Mutations of recombinant rat liver fatty acid-binding protein at residues 102 and 122 alter its structural integrity and affinity for physiological ligands

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Rat liver fatty acid-binding protein (FABP) is able to accommodate a wide range of non-polar anions in addition to long-chain fatty acids. This property means that the liver protein is functionally different from other FABPs from intestine, muscle and adipose tissue that have a more restricted ligand specificity and stoichiometry. The availability of crystal structures for the latter proteins has highlighted the importance of two arginine residues that are involved in the binding of the fatty acid carboxylate. Only one of these arginine residues, arginine-122, is conserved in liver FABP, whereas the other arginine, at position 102, is replaced by a threonine. In order to gain further insight into the nature of ligand interactions with liver FABP these key residues (102 and 122) have been changed by site-directed mutagenesis. The results with an R122Q mutant highlight the critical role of this arginine in determining ligand affinity, while similar but less dramatic effects were observed with the T102Q mutant. The double mutant T102Q/R122Q was expressed but had lost the ability to bind fluorescent ligands. It is concluded that Arg-122 plays a role in accommodating the carboxylate group of at least one fatty acid. It is proposed that physiological ligands with more bulky headgroups, such as lysophospholipids, acyl-CoA and mono-olein, bind with the headgroups in a solvent-exposed location near the portal region of the protein. The portal region is suggested to be more flexible in the mutants (R122Q and to a lesser extent T102Q). The net result is that the ligand specificity of the R122Q mutant changes to that of a protein with enhanced affinity for acyl-CoA, lysophospholipids and mono-olein.

INTRODUCTION

The fatty acid-binding proteins (FABPs) are widely distributed intracellular lipid-binding proteins of low molecular mass and include heart, muscle, intestinal, adipocyte and liver forms [1–4]. Various functions have been proposed for these proteins, including fatty acid transport and targeting, a protective function and mediation of mitogenesis [1–3,5]. Rat liver FABP differs from intestinal, adipocyte and muscle FABPs in terms of ligand-binding characteristics, as follows: (1) it can bind a molar ratio of two fatty acids, as opposed to one for the other proteins [6–10], (2) it binds a wider range of ligands, including acyl-CoA and lysophospholipids [11–16], and (3) NMR studies indicate that fatty acid carboxylate groups are solvent-accessible, located near the protein/solvent interface and do not form electrostatic charge interactions with the protein [6,7].

The structures of various intracellular lipid-binding proteins have been elucidated and consist of two α-helices near the N-terminus and 10 antiparallel β-strands in two orthogonal β-pleated sheets forming a 'β-clam' structure [17–19]. For intestinal, muscle and adipocyte FABPs the fatty acid carboxylate group forms electrostatic and hydrogen-bonding interactions with arginine residues 106 and 126 [17–19], and it has been suggested that intracellular lipid-binding proteins can be separated into two subgroups based on the presence or absence of an arginine at position 106, relative to sequence alignment with intestinal FABP [20]. Proteins that do not contain an arginine at this position, such as liver FABP (threonine-102), are less specific in their ability to bind ligands [20].

Previously we have shown that Arg-122 is not required for fatty acid binding to rat liver FABP. Changing this residue to a glutamine did, however, increase affinity for oleoyl-CoA and lysophospholipids [12,14]. We have now extended these studies by making three further proteins with mutations at residues 102 and 122, i.e. T102R, T102Q and the double mutant T102Q/R122Q. Such mutations would mean that the liver FABP would more closely resemble the other well described FABPs or cellular retinoid-binding proteins at these key residues. The T102R mutant was not expressed, whereas pure protein could be isolated for the other mutants and their binding properties investigated. It is proposed that Arg-122 plays a significant but minor role in the binding of one ligand carboxylate. In addition, the changed binding specificity of the R122Q mutant can be interpreted in terms of increased structural flexibility allowing ligands with more bulky headgroups to be more readily accommodated.

EXPERIMENTAL

Chemicals

The pET-11a plasmid and BL21(DE3) bacterial strain were obtained from Novagen, Madison, WI, U.S.A. Palmitoyl-lysophosphatidylethanolamine (palmitoyl-lysoPE) was purchased from Calbiochem-Novabiochem, Nottingham, U.K. Lysophosphatidylglycerol (lysoPG) (containing primarily palmitic and stearic acids), oleoyl-lysoPE, oleoyl-lysophosphatidylcholine (oleoyl-lysoPC) and oleoyl-lysophosphatidic acid (oleoyl-lysoPA) were obtained from Sigma. 11-(5-Dimethylaminonaphthalenesulphonyl)undecanoic acid (DAUDA) and 1-{*O*-[11-(5-dimethylaminonaphthalenesulphonyl)amino]undecyl}-*sn*-glycero-3-phosphocholine (dansyl-lysoPAF) were purchased from Molecular Probes, Eugene, OR, U.S.A., and isopropyl

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

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β-D-thiogalactoside was from Northumbria Biologicals, Cramlington, Northumbria, U.K. All other chemicals were obtained from Sigma.

