The Crystal Structure of the Liver Fatty Acid-binding Protein

A COMPLEX WITH TWO BOUND OLEATES*

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James Thompson‡, Nate Winters$, Daniel Terwey$, Judy Bratt‡, and Leonard Banaszak‡†

From the ‡Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455 and the §Department of Chemistry, St. Cloud State University of Minnesota, St. Cloud, Minnesota 56301

Liver fatty acid-binding protein (LFABP),1 is a member of the intracellular lipid-binding protein (iLBP) family (1,2). The functions of LFABP, like other family members, are thought to include lipid uptake, lipid transport, regulation of lipid metabolism, and cellular protection by maintaining the concentrations of free cytosolic fatty acids below toxic levels (3). The iLBP family is characterized by their small size of approximately 130 amino acids, their affinity for hydrophobic molecules, and their tertiary structure. Both crystal and solution structures have been determined for different iLBP family members that primarily bind fatty acids in adipocytes (ALBP), intestine (IFABP), heart muscle (HFABP), locust muscle (L-MFABP), myelin (P2), and hornworm midgut (MFB2); retinol in liver (CRBP1) and intestine (CRBPII); or retinoic acid in testis (CRABPI) and skin (CRABPII). References to these structural data for the iLBP family are cited in Table III. Although the homology of the amino acid sequences vary, all iLBPs are composed of a 10-stranded, antiparallel β-barrel with two short antiparallel α-helices positioned near one end of the barrel. Within the confines of the β-strands is a cavity that forms the lipid binding site.

The various genes for iLBPs have expression levels that are related to the cell or tissue type. Several of the different genotypes have been cloned and are available in expression vectors. In some instances, measurements have been made of their expression levels in different cell types. For example, LFABP has been detected in abundance in tissue from liver, adipose deposits, myocardium, kidney, and large and small intestinal epithelia of rats (4,5). In the small intestine, it is expressed at highest concentrations in tips of the villi in the jejunum, where it can make up to 5% of the soluble protein (5,6). In the liver, it also comprises up to 5% of the cytoplasmic protein (7).

The gene for rat LFABP contains three introns, and the initial study was followed by the insertion of the corresponding cDNA into an expression system (8,9). The expression system has been used to provide protein for the structural studies described in this report. Previously, introductory structural data was described for LFABP from chicken (10). Although the crystal structure of chicken liver LFABP suggested that few conformational differences existed between it and other family members, no information was given for any bound fatty acid or on the full structure with side chains (10).

Beyond its ubiquity across a variety of cell types, LFABP is unique within the iLBP family in its broad ligand binding specificity. In addition to fatty acids, their CoA esters, and lysophosphatidic acid, LFABP also binds heme, squalene, cer-tain eicosanoids, bilirubin, and a host of other hydrophobic compounds (3,4,11–14). Whether LFABP binds cholesterol is uncertain. Using radiola beled cholesterol, a Kd of 1.53 μM and a stoichiometry of 0.83 mol cholesterol/mol of protein has been reported (15). However, no cholesterol binding was detected by other workers using a similar technique (11).

Another distinguishing feature of LFABP is its ability to bind two fatty acids per protein molecule; other iLBPs bind only one. For the dissociation of oleate from rat and bovine LFABP, one site has a Kd ranging from 0.009 to 0.2 μM, and the other has a Kd of 0.06–4.0 μM (11,16,60). The weaker binding was determined by titration calorimetry (11,16), while the small Kd values were measured with a fluorescent reagent, itself a fatty acid-binding protein (60). In some studies, a third molecule has even been reported to bind to LFABP (17). Con-

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The atomic coordinates and structure factors (codes 1LFO and R1LFOSP) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

† To whom correspondence should be addressed. Tel.: 612-626-6597; Fax: 612-625-2163; E-mail: len_b@decc.med.umn.edu.

