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An NMR-Based Structural Rationale for Contrasting Stoichiometry and Ligand Binding Site(s) in Fatty Acid-binding Proteins[†]

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Abstract

Liver fatty acid-binding protein (LFABP) is a 14-kDa cytosolic polypeptide, differing from other family members in number of ligand binding sites, diversity of bound ligands, and transfer of fatty acid(s) to membranes primarily via aqueous diffusion rather than direct collisional interactions. Distinct two-dimensional ¹H-¹⁵N NMR signals indicative of slowly exchanging LFABP assemblies formed during stepwise ligand titration were exploited, without solving the protein-ligand complex structures, to yield the stoichiometries for the bound ligands, their locations within the protein binding cavity, the sequence of ligand occupation, and the corresponding protein structural accommodations. Chemical shifts were monitored for wild-type LFABP and a R122L/S124A mutant in which electrostatic interactions viewed as essential to fatty acid binding were removed. For wild-type LFABP the results compared favorably with previous tertiary structures of oleate-bound wild-type LFABP in crystals and in solution: there are two oleates, one U-shaped ligand that positions the long hydrophobic chain deep within the cavity and another extended structure with the hydrophobic chain facing the cavity and the carboxylate group lying close to the protein surface. The NMR titration validated a prior hypothesis that the first oleate to enter the cavity occupies the internal protein site. In contrast, ¹H/¹⁵N chemical shift changes supported only one liganded oleate for R122L/S124A LFABP, at an intermediate location within the protein cavity. A rationale based on protein sequence and electrostatics was developed to explain the stoichiometry and binding site trends for LFABPs and to put these findings into context within the larger protein family.

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SUPPORTING INFORMATION AVAILABLE

Backbone HN chemical shifts (Tables S1 and S2) for wild-type and R122L/S124A LFABP at titration points of 1:0, 1:0.4, and 1:2. Supplemental materials may be accessed free of charge online at <http://pubs.acs.org>.

Cytosolic fatty acid-binding proteins (FABPs) belong to a family from which more than 10 separate gene products have been identified and several classes of fatty acid transport functions have been proposed (1–3). These 14–15 kDa proteins are expressed abundantly in a tissue-specific manner that is essential to lipid metabolism; they have been isolated from mammalian tissues including intestine, liver, heart, adipose tissue, myelin, brain, muscle, and epidermis, respectively, as well as from invertebrates such as locust and hornworm (4). The FABPs have been found to enhance the transfer of long-chain fatty acids (FA) between artificial and native lipid membranes *in vitro*, and also to exert a stimulatory effect on a number of enzymes that control FA metabolism.

Though the amino acid sequence of rat liver FABP is only 22–27% homologous with FABPs from heart, adipose tissue and myelin sheath, these latter proteins are 60–65% homologous to each other (3). Rat intestinal FABP has a sequence that is 29% homologous to liver FABP and also 30–33% homologous to the above three proteins. FABPs bind a variety of hydrophobic and amphipathic ligands with K_d values in the μM - nM range. Although the divergent primary structures of the FABPs could account in principle for the different binding properties of the family members, their respective X-ray crystal structures exhibit an overall similarity, including a “clam-shell-like” configuration with two parallel β -sheets oriented orthogonally to each other and with the hydrophobic ligand(s) bound inside a cavity formed by the β -clam (5–6).

Liver-type Fatty acid-binding protein (LFABP), which is expressed at mM levels in both intestinal absorptive cells and liver hepatocytes, is thought to play an essential role in dealing with increased levels of fatty-acid flux that may occur in the enterocyte with high fat diets, or in the hepatocyte during starvation or uncontrolled diabetes. An important motivation for studying LFABP lies in its unique biochemical properties. First, LFABP binds diverse ligands relative to other FABPs: it can bind several FA as well as endogenous hydrophobic ligands such as lysophospholipids, bile salts and monoacylglycerols, whereas most other FABPs exhibit high-affinity binding only for long-chain FA (2,7). Secondly, LFABP possesses a very large hydrophobic binding cavity, which allows it to accommodate more than one FA ligand (8–11); other FABPs typically possess a single binding site for long-chain FA (12). Thirdly, transport of anthroxyloxyFA from LFABP to model membranes occurs more slowly than from intestinal, heart, or adipocyte family members and is controlled kinetically by aqueous diffusion of the fatty acid rather than being mediated by direct collisional interactions between the FABPs and the “acceptor” bilayer (1,13).

The high-resolution tertiary structure of LFABP in complex with oleic acid (oleate, OLA) has been determined independently by X-ray crystallography (14) and NMR spectroscopy (8); both reports indicate that wild-type LFABP binds two fatty acid molecules. One internally bound oleate molecule (OLA129) has a U-shaped conformation and lies deep within the cavity, inaccessible to solvent, whereas the other ligand (OLA128) adopts an extended conformation and is more exposed to the aqueous interface. It has been proposed that OLA129 enters the cavity first and presents a hydrophobic surface that can facilitate the subsequent binding of OLA128 (14), underscoring the putatively critical role of the first ligand-binding step. Thus in the current NMR study of LFABP, we sought to monitor the progression of the ligand binding events at the molecular level, including the location of each FA with respect to particular protein sites and any attendant overall conformational changes that might reveal the basis for the anomalous binding properties of this protein family member.

Site-directed mutagenesis and chemical modification studies have implicated R122, S124, and S39 in the binding of OLA129 (15–17), which is likely to occur via direct electrostatic interactions with its carboxylate group and through hydrogen bonding networks mediated by

water within the protein cavity (14,18). This rationale is supported by Multi-Conformational Continuum Electrostatics (MCCE) calculations (19–20) showing that R122 and S124 promote ionization of the OLA129 carboxylate, which then establishes favorable electrostatic interactions with a positively charged patch (R122, K125, R126) at the inner surface of the cavity (21). To test how removing such interactions might affect the binding location, orientation, stoichiometry, and affinity of the protein-ligand complex, key positively charged and polar residues could be replaced by nonpolar groups of similar size, e.g. by constructing a R122L/S124A double mutant of LFABP.