Purification of FABP

The T102R, T102Q and T102Q/R122Q FABP mutants were produced by site-directed mutagenesis [21]. The R122Q mutant has been described previously [12]. The T102Q and T102Q/R122Q FABP mutants were purified from *Escherichia coli* by ammonium sulphate fractionation followed by chromatography on DEAE-Sepharose (20 mM Tris/HCl, pH 8.5, 0–500 mM NaCl gradient) and Sephadex G50 (20 mM phosphate, pH 7.5). The wild-type and R122Q proteins were purified on naphthoyl-aminodecylagarose [22,23]. The proteins were subsequently delipidated on Lipidex 1000 [24]. Protein purity was assessed by SDS/PAGE [25] and the protein concentration determined by the dye-binding assay of Bradford [26].

Ligand displacement assays

DAUDA fluorescence (excitation 350 nm; emission 500 nm) was measured in 50 mM phosphate buffer (at the pH indicated) containing 1 μM FABP and 1 μM DAUDA; ligands (in methanol) were added in 0.2–1.0 μl aliquots (25 °C).

Significance levels were calculated using an unpaired (two sample) Student's t test. In the case of significant differences between variances (F-test) the Mann–Whitney test was used.

Calculation of DAUDA binding constants (B_{max}, K_D)

Fluorescence assays contained FABP (0.5 μM) in 50 mM Hepes, pH 7.5, and DAUDA was added at various concentrations. Fluorescence values (excitation 335 nm; emission 500 nm) were corrected for DAUDA fluorescence and fitted to a hyperbolic equation by non-linear regression:

$$F_{CORR} = B_{max} \times [S]/(K_D + [S])$$

where F_{CORR} is the corrected fluorescence, [S] is the DAUDA concentration (μM), B_{max} is the calculated maximum fluorescence (arbitrary fluorescence units) and K_D is the calculated dissociation constant (μM).

Circular dichroism

Spectra were measured in a Jasco J-720 spectropolarimeter using 1 mm pathlength cuvettes (400 μl sample volume; 25 °C) and converted into molar ellipticity values using the software supplied.

Fluorescent ligands

Concentrations of DAUDA, *N*-(5-dimethylaminonaphthalene-sulphonyl)-1-palmitoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-lysoPE) and dansyl-lysoPAF were determined by using a molar absorption coefficient of 4400 $M^{-1} \cdot cm^{-1}$ at 335 nm in methanol [27]. Dansyl-lysoPE was synthesized as described previously [14].

RESULTS

The lack of a crystal structure for liver FABP means that the best approach to an understanding of its structure and function is by comparison with the crystal structures of related FABPs [17–19]. This comparison has revealed the possibly crucial role of the conserved residue Arg-122 in ligand binding. A second arginine is also conserved in related FABPs; however, this arginine is replaced by a threonine (Thr-102) in liver FABP [4]. This change may be a crucial feature in explaining the different binding characteristics of liver FABP [20].

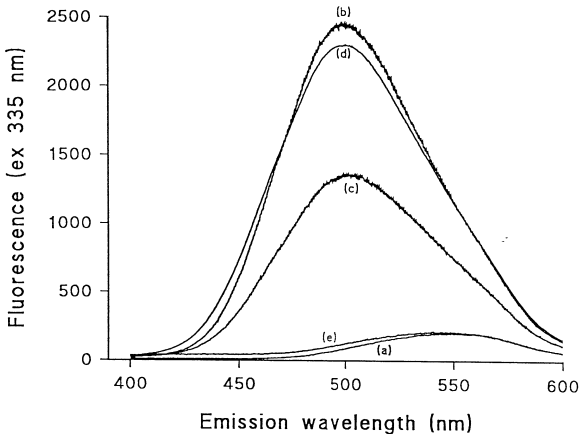

Figure 1 Effect of FABP addition on the fluorescence emission spectra of fluorescent ligands

Samples contained 50 mM Hepes buffer (pH 7.5) and 1 μM DAUDA. The following FABP samples were added: a, none; b, 2 μM wild-type; c, 1.6 μM T102Q; d, 1.5 μM R122Q; e, 3 μM T102Q/R122Q. The fluorescence excitation wavelength was 335 nm.