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The abbreviations used are: LFABP, liver fatty acid-binding protein; iLBP, intracellular lipid-binding protein; ALBP, adipocyte lipid-binding protein; IFABP, intestinal fatty acid-binding protein; HFABP, heart muscle fatty acid-binding protein; L-MFABP, locust muscle fatty acid-binding protein; P2, myelin P2 lipid-binding protein; MFB2, Monedula sexta fatty acid-binding protein; CRBP1, cellular retinol-binding protein I; CRBPII, cellular retinol-binding protein II; CRABPI, cellular retinoic acid-binding protein I; CRABPII, cellular retinoic acid-binding protein II; DAUDA, 11-(5-dimethylaminonaphthalene-1-sulfonyl-amino) undecanoic acid; r.m.s., root mean square.
considering the unusual stoichiometry of fatty acid binding, the variety of lipids that can bind to LFABP, and the greater solvent accessibility of the backbone amides (18), it has been hypothesized that the cavity size of LFABP is larger than that of other members of this family.

The multiple binding sites found in LFABP have distinct chemical shifts from the single site in IFABP according to NMR studies using carboxyl-labeled 13C-fatty acids (17). From the differences it is postulated that the fatty acids are bound to LFABP with their carboxyl termini in a more solvent-accessible conformation than in IFABP. By studying the NMR data as a function of pH, it has also been suggested that the fatty acid carboxylates have relatively normal pKₐ values when bound to LFABP. In IFABP and probably other iLBPs, the carboxylates of bound fatty acids have abnormal pKₐ values because of ion pair formation with arginine residues within the cavity. In the case of LFABP, interpretation of the pH effects are somewhat more difficult due to the fact that there are at least two binding sites (17).

There are a number of other biochemical properties that distinguish LFABP from other iLB family members. For example, the mechanism by which LFABP binds fatty acids also differs from ALBP and HFABP (19). According to fluorescein studies, the transfer of fatty acids from LFABP to liposomes is not dependent on the liposomal concentration. This is not the case for ALBP and HFABP (17). The transfer studies suggest that while ALBP and HFABP form a transient collisional complex with membrane-like acceptors, LFABP favors monomeric soluble fatty acids during the binding reaction (19).

Titration calorimetry demonstrates that a larger proportion of LFABP’s affinity for fatty acids derives from entropic contributions when compared with binding by IFABP (16). This suggests that the hydrophobic effect plays a more important role in ligand binding to LFABP than it does in IFABP and possibly other iLBs that bind fatty acids. In all iLB members that bind negatively charged lipids, two arginines are usually present in the binding cavity. The interaction between the internalized arginine(s) and the carboxylate of the ligand is probably a major factor in the enthalpic contribution to the binding energy. Arg122 of LFABP is homologous with one of these arginines in several other fatty acid-binding proteins. It is also protected from chemical modification by phenylglyoxal when oleic acid is bound (20). Other evidence for Arg122 involvement in ligand binding comes from site mutations where a change to a glutamine weakened LFABP’s affinity for fatty acids (12, 21, 64).

In summary, LFABP is something of an iLB family outcast. It binds more than 1 mol of lipid. The chemical nature of the ligand can vary more widely than most members of the fatty acid-binding proteins. When fatty acids or their analogues are bound, LFABP appears to have different properties from other siblings in the iLB family. Crystallographic data would help explain some of the similarities and differences. Crystallographic studies were undertaken in 1990, but over a period of 3 years very few crystals were obtained. The initial efforts to solve the structure using molecular replacement were unsuccessful until a composite probe was used as described below.

**MATERIALS AND METHODS**

**Expression and Purification**—Unless indicated, the DAUDA and all other chemicals were purchased from Sigma and were reagent grade or better. LFABP fractions were identified by using a DAUDA fluorescence binding (22). LFABP concentrations were determined by absorption at 280 nm using a molar absorption coefficient of 6400 M⁻¹ cm⁻¹.

Rat liver LFABP was obtained from *Escherichia coli* using the pJBL2 expression (8). Although the expression of recombinant LFABP in *E. coli* K12 H1 followed that of Winter et al. (23), the purification procedures differed. A suspension of roughly 110 g of wet cells was made in buffer A, which consisted of 0.05 M Tris, 10% sucrose, 0.05% sodium azide, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 1 mM of a protease inhibitor mixture at pH 7.4. The protease inhibitor mixture contained 100 mM benzamidine, 143 μM pepstatin A, and 200 μM leupeptin in isopropyl alcohol.