Whereas a detailed picture of the protein and its ligand(s) would emerge from solving the tertiary structure of a protein-ligand complex by X-ray or NMR methods, it is desirable to obtain this information more rapidly and cost effectively, particularly when comparing closely related macromolecular assemblies. Although fluorescence (or isothermal titration calorimetry) measurements can provide the ligand stoichiometry and affinities (22), in practice the parameters for the second oleate bound to LFABP are sometimes challenging to determine (21); in any case such methods do not reveal the molecular location(s) adopted by the ligands during the binding process. Amide ^{15}N NMR relaxation, ^1H exchange, and NMR lineshape analyses such as those reported for apo- and holo-intestinal FABP, cellular retinol-binding protein, and bile acid-binding protein provide detailed conformational and motional information but focus exclusively on the protein rather than the protein-ligand complex(es) (23–25), whereas ^{13}C NMR relaxation of FABP-bound fatty acids reveals the dynamics and order of just the ligands (26).

^{15}N -edited heteronuclear single quantum correlation spectroscopy (HSQC) NMR spectroscopy, which efficiently provides a two-dimensional map of amide HN chemical shifts for directly bonded groups in a protein, has been applied extensively to study interactions between proteins and ligands in aqueous solution, for instance to probe ligand binding sites (27–28), predict binding affinity (29), and elucidate structure-activity relationships relevant to drug discovery (30–31). Typically, chemical shift differences between free and bound protein are quantified by obtaining a weighted average of ^1HN and ^{15}N chemical shift perturbations (32). The perturbations arise from changes in chemical environment of particular residues upon addition of ligand (e.g. involving hydrogen bonds, electrostatics, and hydrophobic interactions), revealing either a contiguous binding interface or more global protein conformational changes (33). In the studies presented herein, stepwise addition of 0.4-molar aliquots of unlabeled oleate into ^{15}N -labeled wild-type and mutant LFABPs, respectively, has been monitored using ^{15}N HSQC NMR spectra to determine the stoichiometry of the ligand(s) bound to each protein. Chemical shift resolution of NMR signals for the apo wild-type LFABP and its various OLA-liganded states also makes it possible to deduce the location and sequence of entry of each ligand into the protein cavity, confirming the 1:2 stoichiometry deduced from X-ray and NMR structures of the wild-type protein-ligand complex. A contrasting pattern of chemical shift perturbations is observed for oleate bound to R122L/S124A LFABP, supporting the binding of a single OLA ligand at a site intermediate between those occupied for the wild-type protein. The stoichiometry and ligand binding sites for these LFABPs, along with related family members, are examined in light of their respective protein sequences and electrostatic characteristics.

MATERIALS AND METHODS

Expression and Purification of Wild-type and R122L/S124A ^{15}N -LFABP

The pET-11a vector containing either wild-type or R122L/S124A rat liver FABP cDNA was transformed into *E. coli* host strain BL21(DE3) bacteria on agar plates containing 200 $\mu\text{g}/\text{mL}$ of ampicillin. After rapid growth in unlabeled rich medium to high cell densities

(OD_{600 nm} = 0.6), the transformed cells were harvested and then transferred into M9 minimal medium enriched with ¹⁵NH₄Cl (Isotec, Miamisburg, OH), allowing subsequent growth to higher cell densities. After 1 h incubation, protein expression was induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacterial culture was incubated by shaking at 225 rpm and 37 °C for 3 h. The cells were harvested and resuspended in pH 8.3 Tris buffer, then purified chromatographically by size exclusion, ion exchange, and delipidation (8,13). Protein purity was confirmed by observation of a single band corresponding to a size of 14 kDa in SDS-PAGE gel electrophoresis. Typical yields of pure LFABP were 30 mg/L of bacterial culture.

Preparation of NMR Samples

Unlabeled sodium oleate was obtained from Sigma (St. Louis, MO), and the uniformly ¹⁵N-enriched wild-type and R122L/S124A LFABP proteins were obtained as described above. A 100 mM stock solution of pH 9.2 sodium oleate in H₂O was prepared to permit direct addition to each protein in a pH 7.0 phosphate buffer in a 5-mm NMR tube. For ¹⁵N HSQC titration of wild-type LFABP, 1.8 μL aliquots of 100 mM oleate were added to 579 μL of 0.783 mM apo protein in phosphate buffer (50 mM Na₂HPO₄/NaH₂PO₄, 100 mM NaCl, 5 μM EDTA, and 0.02% NaN₃), corresponding to increments of 0.4 molar equivalent (eq) oleate added successively to the protein. A total of six complexes of ¹⁵N-wild-type LFABP with oleate at ratios of 1:0.4, 1:0.8, 1:1.2, 1:1.6, 1:2.0, and 1:3.0, respectively, were prepared for the NMR titration experiments. For ¹⁵N HSQC titration of R122L/S124A LFABP, 2.0 μL aliquots of 100 mM oleate were added to 550 μL of 0.906 mM apo protein in phosphate buffer (50 mM Na₂HPO₄/NaH₂PO₄, 100 mM KCl, 5 μM EDTA, and 0.02% NaN₃), each representing the addition of 0.4 eq of oleate into the protein. A total of five complexes of ¹⁵N-R122L/S124A LFABP with oleate at ratios of 1:0.4, 1:0.8, 1:1.2, 1:2.0, and 1:3.0, respectively, were prepared for the collection of NMR titration data. No changes in solution pH were observed after addition of successive aliquots of oleate.

