Liver fatty acid-binding protein (FABP) binds a variety of non-polar anionic ligands including fatty acids, fatty acyl CoAs, and bile acids. Previously we prepared charge reversal mutants and demonstrated the importance of lysine residues within the portal region in ligand and membrane binding. We have now prepared several tryptophan-containing mutants within the portal region, and one tryptophan at position 28 (L28W) has proved remarkably effective as an intrinsic probe to further study ligand binding. The fluorescence of the L28W mutant was very sensitive to fatty acid and bile acid binding where a large (up to 4-fold) fluorescence enhancement was obtained. In contrast, the binding of oleoyl CoA reduced tryptophan fluorescence. Positive cooperativity for fatty acid binding was observed while detailed information on the orientation of binding of bile acid derivatives was obtained. The ability of bound oleoyl CoA to reduce the fluorescence of L28W provided an opportunity to demonstrate that fatty acyl CoAs can compete with fatty acids for binding to liver FABP under physiological conditions, further highlighting the role of fatty acyl CoAs in modulating FABP function in the cell.

Liver fatty acid-binding protein (FABP) is a member of a family of structurally related small (14–15 kDa) cytosolic lipid-binding proteins that also include intestinal, heart (muscle), adipocyte, ileal, keratinocyte, and brain FABP (for recent reviews, see Refs. 1–7). The exact physiological functions of these proteins are unclear, although it is generally thought that they may have a potential role in the uptake and targeting of fatty acids to various intracellular organelles and metabolic pathways. All further sites of metabolism of long chain fatty acids in the cell involve membrane proteins. For a targeting role to operate, the FABP must interact with an intracellular structural element, and one tryptophan at position 28 (L28W) has been highlighted as a significant contribution to such binding have been identified as Lys-31, Lys-36, and Lys-57 (10). These residues are strategically placed around the portal region of the proteins in positions of enhanced protein mobility. Our studies also highlighted the role of cationic residues in ligand binding at site 2 of liver FABP particularly ligands with more bulky anionic head groups such as acyl CoAs, lysophospholipids, and bile acids (11).

To investigate further the effect of ligand and membrane binding on the conformation of liver FABP linked to a targeting role for this protein we have used a strategy of tryptophan insertion mutagenesis. Positions 28, 54, and 74 (see Fig. 1) are initially selected for mutagenesis to tryptophan because these positions are strategically placed surrounding the portal region. Fig. 1 is derived from the crystal structure with two bound oleates (12). The oleate at site 1 is buried within the cavity with its carboxyl group neutralized by a hydrogen bonding network linked to Arg-122 (12). The oleate at site 1 is buried within the cavity with its carboxyl group neutralized by a hydrogen bonding network linked to Arg-122 (12). In contrast, the oleate at site 2 has the carboxyl group located in the portal region and exposed to the external environment. Leu-28 is one of the amino acids on helix a2 along with Lys-31 that actually defines the cavity opening in liver FABP (13) and is in contact with the second oleic acid binding site (site 2) (14). The L28W proved to be remarkably effective as a probe for monitoring ligand binding compared with the other tryptophan mutations where fluorescence changes were small (data not shown), an exception being the binding of oleoyl CoA to M74W.
Therefore, the results for the binding of ligands to the L28W mutant are described in detail in this study. These results provide an important insight into the functioning of liver FABP and demonstrate the ability of long chain fatty acids and acyl CoA derivatives to effectively compete for binding to the protein, thus highlighting the potential functional importance of acyl CoA in modulating liver FABP function.

EXPERIMENTAL PROCEDURES

Materials—DAUDA was obtained from Molecular Probes (Junction City, OR.). Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL); fatty acids and laboratory chemicals are from Sigma. Restriction enzymes and other molecular biologicals were from Promega (Southampton, UK) and Sigma. Mutagenic primers were provided by MWG Biotech (Ebersberg, Germany).