Expression of FABP mutants

In order to understand the nature of ligand binding to liver FABP it was decided to mutate the residues at positions 102 and 122, as the equivalent residues are important for ligand interactions with other members of this lipid-binding family [4,17]. Thus intestinal, muscle and adipose FABPs contain arginine at position 102, while the cellular retinoid-binding proteins contain glutamine at both of these positions [4].

We have used a synthetic rat liver FABP gene cloned into the pET-11a vector to produce FABP mutants by site-directed mutagenesis [12,21]. The production and initial characterization of R122Q FABP has been described [12] and here we have produced further mutants: T102Q, T102R and the double mutant T102Q/R122Q. Preliminary studies showed that the T102R protein could not be detected in cell lysates either by SDS/PAGE or by binding of the fluorescent fatty acid analogue DAUDA (results not shown). However, the T102Q, R122Q, T102Q/R122Q and wild-type proteins were isolated and their binding specificities subsequently characterized in more detail.

DAUDA and dansyl-lysophospholipid binding to wild-type, T102Q, R122Q and T102Q/R122Q FABPs

The fluorescence characteristics of dansylated fluorophores are polarity-sensitive [28] and the binding of DAUDA to liver FABP results in a characteristic blue-shift in fluorescence emission and a substantial increase in fluorescence yield [29]. Binding of DAUDA and fluorescent lysophospholipid analogues to R122Q and wild-type FABP has been shown to have a fluorescence emission maximum between 490 and 500 nm [14,30]. The T102Q mutant showed similar binding characteristics, whereas no binding could be detected for the T102Q/R122Q protein (Figure 1; Table 1). In addition, no binding of the fluorescent fatty acid *cis*-parinaric acid was observed using the T106Q/R122Q mutant, although binding could be shown for the other proteins (results

Table 1 Effect of wild-type and mutant FABPs on fluorescence emission maxima of various fluorophores

Cuvettes contained 50 mM Hepes (pH 7.5) and a constant concentration of fluorophore (between 0.5 and 2.0 μM), to which were added aliquots of FABP; the emission maximum was determined until no further change in emission wavelength was detected. Dansyl-lysoPE was added to cuvettes in dioleoyl-phosphatidylglycerol vesicles [14]. N.D., not determined.

	Fluorescence emission maximum (nm)				
Fluorophore	Buffer	Wild-type FABP	T102Q FABP	R122Q FABP	T102Q/R122Q FABP
DAUDA	547.7 ± 2.9	498.5 ± 1.7	502.1 ± 1.8	500.4 ± 2.2	542.6 ± 5.4
Dansyl-lysoPE	516.4 ± 2.5	503.0 ± 0.8	500.5 ± 1.3	N.D.	513.9 ± 2.8
Dansyl-lysoPAF	549.0 ± 3.6	501.5 ± 1.5	500.8 ± 1.6	N.D.	545.4 ± 2.0

Table 2 DAUDA binding constants of FABP mutants

Cuvettes contained 50 mM Hepes (pH 7.5), 0.5 μM FABP and DAUDA at concentrations of 0.25–5 μM. Binding constants were determined by fitting the data to a hyperbolic equation by non-linear regression. B_{max} is in arbitrary fluorescence units.

FABP	K_D (μM)	B_{max}
Wild-type	0.38 ± 0.02	1005 ± 14
T102Q	0.72 ± 0.07	660 ± 22
R122Q	0.53 ± 0.03	1257 ± 26

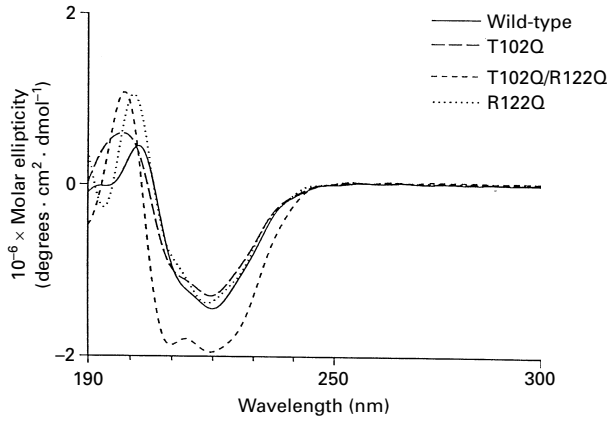

Figure 2 CD spectra of wild-type, T102Q, R122Q and T102Q/R122Q FABPs

The samples were in 20 mM phosphate buffer (pH 7.5) and had been delipidated.

not shown). Although the amino acid residues in the T102Q/R122Q mutant now reflect those seen in cellular retinol-binding proteins, no binding of retinol to this mutant was detected.