¹⁵N HSQC NMR Experiments and Data Analysis

¹⁵N HSQC NMR experiments (34) were conducted on a four-channel Varian UNITYINOVA 600 MHz spectrometer, with observations made at 10 °C to optimize spectral resolution and avoid multiple conformations of the unliganded protein. The H₂O signal (4.91 ppm) was set on resonance in the proton dimension, and the ¹⁵N carrier was set in the middle of the amide region (120.54 ppm). Chemical shifts were referenced following the guidelines of Wishart and Sykes (35). The experiments were carried out with 256 complex points in *t*₁, 1024 complex points in *t*₂, and 32 transients. The NMR spectra were processed with NMRPipe software (36) and analyzed using NMRView (37). Chemical shift perturbations for each backbone HN crosspeak were calculated, for instance, as follows:

$$\Delta(\text{apo} - \text{sgl}) = \{[\delta_{\text{HN}}(\text{apo}) - \delta_{\text{HN}}(\text{sgl})]^2 + [(\delta_{\text{N}}(\text{apo}) - \delta_{\text{N}}(\text{sgl}))/6.5]^2\}^{1/2} \quad (32), \text{ where 'sgl' refers to the single-liganded protein.}$$

Backbone HN Resonance Assignments for Wild-type LFABP Titration

¹H-¹⁵N HSQC spectra were collected at each step of the titration of unlabeled oleate into ¹⁵N-labeled wild-type LFABP. As described previously (8), a single set of backbone HN peaks was observed for both apo-LFABP (without any bound ligand) and holo-LFABP (with two eq of bound oleate). The backbone HN resonance assignments for each of these forms were derived from our previous reports (8,38); minor peak shifts were attributed to different choices of pH or temperature but did not complicate the analysis. At the 0.4 eq titration stage, the backbone HN signals attributed to a single-liganded variant of the protein (sgl-LFABP) were assigned by visual comparison with the HSQC spectrum of apo-LFABP

and confirmed by correlation to both backbone and sidechain ^1H and ^{13}C nuclei using a set of three-dimensional coherence transfer NMR datasets obtained from $\text{U-}^{15}\text{N}/^{13}\text{C}$ -labeled LFABP titrated with 0.4 eq of oleate (data not shown). At other titration stages, the HN assignments were completed by tracking the chemical shift changes visually but unambiguously. Considering all forms of each protein, the completeness of the backbone HN assignments was 98.2% for the wild-type protein.

Backbone HN Resonance Assignments for R122L/S124A LFABP Titration

Sequential assignments of ^1H , ^{15}N , and ^{13}C nuclei in apo- and holo-R122L/S124A LFABP were made by standard through-bond correlation procedures (38), including a set of multidimensional NMR experiments performed on a $\text{U-}^{15}\text{N}/^{13}\text{C}$ -labeled protein (21). The backbone HN assignments at titration stages of 1:0.4, 1:0.8, and 1:1.2, were completed by comparison of the HN peak positions to both apo and 1:2.0 proteins, and by recognition of the peak patterns for preceding or subsequent titration steps. Four residues (G37, M74, G106, and D107) had either missing or ambiguous HN shifts, resulting in 96.0% completeness of the backbone HN assignments for the R122L/S124A LFABP titration.

RESULTS

Observation of Intermediate Oleate-bound States for Wild-type LFABP

Standard chemical shift perturbation methods were extended to map site-specific changes in protein magnetic environment at each stage of the titration with a fatty acid ligand. For wild-type LFABP, stepwise titration monitored through the protein HN resonances in 2D NMR spectra revealed intermediate binding states in addition to the apo and doubly liganded (holo) forms. Moreover, well-resolved backbone resonances attributable to complexes designated as apo, sgl, and holo were found to coexist in slow exchange on the NMR timescale, suggesting the possibility of using $^1\text{H-}^{15}\text{N}$ HSQC to deduce both the location of each OLA molecule that enters the protein cavity and the stoichiometry of the ligand binding.

Figure 1 illustrates protein $^1\text{H-}^{15}\text{N}$ HSQC spectra obtained at various titration points: apo-, 1:0.4, 1:0.8, 1:1.2, 1:1.6, and holo- (1:2), displaying several notable spectral features at each stage. (1) Two sets of HN signals were observed when the first 0.4 eq of oleate was added to LFABP, one set from apo-LFABP and the other from sgl-LFABP. The intensities of the two sets of signals were roughly equivalent at this early titration stage, indicating a preference for the liganded state and consistent with K_d estimates of 9–40 nM (21,22). The composite chemical shift difference between the apo- and sgl-LFABP signals varied significantly along the protein sequence, from 0.002 to 0.850 ppm (32). (2) With the addition of the second 0.4 eq oleate to LFABP, that is, cumulatively 0.8 eq, the HN signals from apo-LFABP were either absent or greatly diminished; the HN peaks from sgl-LFABP were retained with slightly increased intensities; a new set of HN resonances appeared with weaker intensities than sgl-LFABP but identical or near-identical chemical shifts to holo-LFABP, thus designated herein as pre-holo-LFABP. The chemical shift difference between the sgl- and pre-holo-LFABP resonances also varied significantly along the polypeptide chain, from 0.002 to 1.36 ppm. (3) The HN resonances from sgl- and pre-holo forms of LFABP were retained at their respective positions upon addition of 1.2 eq of oleate, but the relative intensities of these slowly exchanging sets of signals were reversed. (4) When the amount of titrated ligand reached 1.6 eq, only one set of HN resonances was observed, attributable to holo-LFABP. (5) At 2.0 eq, the HN resonances from holo-LFABP exhibited the same spectral features as 1.6 eq. The chemical shift difference between pre-holo-LFABP and holo-LFABP forms was typically modest, with 78% of the residues shifted less than 0.02 ppm and only two residues varying by more than 0.05 ppm (K96, 0.075 ppm and F95, 0.085

ppm); these latter protein forms exhibited rapid chemical exchange and thus population-averaged NMR signals. The holo-LFABP designation corresponds to prior crystallographic and NMR determinations of two bound oleate ligands (8,14). (6) Finally, at 3.0 eq the HN peaks were sharpened further, but the resonance positions remained essentially the same as at 2.0 eq, indicating that two ligands remained bound to the protein and the excess ligand was presumably free in solution.