Molecular Biology—The preparation of a synthetic gene for rat liver FABP (15) and expression using a pET-11a vector (9) has been described. Site-directed mutagenesis was performed using standard cloning procedures as described previously (16). The oligonucleotide sequence used for the construction of the L28W mutant was as follows (mutant sequence is underlined), 5'-GGGTCGGCGAAGACTGGATCCAGAAAGGTAAGG-3'. The mutated liver FABP construct was verified by sequence analysis that was carried out by Oswel (Southampton, UK).

Protein Expression and Purification—Recombinant FABP and the mutant were expressed, purified, and delipidated as described previously (9). Purity was confirmed by silver staining after SDS-PAGE with a Silver Stain Plus kit (Bio-Rad) used according to the manufacturer’s instructions.

CD Spectra—The CD spectrum of wild type and L28W mutant FABP were performed using a Jasco J-270 spectropolarimeter. For comparative secondary structure CD, a 0.5 mg/ml solution of protein in 10 mM phosphate buffer, pH 7.4, was measured between 190 and 250 nm.

Ligand Binding Assays—DAUDA binding assays were performed by titrating up to 1 nmol of DAUDA into liver FABP (0.8 nmol) in 10 mM Hepes/NaOH buffer, pH 7.5. The increase in fluorescence at 500 nm was monitored after excitation at 350 nm. Titrations are corrected for dilution and for a blank titration involving addition of DAUDA to buffer only. The $K_d$ values were determined using Sigma Plot software. The binding of fatty acids and other ligands to tryptophan mutants of FABP was performed by titrating ligand in methanol into protein in 10 mM Hepes/NaOH buffer or phosphate-buffered saline as indicated. The final methanol volume did not exceed 2.5% (v/v) and had no effect on the fluorescence measurements other than dilution, which was corrected. Excitation was at 280 nm and emission was at 340 nm using a Hitachi F2000 fluorimeter. Wavelength scans were performed using a Hitachi F2500 fluorimeter.

Urea Denaturation Studies—The tryptophan-containing mutants of liver FABP was equilibrated 50 mM Hepes/NaOH buffer, pH 7.5, and the fluorescence intensity was measured at 1 and 2 hours to ensure that a folding equilibrium had been achieved. Excitation was at 280 nm, and emission was at 340 nm using a Hitachi F2000 fluorimeter.

RESULTS

CD Properties of the Wild Type and L28W Mutant—The CD spectra for wild type liver FABP and L28W mutant are shown in Fig. 2. The spectrum of the L28W mutant is essentially identical to the wild type FABP indicating correct folding. This is confirmed by the ligand binding studies (see below).

Fig. 1. Ribbon diagram of rat liver FABP showing the position of the bound oleic acid (sites 1 and 2) and the amino acids that are mutated to tryptophan. Residues Lys-28, Tyr-54, and Met-74 are shown in Corey-Pauling-Koltun representation, whereas the oleic acids are shown in ball-and-stick representation. Site 1 is the higher affinity fatty acid-binding site where the ligand is buried within the binding cavity, and the carboxyl group of the bound oleate interacts with Arg-122 via a hydrogen bonding network. The carboxyl group of the oleic acid at site 2 extends into the portal region where it is solvent-exposed. The protein molecule is oriented to most clearly show the portal region and associated residues. The model is derived from the crystal structure of liver FABP with two bound oleic acid ligands (12).

Fig. 2. CD spectra of wild type liver FABP and L28W mutant. CD measurements were performed as described under "Experimental Procedures." Solid line, wild type liver FABP; dotted line, L28W mutant.
Fluorescent Characteristics of the L28W Tryptophan Mutant—The fluorescence emission spectrum of the tryptophan mutant was recorded. The fluorescence emission maximum was at a wavelength of 341.7 ± 0.6 nm consistent with the tryptophan being located in a relatively solvent exposed environment. The fluorescence intensity was low compared with other tryptophan-containing mutants of liver FABP measured under identical conditions and having maximum emission at similar wavelengths (data not shown). Although it is difficult to predict precisely how the environment would affect fluorescence intensity, the low fluorescent yield with L28W would be consistent with this tryptophan residue being in close proximity to a quenching agent such as a polar group within the apoprotein.

