The binding of cholesterol and bile salts to recombinant rat liver fatty acid-binding protein

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The physiological role of liver fatty acid-binding protein (L-FABP) has yet to be clarified. An important feature of this member of the family of intracellular lipid-binding proteins is the wide range of compounds that have been identified as potential physiological ligands. By using recombinant L-FABP, the binding of cholesterol, bile salts and their derivatives has been investigated under conditions that allow a direct comparison of the binding affinities of these ligands for fatty acids. The results demonstrate an inability of L-FABP to bind cholesterol, although the anionic derivative, cholesteryl sulphate, will bind under similar assay conditions. Of the bile salts examined, lithocholate and taurolithocholate sulphate showed the greatest binding to L-FABP. It is proposed that an important function of L-FABP is to bind certain physiological amphipathic anions, thus preventing the 'free' concentrations of these compounds from exceeding their critical micelle concentration, which could result in cell damage.

INTRODUCTION

Cholesterol is an important constituent of mammalian plasma membranes and a precursor for bile salt biosynthesis. In the body, cholesterol homoeostasis is maintained by balancing dietary cholesterol absorption and endogenous cholesterol synthesis with cholesterol excretion and bile salt biosynthesis. In the intestinal lumen the detergent properties of bile salts facilitate the absorption of cholesterol, whereas hepatic secretion of bile salts in bile promotes cholesterol removal from the body. Thus the control of cholesterol and bile salt levels is closely linked [1–4].

Cholesterol is poorly soluble in aqueous media, and the transfer of cholesterol between cellular membranes is thought to occur by protein-mediated transport and exchange processes [5]. At least four cytosolic proteins have been implicated in the cellular transport of cholesterol. These are liver fatty acid-binding protein (L-FABP), sterol carrier proteins or non-specific lipid-transfer proteins (SCP and SCP_2) and an unknown soluble protein activator [1,2,5,6]. However, the role of these proteins in cholesterol transport remains controversial [1,2,5–7]. The cytosolic fatty acid-binding proteins are members of the intracellular lipid-binding protein family and include liver, intestinal, heart (muscle) and adipocyte FABP [7–10]. L-FABP can bind a wide range of anionic hydrophobic ligands, including fatty acids, acyl-CoA, lysophospholipids, haem and bile salts [7,8,11–15]. L-FABP is identical with the previously characterized Z-protein, haem-binding protein and aminoazodye-binding protein [7,8] and has been found in liver, intestine, stomach and kidney [10].

In our laboratory we use a bacterial system for the over-expression and purification of recombinant rat L-FABP [16]. The availability of recombinant protein is a major advantage in studying ligand binding, particularly as the protein isolated from liver might be contaminated by other small lipid-binding proteins. This system has previously been used to investigate fatty acid, lysophospholipid and acyl-CoA binding to L-FABP, and its functional identity with rat liver-derived FABP has been confirmed [12,16–18].

In this study the binding of cholesterol, bile salts and their derivatives to L-FABP has been investigated in detail. Cholesterol, cholate, deoxycholate and chenodeoxycholate did not bind to recombinant rat L-FABP under these assay conditions. In contrast, cholesteryl sulphate and cholesteryl glucuronide, lithocholate and lithocholate conjugates display binding affinities that are lower than for oleate. These results support the concept that ligands for L-FABP need to be amphipathic, with a hydrophobic domain as well as a charged (anionic) moiety. We propose that L-FABP probably acts as a 'buffer' in the cell to prevent certain ligands from accumulating to concentrations above their critical micelle concentrations (cmc values), thus protecting the cells from detrimental effects of these ligands.

EXPERIMENTAL

Chemicals

11-(5-Dimethylaminonaphthalenesulphonyl)-undecanoic acid (DAUDA) was purchased from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were obtained from Sigma.

Purification of L-FABP

Recombinant L-FABP was purified on naphthoylamino-decyl-agarose [16,19] and delipidated with Lipidex 1000 at 37 °C [20]. Protein purity was assessed by SDS/PAGE [21] and the protein concentration determined by the dye-binding assay of Bradford [22], with BSA as a standard.