In summary, four unique sets of HN resonances were observed for the protein during the titration process, corresponding to distinct apo-, sgl-, pre-holo-, and holo-LFABP structural forms. In addition to the commonly identified apo form with no ligands and the fully bound holo form with two oleate ligands, it was possible to characterize structural species at intermediate sgl and pre-holo stages of the binding process. As detailed below, the HN resonance assignments of sgl-LFABP are critical for understanding the binding stoichiometry and protein-ligand interactions since they reveal significant site-specific variations with respect to either apo-LFABP (0.002 to 0.85 ppm) or holo-LFABP (0.002 to 1.32 ppm). The stepwise ^{15}N HSQC titration analysis of wild-type LFABP is summarized by HN assignments tabulated for apo-, sgl-, pre-holo-, and holo-LFABP at 10 °C and pH 7.0 (Supporting Information, Table S1), and plots of chemical shift perturbation along the protein sequence (Figure 2A).

Stoichiometry and Entry of Oleates Bound to Wild-type LFABP

Although the presence of two oleates at distinct LFABP binding sites has been demonstrated from X-ray and NMR structures determined for the holo protein complex (8,14) and through our ^{13}C HSQC NMR study of ligand exchange phenomena (18), the current strategy exploits the observation of resolved signals for apo-, sgl-, pre-holo-, and holo-LFABP in ^{15}N HSQC spectra to deduce the stoichiometry of OLA bound to LFABP independently and exclusively from ^{15}N chemical shift perturbation analysis of the protein resonances.

As shown in Figures 2A and 3A, there are 30 amino acid residues with magnitudes of the chemical shift difference $\Delta(\text{apo-sgl})$ above a threshold of 0.15 ppm, 21 of which show $\Delta(\text{apo-sgl}) > \Delta(\text{sgl-holo})$ and thus the bulk of the environmental change occurring upon binding of the first ligand. The quantity $\Delta(\text{apo-sgl})$ exceeds $\Delta(\text{sgl-holo})$ by more than 0.15 ppm for eleven of those backbone sites: S39, L71, T73, E103, N105, G106, D107, T108, I109, K121, and K125. Thus we consider provisionally that these eleven spatially proximal residues comprise the binding site for the first ligand and the corresponding ligand-binding environment of the sgl-LFABP protein structure. By mapping these amino acids onto the NMR structure of the oleate-bound LFABP (Figure 3B, highlighted in red) (8, PDB code: 2ju8), their clustered location near particular β -strands (βB , βE , βI , βJ) and a β - β loop (βH - βI turn) becomes evident, validating our interpretation in terms of ligand location rather than overall protein structural rearrangements. Notably, no residues in the helical regions show any significant chemical shift perturbations when the apo and sgl proteins are compared.

Conversely, there are 41 residues with $\Delta(\text{sgl-holo})$ values above 0.15 ppm (Figures 2A and 3A), 34 of which have $\Delta(\text{sgl-holo}) > \Delta(\text{apo-sgl})$, and 20 for which $\Delta(\text{sgl-holo})$ exceeds $\Delta(\text{apo-sgl})$ by more than 0.15 ppm: Y7, Q8, I29, G32, K33, D34, I35, G37, V38, L50, T51, I52, G55, S56, K57, T102, T112, M113, Y120, and R126. The significant $\Delta(\text{sgl-holo})$ magnitudes indicate that these latter residues are involved in additional ligand binding; a sequential model for transformation of the protein structure from sgl-LFABP to holo-LFABP is proposed below. Figure 3B highlights these sites in green and demonstrates that these latter amino acids are located not only in particular β -strands (βC , βI , βJ , and βC - βD turn), but also in the helical region (αII and αII - βB turn) of the protein structure. Therefore, besides the primary binding site, there exists a second binding locus of the oleate in wild-type LFABP, associated with significant chemical shift perturbations from sgl- to holo-

LFABP -- either directly by ligand entry into the cavity or via repositioning of the first fatty acid.

Given the space available within the protein cavity and the length of the long-chain oleate molecule (Figure 3C), the NMR titration data described above also provide evidence that the stoichiometry of wild-type holo-LFABP to oleate is 1:2. The chemical shift perturbations observed between apo- and sgl-LFABP confirm that the first oleate molecule resides internally and spanning opposite sides of the β -barrel structure (ends of the ligand at the β E- β F and β H- β I turns), consistent with the location of OLA129 reported in the solution- and solid-state structures of the holo-LFABP complex (8, 14). Further titration of the sgl-LFABP form to pre-holo- or holo-LFABP demonstrates that the second oleate molecule has an extended conformation near the surface of the protein (near α II and the β C- β D turn), again in accord with the location of OLA128 reported for the ligand-protein complex. These NMR titration experiments suggest that the interior binding site is occupied first by OLA129 and the surface-accessible site is filled subsequently by OLA128, but alternative sequential and parallel binding models are discussed below.

Chemical Shift Perturbation and Oleate-bound States upon Stepwise Titration of R122L/S124A-LFABP

Figure 4 displays R122L/S124A LFABP ^1H - ^{15}N HSQC spectra observed at several titration points: apo, 1:0.4, 1:0.8, 1:1.2, and 1:2. Chemical shift index analysis (35) showed essentially identical secondary structural elements for both apo and holo forms of the double mutant as compared with the wild-type proteins, arguing against reorganization of the LFABP tertiary structure (21) and in accord with the overall structural similarity of family members that possess a relatively modest degree of sequence homology (4,10,21,39). A single set of backbone HN peaks was evident for each of the apo and holo forms of the double mutant protein, but as for wild-type LFABP, the 1:0.4 titration stage displayed distinct HN signals (in this case with similar integrated intensity) for both apo and liganded proteins. The chemical shift difference between the two sets of signals varied along the protein sequence, from 0.002 to 0.523 ppm (Figure 2B). The average value of $\Delta(\text{apo-holo})$ for the backbone residues of R122L/S124A LFABP was 0.107 ppm, about half the magnitude observed in the wild-type protein for $\Delta(\text{apo-holo})$ and similar to its value of $\Delta(\text{apo-sgl})$. The apo peaks disappeared at and beyond a ratio of 1:0.8, confirming the expected predominance of the liganded form at our 900 μM concentration, given the 450 nM K_d estimated for the first binding event (21). For oleate titrated into the R122L/S124A protein, we designate the 0.4 eq and 0.8 eq complexes as pre-holo-R122L/S124A and the titration points of 1.2 eq and above as holo-R122L/S124A. The pre-holo resonances exhibited very modest changes in chemical shift at the remaining titration steps up to 3.0 eq (maximum variation of 0.06 ppm), but such changes in shift, intensity, and/or linewidth support the presence of two (or more) rapidly exchanging liganded states discussed below. Thus, the oleate-protein binding process differs fundamentally for a R122L/S124A LFABP mutant in which we have removed electrostatic interactions viewed as essential to fatty acid binding: it lacks the slowly exchanging sgl-LFABP stage seen for the wild-type protein and exhibits only two major sets of HN resonances in the ^{15}N HSQC NMR spectra. The stepwise ^{15}N HSQC titration analysis of R122L/S124A LFABP includes HN assignments tabulated at 10 $^\circ\text{C}$ and pH 7.0 (Supporting Information, Table S2) and plots of chemical shift perturbation along the protein sequence (Figure 2B).