Ligand Binding Properties of the L28W Tryptophan Mutant—The binding of the fluorescent fatty acid derivative, DAUDA, to the wild type and mutant liver FABP was determined by making use of the increased fluorescence intensity when this probe binds to liver FABP. This enhanced fluorescence is a direct measure of ligand binding and is used to determine the $K_d$ and the fluorescence properties of the bound probe. The $K_d$ was 0.18 ± 0.04 μM and this compares with a $K_d$ for the wild type liver FABP of 0.12 ± 0.02 μM. The wavelength of maximum fluorescence intensity of FABP-bound DAUDA was the same for the wild type and L28W mutant at 498 nm indicating no significant change in the polarity of the binding cavity.

Effect of Ligand Binding on the Fluorescent Characteristics of L28W—The ability of liver FABP to bind a variety of physiological ligands in addition to long chain fatty acids provided an opportunity to compare the effect of ligand binding on the fluorescent properties of this tryptophan mutant. The ligands oleic acid and oleoyl CoA were chosen together with lithocholic and the conjugated bile acids lithocholic acid 3-sulfate and taurolithocholic acid 3-sulfate as these bile acid derivatives show highest affinity for liver FABP (17, 18) and may be physiological ligands (19).

Fatty Acids—The effects of oleic acid addition on the fluorescent properties of L28W are shown in Fig. 3 and summarized in Table I. A number of important features emerged from this study. First, oleic acid produced a 4-fold increase in fluorescence intensity, and the shape of this titration with the L28W was sigmoidal suggesting binding cooperativity. In fact, binding cooperativity was seen for all long chain fatty acids that were tested (see below). The L28W mutation is remote from site 2 (12), therefore cooperativity was seen for all long chain fatty acids that were tested (see below). The L28W mutation is remote from site 2 (12), therefore cooperativity was seen for all long chain fatty acids that were tested (see below).
Titration of long chain fatty acids into the L28W mutant result in a considerable increase in fluorescence intensity for all fatty acids that were studied. The titration curves are shown (Fig. 4). The greatest fluorescence enhancement was 4-fold with oleic acid, whereas the polyunsaturated fatty acids produced slightly lower enhancement values. The value for palmitic acid was limited by the low solubility of this ligand and reached a plateau above $10^{-2}$ M fatty acid. Light scattering confirmed aggregation of palmitic acid above this concentration, which was not seen with oleic acid (data not shown). As expected, myristic acid showed a much lower affinity consistent with previous published work (12).

Bile Acids—The binding of lithocholic acid and its conjugates was accompanied by an increase in fluorescence that was larger with the two sulfate containing derivatives, lithocholic acid 3-sulfate and taurolithocholic acid 3-sulfate (Fig. 3). In contrast, lithocholic acid and taurolithocholic acid produced a modest increase in fluorescence (Fig. 3). When a very high concentration of lithocholic acid 3-sulfate was used to saturate the FABP, an almost 4-fold fluorescence enhancement was observed.

FIG. 4. The effect on fluorescence intensity of the binding of various long chain fatty acids to the L28W mutant of liver FABP. To a sample of L28W in Hepes buffer was added up to 5 nmol of fatty acid. Results are expressed as the increase in fluorescence. Titrations were performed in the presence of 1.48 nmol of FABP. ●, oleic acid; ■, myristic acid; △, palmitic acid; ▽, linoleic acid; ●, arachidonic acid; ○, linolenic acid; □, docosahexaenoic acid.
observed essentially identical to that seen for oleic acid binding (Table I). No suggestion of cooperativity was seen for any bile acid derivative when binding to the L28W mutant consistent with only one ligand molecule being accommodated within the binding cavity of the protein.

Overall, the spectral properties of the L28W mutant with bound fatty acids, oleoyl CoA, or lithocholic acid 3-sulfate are summarized in Table I. No significant change in the wavelength of maximum fluorescence is seen for any ligand, however the increase in fluorescence intensity is greatest for oleic acid and lithocholic acid 3-sulfate using saturating concentrations of ligand. In contrast, a 25% reduction in fluorescence intensity is observed with binding of oleoyl CoA.

