Fatty Acid Induced Remodeling within the Human Liver Fatty Acid-binding Protein*5

Ashwani Sharma and Amit Sharma1
From the Structural and Computational Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Road, 110067 New Delhi, India

We crystallized human liver fatty acid-binding protein (LFABP) in apo, holo, and intermediate states of palmitic acid engagement. Structural snapshots of fatty acid recognition, entry, and docking within LFABP support a heads-in mechanism for ligand entry. Apo-LFABP undergoes structural remodeling, where the first palmitate ingress creates the atomic environment for placement of the second palmitate. These new mechanistic insights will facilitate development of pharmacological agents against LFABP.

Liver fatty acid-binding protein (LFABP)2 is an intracellular lipid chaperone belonging to a family of ~15 kDa intracellular lipid-binding proteins (iLFABP). LFABP, along with the other iLFABP family members, appears to maintain physiologically relevant concentrations of cytosolic fatty acids (1–6). Increased expression of LFABP in humans is associated with insulin-dependent diabetes and gestational diabetes (7–9). Studies with LFABP gene-ablated mice demonstrate a physiological role for LFABP in hepatic fatty acid metabolism and in diet-induced obesity (10–12). Mechanistic details of LFABP-fatty acid interaction have not as yet resolved the mode of binding for both fatty acid molecules. This is in part due to the lack of high resolution crystal structures of ligand-free and ligand-bound forms of human LFABP. Unlike other members of the iLFABP family, LFABP is unique in binding two fatty acid molecules (13, 14). It can also bind bile salts, acyl-CoA esters, and other hydrophobic compounds (15–19). LFABP shares a common structural motif with other iLFABPs comprising a 10–11-stranded β barrel that forms a ligand binding cavity, which is covered by a helix-turn-helix (HTH) motif (20). This HTH along with neighboring β turns between β strands is hypothesized to form a portal to the protein cavity, allowing ligand trafficking without significant structural rearrangements in the β barrel (21–24). The terminology of apo- and holo- used in this work refers to ligand-bound and ligand-unbound states of FABP, and this terminology is used here to remain consistent with previously published works on FABP (21). We crystallized the human LFABP in apo, holo, and in intermediate states of fatty acid (palmitate) binding to obtain structural snapshots of fatty acid engagement, entry, and final docking within LFABP. Our data provide crystallographic evidence for hitherto unexplored heads-in modes of entry for fatty acids in LFABP. The analyses are supported by earlier mutagenesis experiments (13), and together these data reveal the atomic reconstruction required within LFABP to accommodate two chains of fatty acids. Targeting the newly identified critical residues within LFABP, which undergo conformational alterations to accommodate two fatty acids, with small molecule inhibitors may be a potent new strategy for developing anti-FABP drugs.

EXPERIMENTAL PROCEDURES

Overexpression and Purification of LFABP—The full-length human LFABP was cloned and overexpressed as described previously (25). Delipidation of LFABP was performed by the protocol of Velkov et al. (26). Briefly, bacterial cells were lysed by sonication in a buffer containing protease inhibitors and 1 mM DTT. Affinity purification was performed on a Ni-NTA column on an AKTA FPLC system (GE Biosciences). Fractions containing LFABP were identified by SDS-PAGE and were pooled and brought to 65% saturation with ammonium sulfate at 4 °C. Delipidation was achieved by HIC on a phenyl FF 16/10 column (26). 6× His tag was cleaved using thrombin protease in 1× PBS at 20 °C overnight. Uncleaved protein and thrombin protease were removed by passing through a Ni-NTA and benzamidine column attached serially using a peristaltic pump. The cleaved protein was further purified by gel permeation chromatography (GPC) on an S-75 Sepharose column (GE Biosciences) in a low salt buffer containing 25 mM Tris (pH 8.0) and 25 mM NaCl. Complexes with palmitic acid were prepared by mixing delipidated LFABP with palmitic acid (Sigma Chemicals) and incubating overnight at 4 °C. The protein was concentrated using 5 kDa cutoff centrifugal devices (Millipore) followed by another step of GPC on a S-75 Sepharose column.