The dissociation constants (K_Ds) for DAUDA binding to the three viable proteins were similar and in agreement with published values [12,15], but there was a significant difference in the calculated maximum fluorescence yield (Table 2). These differences in B_{max} are difficult to explain, but the increased B_{max} for the R122Q protein relative to the wild-type FABP has been observed before for DAUDA and the fluorescent lysophospholipids [12,14]. Although fluorescence solvent effects are complex [31], we have previously speculated that an increase in fluorescence yield observed for dansyl-lysoPAF binding was the result of decreased hydrogen-bonding or van der Waals interactions with the R122Q FABP [14]. Similar effects could explain the decreased fluorescence observed for DAUDA binding to the T102Q mutant (Table 2).

Circular dichroism

CD spectra of the purified FABP proteins showed that the secondary structure characteristics of the T102Q and R122Q mutants were similar to those of the wild-type protein (Figure 2). However, there was a significant difference for the T102Q/R122Q protein, seen as an apparent increase in predicted α-helical content, consistent with a measure of incorrect protein folding of a predominantly β-barrel structure [17,32]. Thus both ligand-binding and CD studies indicate structural changes in this mutant protein.

Ligand displacement of DAUDA

We have used a relatively simple method involving the displacement of DAUDA from FABP, with a resulting loss of fluorescence, to assess the apparent binding affinities of the T102Q, R122Q and wild-type FABPs (Figure 3; Table 3). This assay has been used to demonstrate that the wild-type protein preferentially binds oleate relative to the R122Q FABP, whereas the opposite effect is observed for oleoyl-CoA [12] and lysophospholipids [14]. The T102Q protein consistently displayed affinities between those of the R122Q and wild-type proteins (Figure 3), and further binding studies were concentrated on the latter proteins.

The relative effects of a variety of physiological ligands on DAUDA displacement from wild-type and R122Q FABP are shown in Table 3. Ligands with greater affinity for the wild-type protein included long-chain fatty acids, bilirubin and haem. These ligands contain one or more carboxylate moieties and therefore it would appear that Arg-122 plays a role in determining the affinity of normal carboxylate-containing ligands for the wild-type FABP. By contrast, lysophospholipids, oleoyl-CoA and mono-olein bound with higher affinity to R122Q FABP (Table 3). In the case of lysophospholipids it is also apparent that increased binding to both proteins was observed with decreased headgroup size, although this effect was most significant with the R122Q mutant. The ability of the R122Q protein to bind more bulky ligands is especially relevant for oleoyl-CoA and mono-olein. Binding of a fluorescent anthroyloxy analogue of mono-olein has been reported [33,34], but, in the present investigation, fluorescence displacement studies with unmodified mono-olein indicated that binding to the wild-type protein is minimal (Table 3).

The nature of the interaction of oleoyl-CoA, lysophospholipids and mono-olein with FABP must be speculative. It has been previously proposed that the acyl chain of a lysophospholipid is