Ligand displacement assays

The fluorescence displacement assays were performed at 20 °C in 50 mM Hepes, pH 7.5, containing 1 μM L-FABP and 1 μM DAUDA. Ligands (1 mM in methanol) were added in 0.5–1.0 μl

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aliquots. DAUDA fluorescence was measured at an excitation wavelength of 335 nm (slit 10 nm) and an emission wavelength of 500 nm (slit 10 nm) [23,24]. The percentage initial fluorescence was calculated as $100 \times$ (fluorescence in the presence of ligand) divided by (fluorescence in the absence of added ligand). Statistical analysis was performed with Student's t test. Concentrations of DAUDA were determined in methanol by using a molar absorption coefficient of 4400 $M^{-1} \cdot cm^{-1}$ at 335 nm [24]. No fluorescence corrections were required for inner-filter effects.

Dehydroergosterol fluorescence was measured at an excitation wavelength of 325 nm (slit 5 nm) with emission at 375 nm (slit 10 nm) [25]. Fluorescence energy transfer between dehydroergosterol and a tryptophan mutant, L-FABP[F18W], was determined by excitation at 295 nm and emission at both 330 and 375 nm [25,26].

Dielectric constants for water and organic solvents were obtained from [27]. A proportional relation was assumed in calculating the dielectric constant when solvents were mixed.

Preparation of small unilamellar vesicles

Phospholipids were obtained as a solution in chloroform, dried by nitrogen aspiration and redissolved in methanol at a concentration of 10 mg/ml. Small unilamellar vesicles were prepared by methanol injection into buffer [28].

RESULTS

Binding protocols

The availability of pure recombinant rat L-FABP [16] allows a detailed investigation of the binding of cholesterol and bile salts to this lipid-binding protein. The measurement of the binding of such ligands to proteins is difficult, because of poor ligand solubility and the technical problems of separating bound and free ligands. To overcome some of these obstacles, we have used a competitive displacement method for assaying ligand binding with the fluorescent fatty acid probe DAUDA. However, because DAUDA binds with a K_d below 1 μM [17], the competitive nature of the assay would mean that ligands with a K_d above approx. 10 μM would not give significant DAUDA displacement. The direct binding of a fluorescent cholesterol analogue, dehydroergosterol, to L-FABP was also investigated and compared with the binding characteristics of DAUDA.

Sterol binding

Cholesterol and several of its derivatives do not show significant displacement of DAUDA (Table 1), suggesting that these ligands do not bind to L-FABP under the conditions of this binding assay and, for cholesterol, confirm similar observations with FABP isolated from rat liver [7,13,29,30]. The absence of cholesterol binding to L-FABP was further validated by using a fluorescent analogue, dehydroergosterol, which has been shown to bind to L-FABP, SCP and SCP$_2$ [25,31]. By using dielectric constants as a measure of 'apparent hydrophobicity', it could be shown that DAUDA shows similar fluorescence characteristics in butanol and when bound to L-FABP (Figure 1). It can therefore be reasoned that the DAUDA-binding site in L-FABP displays similar solvation properties to butanol. If dehydroergosterol binds to L-FABP, it should therefore also show similar fluorescence characteristics, assuming that all ligands bind within the hydrophobic binding cavity of this protein [9]. However, with dehydroergosterol different fluorescence characteristics were observed. When compared with water, the fluorescence of dehydroergosterol increased by approx. 200 and 30 fluorescence

Table 1   Displacement of DAUDA from L-FABP by cholesterol analogues

The values shown were calculated at a 5-fold excess of ligand over DAUDA and FABP, as described in the Experimental section. The values for methanol are shown as a volume equivalent to that added with the ligands. Significant differences from results with an equivalent amount of methanol are indicated as *$P < 0.01$, **$P < 0.001$. Results are shown as means $\pm$ S.D. for the number of data sets shown in parentheses.