Crystallization of LFABP—Initial crystallization screening was performed at room temperature (293 K) using Hampton Research Screen kits (Crystal Screen, Crystal Screen II, and Index) by the hanging-drop vapor-diffusion method. Crystallization drops were prepared by mixing of 100 nl of protein (10 mg ml−1) with 100 nl of reservoir solution. Crystals suitable for data collection were obtained using a condition containing

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6 The atomic coordinates and structure factors (codes 3STK, 3STN, and 3STM) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
1 To whom correspondence should be addressed. Tel.: 91-11-26741731; E-mail: amit.icgeb@gmail.com.
2 The abbreviations used are: LFABP, liver fatty acid-binding protein; OLA, oleate; iLFABP, intracellular lipid-binding proteins; HTH, helix-turn-helix.
0.15 M potassium bromide and 30% polyethylene glycol monomethyl ether (PEG MME) 2000. The crystallization droplet contains 1/9262 l of protein and 1/9262 l of reservoir solution and was equilibrated against 200/9262 l of well solution. Rectangular shape crystals were transferred to paratone oil (Hampton Research) for removing aqueous solvent prior to freezing.

Data Collection and Processing—Data sets were collected at 100 K using Cu Kα radiation (λ = 1.54 Å) on a MAR345 image plate detector mounted on a Rigaku MicroMax 007 rotating anode x-ray generator operated at 40 kV and 20 mA with Osmic mirrors. Data sets were reduced with the HKL2000 program (27), and relevant statistics are summarized in Table 1. Structure was solved by molecular replacement using a LFABP model solved by Sulfur-SAD3 using Molrep in CCP4 (28). Manual model building, including placement of palmitate molecules, was performed using COOT (29). The structure was refined, and waters were added by phenix.refine in the PHENIX (30). The figures and movies were generated using Chimera (31) and PyMOL.

RESULTS AND DISCUSSION

Structure of LFABP in Complex with Two Molecules of Palmitic Acid (PLM)—The structure of LFABP in complex with two molecules of palmitic acid (PLM) at 1.54 Å (Table 1) displays the overall fold of FABP consisting of a β barrel formed by 11 antiparallel β sheets and two α helices closing one end of the barrel (Fig. 1a). One molecule of PLM is bound in a “U” shaped conformation in an interior cavity within LFABP β barrel (called inner binding site from here-on). PLMici (for the fatty acid that occupies the inner binding site) head group makes ionic contacts with side chains of Arg-122 and Ser-39. The PLMici carboxyls are also involved in H-bond interactions with three waters: HOH3, HOH19, and HOH129 (Fig. 1c). PLMici acyl chain is accommodated in a hydrophobic cavity lined by Phe-50, Ile-52, Phe-48, Phe-63, Leu-71, Phe-95, and Ile-59. The U-shaped curved orientation of PLMici is stabilized by the above hydrophobic contacts (Fig. 2). A molecule of oleate (OLA) was believed to attain this “U shaped” conformation in rat LFABP because of the C9-C10 double bond in its structure (16). Our data show that PLMici attains its characteristic U-shape conformation for palmitate despite the absence of a double bond. This confirms that fatty acid conformation is a characteristic of the

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### TABLE 1

| Data collection | apo-LFABP | holo-LFABP | LFABP-intermediate |
|----------------|-----------|------------|-------------------|
| Wavelength (Å) | 1.54      | 1.54       | 1.54              |
| Space group    | P2₁₂₁₂₁   | P2₁₂₁₂₁    | P2₁₂₁₂₁           |
| Cell dimensions| 30.14, 57.07, 74.24 | 30.28, 57.09, 74.86 | 30.24, 57.33, 74.40 |
| a, b, c (Å)    | 90, 90, 90 | 90, 90, 90 | 90, 90, 90        |
| Resolution (Å) | 50.0, 1.54 | 50.0, 1.54 | 28.0, 1.216       |
| Rmerge/Rfree   | 0.056(0.43)| 0.046(0.38)| 0.077 (0.45)      |
| i/σ(I)         | 62.02 (5.3)| 58.3 (6.2) | 33.2 (3.08)       |
| Completeness (%) | 99.3 (92.7) | 92.2 (71.8) | 98.2 (86.5) |
| Redundancy     | 16.4 (11.7)| 10.7 (9.9) | 6.6 (5.0)        |
| Refinement     |           |            |                   |
| Resolution (Å) | 9.979-2.595| 25.19-1.55 | 19.17-2.216       |
| Number of reflections | 4163 | 18190 | 6770 |
| Rmerge/Rfree   | 0.2447/0.2667| 0.2073/0.2301| 0.2402/0.2815 |
| Number of proteins | 991 | 1002 | 982 |
| Ligand         | - | 36 | 18 |
| Water          | 11 | 224 | 55 |
| B-factors      | 66.00 | 23.31 | 40.35 |
| Water          | 39.51 | 31.61 | 39.66 |
| Rmsd           |           |            |                   |
| Bond lengths (Å) | 0.003 | 0.006 | 0.01 |
| Bond angles (°) | 0.23 | 1.02 | 1.22 |
| Dihedral angles (°) | 23.85 | 16.67 | 19.08 |
| Ramachandran plot | 91.8 | 98.43 | 97.6 |
| Favored regions | 8.2 | 157 | 2.4 |
| Allowed regions | 8.2 | 0.0 | 0.0 |