The large increase in fluorescence seen in particular with fatty acid binding requires a molecular explanation. The intrinsic fluorescence of the L28W is low compared with other tryptophan insertion mutants. An explanation is that the tryptophan in the apoprotein is quenched by another group within the protein that is in close proximity to Trp-28. Ligand binding must change the environment of Trp-28 reducing the quenching of this tryptophan and producing the large increase in fluorescence intensity that is observed. If this is the case, then loss of protein structure as a result of unfolding should, at least in terms of partial unfolding, also result in an increase in fluorescence. When the L28W mutant was equilibrated in urea concentrations it demonstrated a significant increase in fluorescence on ligand addition is reversed, a reciprocal plot is obtained in which the expected fall in fluorescence on addition of oleoyl CoA is followed by an equally large fall in fluorescence immediately following the addition of oleoyl CoA. Moreover, when the order of ligand addition is reversed, a reciprocal plot is obtained in which the expected fall in fluorescence on addition of oleoyl CoA is followed by a large rise in fluorescence on the subsequent addition of oleic acid. Control titrations using only oleic acid or oleoyl CoA over these ranges of ligand concentration (Fig. 6) confirm the fluorescence response seen in Fig. 3.

These results provide the first direct illustration of how, when present at similar concentrations, oleic acid and oleoyl CoA compete effectively for binding to liver FABP as a consequence of similar affinities. The unique fluorescence properties of L28W allow the binding of the natural ligands rather than fluorescent analogues to be measured, and these observations have important implications when discussing the physiological role of liver FABP.

This experimental protocol allowed the binding of other fatty acids to be compared with oleic acid, and the experiment was repeated with a saturated fatty acid, palmitic acid, and a polyunsaturated fatty acid, linoleic acid. The results (data not shown) again showed displacement of oleoyl CoA by the fatty acid with an increase in fluorescence and displacement of fatty acid by oleoyl CoA with a decrease in fluorescence. Similarly, lithocholic acid 3-sulfate was an effective displacer of oleoyl CoA and vice versa as monitored by the changes in fluorescence of L28W (Fig. 7), producing very similar responses to that seen in when oleic acid and oleoyl CoA compete (Fig. 6).
Fatty Acyl CoAs Can Compete with Fatty Acids for Binding to Liver FABP—The significant decrease in fluorescence of L28W on binding of oleoyl CoA permitted a number of important cooperativity between sites 1 and 2. In fact, the crystal structure of liver FABP with two bound oleates had already indicated that a fully functional (high affinity) site 2 appeared to depend on the presence of oleic acid at site 1, the primary site (12). The $K_d$ values range from 0.009–0.2 $\mu M$ for the primary site and from 0.06–4.0 $\mu M$ for the secondary site (27–29). It has been proposed that the very low $K_d$ values that have been quoted result from using fatty acid salts rather than free acids as ligands (30).

At least two models explain the observed cooperativity for fatty acid binding resulting, both requiring that ligand binding at site 2 and not the more remote site 1 produces the fluorescence change seen with L28W. The first model involves sequential ligand binding in which site 2 is only fully formed after occupancy of site 1. This cooperativity would become increasingly more dramatic with increasing differences in affinity between the two sites for fatty acid, as site 1 is compulsorily occupied prior to site 2. However, the observed sigmoidal nature of the fluorescence response is modest and not consistent with compulsory sequential binding. Therefore we feel that a more likely explanation is that the first molecule of oleic acid can bind at either site 1 or site 2. Cooperativity will be achieved because the addition of further ligand will preferentially bind to site 2 of FABP molecules, in which site 1 is already occupied, rather than to apo-FABP. This model is in line with recent NMR studies using $^{13}$C-labeled long chain fatty acids where the NMR resonances are interpreted as involving exchange of fatty acid between sites 1 and 2 (31).

All fatty acids that were studied demonstrated cooperativity, which was most apparent when oleic acid was the ligand. No major differences were observed between fatty acids other than those anticipated based on different affinities of individual ligands as a result of differences in hydrophobicity or solubility. For example, essential polyunsaturated fatty acids did not produce major differences in terms of fluorescence response as compared with saturated and monounsaturated fatty acids. These results suggest that there would be no specific differences in the metabolism of particular fatty acids as a result of being bound to liver FABP.