Ligand	Percentage of initial fluorescence
Methanol	95.7 ± 2.1 (3)
Oleate	13.3 ± 2.2 (3)**
Cholesterol	97.1 ± 1.6 (3)
25-Hydroxycholesterol	94.5 ± 2.0 (3)
4-Cholesten-3-one	93.7 ± 2.3 (3)
Ergosterol	95.7 ± 0.2 (3)
Oestrone sulphate	94.6 ± 2.6 (4)
Cholesteryl sulphate	42.6 ± 7.8 (5)**
Cholesteryl glucuronide	72.3 ± 7.0 (3)*
Cholesteryl hemisuccinate	74.5 ± 2.3 (3)**

units in butanol and in the presence of L-FABP respectively, at an emission wavelength of 370 nm (Figure 1). Moreover, the increase in protein-dependent fluorescence does not seem to be saturable and is directly proportional to the L-FABP concentration (Figure 1); it probably indicates a non-specific effect. In addition, dehydroergosterol binding could not be detected in fluorescence energy transfer experiments with a tryptophan mutant, L-FABP[F18W] (results not shown). The results described above demonstrate by two different methods that sterols such as cholesterol are not ligands for L-FABP under assay conditions *in vitro*.

Binding of anionic sterol derivatives

There are several reasons why a non-polar ligand such as cholesterol does not bind to L-FABP. Steric constraints could prevent the rigid sterol nucleus from being accommodated in the hydrophobic binding domain of L-FABP. Alternatively, because an anionic group is a normal feature of ligands for L-FABP, the absence of such a group might adversely affect binding affinity. The anionic moiety might be a requirement for high-affinity binding to L-FABP, or it might simply increase the aqueous solubility of such a ligand. Therefore it was decided to investigate cholesteryl sulphate, cholesteryl hemisuccinate and cholesteryl glucuronide as potential ligands. These compounds showed significant binding to L-FABP, indicating that steric constraints do not prevent cholesterol from binding to L-FABP (Table 1).

In the, cell cholesterol and cholesteryl sulphate are found predominantly in the plasma-membrane bilayer; under such physiological conditions the aqueous concentration of these ligands is minimal. When cholesteryl sulphate was presented in dioleoylphosphatidylcholine (DOPC) vesicles, its capacity to bind to L-FABP was greatly reduced (Table 2; compare expt. 2 with expt. 4) compared with presentation in aqueous solution (Table 1), whereas cholesterol remained ineffective as a ligand for L-FABP under these conditions (Table 2; compare expt. 2 with expt. 5). Similarly, the anionic probe DAUDA also seems to partition into DOPC vesicles, as demonstrated by the increased fluorescence observed (Table 2; compare expt. 1 with expt. 2). Thus it seems that L-FABP is unable to access membrane-bound ligands directly, but instead binds ligands from the aqueous phase. The increased solubility of cholesteryl sulphate relative to
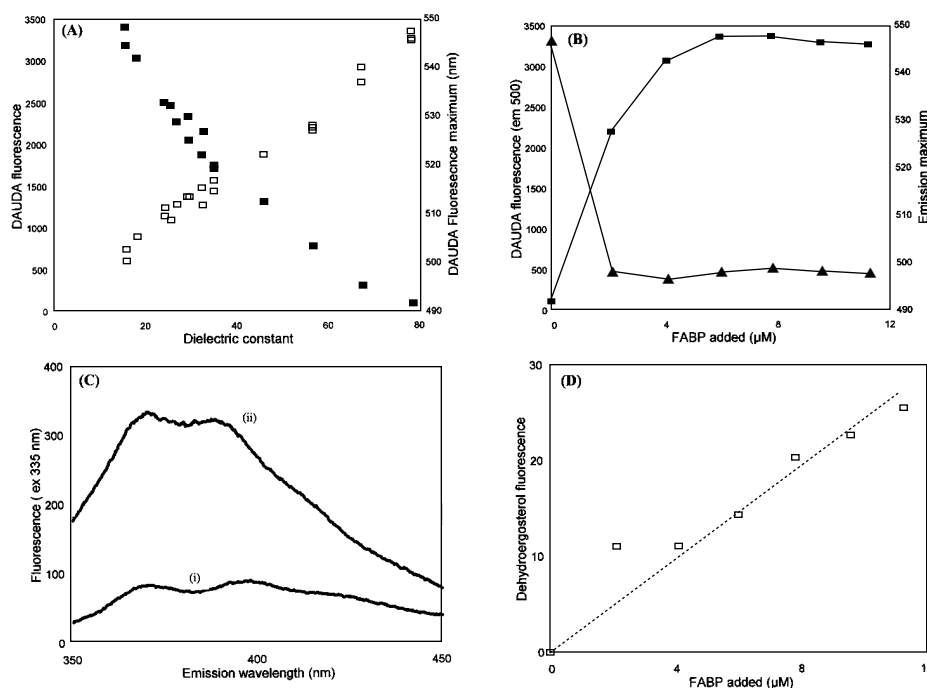
Figure 1   Fluorescence properties of DAUDA and dehydroergosterol

