The binding of natural and fluorescent lysophospholipids to wild-type and mutant rat liver fatty acid-binding protein and albumin

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Rat liver fatty acid-binding protein (FABP) is able to bind a wide range of non-polar anionic ligands, including lysophospholipids. In order to understand the nature of lysophospholipid interactions with liver FABP, the binding of natural lysophospholipids and two fluorescent analogues, *N*-(5-dimethylaminonaphthalenesulphonyl)-1-palmitoyl-*sn*-glycero-3-phosphoethanolamine (dansyl lysoPE) and 1-*O*-[11-(5-dimethylaminonaphthalenesulphonyl)amino]undecyl]-*sn*-glycero-3-phosphocholine (dansyl- C_{11} -lysoPAF), has been investigated. The results confirmed the ability of liver FABP to bind lysophospholipids with K_D values in the range of 1–2 μ M, and a 1:1 binding stoichiometry was

indicated. Binding of fluorescent lysophospholipids was enhanced with the FABP mutant, R122Q, possibly due to increased flexibility of the binding cavity as a result of reduced hydrogen-bonding constraints. The fluorescent lysophospholipids also bound to albumin, with K_D values in the range 0.1–1.0 μ M, and could be displaced by oleic acid. The fluorescence characteristics of the dansyl lysophospholipid analogue dansyl- C_{11} -lyso-PAF suggested that this probe binds to the same site(s) on albumin as the fluorescent fatty acid probe 11-(5-dimethylaminonaphthalenesulphonylamino)-undecanoic acid (DAUDA).

INTRODUCTION

Lysophospholipids, together with fatty acids, are products of phospholipid hydrolysis by phospholipases A and are found at low concentrations in most biological membranes [1]. Increased levels of lysophospholipids are associated with some disease states (e.g. myocardial ischaemia), while free radical damage and lysis of membranes is also associated with increased concentrations of these phospholipid metabolites [1–4]. In the cell lysophospholipids play an important role in phospholipid metabolism and lysophosphatidic acid (lysoPA) has been shown to be involved in cellular signalling [4,5]. It has also been proposed that lysophosphatidylcholine (lysoPC) secretion may provide a novel transport system for polyunsaturated fatty acids and choline [6]. Recently it has been suggested that lysophospholipids may play

a crucial role in cell infection by the yeast *Candida tropicalis* [7]. Thus there is increasing interest in the physiological and pathological role of lysophospholipids which highlights the importance of proteins that can bind these biologically active molecules.

The intracellular lipid-binding protein family is presumed to play an important role in the transport and metabolic control of fatty acids and related lipids, and includes such proteins as myelin P2, adipocyte lipid-binding protein, cellular retinol- and retinoic acid-binding proteins, and various fatty acid-binding proteins (FABP), including heart, intestinal and liver isoforms [8–10]. These proteins are relatively small, with approximately 130 residues [8,10] and display a β -clam, or β -barrel, structure together with two α -helices forming part of a proposed ligand entry portal [11]. The structural homology within this protein family is high, although sequence similarity is highly variable [8,10]. No structure for liver FABP has been published, but ligand binding to this protein appears to differ significantly from that of the muscle or intestinal isoforms. Thus, liver FABP can bind two fatty acids per protein molecule, displays the capacity to bind a wider variety of ligands and, in the case of fatty acids, the carboxylates appear to be solvent-exposed rather than buried [12–16].

In a previous study we have shown by mutagenesis that Arg-122, a conserved residue, is not directly involved in fatty acid binding to liver FABP. It is of interest, however, that a glutamine mutation at this position increased apparent affinity for oleoyl coenzyme A [17]. Subsequently, using tryptophan mutants, it was shown that ligand binding resulted in subtle protein fluorescence differences with a variety of different ligands. In addition the dansyl moiety of the fluorescent fatty acid analogue DAUDA bound within the β -clam cavity at similar distances from the three tryptophans mutated at positions 3, 18 and 69 [18].