Stoichiometry and Location of Oleate Bound to R122L/S124A-LFABP

The observation of just two sets of ^{15}N HSQC signals, coupled with an average $\Delta(\text{apo-holo})$ that is half the magnitude of wild-type LFABP and the failure to observe chemical shift perturbations at any additional sites for titration points beyond 0.4 eq of added ligand,

together suggest that the stoichiometry is 1:1 for the mutant protein-OLA complex. The observed pattern of protein spectral changes is also in accord with observations of the ligand in NMR titration experiments, for which addition of two equivalents of [U- ^{13}C]-labeled oleate produces two sets of ^1H and ^{13}C resonances in ^{13}C HSQC for wild-type LFABP (18) but only one such set for the mutant protein (21). Possible interpretations of small chemical shift changes observed at later stages of the titration are discussed below.

As shown in Figure 2B, 24 polypeptide residues exhibit an HN chemical shift difference (between apo- and holo-R122L/S124A LFABP) above 0.15 ppm: I29, G32, K33, K36, Y54, G55, K57, I59, H60, F63, T75, K78, V83, M85, V92, T93, T94, K99, S100, I109, T110, N111, T112, and L122. Because the holo structures are quite similar for wild-type and R122L/S124A LFABP (8, 21), highlighting the perturbed residues on the wild-type protein solution structure made it possible to compare the ligand binding sites of the two proteins (Figure 3D). T75, I109, N111, T110, and L122 are situated close to the first oleate binding site of wild-type LFABP, whereas I29, G32, K33, K36, Y54, G55, K57, I59, H60, K99, S100, and T112 are close to the second oleate binding site of the wild-type protein. Five additional perturbed residues, V83, M85, V92, T93, and T94, which are located along the βF and βG strands, define a binding region for oleate that is unique to the mutant protein. As a group, the 22 out of 24 perturbed residues (excluding F63 and K78) comprise a localized binding region that supports the presence of a single OLA molecule in the binding cavity of R122L/S124A LFABP and allows us to deduce with reasonable certainty the location and conformation of this bound ligand. As designated by the brown oval in the Figure 3D, we propose that the oleate ligand is bound to the mutant protein in an extended conformation, with its carboxylate head pointing toward the helical region (near K33 or K36) and its methyl tail close to the hydrophobic amino acids, V83 and/or V92. Docking calculations with chemical shift perturbation and intermolecular NOE restraints are in progress to test this hypothesis. Comparisons with wild-type LFABP, other members of this protein family, and an electrostatic rationale for these trends are outlined below.

DISCUSSION

Chemical Shift Mapping vs. Structure of Wild-type Oleate-LFABP Complex

As demonstrated above, monitoring the protein ^{15}N HSQC spectra during stepwise ligand titration allowed for an independent determination of the 1:2 wild-type LFABP-to-OLA stoichiometry deduced previously by full structure determinations of the complex (8,14). Moreover, the observation of an intermediate sgl-LFABP liganded structure allowed us to discriminate between the internal and surface-exposed binding sites that are occupied by OLA molecules within the protein cavity.

An internal binding site for the first oleate was proposed to include the following residues: S39, L71, T73, E103, N105, G106, D107, T108, I109, K121, and K125. Although a structure for the singly-liganded complex has not yet been reported, it is possible to evaluate this proposal by reference to the amino acid residues located proximally to the internal OLA129 ligand in the reported structures of holo-LFABP (8,14). For example, S39, R122, and S124 are involved in hydrogen-bonding interactions with the carboxyl group of OLA129 in holo-LFABP crystals (40); residues near the βH - βI turns (N105, G106, D107, T108, and I109) are spatially close to only the primary OLA129 ligand (8,14); I41, E72, T73, and T102 are recognized as packing closely with OLA129 in conjunction with entropically favorable displacement of ordered water molecules from the rat LFABP protein cavity (40); intermolecular nuclear Overhauser effects (NOEs) are observed by solution NMR between proton pairs of OLA129 with I41, L71, and I109 (8). Thus, these comparisons of sgl- and holo-LFABP suggest that many segments of the first oleate reach

their final locations within the protein cavity upon initial entry and do not contribute to the chemical shift changes measured subsequently as $\Delta(\text{sgl-holo})$.

Analogously, a binding site for the surface-accessible oleate was proposed to involve amino acids closer to the aqueous interface: Y7, Q8, I29, G32, K33, D34, I35, G37, V38, L50, T51, I52, G55, S56, K57, T102, T112, M113, Y120, and R126. Among the above residues and their neighbors, K31, Y54, and S56 are involved in the hydrogen bonding network of OLA128 (38); several sidechain atoms of the predominantly non-polar residues L28, G32, I35, I52, Y54, G55, and M113 are in contact with the hydrophobic chain of that ligand (40); intermolecular NOEs are observed between OLA128 and L28, G32, I35, G37, I52, Y54, K57, I59, and Y120.