(**A**) Effect of dielectric constant on DAUDA fluorescence emission at 500 nm (■) and fluorescence emission maximum (□) (excitation at 335 nm). (**B**) DAUDA fluorescence characteristics (excitation at 335 nm) in the presence of L-FABP: ■, fluorescence emission at 500 nm; ▲, fluorescence emission maximum. (**C**) Dehydroergosterol fluorescence emission in the presence of (i) water and (ii) butanol (excitation at 325 nm). (**D**) Dehydroergosterol fluorescence in the presence of L-FABP (excitation at 325 nm; emission at 370 nm). The concentrations of DAUDA and dehydroergosterol were 1 μM in all cases. Results are corrected for fluorescence of L-FABP alone.

Table 2   Displacement of DAUDA from L-FABP by cholesterol and cholesteryl sulphate in the presence of phospholipids

The values shown were calculated at a 5-fold excess of ligand (cholesteryl sulphate or cholesterol) over DAUDA and FABP, as described in the Experimental section. The values for methanol, DOPC and DOPC + 20 % DPPS are shown as a volume and concentrations equivalent to that added with the ligands. Significant differences from results with an equivalent concentration of DOPC (expt. 2) are indicated as $*P < 0.01$, $**P < 0.001$; significant difference from DOPC + 20 % DPPS (expt. 3), $\dagger P < 0.001$; significant difference from DOPC + cholesterol (expt. 5), $\ddagger P < 0.001$. Results are shown as means $\pm$ S.D. for the number of data sets shown in parentheses.

Expt.	Ligand	Percentage of initial fluorescence
1	Methanol	$94.6 \pm 1.0\%$ (3)**
2	DOPC	$124.6 \pm 1.5\%$ (3)
3	DOPC + 20 % DPPS	$85.7 \pm 2.1\%$ (3)**
4	DOPC + cholesteryl sulphate	$110.5 \pm 1.2\%$ (4)**
5	DOPC + cholesterol	$132.5 \pm 1.2\%$ (3)*
6	DOPC + 5 % DPPS + cholesteryl sulphate	$99.5 \pm 1.1\%$ (3)**‡
7	DOPC + 10 % DPPS + cholesteryl sulphate	$78.1 \pm 1.8\%$ (3)**‡
8	DOPC + 20 % DPPS + cholesteryl sulphate	$61.5 \pm 1.1\%$ (3)**†‡
9	DOPC + 20 % DPPS + cholesterol	$84.3 \pm 3.6\%$ (3)**

cholesterol might be more important for binding to L-FABP than the presence of a negative charge, as aqueous solubility has been shown to be a limiting factor in the transfer of fatty acids from phospholipid membranes to L-FABP [32,33]. The inclusion of negatively charged dipalmitoylphosphatidylserine (DPPS) into

the DOPC vesicles reduces the partitioning of cholesteryl sulphate (and DAUDA) into the bilayer, owing to electrostatic repulsion. Under these conditions the displacement of DAUDA from L-FABP was enhanced for cholesteryl sulphate (Table 2; compare expt. 3 with expt. 8), but not cholesterol (Table 2; compare expt. 3 with expt. 9), and presumably reflects the increased aqueous-phase concentration of cholesteryl sulphate.