Cell lysis resulted from sonication for 5 min at 4–15°C. After removal of cellular debris by centrifugation at 5000 g, insoluble nucleic acid and some protein were extracted at 25,000 × g in two steps with 1% protamine sulfate and 65% ammonium sulfate in buffer A. The supernatant was dialyzed overnight into buffer B containing 20 mM potassium phosphate, 10 mM 2-mercaptoethanol, 0.05% sodium azide, 1 mM EDTA, and 1.4 ml of the protease inhibitor mixture at pH 7.4. It was then concentrated to 40 ml by ultrafiltration with an Amicon YM5 membrane. Pure LFABP was obtained after passage over a Sephadex G50 column (1 × 5 cm), equilibrated in buffer B and run at 17 ml/h.

The purified LFABP was run through a hydroxalkoxypropyl dextran column (Lipidex 1000, VI) at 37°C to remove bound endogenous *E. coli* lipids. SDS-PAGE (24) of the sole eluant showed a single band at 14 kDa. The first 10 amino acids match that of LFABP but with an additional amino-terminal methionine present: MNFSGKQYQQ (Applied Biosystems gas phase sequenator). Since the x-ray results to be described herein indicate an N-formyl group at the amino-terminal, the amino acid sequence data suggest that some of the purified LFABP was deamidylated by *E. coli* deformylase partially unfolding the amino terminus. However, for unknown reasons, a relatively large percentage of the crystalline protein still contains the N-formyl group on the terminal methionine as is evident in the electron density maps.

**Crystallization**—The LFABP is equilibrated with a 10 × molar excess of oleic acid (Nu Check Prep, Elysian, MN) overnight at 5°C in 100 ml of buffer C (20 mM potassium phosphate and 30 mM sodium chloride at pH 7.4). Unbound lipid is removed by ultrafiltration, and the LFABP-oleate complex is concentrated to about 10–15 mg/ml. The protein stock was stable at 4°C for up to 5 months. A single 0.5 × 0.25-mm hexagonal rod crystal grew by hanging drop diffusion against a 1:ml well volume of 3 mM ammonium sulfate, 200 mM LiSO₄, 100 mM citrate, at a pH of 5.6. The 10-μl drop was a 1:1 mixture of the protein stock (13 mg/ml) and the well contents.

**Data Collection**—This crystal was mounted 12 cm from a Siemens X-1000 multiwire area detector operating at room temperature. A Rigaku RU-200 generator was operating at 45 kV and 200 mA and fitted with a graphite monochromator produced a Cu Kα beam collimated to 0.5 mm. Data frames were counted for 3 min before each crystal rotation of 0.25°. An attempt was made to measure a complete P1 data set to the maximum extent of diffraction at 2.1 Å, although this was not possible due to radiation decay and the availability of only one crystal. The indexing, integrating, scaling, and merging of the diffraction data were handled with the XENGEN package of programs (25). Data collection statistics are given in Table I.

Reflections were present from a resolution range of 35–2.1 Å. However, for the resolution range of 2.3–2.1 Å, only about one-third of the x-ray data has been recorded. The DATAMAN program (26) (version 4.1.2) indicates an effective resolution of roughly 2.3 Å with |F|/σ(F) ≥ 1 for all reflections. The data from 20 to 2.3 Å are complete, well measured, and uniformly distributed in reciprocal space. Only reflections from 20 to 2.3 Å were used in the structural study.

The symmetry of the x-ray data indicated that the space group was either P3₁21 or P3₂1 with unit cell dimensions of a = b = 84.34 Å and c = 46.8 Å. The data were isomorphous (Rmerge = 0.12) to that described for an earlier LFABP x-ray data set (26). Calculation of the Matthews’ coefficient (27), Vm = 2.6 Å³Da⁻¹, suggested 1 LFABP molecule/asymmetric unit.

**Structure Determination**—The initial phases were determined by molecular replacement using X-PLOR (version 3.1) (28). Cross-rotation and translation function computations with various resolutions and integration radii were unsuccessful when a variety of complete, poly-alanine, or fragmented iLB coordinates were used as search probes.