Figure 1 The structures of (a) dansyl lysoPE and (b) dansyl- C_{11} -lysoPAF

Dansyl lysoPE may contain some 2-acyl conformer.

Abbreviations used: FABP, fatty acid-binding protein; lysoPE, lysophosphatidylethanolamine; lysoPA, lysophosphatidic acid; lysoPG, lysophosphatidylglycerol; lysoPC, lysophosphatidylcholine; lysoPS, lysophosphatidylserine; dansyl lysoPE, *N*-(5-dimethylaminonaphthalenesulphonyl)-1-palmitoyl-*sn*-glycero-3-phosphoethanolamine; dansyl- C_{11} -lysoPAF, 1-*O*-[11-(5-dimethylaminonaphthalenesulphonyl)amino]undecyl]-*sn*-glycero-3-phosphocholine; HSA, human serum albumin; DAUDA, 11-(5-dimethylaminonaphthalenesulphonylamino)-undecanoic acid.

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The binding of lysophospholipids (e.g. lysoPC) to liver FABP *in vitro* was first demonstrated by Burrier and Brecher [19] and a physiological role as a lysophospholipid transfer protein has been suggested [20]. We have previously investigated the binding of lysophospholipids to liver FABP and albumin [21]; however, in the case of FABP only indirect methods were used to demonstrate lysophospholipid binding. We have now extended this work using FABP mutants and, in particular, have investigated the binding of two fluorescent lysophospholipids, 1- $\{O$ -[11-(5-dimethylaminonaphthalenesulphonyl)amino]undecyl $\}$ -*sn*-glycero-3-phosphocholine (dansyl- C_{11} -lysoPAF) and *N*-(5-dimethylaminonaphthalenesulphonyl)-1-palmitoyl-*sn*-glycero-3-phosphoethanolamine (dansyl lysoPE) (Figure 1), to liver FABP, BSA and human serum albumin (HSA).

MATERIALS AND METHODS

Materials

Palmitoyl lysophosphatidylethanolamine (lysoPE) was obtained from Calbiochem-Novabiochem, Nottingham, U.K. Dansyl chloride, BSA (essentially fatty acid free), HSA (essentially fatty acid free), oleoyl-lysoPE, oleoyl-lysoPC, lysophosphatidylglycerol (lysoPG) (contains primarily palmitic and stearic fatty acids) and oleoyl-lysoPA were obtained from Sigma. DAUDA and dansyl- C_{11} -lysoPAF were obtained from Molecular Probes, Eugene, OR, U.S.A. Isopropyl- β -D-thiogalactoside was obtained from Northumbria Biologicals, Cramlington, Northumberland, U.K.

All other chemicals were obtained from Sigma.

FABP purification

Recombinant rat liver FABP was produced in *Escherichia coli* by expression of a synthetic gene [22]. The bacteria were diluted 10–100-fold into Luria–Bertani broth containing 50 μ g/ml ampicillin (37 °C) and grown for approx. 8 h before overnight induction with 0.5 mM isopropyl- β -D-thiogalactoside [23]. A 60% (w/v) ammonium sulphate suspension was prepared [17], dialysed overnight against 20 mM phosphate/5% glycerol, pH 7.5, and concentrated on an Amicon Diaflo YM3 membrane before loading on to a Sephadex G-50 column (100 cm $\times$ 3 cm). Fractions showing DAUDA binding were pooled and, after dialysis, loaded on to a DEAE column (20 cm $\times$ 2.5 cm) equilibrated with 10 mM Tris/HCl, pH 8.5 [13,24]. The protein was eluted with a 0–0.2 M NaCl gradient and DAUDA binding fractions collected. The pooled sample was dialysed overnight against 10 mM phosphate, pH 7.4, delipidated [25], concentrated on a YM3 membrane and stored at –20 °C. The purification of the R122Q mutant of FABP and three tryptophan mutants (F3W, F18W, C69W) has been described previously [17,18].

Protein purity was assessed by FPLC on a Superdex G-75 column and by SDS/PAGE [26]. The protein concentration was determined by the dye-binding assay of Bradford [27].