Binding of bile salts to L-FABP

Bile salts are considerably more soluble than cholesterol and should bind to L-FABP if solubility is the most important constraint to binding. With the DAUDA displacement assay, lithocholate displayed the greatest binding of the unconjugated bile salts investigated (Table 3). The taurine and glycine conjugates of these bile salts showed a lower affinity for L-FABP, as demonstrated in particular for the lithocholate conjugates (Table 3). These results are similar to those previously obtained with L-FABP purified from rat liver [30,34] and are consistent with the need for L-FABP ligands to have a major non-polar interaction with the protein that will be decreased in those bile salts having additional hydroxy groups. Recently it has been shown that a taurolithocholate sulphate photoaffinity analogue exclusively labelled a hepatic protein identified as L-FABP, although tauro-cholate and taurolithocholate derivatives did not bind [14]. Taurolithocholate 3-sulphate is also a good ligand in our system (Table 3), and the addition of a hydrophobic diazo moiety in the photoaffinity probe may explain the high selectivity for L-FABP. The lack of binding for the taurolithocholate derivative [14] is more difficult to explain, although L-FABP affinity for this

Table 3   Displacement of DAUDA from L-FABP by bile salts

The values shown were calculated at a 5-fold excess of ligand over DAUDA and FABP, as described in the Experimental section. The values for methanol are shown as a volume equivalent to that added with the ligands. Significant differences from results with an equivalent amount of methanol are indicated as $*P < 0.05$, $***P < 0.001$; comparison of conjugated with unconjugated bile salts is indicated as $†P < 0.05$, $††P < 0.01$, $†††P < 0.001$. Results are shown as means $\pm$ S.D. for the number of data sets shown in parentheses.

Ligand	Percentage of initial fluorescence
Methanol	$95.7 \pm 2.1\%$ (3)
Cholate	$87.4 \pm 1.8\%$ (4)*
Deoxycholate	$88.6 \pm 3.2\%$ (4)*
Chenodeoxycholate	$90.2 \pm 7.7\%$ (3)
Lithocholate	$45.7 \pm 1.1\%$ (4)***
Glycocholate	$93.0 \pm 2.5\%$ (3)†
Glycodeoxycholate	$98.6 \pm 4.8\%$ (3)†
Glycochenodeoxycholic acid	$93.3 \pm 1.7\%$ (3)
Glycolithocholate	$83.2 \pm 1.6\%$ (3)*†††
Taurocholate	$97.6 \pm 3.2\%$ (3)††
Taurodeoxycholate	$96.1 \pm 0.6\%$ (3)†
Taurolithocholate	$67.6 \pm 1.3\%$ (3)***†††
Taurolithocholate 3-sulphate	$44.2 \pm 2.0\%$ (3)***

Figure 2   Proposed scheme for the role of liver FABP in the control of cytoplasmic levels of lipid metabolites which bind to this protein

The presence of liver FABP will prevent the levels of 'free' ligand from reaching micelle-forming concentrations, which can cause cell damage.

ligand is lower than for lithocholate or taurolithocholate sulphate (Table 3).

DISCUSSION

Liver FABP can bind a wide range of ligands, including fatty acids, acyl-CoA, lysophospholipids, haem, bilirubin and bile salts [7,8,11–15]. The binding of cholesterol to L-FABP is controversial: generally a lack of cholesterol binding to L-FABP has been observed [7,13,29,35]. However, affinities similar to those obtained for lysophospholipids and long-chain fatty acids (0.8–1.5 μM) have been published [25,36,37]. It has been argued by Scallen et al. [6], among others, that the observed effects of L-FABP in sterol metabolism and transport are the result of SCP contamination, especially as FABP and SCP were thought to be the same protein by early workers. We have therefore investigated cholesterol and bile salt binding by using a recombinant L-FABP system.