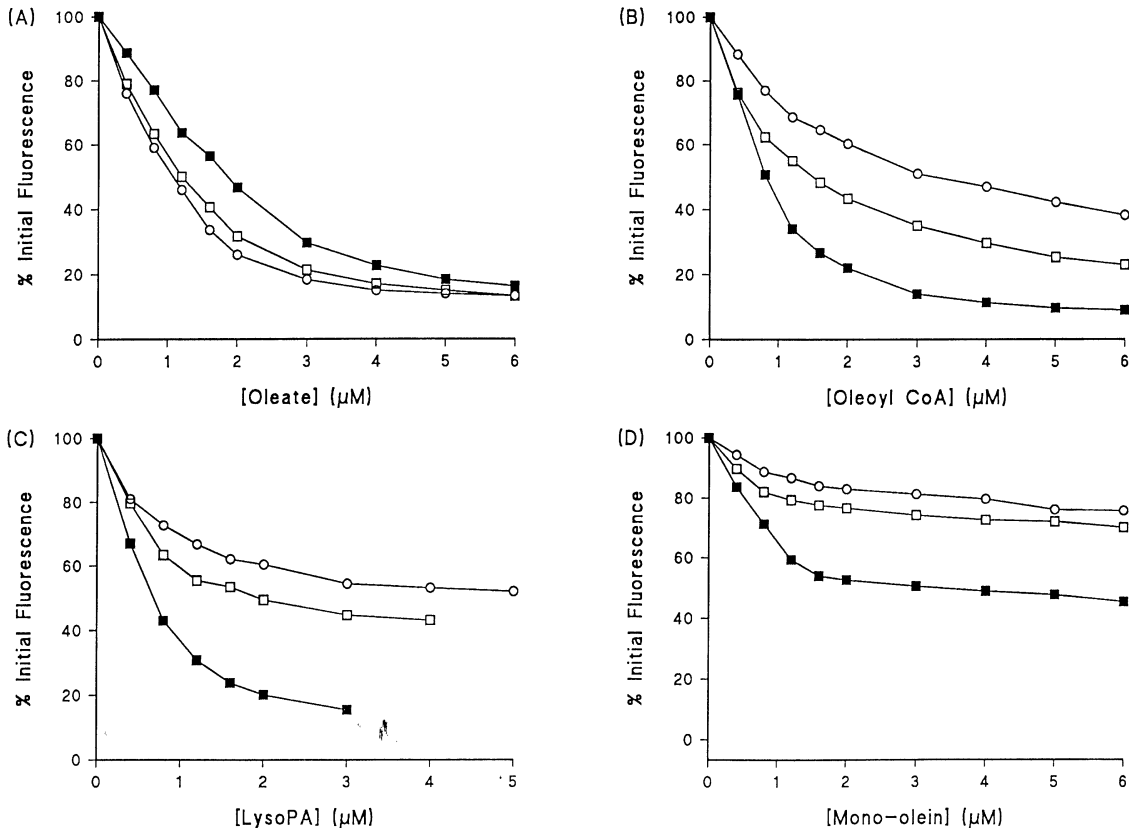

Figure 3 Displacement of DAUDA from FABP by (A) oleate, (B) oleoyl-CoA, (C) lysoPA and (D) mono-olein

Cuvettes contained 50 mM phosphate buffer (pH 7.5), 1 μM FABP and 1 μM DAUDA, to which aliquots of the second ligand were added (0.2 mM stock solutions in methanol). ○, Wild-type; □, T102Q; ■, R122Q. Results are given as percentages of the initial fluorescence (before addition of the second ligand).

Table 3 Ligand displacement of DAUDA from wild-type and R122Q FABPs at pH 7.2

Cuvettes contained 50 mM phosphate (pH 7.2), 1 μM FABP and 1 μM DAUDA, with the second ligand added in aliquots of 0.2–1.0 nmol. Data are shown for ligand concentrations of 2 μM, and are means ± S.E.M. for the numbers of determinations given in parentheses. Significant differences between wild-type and R122Q: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

	Fluorescence (% of initial)		
Ligand	Wild-type FABP	R122Q FABP	Fluorescence ratio (wild-type/R122Q)
Methanol	77 ± 5 (13)	80 ± 6 (13)	0.96
Oleate	24 ± 3 (18)	40 ± 7 (18)	0.60***
Arachidonate	27 ± 1 (4)	35 ± 3 (4)	0.77*
Haem	29 ± 4 (5)	49 ± 7 (5)	0.59***
Bilirubin	57 ± 4 (4)	72 ± 6 (4)	0.79*
LysoPC	70 ± 4 (3)	54 ± 4 (3)	1.30**
LysoPG	62 ± 3 (3)	39 ± 7 (3)	1.59**
LysoPE	69 ± 1 (3)	41 ± 1 (3)	1.68**
LysoPA	56 ± 8 (5)	24 ± 5 (5)	2.33**
Oleoyl-CoA	48 ± 8 (5)	17 ± 3 (5)	2.82***
Mono-olein	77 ± 1 (3)	48 ± 1 (3)	1.60***

located within the hydrophobic domain of the protein [14]. In the case of oleoyl-CoA the pantatheine moiety could also be buried, leaving the 5′-phosphates of the ADP to provide an anion. Although the adenine ring structure has some non-polar character this is unlikely to be bound, since ATP is not a ligand for this protein [35]. The enhanced binding of mono-olein to the R122Q mutant could be explained by the glycerol headgroup being more easily accommodated in the portal region of the protein.

Effects of pH and ionic strength on ligand displacement from wild-type and R122Q FABPs

To further assess the possibility that ligand carboxylate anions interact with cationic amino acids in liver FABP, the effects of pH and ionic strength on ligand binding were investigated. Between pH 4 and pH 9 only small effects on DAUDA fluorescence yield and emission maximum were observed (Figure 4). However, at pH levels below 6 there was a significant decrease in DAUDA displacement by oleate from wild-type FABP that was not observed with the R122Q mutant (Figures 4 and 5). By contrast, oleoyl-CoA did not show the same pH effect, although there appeared to be a small but consistent decrease in binding to both proteins with lowered pH (Figure 5).