Tryptophan fluorescence

Changes in tryptophan fluorescence upon addition of ligands to FABP tryptophan mutants were measured at an excitation wavelength of 295 nm and an emission wavelength of 330 nm [28]. FABP (1 μ M final concentration) was added to 50 mM Hepes, pH 7.5, and the sample allowed to equilibrate, as determined by fluorescence intensity. Ligands (0.75–2.7 mM stock solution in methanol) were added to the FABP solution in

aliquots (0.2–1 μ l) and the fluorescence measured after apparent equilibration.

Fluorescence energy transfer

Fluorescence energy transfer entails the transfer of electronic excitation energy from a fluorescent energy donor to a suitable acceptor [28,29]. The extent of transfer can be related to the distance between the acceptor and the donor [30] and thus this methodology can be used in the 10–60 Å range as a spectroscopic ruler [29,31].

This technique requires a spectral overlap between donor emission and acceptor absorption [28,29]. Tryptophan emission is centred at approx. 330 nm [29] and can be used as a fluorescence donor for dansyl compounds which have an excitation wavelength of 330–350 nm [32]. Thus this technique can be used to determine the distance between tryptophans and dansyl-containing ligands.

Liver FABP does not contain tryptophan and therefore three recombinant tryptophan mutants (F3W, F18W, C69W) were used. To exclude possible interference by phenylalanine or tyrosine the tryptophan fluorescence was selectively excited at 295 nm [28] and the emission spectrum measured between 300 and 600 nm to assess both a decrease in tryptophan emission and an accompanying increase in dansyl lysophospholipid emission.

Fluorescence polarization

The excitation and emission wavelengths were 350 and 500 nm respectively, and polarization was calculated using the following equations [28]:

$$\rho = (I_{vv} - GI_{vh}) / (I_{vv} + GI_{vh})$$

$$G = I_{hv} / I_{hh}$$

where ρ refers to the fluorescence polarization, I is the fluorescence intensity with the subscripts referring to the positions of the excitation or emission polarizers set in the vertical (v) or horizontal (h) positions. G is a correction factor for the detection system sensitivity for vertically and horizontally polarized light. An SLM 8000 fluorimeter was used for these studies.

Synthesis of dansyl lysoPE

Dansyl lysoPE was synthesized by the reaction of palmitoyl lysoPE (0.3 mmol) with 1.5 mmol of dansyl chloride [33]. The two compounds were dissolved in 5 ml of acetone containing 500 μ l of triethylamine and stirred for 24 h in the dark (pH > 10). The pH was then adjusted to 3.5 with citric acid and the product extracted into ethyl acetate. Water was removed from the organic phase by filtration over anhydrous sodium sulphate and the sample dried by rotary evaporation. The sample was redissolved in chloroform and applied to a silica column equilibrated with chloroform. Dansyl lysoPE was eluted with 1% acetic acid/chloroform. Acetic acid was removed by rotary evaporation in the presence of ethanol. Sample purity was verified by TLC (chloroform/methanol/acetic acid; 89:10:1, by vol.) and the identity confirmed by fast-ionization-bombardment mass spectrometry using a VG TS-250 spectrometer. We assume that the material may contain up to 10% of the 2-acyl conformer, due to acyl migration.

Fluorescence measurements and data fitting

Fluorescence measurements were determined in 50 mM Hepes buffer (pH 7.5) and fluorescence intensity measurements (not

involving fluorescence polarization) were performed on a Hitachi F2000 fluorimeter at 25 °C.

Concentrations of all dansylated compounds were determined by absorbance measurements, using an absorption coefficient of $4400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 335 nm in methanol [32]. Dansyl lysoPE was usually added to buffer in dioleoyl phosphatidylglycerol (5 mol % dansyl lysoPE). Concentrations of BSA and HSA were calculated by using the following absorption coefficients, assuming a molecular mass of 66000 Da: BSA, 6.67 cm^{-1} (1 %) at 279 nm [34]; HSA, 5.31 cm^{-1} (1 %) at 279 nm [35].

