBD and 160 ml/l of BBS-3 Biosolv. All determinations were performed in triplicate. All measurements were carried out on at least three separate proteoliposome preparations. The observed standard error was less than 10%. The non-specific binding of taurocholic acid to the proteoliposome was estimated by added the substrate at 0°C followed by chromatography at 2°C at zero time as described above. All data reported have been corrected for this value which was approx. 15% of the total mediated uptake. The amount of taurocholic acid associated with liposomes in the absence of protein has also been determined as described above. The taurocholic acid associated with the protein in the absence of lipid was determined by Millipore filtration on a 0.45 μm GSWP filter.

Analytical procedures. Membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis as previously described [27]. Protein concentrations were determined by the method of Lowry et al. [28]. Radioactivity was determined on a Beckman LS-345 liquid scintillation counter. Proteoliposomes were visualized on a Phillips 3000 electron microscope by negative staining with 2% phosphotungstic acid.

Materials. [^{3}H]Taurocholic acid (6.8 mCi/mmol) was purchased from New England Nuclear. Soybean phospholipids (asolectin) and Triton X-100 were obtained from Sigma Chem. Co. Activated CH-Sepharose 4B was obtained from Pharmacia.

Results

Affinity chromatography procedures were used to substantially purify the bile acid carrier protein. Cholic acid was converted to (3α,7α,12α-trihydroxy-5β-cholan-24-oyl)-N^{α}-lysine (lysylcholic acid) by coupling the parent compound to N^{ε}-*t*-Boc-lysine and the resulting *t*-Boc intermediate treated with trifluoroacetic acid. This product was characterized by thin layer chromatography and elemental analysis, which confirmed its purity and structure. The ε-amino group on the lysyl moiety was then coupled to activated CH-Sepharose to form the affinity resin as shown in Fig. 1. Hepatocyte plasma membranes derived primarily from the sinusoidal surface domain as assessed by enzyme markers were solubilized in Triton X-100 and the resultant supernatant chromatographed on this affinity resin. Non-adsorbed proteins were

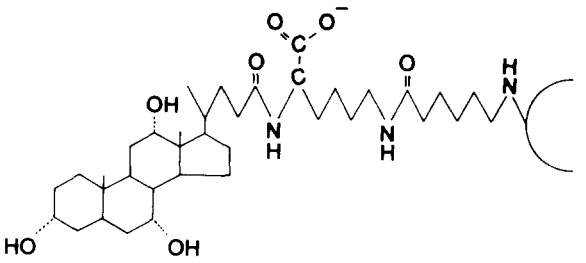

Fig. 1. Structure of immobilized glycocholic acid. Lysylcholic acid was coupled to activated CH-Sepharose 4B.

eluted with a 0.1% Triton X-100 buffer (GCS-I). The detergent concentration was then raised to 3% at which point the absorbed material (GCS-II) was eluted. The chromatographic profile of the sinusoidal membrane components is shown in Fig. 2. Approx. 75% of the total recovered protein was eluted with 0.1% detergent. The use of 3% Triton X-100 resulted in the elution of 25% of the recovered protein. The total recovery was approx. 65% of the protein applied to the column. Similar results were obtained when 2% cholic acid in 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0) was used for elution (data not shown). Essentially all of the membrane proteins were eluted at 0.1% Triton X-100 when underivatized Sepharose was utilized, demonstrating the specificity of the affinity column.

The protein fractions from this affinity column

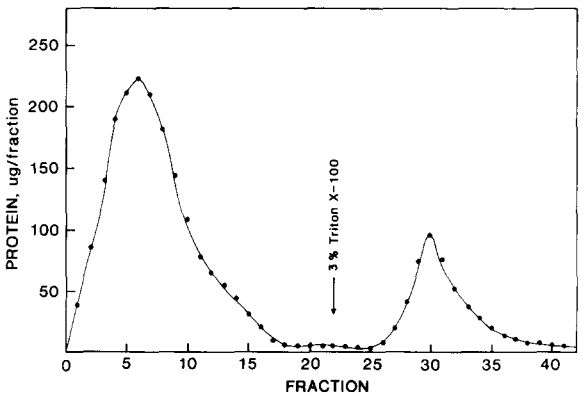

Fig. 2. Affinity chromatography of Triton X-100 solubilized sinusoidal plasma membranes on a glycocholate-Sepharose 4B resin. Solubilized protein was first eluted with 0.1% Triton X-100, 0.5 N NaCl, 10 mM Tris-HCl (pH 8.0). Absorbed material was then removed with the addition of 3% Triton X-100 to the eluting buffer as described in Experimental Procedures.

as well as sinusoidal plasma membranes were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 3. As seen in lane B, essentially all of the membrane proteins were solubilized by the detergent. The protein adsorbed on the affinity column (lane D) was highly enriched in components with molecular weights of approximately 49 000–54 000.