**Fatty Acid Binding and Binding Site Cooperativity**—The fatty acid binding studies with the L28W mutant demonstrated

**DISCUSSION**

There is considerable evidence that highlights the important role of the portal region of FABPs in ligand and membrane binding and cellular function (7) including deletion of the helical motif of intestinal FABP (22, 23) and helical swapping with liver FABP (24). In the case of murine adipocyte FABP, the effect of enlargement of the portal on ligand access was evaluated after multiple glycine insertion mutagenesis (25). In this paper we used tryptophan insertion mutagenesis to produce the portal mutant, L28W, with fluorescence properties that are very sensitive to ligand binding. The position of this mutation is consistent with the tryptophan reporting at ligand-binding site 2, where the anionic group of ligands such as fatty acids is located within the portal (12).

We propose that the increase in fluorescence intensity on binding fatty acids is caused by conformational changes at site 2 that reduces the quenching environment. It has been proposed that quenching of tryptophan by nearby amino acid residues is a common occurrence in folded proteins (26). Inspection of the structure (Fig. 1) suggests that residues in the vicinity of Tyr-54 at the 5BCBD turn are appropriately placed. The unfolding of the protein as a result of urea treatment should remove such amino acid residues from the vicinity of Trp-28 resulting in an increase in fluorescence. This was indeed the case and also argues against quenching of Trp-28 by solvent in the apoprotein but not after fatty acid binding because urea treatment should enhance solvent exposure resulting in a decrease in fluorescence.

The binding of oleoyl CoA reduced the fluorescence intensity of L28W, indicating that in contrast to the binding of fatty acids this tryptophan is in a more quenched environment in the holoprotein. Oleoyl CoA has been modeled into the binding cavity of liver FABP (13), and the pyrophosphate backbone of CoA is adjacent to Leu-28. This pyrophosphate group would provide such a quenching environment, whereas when oleic acid is bound at site 2, the non-quenching alkyl chain of the fatty acid is adjacent to Leu-28 (12). The fluorescence studies with the L28W mutant have clarified three important ligand binding properties of liver FABP that are relevant to physiological function.

**Fatty Acid Binding and Binding Site Cooperativity**—The fatty acid binding studies with the L28W mutant demonstrated

![Graph](Image)
properties of liver FABP to be investigated with respect to this ligand.

First, it provided an opportunity to directly measure the binding affinity of oleoyl CoA rather than using indirect methods or relying on fluorescent analogues. The binding data produced an apparent $K_d$ value of 0.68 $\mu M$, a figure that is similar to that obtained using DAUDA displacement studies (11) and in the same affinity range as that reported for fatty acids binding at the secondary site (27–29). A second tryptophan mutant, M74W, was also used to confirm these observations. The M74W mutant produced a larger decrease in fluorescence than that seen with L28W. The apparent $K_d$ value obtained, 0.66 $\mu M$, was very similar to that using L28W whereas both calculations assumed a 1:1 molar binding stoichiometry.

Second, the opposite effects on fluorescence intensity of binding oleic acid and oleoyl CoA allowed a direct measurement of the competition between fatty acid and fatty acyl CoA binding when added successively to liver FABP (Fig. 6). The titration profiles are not compatible with the apparent $K_d$ values that were determined in previous studies. Thus, using DAUDA displacement, an apparent $K_d$ value of 1 $\mu M$ was obtained for oleoyl CoA (11), whereas in contrast the apparent $K_d$ value for oleic acid was 0.04 $\mu M$ (10). A greater than 20-fold difference in $K_d$ between oleic acid and oleoyl CoA should dramatically affect the shape of the fluorescence changes with the L28W mutant with oleoyl CoA being a poor displacer of oleic acid. In fact, the shape of the curves in Fig. 6 indicates that oleoyl CoA and oleic acid have similar affinities for liver FABP of considerable physiological relevance.