Apparent dissociation constants (K_D) and fluorescence intensities (B_{max}) were determined by fitting the relevant data to a hyperbolic equation using non-linear regression [36]. All titrations show results from a single experiment which are representative of several measurements.

RESULTS

Lysophospholipid-induced fluorescence changes in FABP tryptophan mutants

Tryptophan fluorescence is sensitive to the polarity of the local environment [28] and can thus potentially be used to determine changes in protein structure upon ligand binding. As a result of this phenomenon, the binding of lysophospholipids to albumin could be demonstrated [21,37]. Although wild-type liver FABP does not contain tryptophan, we have previously shown that the three FABP tryptophan mutants used in this study, F3W, F18W and C69W, are sensitive to ligand binding while the affinity for fatty acids and fatty acid analogues has not been significantly affected by the mutations [18]. Figure 2 shows that addition of various lysophospholipids to FABP affects tryptophan fluorescence in a manner dependent on the position of the tryptophan. The tryptophan located in the presumptive α -helical portal domain, Trp-18, shows a very small enhancement in fluorescence emission upon addition of lysophospholipids, whereas a decrease is observed for the tryptophans in the β -sheet domain, Trp-3 and Trp-69 (Figure 2a). Using the C69W mutant the binding of various lysophospholipids was monitored by changes in tryptophan fluorescence (Figure 2b). All the lysophospholipids tested showed similar binding characteristics with the smallest change in tryptophan fluorescence observed with lysoPG. The dissociation constants for lysoPA, lysoPE and lysoPC calculated from these titration curves are in the range of 1–2 μM . It should be noted that lysoPA, lysoPE and lysoPC were synthetic oleoyl derivatives, whereas lysoPG was derived from natural sources and consists primarily of palmitic and stearic acid derivatives.

The binding of dansyl lysophospholipids to liver FABP and albumin

Dansyl- C_{11} -lysoPAF may be regarded as a good fluorescent analogue of lysoPC in which the dansyl group is attached to the alkyl chain, whereas dansyl lysoPE has the fluorophore linked to the ethanolamine headgroup. The fluorescence emission characteristics of dansyl- C_{11} -lysoPAF and dansyl lysoPE, both in solution and bound to liver FABP or albumin, are shown in Table 1. Dansyl- C_{11} -lysoPAF displays an emission maximum in buffer and bound to FABP which is similar to that for the fluorescent fatty acid probe DAUDA [17]. Binding to albumin shows a greater blue-shift when compared with FABP, while HSA displayed a lower emission maximum than BSA (Table 1). These spectral characteristics are very similar to those of DAUDA binding to albumin [38,39].

The emission maximum obtained for dansyl lysoPE in buffer

Figure 2 Effect of lysophospholipids on fluorescence of FABP tryptophan mutants

Fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 330 nm. Cuvettes contained 50 mM Hepes, pH 7.5, and 1 μM protein. (a) LysoPC from a 0.2 mM stock solution (in methanol) was added to cuvettes containing FABP mutants: F3W (●), F18W (■), C69W (□). (b) Lysophospholipids from a 0.2 mM stock solution (in methanol) were added to cuvettes containing FABP(C69W): lysoPC (●), lysoPE (■), lysoPA (○), lysoPG (□).

(Table 1), which is lower than that obtained for dansyl- C_{11} -lysoPAF, would be consistent with the formation of hexagonal (H_1 and H_{11}) lamellar and undefined isotropic phases by lysoPE [1,40,41]. Phosphatidylglycerol and phosphatidylserine phospholipids have been shown to stabilize the bilayer structure of ethanolamine phospholipid bilayers [41,42] and thus dansyl lysoPE was added to the cuvettes as a solution in dioleoyl phosphatidylglycerol. This increased the emission maximum of dansyl lysoPE, although not to the values obtained with dansyl- C_{11} -lysoPAF (Table 1). Addition of dansyl lysoPE in a phospholipid solution did not appear to change the liver FABP-binding characteristics of dansyl lysoPE, displaying an emission maximum of approx. 500 nm (Table 1). The binding of dansyl lysoPE to albumin was similar for both BSA and HSA, although showing a considerably smaller blue-shift than obtained for dansyl- C_{11} -lysoPAF (Table 1).