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**Table I**

| X-ray diffraction data from LFABP crystals |
|-------------------------------------------|
| Resolution (Å)                             | 38.8–2.1 |
| Crystal number                            | 1       |
| Number of reflections                     | 8640    |
| Completeness to 2.3 Å (%)                 | 95.0    |
| Completeness to 2.1 Å (%)                 | 79.3    |
| Redundancy                                | 5.3     |
| Rmerge (%)                                | 10.0    |
| Average I0/I                             | 10.7    |

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However, when a composite structure consisting of six superimposed iLBPs was used as the search probe (29), the solution for the cross-rotation and translation searches was easily determined. The composite structure was composed of the complete polyalanine coordinates of ALBP, CRABPI, CRBP1, CRBPII, IFABP, and HFABP. Patterson searches were used with a 2 or cut-off on $F_i^*$. Patterson search vectors between 5 and 20 Å sampled one asymmetric unit of rotational space with a step size of 3.5°. The highest rotation function peak, 3.2 $\sigma$ above the mean, was correct. Patterson correlation refinement (30) was used to refine this orientation using 10–4 Å data. The translation function was calculated over the same resolution range with an $F_i^*$ cut-off on $F$. The best peak was 5.7 $\sigma$ over the mean value. At this point, the crystallographic symmetry was confirmed as P5$_2$1. Rigid body refinement in X-PLOR was used to optimize this molecular replacement solution using 1115 $F_i^*$ values $\geq 2 \sigma$ between 6 and 4 Å. The resulting $R$ factor was 49.3%. A $\Delta [2F_o - |F_i|]$ map (8–2.3 Å) showed appropriate electron density for 87% of the omitted amino acid side chains. Furthermore, unbiased electron density for two oleate molecules was found within the binding cavity, confirming the completion of the initial phase determination.

**Refinement**—A 126-residue model was built using the main chain fragments that best fit the electron density from the six protein models in the search probe. The initial electron density for LFABP side chains was also modeled. Extensive use was made of the rotamer data base provided in the program (version 5.10) (31). Temperature factors were preset to 20 Å$^2$. A randomly selected set of 496 or 5.3% of the reflections were set aside for $R_{	ext{free}}$ calculations (32) to monitor the refinement process and the appropriate weights for the x-ray pseudoenergy terms in X-PLOR. The Engh and Huber force field was used (33). No ionic charges were assigned to protein side chains. Reflections from 8 to 2.3 Å with $|F_i^*|$ values $\geq 1 \sigma$ were used. The first cycle of rigid body and energy minimization reduced the $R$ factor to 37.6% ($R_{	ext{free}} = 42.0\%)$. Next, one round of slow cooling-simulated annealing (34) was carried out, starting from 4000 K with 25 K temperature decrements. Finally, the structure was subjected to several macrocycles of energy minimization, group B factor refinement, and model rebuilding before completion. The atomic occupancies were not refined.

Data from 20 to 2.5 Å ($>1 \sigma$), modified by a bulk solvent correction, were used in refinement macrocycles run in parallel with the 8–2.3 Å refinements described above. The resultant coordinates and maps from both processes were then viewed together during remodeling. However, only the 8–2.3 Å-refined models were actually manipulated and rebuilt. The bulk solvent correction was accomplished by a modified X-PLOR script. It utilized the available solvent mask calculation to generate $F_{	ext{obs}}$ values for the addition to the model. Required parameters such as solvent density (0.36 e/Å$^3$), radii of mask probe (0.25 Å), and $B_{	ext{solvent}}$ (50 Å$^2$) were methodically optimized by evaluation of the $R_{	ext{free}}$. The plot of R factor and $R_{	ext{free}}$ in the resolution range of 20–2.3 Å closely matched theoretical Luzzati plots (35) in slope and value only after the bulk solvent correction was applied.

At each stage, LFABP models were critically examined with help from PROCHECK (36). When structural adjustments were contemplated, omit maps were calculated with and without the solvent-corrected low resolution data for every five residues along the protein’s sequence to help verify the map interpretation. In the initial refinement cycles, 20% $|F_o^*| - |F_i^*|$ and 80% $|F_o^*| - |F_i^*|$ omit maps (37) were used to help guarantee unbiased electron density with respect to the model being fit; $m$ is a figure of merit based on the similarity of $|F_o^*|$ to $|F_i^*|$. $D$ is estimated from the coordinate error. After the $R$ factor was reduced to 23.7% (29.8% $R_{	ext{free}}$) and the model displayed acceptable geometry, $2|F_o^* - |F_i^*|$ and $|F_o^*| - |F_i^*|$ omit maps were used instead. Simulated annealing omit maps (38) calculated for residues 21–25, 26–30, 71–75, and 76–80 were nearly equivalent to these later omit maps.