Previous studies of pH effects on this protein have involved IR, CD and NMR analysis. IR spectra of liver holo-FABP show a conformational change with a pK of 4.5, which suggests that the α-helical segments lose intensity and denature into unordered structures [36], while both CD and NMR analysis indicated a pH-sensitive conformational change and fatty acid dissociation from liver FABP [6,7]. The dissociation of ligand probably results in a conformational change, since: (1) differences in CD

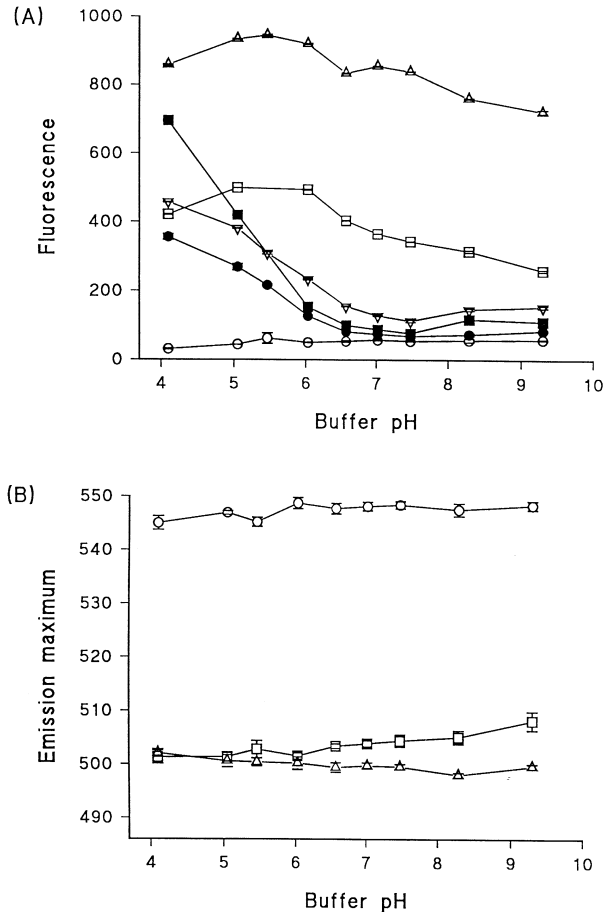

Figure 4 Effect of pH on the fluorescence characteristics of DAUDA in the presence of FABP and oleate

Cuvettes contained 10 mM sodium phosphate buffer and 0.5 μM DAUDA, to which were added 0.5 μM FABP and 5 μM oleate. Fluorescence emission characteristics were measured at an excitation wavelength of 335 nm: (**A**) fluorescence yield (emission = 500 nm); (**B**) fluorescence emission maximum (nm). ○, DAUDA; ●, DAUDA + oleate; □, wild-type FABP + DAUDA; ■, wild-type FABP + DAUDA + oleate; △, R122Q FABP + DAUDA; ▽, R122Q FABP + DAUDA + oleate.

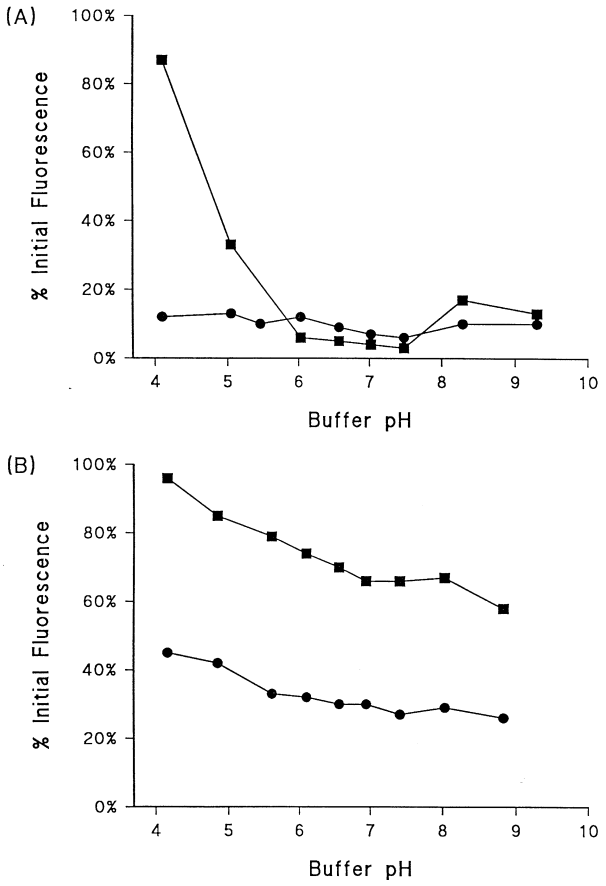

Figure 5 Effect of pH on the displacement of DAUDA from FABP by (A) oleate and (B) oleoyl-CoA