Binding of dansyl lysophospholipids to FABP displays a 3-fold higher apparent affinity (K_D) for the R122Q mutant than the

Table 1 Fluorescence characteristics of dansyl lysophospholipids bound to FABP and albumin

Fluorescence emission maxima (nm) were measured upon excitation at 350 nm. The relevant proteins were added to the cuvettes until a maximum emission wavelength was attained, at which point the data were averaged ($\pm$ S.D.). The numbers of data points are shown in parentheses. Abbreviations: WT, wild type; N.D., not determined.

Protein	Emission maximum (nm)		
	Dansyl-C ₁₁ -lysoPAF (0.65 μ M)	Dansyl lysoPE (1.35 μ M)	Dansyl lysoPE* (0.5 μ M)
Buffer	540 $\pm$ 1.9 (4)	515 $\pm$ 1.7 (7)	525 $\pm$ 1.5 (6)
FABP(WT)	489 $\pm$ 1.5 (5)	501 $\pm$ 1.5 (4)	495 $\pm$ 1.5 (6)
FABP(R122Q)	490 $\pm$ 1.1 (4)	500 $\pm$ 2.1 (4)	504 $\pm$ 1.7 (9)
BSA	476 $\pm$ 2.1 (5)	N.D.	489 $\pm$ 1.3 (9)
HSA	460 $\pm$ 0.7 (6)	N.D.	488 $\pm$ 0.8 (9)

* Dansyl lysoPE (0.5 μ M final concentration) was added to cuvettes in dioleoyl phosphatidylglycerol vesicles.

Table 2 Characteristics of dansyl lysophospholipid binding to FABP and albumin

Fluorescence was determined at an excitation wavelength of 350 nm and emission wavelength of 500 nm. Protein concentrations were kept constant at 0.1 μ M. Dansyl lysoPE (0.5 μ M final concentration) was added to cuvettes in dioleoyl phosphatidylglycerol vesicles. $B_{\max}$ is shown in arbitrary fluorescence units and K_D in μ M of the dansyl lysophospholipid ($\pm$ S.E.M.). The number of data points is shown in parentheses.

Protein	Ligand	$B_{\max}$	K_D (μ M)
FABP(WT)	Dansyl-C ₁₁ -lysoPAF	50 $\pm$ 5	1.7 $\pm$ 0.4 (40)
FABP(R122Q)	Dansyl-C ₁₁ -lysoPAF	146 $\pm$ 16	0.6 $\pm$ 0.2 (18)
BSA	Dansyl-C ₁₁ -lysoPAF	35 $\pm$ 3	0.52 $\pm$ 0.18 (8)
HSA	Dansyl-C ₁₁ -lysoPAF	109 $\pm$ 9	0.74 $\pm$ 0.16 (10)
FABP(WT)	Dansyl lysoPE	54 $\pm$ 3	0.9 $\pm$ 0.1 (18)
FABP(R122Q)	Dansyl lysoPE	63 $\pm$ 4	0.3 $\pm$ 0.1 (25)
BSA	Dansyl lysoPE	114 $\pm$ 6	0.14 $\pm$ 0.03 (8)
HSA	Dansyl lysoPE	243 $\pm$ 5	0.19 $\pm$ 0.02 (8)

wild-type protein (Table 2). A small increase in the maximum fluorescence intensity ($B_{\max}$) was observed for dansyl lysoPE binding to the R122Q mutant, relative to the wild-type protein, but there was a significant increase in this parameter with dansyl-C₁₁-lysoPAF binding. The dissociation constant calculated for dansyl-C₁₁-lysoPAF binding to wild-type FABP (Table 2) is similar to the value of 1.35 μ M determined for lysoPC binding to this protein [21]. Fitting the data to the Hill equation indicated a 1:1 binding ratio (data not shown). The ability of oleic acid to displace bound dansyl-C₁₁-lysoPAF or DAUDA was compared (Figure 3a) and both fluorescent probes were displaced to a similar extent by oleic acid, suggesting a shared binding topology.