Taken together, these results demonstrate the usefulness of extending the well-established chemical shift mapping method for identification of protein-ligand binding sites to delineate the binding events that occur when multiple ligands are bound to a protein target, provided that the various forms are resolved spectroscopically. Such analyses of NMR chemical shifts or lineshapes can be useful, for instance, to gain molecular insights into allosteric regulation of protein function (25). Chemical shift mapping of environmental perturbations for a protein may also complement assessments of binding energetics derived from isothermal titration calorimetry or NMR of the ligands, as demonstrated in the human ileal bile acid binding protein multisite system (41).

For doubly liganded wild-type LFABP, both sequential and parallel step-wise binding models may be considered (25,41). The latter model would require formation of a protein complex with a single oleate bound at one site as well as a complex with a single oleate bound at the other site. Tight binding of long-chain FA ($K_d \sim 9\text{--}60\text{ nM}$) has been reported at both sites (22). Thus if binding were to occur in parallel fashion at both of these sites, the exchange rate between the two singly liganded complexes would likely be too slow to average the NMR signals and we should observe distinct HSQC crosspeaks. No such peaks were observed for any protein backbone residues, though the smaller population of the more weakly-liganded state could make it challenging to detect in the NMR spectrum. In addition, the x-ray structure of doubly-liganded LFABP shows that the protein has only one contiguous cavity instead of two distinct binding pockets (14), making it difficult to envision the existence of a complex that is singly-liganded at a much weaker site. Finally, upon titration of LFABP with $[\text{U-}^{13}\text{C}]$ -oleate, we previously observed an α -methylene ^1H -resonance at 1.89 ppm that appeared initially, and two resonances at 1.72 and 2.22 ppm at the end point of the titration (18). Both of the upfield resonances (1.72 and 1.89 ppm) have quite different shifts from bulk oleate; they may be attributed to a methylene group next to a carboxylate that is salt-bridged with a basic residue. Taken together, these observations support a sequential model in which the first long-chain fatty acid molecule binds at the interior wild-type LFABP site via a salt-bridge tether to R122 and the second oleate binds at an aqueous-accessible portal protein site. Chemical shift variations observed for both ligand and protein resonances at the later titration stages may reflect environmental changes for the protein, the ligand, or both partners.

Accommodation of the Two Oleate Ligands within the Wild-type LFABP Binding Cavity

An intermediate state designated as pre-holo-LFABP was observed at titration stages between 0.8 and 1.2 eq of oleate, suggesting an “accommodation” of the sgl-protein to the entry of the second oleate ligand (OLA128) into the protein cavity. The pre-holo and holo states exhibit small chemical shift differences ($<0.02\text{ ppm}$ for 78% of the residues) but undergo exchange averaging rather than displaying separate resonances, a reasonable result in light of the weaker binding reported for the second ligand (21,22,42). Among the 2% of residues that have HN shift differences exceeding 0.05 ppm between pre-holo- and holo-

LFABP, F95 (0.085 ppm) and K96 (0.075 ppm) are located near the β G- β H turn and close to the first oleate ligand, although they do not exhibit significant values of $\Delta(\text{apo-sgl})$. Such shift changes could reflect adjustments of the OLA129 segments proximal to F95 and K96 that occur only upon entry of the OLA128 ligand into the protein cavity. In contrast, G32, G37, and Y54 exhibit substantial values of both $\Delta(\text{sgl-holo})$ (≥ 0.50 ppm) and $\Delta(\text{pre-holo-holo})$ (~ 0.04 ppm) as well as intermolecular NOEs with OLA128. Together these observations suggest that these latter residues are not only contributing directly to binding of the second OLA128 ligand, but that they also experience the effects of accommodation by the first OLA129 ligand, after both fatty acids have entered the binding cavity. The observation of small protein chemical shift perturbations as the second fatty acid binding site becomes occupied is also in accord with the hydrophobic interactions that have been postulated to occur between OLA128 and OLA129 (38) and thought to be enhanced by a swiveling of the polar K90 side chain outward toward the aqueous solvent (8). As will be discussed below, this capacity for subtle accommodation of the LFABP protein structure to adjust the electrostatic character of the binding cavity may be a key prerequisite for recruitment of diverse or multiple ligands.

Molecular Environment and Orientation of the Oleate Bound to R122L/S124A LFABP

Unlike doubly liganded wild-type LFABP, R122L/S124A LFABP has a binding region defined by residues I29, G32, K33, K36, Y54, G55, K57, I59, H60, T75, V83, M85, V92, T93, T94, K99, S100, I109, T110, N111, T112, and L122 as judged by stepwise ^{15}N HSQC NMR titration. A likely oleate orientation and conformation may also be proposed: the carboxyl group will point toward K33 or K36 because a hydrogen bond can form between the ligand COO group and the side-chain NH_3 of the lysine residue. The hydrocarbon tail of the oleate could then approach V83 and V92, promoting hydrophobic interactions between the protein and the ligand. In this scenario, the bound oleate would adopt an extended conformation within the R122L/S124A LFABP cavity, spanning strands β J to β F in a way that perturbs chemical shifts of the residues listed above. The rather modest degree of conformational accommodation evidenced by chemical shift and intensity changes beyond the addition of 0.4 eq of oleate ligand is also a reasonable result if only a single fatty acid binds to the protein.

Stoichiometry and Electrostatics of Oleate Binding to LFABPs

Though the solution structures of wild-type and R122L/S124A LFABP are similar (21), our NMR titration results suggest that the mutant binds a single oleate; only the wild-type protein is able to accommodate two fatty acid molecules. Slowly exchanging apo-, single- and holo-protein resonances are evident for the wild-type protein, which also displays separate ligand resonances in the doubly liganded state (18). For the double mutant, no distinct NMR signals are observed for multiple liganded states or multiple fatty acid ligands (21). Nonetheless, modest changes in chemical shift observed at the later titration stages support the presence of two (or more) rapidly exchanging liganded states for R122L/S124A-LFABP. If the late-appearing state were a doubly liganded protein, the progressively changing shifts would indicate weak binding ($K_d \geq 1$ mM) and the second ligand would be expected to perturb a host of additional protein backbone sites. A more likely explanation for the late-stage spectral changes invokes conformational accommodation of a singly liganded protein: (a) the total shift perturbation is only half as large as for doubly liganded wild-type (wt) LFABP (Figure 2); (b) titration beyond the 1:0.4 stage results in no additional perturbed sites and maintains a smaller locus of affected residues than for wt-LFABP (Figure 3); (c) conformational accommodation is plausible because the chemical shift changes occur at protein sites near the portal region, which often displays greater flexibility in FABPs and where oleate is estimated to bind six times less tightly in wt-LFABP (21,22).