Data were corrected for DAUDA or DAUDA + oleate. The results are presented as percentages of the initial fluorescence (in the absence of ligand), and were calculated as: [(FABP + DAUDA + ligand)/(FABP + DAUDA)] × 100. ■, Wild-type; ●, R122Q FABP

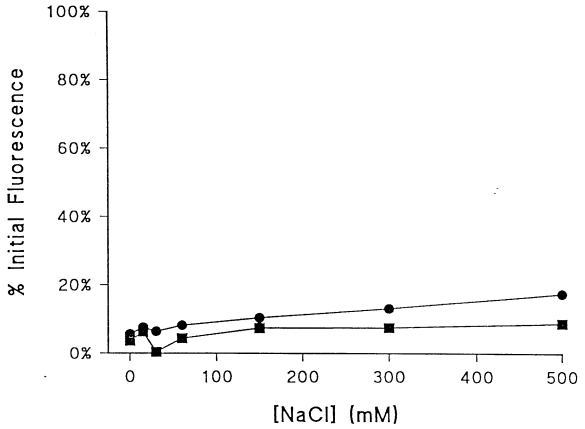

Figure 6 Effect of NaCl on the displacement of DAUDA from FABP by oleate

Cuvettes contained 10 mM sodium phosphate buffer (pH 7.5), NaCl (in the same buffer), 0.5 μM DAUDA and 0.5 μM FABP, to which was added 5 μM oleate. The results are presented as percentages of the initial fluorescence (in the absence of ligand; excitation 335 nm, emission 500 nm), and were calculated as: [(FABP + DAUDA + oleate)/(FABP + DAUDA)] × 100. ■, Wild-type; ●, R122Q FABP.

spectra have been observed for apo- and holo-FABPs [37], and (2) the apparent pK_a of oleate bound to liver FABP (4.8) is similar to the pK observed for conformational changes to liver holo-FABP [6,7,36,38].

Electrostatic interactions between fatty acid carboxylates and arginine side-chains have been demonstrated for intestinal FABP by NMR and structural studies [6,7,17], and a strong ionic strength effect on oleate binding to acrylodan-labelled intestinal FABP is indicative of ionic interactions [39,40]. However, no such effects could be demonstrated for either the wild-type or R122Q FABP (Figure 6), suggesting that ionic interactions are not the dominant feature of ligand binding to this protein.

The overall conclusions from these studies involving pH and ionic strength is that hydrophobic interactions, which are not affected by pH, play a dominant role in ligand binding, particularly for the more bulky ligands. However, with fatty acids and other carboxylate-containing ligands there is a significant contribution from a carboxylate group that interacts with Arg-122. As a result the binding of fatty acids such as oleate is affected by a decrease in pH. Surprisingly, DAUDA binding is

not affected by a potential disruption of carboxylate interactions at lower pH. A possible explanation is that the contribution of the carboxylate to DAUDA binding is minimal as the bulky hydrophobic group is the dominant feature. A similar conclusion was drawn with regard to the binding of anthroyloxy fatty acids to wild-type and mutant forms of adipocyte FABP [41].

DISCUSSION

A general feature of the intracellular FABPs is that they display a conserved arginine at positions equivalent to residue 122 of liver FABP. The intestinal, muscle and adipose FABPs, which demonstrate a higher ligand specificity than the liver FABP, also contain an arginine at the position equivalent to residue 102 in liver FABP, whereas this is a threonine in the liver protein [4]. Another lipid-binding protein with a broader ligand specificity, ileal lipid-binding protein, also lacks an arginine at position 102 [42]. In contrast, the structurally related cellular retinol-binding proteins contain glutamines at both positions, while cellular retinoic acid-binding protein retains the two arginines. Thus these two residues play a crucial role in ligand binding and specificity, as confirmed by X-ray crystallography studies [17–19]. In the present investigation our original aim was to change the binding affinity of wild-type liver FABP to resemble that of the intestinal, muscle and adipose FABPs (i.e. T102R) or the cellular retinoid-binding proteins (i.e. T102Q and T102Q/R122Q) [4], as a means of trying to define more clearly the nature of ligand binding to liver FABP.

Preliminary investigations showed that the T102R mutant was not expressed in our system, possibly as the result of incorrect protein folding. Since expression of the T102Q mutant was successful, it is presumed that the introduction of a positive charge at position 102 has an adverse effect on protein folding, resulting in bacterial degradation of the expressed protein. However, we were able to purify the other mutants and these were characterized by a variety of methods.