Binding affinities of BSA and HSA for dansyl-C₁₁-lysoPAF and dansyl lysoPE were of the same order of magnitude, although the maximum fluorescence intensity ($B_{\max}$) was significantly higher for HSA (Table 2). No unambiguous results could be obtained for the number of binding sites on BSA or HSA; however, between one and two sites were indicated by data fits to the Hill equation (data not shown). The displacement of dansyl-C₁₁-lysoPAF by oleic acid was very similar for both BSA and HSA (Figure 3b) and confirms the similar affinity of these two proteins for the ligand (Table 2). Comparison of DAUDA and dansyl-C₁₁-lysoPAF displacement suggests that dansyl-C₁₁-

Figure 3 Oleic acid displacement of DAUDA and dansyl-C₁₁-lysoPAF from (a) wild-type FABP and (b) albumin

(a) Cuvettes contained 1 ml of buffer, 1 μ M FABP and 1 μ M DAUDA (●) or dansyl-C₁₁-lysoPAF (■). (b) Cuvettes contained 1 ml of buffer, 1 μ M BSA (squares) or HSA (circles) and 1 μ M DAUDA (open symbols) or dansyl-C₁₁-lysoPAF (solid symbols). Oleic acid was added in 0.5 μ l aliquots. Fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 500 nm.

lysoPAF binds to its primary binding site on BSA with somewhat lower affinity than DAUDA (Figure 3b).

Fluorescence polarization

The fluorescence polarization experiments showed no significant difference between wild-type FABP and the R122Q mutant, or between BSA and HSA, for the binding of dansyl-C₁₁-lysoPAF. However, the polarization values were significantly higher for albumin than for FABP (Table 3) and appear to correlate with the enhanced hydrophobicity of the albumin-binding site as demonstrated by the emission wavelength maxima (Table 1).

Fluorescence energy transfer

Using three tryptophan mutants of rat liver FABP we were able to measure the relative fluorescence energy transfer between single, strategically positioned tryptophans and the dansyl moieties of the modified lysophospholipids. The tryptophans are positioned near the α -helical entry portal (F18W) and in the

Table 3 Fluorescence polarization of dansyl-C₁₁-lysoPAF binding to FABP and albumin

Fluorescence was determined at an excitation wavelength of 350 nm and an emission wavelength of 500 nm. Samples contained 50 mM Hepes, pH 7.5 buffer (2 ml), 0.5 μ M dansyl-C₁₁-lysoPAF and protein. Results were averaged ($\pm$ S.D.) for data obtained with the following protein concentrations: 1.0, 1.5, 2.5 μ M, FABP(WT); 0.25, 0.5 μ M, FABP(R122Q); 0.25, 0.5 μ M, BSA; 0.25, 0.5 μ M, HSA. Fluorescence polarization is shown in arbitrary fluorescence units. The number of data points is shown in parentheses.

Protein	Polarization (arbitrary units)
Buffer	0.028 $\pm$ 0.005 (6)
FABP(WT)	0.083 $\pm$ 0.003 (6)
FABP(R122Q)	0.089 $\pm$ 0.001 (5)
BSA	0.190 $\pm$ 0.017 (4)
HSA	0.214 $\pm$ 0.005 (4)

Figure 4 Fluorescence energy transfer between FABP tryptophan mutants and (a) dansyl-C₁₁-lysoPAF or (b) dansyl lysoPE, as displayed by a decrease in tryptophan fluorescence at 330 nm

Fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 330 nm (energy transfer was confirmed by an increase in fluorescence emission at 500 nm in a control experiment). Cuvettes contained 50 mM Hepes, pH 7.5, and 1 μ M of the relevant protein: F3W (●), F18W (■), C69W (○). Dansyl-C₁₁-lysoPAF or dansyl lysoPE in dioleoyl phosphatidylglycerol was added in aliquots from a methanol stock solution.