The detergent solubilized membrane proteins with or without fractionation on the glycocholate-Sepharose 4B affinity column were reconstituted into phospholipid liposomes as previously described [20]. This process was extremely sensitive to the time of sonication and the amount of protein added. All studies were performed using 50–150 μg protein/ml of reconstitution buffer. The proteoliposomes were visualized by electron microscopy as shown in Fig. 4. Vesicle populations ranged in size from 500 to 2500 Å and were used in an unfractionated form for the transport studies.

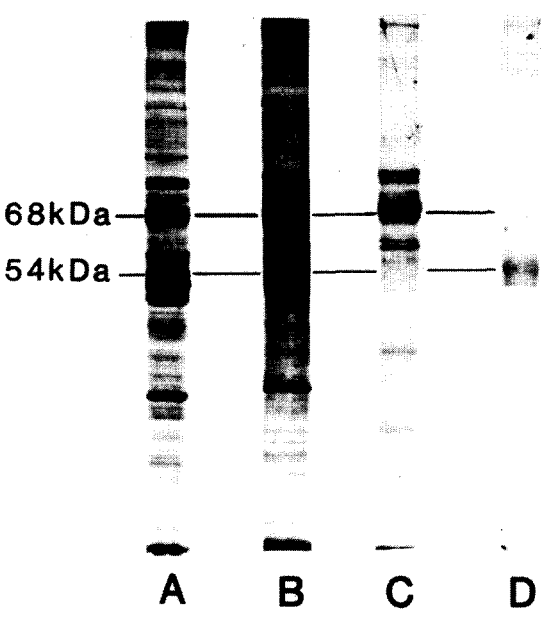

Fig. 3. SDS-polyacrylamide gel electrophoresis. A, hepatocyte sinusoidal plasma membranes (100 μg); B, Triton X-100 extract of plasma membranes (120 μg); C, membrane proteins that were not adsorbed by the glycocholate-Sepharose 4B affinity column (GCS-I), (25 μg); D, membrane proteins that are adsorbed by the glycocholate-Sepharose 4B affinity column (GCS-II) and subsequently eluted with 3% Triton-X-100 (10 μg). Gels were stained with Coomassie blue.