Binding studies with DAUDA and dansyl-lysophospholipids showed that the T102Q/R122Q mutant did not bind any of the fluorophores (Figure 1; Table 1). Although the ability of this mutant to bind fatty acids such as oleic acid was not investigated, no binding of the fluorescent fatty acid *cis*-parinaric acid was detected. CD spectra also showed a significant difference between the T102Q/R122Q protein and wild-type FABP (Figure 2), indicating a measure of incorrect folding.

The T102Q and R122Q mutants were investigated in more detail. These proteins showed similar fluorescence emission spectra and dissociation constants (K_Ds) for DAUDA, although there were significant differences in fluorescence yield (Figure 1; Tables 1 and 2). A simple fluorescence displacement assay using DAUDA showed that wild-type FABP favours ligands containing carboxylate moieties, whereas the R122Q mutant appeared to prefer more bulky ligands (Figure 3), with the affinity of the T102Q mutant being intermediate between these two proteins. More detailed comparisons of the wild-type and R122Q proteins highlighted further the difference in ligand-binding specificity between them (Table 3).

The apparent preference of wild-type FABP for carboxylate-containing ligands could indicate that some type of involvement of the arginine residue is significant in binding this type of ligand. Investigations by varying the pH showed a significant decrease in oleate binding to wild-type but not R122Q FABP below pH 6, whereas both proteins displayed a consistent effect with oleoyl-CoA (Figures 4 and 5). However, at pH 7.5 no ionic strength effects were observed (Figure 6). We therefore postulate that ligand carboxylate groups interact weakly with Arg-122 in wild-type FABP, probably via a hydrogen-bonding water network, and this interaction is decreased in the R122Q mutant. However, the carboxylate interaction is only a secondary component in the overall binding process, which is dominated by hydrophobic effects.

Work from Spener's laboratory first demonstrated the binding of two fatty acids to bovine liver FABP [10]. We would suggest that it is the interaction with the primary fatty acid-binding site which involves Arg-122. The second fatty acid site accommodates the acyl chain within the hydrophobic core of the protein, with the carboxylate group in a more solvent-exposed location near the portal region. This would place the carboxylate in a similar location to the more bulky headgroups of other physiological ligands which also bind at this position. Similarly, haem and bilirubin would bind with one carboxylate near Arg-122 and the second carboxylate in a solvent-exposed environment. As previously mentioned the less rigid structure of R122Q FABP results in a more flexible portal region which permits ligands with more bulky headgroups to bind with higher affinity [14].

This hypothesis can be supported by the following evidence. (1) Fatty acid carboxylate groups are solvent-accessible and not implicated in electrostatic interactions with liver FABP, as shown by NMR studies [6,7]. (2) Fluorescence quenching experiments with tryptophan mutants indicate that the carboxylate of at least one fatty acid is close to the portal domain [43]. (3) Studies with R122Q FABP suggested that ligand affinity is only decreased 2–5-fold relative to wild-type FABP, a smaller decrease being observed for DAUDA relative to oleate [12]. By contrast, an R106Q mutant of intestinal FABP showed a 20-fold decrease in affinity [20]. (4) Studies of bovine liver FABP isoforms showed a single binding site, comprising both polar and hydrophobic regions, which can accommodate two straight-chain fatty acids. The importance of hydrophobic interactions in ligand binding, especially for the second fatty acid, was highlighted in this study [10]. (5) Thermodynamic studies show a strong enthalpic component for fatty acid binding to intestinal FABP which is consistent with electrostatic interactions between the ligand carboxylate group and Arg-106 [17,20,44]. The two fatty acid sites in liver FABP show distinct thermodynamic properties. The high-affinity site has an entropic component of approx. 60%, compared with 10% for intestinal FABP, whereas the low-affinity site has an entropic component of approx. 80% [20,44]. The latter site can be compared with a T106Q mutant of intestinal FABP with an entropic component of approx. 85% [20,44]. Entropic components are positively influenced by loss of water from the binding cavity as well as loss of solvent ordering, and can be explained by a hydrophobic effect [39,45], which would be the main contribution to binding at both fatty acid-binding sites of liver FABP.

In conclusion, it would appear that the residue at position 122 in liver FABP may be important in determining ligand affinity and specificity, although ligand binding is driven primarily by a hydrophobic effect. The broader specificity of liver FABP would allow the binding and transport of the lysophospholipid products of phospholipase A activity of the intestine (pancreatic phospholipase A_2) and liver (hepatic lipase), and thus may prevent the deleterious effects of these biologically active lipid products within the cell. Therefore liver FABP appears to perform a role in the cell similar to that of albumin in the serum and further studies are required to determine its precise physiological function. Understanding of the exact nature of ligand interactions would be facilitated by the elucidation of the tertiary structure of liver FABP.

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