Figure 5 Fluorescence energy transfer between dansyl lysophospholipids and albumin tryptophans as displayed by a decrease in tryptophan fluorescence at 330 nm

Fluorescence was determined at an excitation wavelength of 295 nm and an emission wavelength of 330 nm (energy transfer was confirmed by an increase in fluorescence emission at 500 nm in a control experiment). Cuvettes contained 1 ml of 50 mM Hepes buffer, pH 7.5, and 1 μ M of BSA (circles) or HSA (squares). Dansyl-C₁₁-lysoPAF (open symbols) or dansyl lysoPE in dioleoyl phosphatidylglycerol (solid symbols) was added in aliquots from a methanol stock solution.

β -clam structure (F3W, C69W), as deduced from a comparison between the intestinal FABP structure and the liver FABP amino acid sequence [18]. As shown in Figure 4, the relative decrease in tryptophan fluorescence observed on addition of both dansyl-C₁₁-lysoPAF and dansyl lysoPE is very similar for the three tryptophans, which correlated with enhanced emission at longer wavelengths (data not shown). The efficiency of fluorescence energy transfer can be directly related to the distance between the fluorescence donor and acceptor [29]. Hence, it is deduced that the dansyl moieties on both dansyl lysophospholipids are probably similar distances from each of the tryptophans, which is consistent with the dansyl residues being located within the binding cavity of the protein.

The relative fluorescence decrease by dansyl-C₁₁-lysoPAF or dansyl lysoPE of the single HSA tryptophan was approximately equivalent (Figure 5). By contrast, a greater decrease in BSA fluorescence intensity was observed with dansyl-C₁₁-lysoPAF binding, as compared with dansyl lysoPE (Figure 5). HSA possesses only a single tryptophan, at position 214, as opposed to two tryptophans at positions 135 and 214 in BSA [43]. These results appear to indicate that the binding of both lysophospholipids is equidistant from the single HSA tryptophan, assuming that BSA and HSA have a similar structure. On the other hand, the lysophospholipid acyl chain is closer than the headgroup to at least one tryptophan in BSA, as indicated by the dansyl-C₁₁-lysoPAF and dansyl lysoPE results respectively.

DISCUSSION

We have investigated the binding of lysophospholipids to rat liver FABP and albumin. The ligand-binding properties of liver FABP are significantly different from those of the heart or intestinal FABP isoforms [12–16]. We have recently confirmed, using displacement studies, that liver FABP and albumin can bind lysophospholipids as well as fatty acids [21,38,44], both ligands being products of phospholipid hydrolysis by phospho-

lipases A. Using liver FABP mutant proteins [17,18] and lysophospholipid fluorophores we have been able to investigate the binding characteristics of lysophospholipids in more detail. Similar investigations were performed with albumin, using both BSA and HSA.

Addition of lysophospholipids to three FABP tryptophan mutants displayed a decrease in fluorescence intensity for the two fluorophores, F3W and C69W, located in the β -clam binding cavity (Figure 2a), which indicates that the binding of lysophospholipids caused a structural change within the β -clam cavity. The fluorescence characteristics of dansyl- C_{11} -lysoPAF and dansyl lysoPE bound to both wild-type FABP and the R122Q mutant were similar, displaying emission maxima of 490–500 nm (Table 1), which are comparable with those shown previously for the fatty acid probe DAUDA [44]. The approx. 3-fold increase in binding affinity by the R122Q protein for both dansyl- C_{11} -lysoPAF and lysoPE (Table 2) is difficult to explain, but parallels the enhanced binding of oleoyl coenzyme A [17]. By comparison with intestinal FABP it can be predicted that Arg-122 in liver FABP is relatively close to the hypothesized ligand entry portal [11,45]. Thus, the removal of hydrogen bonds and the smaller size of glutamine, as compared with arginine, could make the entry portal region more flexible, thereby increasing the affinity for bulky ligands. Increased reactivity of Cys-69 and decreased stability of the R122Q mutant [17] indicate that increased flexibility is feasible.