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3 A. Sharma, manuscript under submission.
binding site within LFABP, and is not a structural feature of the ligand.

In contrast to the orientation of bound OLA in rat LFABP (16, 21), human LFABP binds second molecule of PLM (PLMo) in an orientation in which its head group is buried in the protein cavity (“heads-in”), where the head carboxyls make polar contacts with the positive amide of Asn-111 and with \( \frac{\xi}{H} \)NH of Arg-122, at the interface of the two binding sites (Fig. 1b). The PLMo alkyl tail is stabilized by LFABP hydrophobic residues, which line the PLM binding site. The heads-in orientation is confirmed from our high resolution structures, where the distinctive Y-shaped head group is easily visualized in the electron density maps (Fig. 1, d and e). This, therefore, stands in contrast to the prevalent view in the literature that supports a heads-out model, which has an opposite orientation for the fatty acid ligand.

**Structure of Apo-LFABP**—Apo-LFABP, which was unable to be crystallized before this report, shows unique structural features not observed in other FABP family members (Fig. 3, Table 1). The two fatty acid binding sites in apo-LFABP are not inter-
connected, as the entrance to the second binding site is partially blocked. This obstruction is due to the unique conformation of two pivotal residues, Arg-122 and Met-74, which together seem to create (and later open) the barrier that controls translocation of fatty acids (Fig. 3, a and b). Between apo- and holo-LFABP structures, it is evident that Arg-122 side chain is conformationally altered by \( \sim 90^\circ \) rotation at the \( \delta \)CH2. Further, the Arg-122 \( \zeta \)CH2 is also displaced by \( \sim 3 \) Å. The second key driver for binding site remodeling is residue Met-74. Again, between apo- and holo-LFABP states, Met-74 undergoes a conformational flipping of \( \sim 90^\circ \) at the \( \gamma \)CH2, resulting in displacement by \( \sim 3.9 \) Å. Because of the binding site reconstruction by Arg-122 and Met-74, the distance between the \( \zeta \)CH2 of Arg-122 and \( \zeta \)CH3 of Met-74 expands from \( \sim 4.4 \) Å to \( \sim 10.1 \) Å (Fig. 3, d, e, and f).

This rearrangement occurs close to the conformationally invariant Ile-52, and leads to partial blockage at the gate of the inner binding site in apo-LFABP. Because of the Arg-122 head group orientation in apo-LFABP, the outer binding site has a positive base (Fig. 3a). However, in holo-FABP, the Arg-122 is sufficiently displaced to result in an opening (Fig. 3b). In apo-LFABP, therefore, the first PLM molecule cannot bind in a heads-out orientation as its alkyl tail will directly clash with the charged Arg-122 head group. Therefore, the heads-in scenario (i.e. where the PLM carboxyl head group makes first advances towards the oppositely charged NH\(_2\) of Arg-122), that we propose here seems the most reasonable mode for initial recognition of fatty acids by human LFABP. Another major structural difference between apo- and holo-LFABP is in the internal binding site where the side chain of Phe-50 flips by \( \sim 90^\circ \) (Fig. 3c). So, unless there is flipping of the Phe-50 benzene ring, the apo-LFABP will sterically clash with PLMi. The \( \sim 90^\circ \) reorientation in the Phe-50 side chain seems critical for lacing the hydrophobic cavity where the PLMi acyl chain can fit.