Examination of the electrostatic potential surfaces for both apo proteins provides a rationale for the entry of oleate molecule(s) into the respective LFABP cavities. As noted previously (8), the inner surface of the wt apo-LFABP cavity is studded with positively charged K90 and R122 groups (Figure 5A). K90, with a positive charge located deep within the binding cavity (near the flexible β F- β G turn), may be able to recruit the first OLA129 ligand. The structure of the wt oleate-bound complex (8,14) provides evidence for a hydrogen bond between the carboxyl oxygen of the internal ligand OLA129 and the side-chain NH_2 proton of R122 that could play a key role in establishing the first ligand binding site. If, as we hypothesized previously (8), the K90 side chain then swivels outward, it would be possible to create a less crowded and more hydrophobic local environment for the second OLA128 ligand (Figure 5B). For the latter oleate, an extended conformation is favored by electrostatic interactions between the carboxyl group and K31, as well as by hydrophobic interactions with the ω -methyl group and the OLA129 chain.

Not surprisingly, formation of the key hydrogen bond is precluded for a mutant LFABP in which R122 and nearby S124 residues are both replaced by hydrophobic residues of similar size. As noted above, Multi-Conformational Continuum Electrostatics (MCCE) calculations (19–20) also indicate that R122 and S124 promote ionization of the OLA129 carboxyl group, so in the absence of these residues both electrostatic attraction and hydrogen bonding between protein and ligand could well be compromised. Thus, the oleate molecule is not likely to reside deeply within the R122L/S124A β -clam pocket, and presumably no hydrophobic surface is formed within the protein cavity to promote binding of a second ligand.

Moreover, the K90 side chain of the double mutant apo protein faces outward from the surface (Figure 5C) (21), as in wild-type holo-LFABP and as previously reported for analogous residues in ILBP, CRABP(II), and IFABP (44–46). Because the R122L/S124A mutation is likely to create a more hydrophobic environment within the cavity compared with wild-type LFABP, swiveling of the positively charged K90 side chain outward toward the aqueous interface could achieve a lower energy state. Although such a structural change could promote hydrophobic interactions with an oleate chain, it may also diminish the electrostatic forces helping to recruit the carboxylate out of water and into the protein cavity. Nonetheless, R122L/S124A LFABP can bind a single oleate without experiencing significant electrostatic perturbations (Figure 5D), much as reported for CRABP(II) and IFABP (44–46). Like the double mutant, the potential surfaces for apo forms of CRABP(II) and IFABP lack positively charged residues like R122 or K90 facing the interior of the apo-protein cavity but have other positively charged groups capable of recruiting ionized fatty acid molecules; ILBP has a negatively charged interior and different ligand specificity (data not shown). Overall, our analysis suggests that although oleate binding to FABPs should be accompanied by favorable entropic effects for expelled water molecules and is likely influenced by contrasting cavity sizes (11), the binding of multiple fatty acid molecules may also demand favorable protein-ligand energetics at each stage of the binding process.

Bound Oleate Environment in Diverse FABPs

It is well established that despite the somewhat divergent primary sequences of the various FABPs, their tertiary structures exhibit a common “clam shell-like” configuration, with two parallel β -sheets oriented orthogonally to each other to form a deep cavity in which hydrophobic ligands can be bound (39). The three-dimensional NMR-based structures of two oleate-bound FABP complexes have been reported: oleate-bound locust muscle *Lm*-FABP (133 residues, pdb code: 2flj, 4) and oleate-bound wild-type LFABP (127 residues, pdb code: 2ju8, 8). Based on the two superimposed backbone traces (Figures 6A and 6B), the sequence KR¹²²VS¹²⁴KR in LFABP is aligned structurally with the sequence TR¹²⁸IY¹³⁰KA in *Lm*-FABP. In both complexes, a hydrogen bond is formed from the

carboxyl oxygen of the oleate ligand (OLA129 in LFABP, OLA134 in *Lm*-FABP) to an arginine side-chain NH₂ group (R122 in LFABP, R128 in *Lm*-FABP), establishing a similar location and orientation for the respective oleate carboxyl groups. However, in the *Lm*-FABP-oleate complex, the formation of an additional hydrogen bond between the COO of OLA134 and the side-chain NH₂ group of R108 tethers the carboxyl head of OLA134 between R128 and R108, effectively preventing a repositioning to accommodate the entry of a second OLA ligand (Figure 6D). Additionally, the different sequences in the α II region (IQK³¹GKD in LFABP, RK³²A³³GLA in *Lm*-FABP) produce slightly different lysine locations (K31 and K33 in LFABP, K32 in *Lm*-FABP) that could nevertheless account for different binding stoichiometries: two oleate ligands bind to LFABP whereas only one oleate binds to *Lm*-FABP. As noted above, the OLA128 carboxyl of LFABP exhibits favorable electrostatic interactions with the ammonium sidechain of K31 (Figure 6A). In *Lm*-FABP, however, the K31 side-chain is replaced by the hydrophobic methyl group of A33.