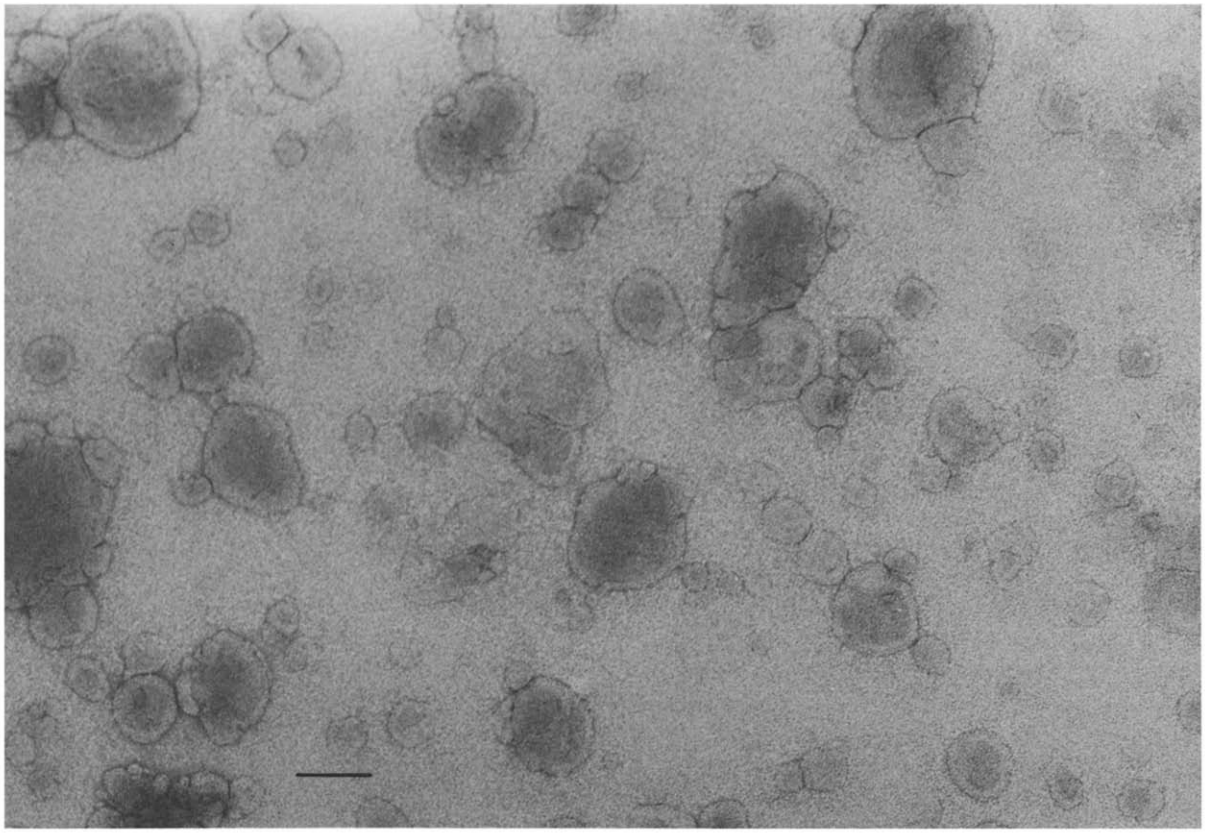

Fig. 4. Electron micrograph of proteoliposomes prepared from the Triton X-100 extracts of sinusoidal plasma membranes and soybean phospholipids as described in Experimental Procedures. Vesicles were stained with 2% phosphotungstic acid. Bar, 100 nm.

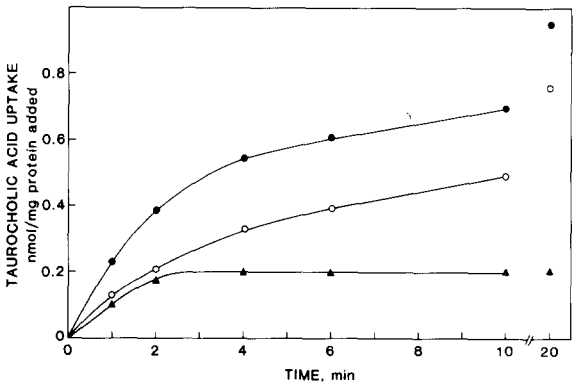

Fig. 5. Uptake of taurocholic acid by proteoliposomes prepared from Triton X-100 solubilized hepatocyte sinusoidal membrane proteins. Proteoliposomes prepared in a K^+ buffer were suspended in a Na^+ (●) or K^+ (○) buffer and incubated with 5 μM taurocholic acid. At the indicated times aliquots were analyzed for taurocholic acid uptake by gel chromatography as described in Experimental Procedures. The sodium-dependent uptake component is indicated (▲).

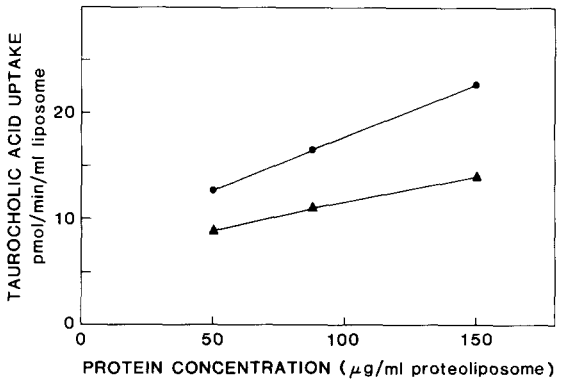

Fig. 6. The effect of Triton X-100 extracted protein concentration on the transport capacity of the reconstituted proteoliposomes. Proteoliposomes were prepared with 50–150 μg protein/ml liposome and the uptake of taurocholic acid (5 μm) was measured in the presence (●) and absence (▲) of sodium as described in Experimental Procedures.

TABLE I

RECONSTITUTION REQUIREMENTS

	Total taurocholic acid uptake (pmol/min per mg protein added)
Proteoliposome	215
Membrane protein [a]	6
Asolectin liposome	28

[a] Membrane protein was solubilized in 0.6% Triton X-100 and detergent subsequently removed with Bio-Beads SM-2 as described in Experimental Procedures.