Binding of dansyl- C_{11} -lysoPAF to the R122Q protein shows a significant increase in B_{max} (Table 2) although no change in the fluorescence emission maximum was detected (Table 1). Fluorescence solvent effects can result from a change in polarity, dielectric constant or polarizability, while solvent quenching can be the result of an increase in phosphorescence, formation of non-fluorescent complexes via hydrogen-bonding, van der Waals interactions or ionization to a non-fluorescent species [46]. Replacement of Arg-106 by a glutamine in intestinal FABP has been shown to decrease the number of hydrogen bonds, ion-pair and van der Waals interactions in the binding cavity [47]. Both proteins show similar fluorescence polarization values (Table 3) and a change in binding-site polarity can be ruled out as the latter phenomenon would be indicated by a change in emission maxima between the two FABP proteins [28]. Therefore, the increased B_{max} observed for dansyl- C_{11} -lysoPAF binding to the R122Q mutant can be explained by the loss of hydrogen bonding or van der Waals interactions. By contrast, no significant difference in fluorescence intensity was observed with lysoPE binding (Table 2), suggesting that the dansyl group of this lysophospholipid analogue may be in a different location within the binding cavity from the dansyl group on dansyl- C_{11} -lysoPAF.

Fluorescence energy transfer studies with three liver FABP tryptophan mutants (F3W, F18W, C69W) and the dansyl lysophospholipids indicated that the fluorescent moieties are at similar distances from the three tryptophans (Figure 4). This observation is consistent with the fluorophores being located in a non-polar environment. In the case of dansyl lysoPE a bent or U-shaped conformation is suggested, with both the acyl chain and lysophospholipid headgroup being buried within the β -barrel cavity of the protein, while we anticipate that the phosphate anion would be more solvent-exposed. Previously we have shown that the dansyl moiety of DAUDA is also bound within this cavity [18].

Albumin is a ubiquitous transport protein which is able to bind a wide range of ligands, including lysophospholipids, at several different sites on the protein [37,43,48,49]. Both BSA and HSA show similar binding affinities for dansyl lysoPE and dansyl- C_{11} -lysoPAF, the latter compound binding with slightly

lower affinity (Table 2). The increased fluorescence intensity (B_{max}) observed with ligand binding to HSA (Table 2) can readily be explained by the increased blue-shift, or apparent hydrophobicity, observed with HSA (Table 1) and the same phenomenon was observed with the binding of DAUDA to HSA, as compared with BSA [38,39]. We have previously proposed that, with the exception of lysoPA [21], lysophospholipids and the fluorescent fatty acid probe DAUDA [38,39] bind to albumin at a site(s) which is distinct from the high-affinity fatty acid-binding sites. In the case of dansyl- C_{11} -lysoPAF the fluorescence emission characteristics of the dansyl group bound to BSA and HSA are very similar to those for DAUDA and therefore it is tempting to speculate that the sites share considerable identity. The relatively large blue-shift of the fluorescence emission maximum indicates that this is a low-polarity site, particularly in the case of HSA, and would fit in with the proposal that the interaction of lysoPC with albumin is mainly due to hydrophobic effects [48,49].

In summary, we have directly demonstrated the binding of lysophospholipids to liver FABP with a 1:1 stoichiometry. Displacement studies with oleic acid indicate that at least part of the binding site is shared with long-chain fatty acids. The fluorescent lysophospholipids are also able to bind to albumin in competition with long-chain fatty acids and hence appear to behave very similarly to normal lysophospholipids. These fluorescent probes should prove useful in evaluating the precise role of liver FABP in lysophospholipid binding in liver and other tissues that contain this protein.

Financial assistance from the Wellcome Trust is gratefully acknowledged. We thank Dr. D. Corina for performing the mass spectrometry measurements.

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