Earlier, the helix-turn-helix portal region in rat LFABP was shown to undergo major conformational changes from a so-called open gate state to a closed state (21). We are unable to confirm this alteration in human LFABP as we observe only very small, possibly insignificant differences between the apo- and holo-LFABP structures that we have solved. Nonetheless, the HTH portal seems more mobile in apo-LFABP as evidenced by overall higher isotropic temperature factors.

Crystal Structure of a Binding Intermediate—To crystallographically capture binding intermediates in LFABP-ligand interaction, we incubated apo-LFABP with various concentrations of palmitic acid. In one crystal, we encountered only one molecule of PLM bound in the inner binding site (Table 1, Fig. 4). The outer binding site in LFABP was empty, and there were no conformational changes noted with comparison with holo-LFABP. The lack of structural differences with holo-LFABP suggests that structural remodeling in the binding sites is concurrent with recognition and placement of the first PLM. The entry of the second PLM, therefore, likely occurs without additional changes. This is further supported by the work of He et al. (13) where they observed comparatively large conformational changes in LFABP upon binding the first ligand when compared with entry of the second ligand, based on NMR titration experiments. Consistent with this scenario is the fact that no structures of LFBAP have been resolved where only the outer binding site is occupied with a fatty acid, highlighting the rapid transfer of bound fatty acid to inner binding site. These analyses concur with a measured 20-fold higher affinity of the inwardly bound oleic acid (\( K_d \sim 0.26 \mu M \)) when compared with the second occupant (\( K_d \sim 5 \mu M \)) (32). However, a rat LFABP mutant, where Arg-122 is replaced by Leu, binds only one molecule of oleate in the outer binding site, highlighting the crucial role played by Arg-122 for fatty acid binding in the inner binding site (13). Structures were deposited in the Protein Data Bank as holo-LFABP (3STK), apo-LFABP (3STN), and intermediate-LFABP (3STM).

A Mechanism of Fatty Acid Binding by LFABP—Based on the above observations, we propose a mechanism for fatty acid uptake by LFABP (Fig. 5). In the apo state, the two PLM binding sites are partitioned by side chains of three gatekeeper residues: Arg-122, Met-74, and Ile-52 (Figs. 3d and 5a). In the apo conformation, part of the outer binding site has a positive character to it (because of Arg-122) (Fig. 3a), and therefore a fatty acid can only enter in a heads-in orientation, the opposite will result in
direct clash of fatty acid hydrophobic tails with the resident Arg-122. The first PLM must therefore enter with heads-in directionality, allowing its head group to be accommodated in the positively charged (because of Arg-122) binding pocket. Our insights therefore suggest the following scenario: encounter of first fatty acid (PLMi) with Arg-122 in LFABP triggers a conformational change in this residue, which then shuts the hydrophobic cargo to the inner fatty acid binding site in LFABP. This PLMi acyl stem docks in a U-shaped form within LFABP, where its shape is dictated by LFABP residues that line the inner fatty acid binding site (Figs. 3c and 2). PLMi internalization marks transition from apo to holo states of LFABP (supplemental Movie S1). Arg-122 plays a pivotal role in this scheme, and consistent with our model a mutation of this residue results in an LFABP capable of engulfing only one fatty acid ligand in rat-LFABP (13). Once the inner binding site is occupied, the outer one is free to receive the second fatty acid cargo (Fig. 4), which again binds in a heads-in orientation stabilized by interactions with Asn-111, Arg-122, and a structured water (Fig. 1, a and b). In summary, our mechanistic model, supportive of cooperativity, proposes that sequential binding of fatty acids to LFABP is marked by conformational changes accompanying placement of the first fatty acid, which creates the atomic environment for accommodation of the second ligand.

Our data provide intricate details of fatty acid-induced remodeling of the binding site in human LFABP. These insights provide a platform for structure-guided design of small molecule inhibitors of LFABP. Blockage of side chain remodeling events in LFABP with small molecule inhibitors presents a new focus for specifically inhibiting the human liver forms of FABP. Pharmacological strategies that reduce the LFABP activity might be effective in prevention or reversal of diet-induced obesity and diabetes (7–12).

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