TABLE II

EFFECT OF TAUROCHOLIC ACID AND TAUROCHENODEOXYCHOLIC ACID ON TAUROCHOLIC ACID (5 μM) UPTAKE INTO PROTEOLIPOSOMES

Proteoliposomes were prepared in a K^+ buffer using sinusoidal membrane proteins that were solubilized in 0.6% Triton X-100 as described in Experimental Procedures.

Reagent	Total uptake (pmol/min per mg protein added)	Inhibition (%)
None	215	0
Taurocholic acid (100 μM)	137	36
Taurochenodeoxycholic acid (50 μm)	148	32

The functional integrity of the reconstituted system using total Triton X-100 solubilized membrane protein was evaluated by measuring the time-course of taurocholic acid uptake in the presence and absence of a sodium gradient as shown in Fig. 5. The effect of sodium on the stimulation of transport is similar to that observed in hepatocyte membrane vesicles [9]. Na^+-dependent uptake reached a maximum value in approximately 2 min. The transport activity of the reconstituted system was also dependent on the amount of protein added to the liposome during reconstitution as shown in Fig. 6. Numerous attempts using chromatographic and density gradient centrifugation procedures failed to quantitatively separate insoluble membrane proteins following Triton-X 100 removal, from proteoliposomes. All data is thus reported on the basis of protein added as opposed to protein incorporated. Using protein concentrations greater than 250 μg/ml appeared to perturb the liposome structure, resulting in transport that was insensitive to sodium suggestive of the appearance of a substantial non-mediated uptake process. The concentration of taurocholic acid was also maintained at 5 μM to prevent perturbation of the liposome by the detergent activity of the bile acid. In the absence of added protein a greatly diminished amount of taurocholic acid was bound and/or taken up by the liposome system as shown in Table I. In the absence of lipid, the protein preparations used in the reconstitution procedures were observed to bind less than 3% of the radioactivity associated with the proteoliposome (Table I). In an effort to establish the specificity of the reconstituted system, transport was measured in the presence of excess non-radioactive taurocholic acid as well as taurochenodeoxycholic acid. As shown in Table II both bile

TABLE III

TAUROCHOLIC ACID UPTAKE BY PROTEOLIPOSOMES

Proteoliposomes were prepared in a K^+ buffer using Triton X-100 solubilized sinusoidal membrane proteins before and after fractionation on a glycocholate-Sepharose 4B affinity column as described in Experimental Procedures. GCS-I and GCS-II refer to non-adsorbed and adsorbed proteins, respectively. Taurocholic acid uptake was then measured in a K^+ or Na^+ buffer in the presence or absence of taurochenodeoxycholic acid (TCDC).

Addition to liposome	Uptake [a]		Na^+-dependent uptake ($Na^+ - K^+$)	% Na^+ dependency	Uptake Na^+/ 50 μM TCDC	Inhibition %
	Na^+	K^+				
Plasma membrane	233	130	103	44	148	37
GCS-I	150	143	7	5	140	7
GCS-II	738	357	381	52	395	47

[a] Uptake reported as pmol/min per mg protein added.

acids significantly inhibited the uptake of [^{3}H]taurocholic acid.

The functional properties of the proteins isolated from the glycocholate-Sepharose 4B affinity column were also evaluated by the reconstitution procedure described above. The addition of the non-adsorbed protein (fraction GCS-1) (Fig. 3C), to the liposome system resulted in a reduced uptake of taurocholic acid when compared to the values obtained from total membrane protein, as well as the loss of sodium stimulatory effect and the sensitivity to inhibition by taurochenodeoxycholic acid (Table III). In contrast, reconstitution with the protein (fraction GCS-II) that was adsorbed to the affinity column (Fig. 3D) resulted in a 3.7-fold increase in sodium-dependent uptake capacity. There was also significant inhibition of taurocholic acid transport by taurochenodeoxycholic acid in this system, similar to that observed in proteoliposomes prepared with unfractionated plasma membranes. These results suggest that the fractionation leading to a purification of proteins in the 54 kDa zone results in a significant enrichment of the bile acid carrier.