Cholesteryl sulphate, cholesteryl hemisuccinate and cholesteryl glucuronide, but not cholesterol and a number of structural analogues, can bind to L-FABP (Table 1). It is therefore concluded that cholesterol binding is not prevented by steric constraints, but can be impeded by its low solubility and/or lack of a charged moiety. Cholesteryl sulphate in DOPC vesicles binds to L-FABP at much lower levels than cholesteryl sulphate in free solution (Tables 1 and 2), but the use of negatively charged vesicles containing DPPS enhances binding, probably by increasing the aqueous-phase concentration of this ligand (Tables 1 and 2). Storch and co-workers [32,33] have shown that the transfer of fluorescent fatty acids from model membranes to L-FABP takes place by aqueous-phase diffusion, which implies that aqueous solubility might be an intrinsic requirement for L-FABP ligands.

The importance of hydrophobic interactions in ligand binding to L-FABP [38–41] is borne out by a comparison of the binding of various bile salts. The least polar bile salt investigated, lithocholate, showed a higher affinity for L-FABP than the more polar cholate, deoxycholate and chenodeoxycholate, whereas affinity was also decreased by conjugation of bile salts with
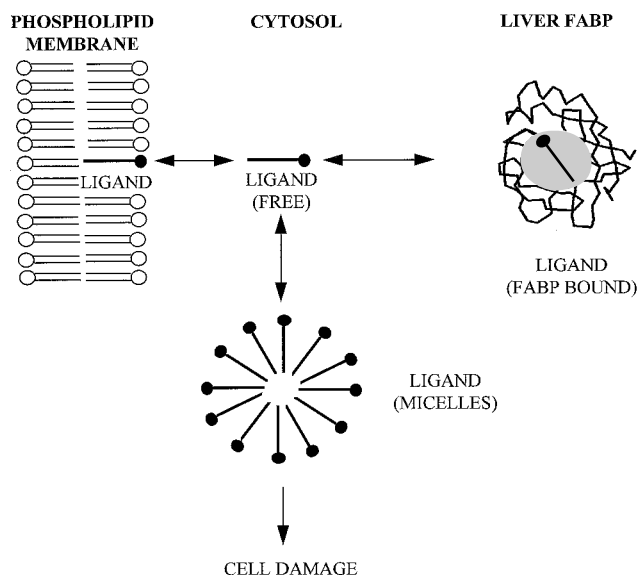
taurine and glycine (Table 3). Thus a balance of polar and non-polar properties is important for binding to L-FABP, and this point is emphasized by the relatively narrow range of cmc values displayed by L-FABP ligands such as long-chain fatty acids, acyl-CoA and lysophospholipids. A micromolar range of cmc values seems to be optimal for ligand binding to L-FABP, whereas cholesterol has a nanomolar cmc and most bile salts display cmc values in the millimolar range.

An intriguing question is therefore why L-FABP displays this apparent selectivity for amphipathic ligands with cmc values in the micromolar range. No physiological role has been directly identified for the FABPs, but the following functions have been suggested: (1) fatty acid uptake, transport and metabolism; (2) ligand targeting; (3) a mediator of mitogenesis, cell growth and differentiation; and (4) protection against the detrimental effects of fatty acids [7,8,10,42]. The cytosolic concentration of L-FABP in the liver is relatively high at 0.1–0.4 mM, and this protein is abundant in tissues with a high lipid flux, such as the liver and intestine [7,10]. We hypothesize that L-FABP might act as a physiological buffer to prevent the 'free' (unbound) concentration of certain ligands from exceeding their cmc and the resulting micelles from causing cell damage. A model illustrating the buffering role of L-FABP in the cell is shown in Figure 2. Ligands for L-FABP such as fatty acids, lysophospholipids and acyl-CoA are soluble in both a hydrophobic phospholipid membrane domain and the cytosol. The total cytosolic concentration of these ligands would be enhanced by the presence of L-FABP, which would maintain an optimal steady-state concentration of 'free' ligands for further metabolism. Under conditions of increased lipid metabolism L-FABP would prevent accumulation of excess micelle-forming 'free' ligands and therefore have a protective role in preventing cell damage. This role for L-FABP does not preclude other specific functions for this protein in the cell, but provides an explanation for the presence of L-FABP in tissues exposed to high lipid flux.

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