The discrepancy with DAUDA displacement studies probably reflects the fact that L28W is reporting on site 2 with an $K_d$ value of 0.009 to 0.2 $\mu M$, whereas a 2:1 stoichiometry is proposed for both oleoyl CoA and oleic acid. In fact we observed similar titration curves to those involving oleic acid and oleoyl CoA, and this reflects the fact that these ligands must bind at site 2 with similar $K_d$ values. We have reported $K_d$ values of the order of 1 $\mu M$ for both oleoyl CoA and oleic acid at site 2. Presumably, the replacement of a fatty acid at site 2 by oleoyl CoA compulsorily also results in loss of the fatty acid including DAUDA from a per-turbed site 1 to allow the bulky acyl chain of the oleoyl CoA to be accommodated within the binding cavity.

When the fluorescent fatty acid paranicaric acid and its CoA derivative are used to measure bind affinities to liver FABP, $K_d$ values in the nm range were obtained for both the free acid and CoA ester (30), whereas a 2:1 stoichiometry is proposed for both ligands. Although these studies are reporting much higher affinities than seen by others, the authors conclude that both fatty acid and fatty acyl CoA can effectively compete for liver FABP binding sites consistent with our observations with the L28W mutant.

Liver FABP with bound fatty acyl CoA will have significantly different structural properties compared with the apoprotein or with fatty acid as the ligand. This is because the highly anionic phosphopanthenyl part of the molecule is predicted to be surface exposed within the portal region (13) and should dramatically affect potential protein-protein or protein-membrane interactions. It remains to be established if these structural differences in properties affect ligand targeting or if the holo-protein has more specific regulatory affects on lipid metabolism such as interacting with peroxisome proliferator-activated receptor $\alpha$ (32). A recent study involving liver FABP knock-out mice has highlighted the significant role of this protein in binding fatty acyl CoA in vivo while the relative contributions of liver FABP and acyl CoA binding protein under varying conditions were discussed (8). Overexpression of the liver FABP gene enhanced the targeting of both fluorescent fatty acids and acyl CoAs to the nucleus (33).

Third, we have demonstrated that bile acids derived from lithocholic acid are good ligands for FABP and that lack of cooperativity is consistent with liver FABP binding only one molecule of ligand. The binding of these bile acids and their conjugates could involve two possible orientations of ligands in the protein cavity. We have previously argued that more bulky anionic groups occupy site 2 where solvent exposure allows such groups to be more readily accommodated and stabilized (11). We propose that the identical and small effect of lithocholic acid and taurolithocholic acid on L28W fluorescence is caused by the internalization of the bile acid side chain so that its anionic charge interacts with site 1 or may exchange between the two sites. In contrast we argue that the 3-sulfate group is located at site 2 and produces the dramatic fluorescence enhancement as a result of conformational changes in the region of tryptophan at position 28. Thus lithocholic acid 3-sulfate can be accommodated at site 2 with the anionic sulfate located in the portal region, whereas the side chain anionic carboxyl group would be in the vicinity of the site 1 anion binding site of liver FABP.

We have argued that lithocholic acid-3-sulfate binds at site 2 and competes with oleoyl CoA binding. In fact we observed similar titration curves to those involving oleic acid and oleoyl CoA, and this reflects the fact that these ligands must bind at site 2 with similar $K_d$ values. We have reported $K_d$ values of the order of 1 $\mu M$ for both oleoyl CoA and lithocholic acid 3-sulfate (11), whereas a similar value has previously been reported for lithocholic acid 3-sulfate (18). Thus, in theory, such bile acid derivatives could affect the physiological function of liver FABP.

In summary, fluorescence studies with the tryptophan mutant L28W have highlighted conformational changes in liver FABP as a result of ligand binding. These studies have provided more details as to how specific ligands, particularly fatty acids and acyl CoAs, interact with the protein. It is proposed that competition between fatty acids and fatty acyl CoAs for liver FABP should play an important role in ligand targeting within the cell.

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Conformational Changes in Liver FABP

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Tryptophan Insertion Mutagenesis of Liver Fatty Acid-binding Protein: L28W MUTANT PROVIDES IMPORTANT INSIGHTS INTO LIGAND BINDING AND PHYSIOLOGICAL FUNCTION

Robert M. Hagan, Jane Worner-Gibbs and David C. Wilton

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