Discussion

Numerous studies have characterized the transport of bile acids into hepatocytes as well as into hepatocyte plasma membrane vesicles [2–11]. In this study we have used liposomal reconstitution techniques in order to characterize the functional properties of the bile acid carrier system in hepatocyte sinusoidal plasma membranes. Using the methods initially described by Kasahara and Hinkle [20], the proteoliposomes formed using unfractionated membrane proteins afforded taurocholic acid permeable lipid vesicles. Proteoliposome transport was dependent in part on a sodium gradient and could be substantially inhibited by both taurocholic acid and taurochenodeoxycholic acid, a bile acid which is an effective inhibitor of a taurocholic acid uptake in hepatocytes [4] and hepatocyte plasma membrane vesicles (Von Dippe, P. and Levy, D., unpublished results). These studies indicate the high specificity of the reconstituted transport system. Negligible amounts of taurocholic acid were associated with the membrane proteins in the absence of lipid. Similarly, the association of taurocholic acid with phospholipid in the absence of protein indicated that there was very little binding and/or uptake of the substrate by this system. In addition, studies of the binding of taurocholic acid to hepatocyte plasma membranes have demonstrated that this process is insensitive to sodium ion [12]. The results reported in this study thus suggest that the radioactivity associated with the proteoliposomes is dependent on a protein mediated transport process, with negligible contributions from substrate binding. Further support for the integrity of the reconstituted system comes from the observation that the Na^+-dependent initial uptake (Table III) by the proteoliposomes was estimated to be approx. 80% of that observed for sinusoidal plasma membrane vesicles under comparable substrate conditions (Von Dippe, P. and Levy, D., unpublished data).

In order to further characterize the functional properties of the bile acid transport protein we have developed a chromatographic procedure for the substantial purification of this integral membrane protein. Previous studies [29–31] utilizing bile acids coupled through the carboxyl group to Agarose or Sepharose resins resulted in affinity resins which bound several different proteins suggesting a possible reduction of specificity because of the loss of the negative charge on the bile acid. We have thus attached a lysine-bile acid conjugate to Sepharose beads with the retention of the carboxyl group. This has resulted in a ligand, essentially identical to glycocholic acid (Fig. 1), which is attached to the resin via the α-carbon atom of glycine. Chromatography of a Triton X-100 solubilized sinusoidal plasma membrane preparation on this affinity resin resulted in the retention of proteins in the 49000–54000 molecular weight range. Membrane associated serum albumin which binds bile acids was not absorbed on the column, presumably because of the presence of the detergent. Serum albumin was, however, retained by this column in the absence of Triton X-100.

Using identical reconstitution techniques to those described for the total detergent extracted proteins, the nonadsorbed proteins afforded proteoliposomes which exhibited reduced taurocholic acid uptake, the loss of sodium dependency and

insensitivity to inhibition of taurochenodeoxycholic acid. Substrate transport by this system may thus be the result of protein perturbation of the liposome structure at the protein/lipid interface or protein aggregation leading to the creation of a non-mediated passive diffusion process [32]. In contrast, the proteoliposomes formed from the material adsorbed to the affinity column showed a significant increase in substrate transport capacity which retained its sensitivity to sodium and to taurochenodeoxycholic acid as observed in membrane vesicles, results indicating the existence of a mediated transport process.

The transport capacity of a proteoliposome reconstituted system will depend on the purity of the transport protein; however, studies have indicated that membrane proteins can lose all or part of their functional activity during the solubilization procedure while exposed to various detergents as a result of some degree of denaturation [33]. In addition, the activity of a carrier protein may also be significantly affected by the loss of native lipids during the purification procedure and by exposure to other lipids that constitute the liposome environment. These lipid environmental factors may play a critical role in determining (a) the extent of protein incorporation into the liposome, (b) the conformation of the transport protein and (c) the orientation of the protein in the lipid bilayer. Thus the observed increase in the specific activity of this reconstituted system may not parallel the physical purification of the carrier system as a result of one or more of the above described factors.

In summary, these studies suggest that the bile acid carrier system can be reconstituted in phospholipid vesicles. Utilization of partially purified membrane components, identified originally by photoaffinity labeling as components of this transport system, resulted in a reconstituted proteoliposome exhibiting an increased capacity to transport bile acids in a sodium-dependent mediated fashion. These results suggest that protein(s) in the 54 kDa zone possess functional properties consistent with the bile acid carrier system. Reconstitution of this system will be useful in monitoring the specific activity of the carrier system at different stages of its further purification.

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