CONCLUSIONS

By monitoring the stepwise titration of liver fatty acid-binding proteins using two-dimensional ¹⁵N HSQC NMR, it was possible to identify slowly exchanging structural variants that differ with respect to the number of bound ligands and associated conformational accommodations of the protein. These measurements revealed the number of bound fatty acids, the location of each ligand in the cavity of its protein binding partner, the sequence of binding sites that were occupied by successively entering ligands, and the protein structural accommodations associated with their respective entry; solving the structure of the macromolecular complex was not required. HSQC evidence for a protein-to-ligand stoichiometry of 1:2, with binding of the first oleate deep within the cavity followed by the second fatty acid near the aqueous interface, was validated for wild-type LFABP by reference to published structural data (8,14). An analogous titration conducted for an R122L/S124A LFABP mutant revealed 1:1 stoichiometry and an intermediate ligand location within the protein cavity.

These contrasting modes of oleate binding for structurally similar FABPs (wild-type LFABP, R122L/S124A LFABP, and *Lm*-FABP) may be attributed to a variety of energetic and dynamic factors: both recruitment of the first oleate ligand and accommodation of a second one appear to require favorable electrostatic interactions with positively charged amino acid sidechains, whereas the capacity of a protein to accommodate two ligands is likely to rely upon both the cavity size and the facility for structural accommodations that promote hydrophobic interactions between the fatty acyl chains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LFABP rat liver fatty acid-binding protein

HSQC	heteronuclear single-quantum coherence spectroscopy
FA	long-chain fatty acid
OLA	oleate
eq	molar equivalent

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FIGURE 1.

Expanded regions of ^1H - ^{15}N HSQC contour plots for selected wild-type LFABP residues upon titration with oleate ligands up to a protein-to-ligand ratio of 1:2.0. Chemical shifts are referenced according to the guidelines of Wishart and Sykes (35). NMRView is used to illustrate chemical shift changes between the apo peaks centered at the black cursor, the holo peaks centered at the red cursor, and other labeled peaks corresponding to various titration stages. The top panels illustrate amino acid residues with $\Delta(\text{apo-sgl})$ larger than $\Delta(\text{sgl-holo})$, whereas the bottom panels show examples with $\Delta(\text{sgl-holo})$ larger than $\Delta(\text{apo-sgl})$.

FIGURE 2.

Composite ^1H - ^{15}N chemical shift perturbation plotted along the protein sequence and with respect to secondary structural elements for (A) wild-type LFABP and (B) R122L/S124A LFABP. Note that the y-axes differ for wild-type and double mutant proteins; with average perturbations nearly twice as large for wt-LFABP. The apo, sgl, and holo notations refer to protein-ligand molar ratios of 1:0, 1:0.4, and 1:2.0, respectively. Chemical shift perturbations are calculated, for instance, as

$\Delta(\text{apo} - \text{sgl}) = \{[\delta_{\text{HN}}(\text{apo}) - \delta_{\text{HN}}(\text{sgl})]^2 + [(\delta_{\text{N}}(\text{apo}) - \delta_{\text{N}}(\text{sgl}))/6.5]^2\}^{1/2}$ (32). The dashed lines at 0.15 ppm, set slightly below the mean plus standard deviation for each pair of protein forms, serve as a guide to identify backbone sites with structurally significant chemical shift changes.

FIGURE 3.

Structural view using Rasmol (www.RasMol.org) for HN chemical shift perturbations of oleate titrated into wild-type LFABP (A–C) and R122L/S124A LFABP (D) [8,21, pdb code 2JU8 for the protein-ligand complex]. Residues are highlighted in terms of the degree of chemical shift perturbation upon ligand binding. In panel A, residues are colored in red when $\Delta(\text{apo-sgl}) > 0.15$ ppm, in green when $\Delta(\text{sgl-holo}) > 0.15$ ppm, and in blue when both $\Delta(\text{apo-sgl})$ and $\Delta(\text{sgl-holo})$ exceed 0.15 ppm. Panel B compares the impact of binding the first and second ligand to wild-type LFABP: in red when $\Delta(\text{apo-sgl})$ exceeds $\Delta(\text{sgl-holo})$ by 0.15 ppm so the effect of the first ligand predominates; in green when $\Delta(\text{sgl-holo})$ exceeds $\Delta(\text{apo-sgl})$ by 0.15 ppm so the effect of the second ligand predominates. Panel C highlights the residues in blue if $\Delta(\text{apo-holo}) > 0.15$ ppm when both oleates bind to wild-type LFABP and shows the ligand locations derived from NMR measurements (8). Panel D colors the residues in blue if $\Delta(\text{apo-holo}) > 0.15$ ppm when oleate binds to R122L/S124A LFABP, showing the deduced binding region for the sole ligand circled in brown. Additional explanations appear in the text.

FIGURE 4.

Expanded regions of ^1H - ^{15}N HSQC contour plots for R122L/S124A LFABP upon titration with oleate, highlighting the same residues shown in Figure 1 for the wild-type protein. Chemical shifts are referenced according to the guidelines of Wishart and Sykes (35). NMRView is used to visualize the apo peaks centered at the black cursor and the 1:2 holo peaks centered at the red cursor. Very modest chemical shift perturbations were observed for titration beyond a protein-to-oleate molar ratio of 1:0.4.

FIGURE 5.

Electrostatic potential surface views inside the cavities of wild-type apo-LFABP and the OLA-LFABP complex (A–B, 8), and for apo- and holo-R122L/S124A LFABP (C–D, 21), displayed with DS ViewerPro. Calculations were performed at pH 7 assuming that the Asp, Glu, Arg, and Lys residues are ionized (43). The inner protein surfaces are displayed for residues 1–37 and 81–127, respectively. Surface charges are shown as blue (positive), red (negative), and white (neutral). Protein backbone traces of the β -strand residues 38–80 are shown in pink; ligands are shown in green. K90 is not visible in (B–D) because its side chain faces the outer surface of the protein.

FIGURE 6.

(A–B) Superposition with PDB Viewer for oleate-bound complex structures of LFABP (8, blue) and locust muscle *Lm*-FABP (4, green), displayed with DS ViewerPro. (A) Locations for the first oleate (OLA129) and the second oleate (OLA128) within LFABP; (B) location for the sole oleate (OLA134) within *Lm*-FABP; (C–D) electrostatic potential surface views (same scheme as Figure 5) for LFABP (C) and the *Lm*-FABP complex (D).