At this point, the fitting of $|F_o^*| - |F_i^*|$ electron density for oleic acid inside the LFABP cavity was begun. An amino-terminal formyl-methionine and modified cysteine, residue 69, were also placed in available electron density. Nonolvent $|F_o^*| - |F_i^*|$ electron density over 5 $\sigma$ and roughly 80 Å$^2$ in volume was found for an unknown molecule near the amino-terminal end of the first LFABP $\alpha$-helix. This density was ultimately modeled by one molecule of butanoic acid. Additional $|F_o^*| - |F_i^*|$ electron density (39) found adjoining that of one of the oleic acid molecules near its $\omega$-terminal end was also definitely nonolvent in nature. This density proved difficult to interpret and was modeled by a single carbon pseudoatom. The carbon atom is residue 131 in the deposited list of atomic coordinates. During the ligand modeling and refinement process, the $B$ factors of individual atoms were refined. Moreover, preliminary stereochemical restraints applied to the fatty acid chains were relaxed in the last stages of refinement to allow a better fit of the available electron density. Sixty-one ordered solvent molecules were added, and waters were restrained in their position by a constraint of 10 kcal Å mol$^{-1}$. Solvent molecules were removed after either 8–2.3 Å or solvent-corrected 20–2.3 Å refinement if the volume of the electron density became insufficient, their temperature factors surpassed 90 Å$^2$, or their location moved by more than 2.0 Å. The electron density clearly displayed discrete disorder for Asp$^{131}$, and two conformations were modeled. Multiple conformations for four solvent-surface residues Thr$^{34}$ and Thr$^{134}$ were weakly observable but not modeled.

The atomic coordinates for the 20–2.3 Å and 8–2.3 Å-refined LFABP-oleate complexes have been deposited in the Protein Data Bank (39). The identification code is 1lfo. The residue-numbering scheme begins with the formyl-methionine as number 1 and is complete for 127 amino acids or the entire LFABP polypeptide chain. The two complete oleate molecules, the butanoic acid, a single carbon atom, and 61 waters are consecutively numbered 128–192 following the protein residues.

**RESULTS**

**Model and Map**—The results from refinement and the model statistics are contained in Table II. The final structure of LFABP in complex with oleate residues in an $R$ factor of 19.0% (20–2.3 Å) or 20.2% (8–2.3 Å), while the $R_{	ext{free}}$ is 25.1% (20–2.3 Å) or 26.2% (8–2.3 Å). The differences between the $R$ factor and $R_{	ext{free}}$ are centered within the empirically observed range (40). Initially, the $R$ factor and $R_{	ext{free}}$ determined from bulk solvent-corrected 20–2.3 Å refinement were 3 and 5% less, respectively, than the corresponding 8–2.3 Å refinement. These differences decreased to 1.1%, shown here, commensurate with the significant improvements in the LFABP model.

The inclusion of the corrected low resolution data had a number of effects. (a) The increase in electron density contrast facilitated the correct placement of amino acid side chains, particularly the loop containing residues 73–76. (b) Alternative side chain conformations were resolved and tested, which removed problematic errors in placement. (c) There was a generalized reduction of electron density for solvent-facing lysines, glutamates, and side chains of the second helix. (d) As expected, differences between the atomic coordinates were small in comparison with r.m.s. deviations always less than 0.6 Å but generally around 0.2 Å. A conservative estimate of the average coordinate error is about 0.47 Å, obtained from a Luzzati plot with the $R_{	ext{free}}$ rather than the $R$ factor as one of the variables (41). (e) Finally, the use of the solvent correction systematically increased the average B factor of protein + $6 \AA^2$ and oleate (+7 $\AA^2$) atoms but decreased that of nonolvent atoms (5 $\AA^2$) compared with a trial refinement using just the 5–2.3 Å data. The overall average B factor for all atoms is 5 $\AA^2$ higher. The systematic discrepancy between $|F_o^*|$ and $|F_i^*|$ is expected to be slight for those reflections higher than 5 Å in resolution because the amplitudes are not as dependent on the contrast between the protein and the solvent. We currently have no...
Lys47, Lys57, Glu62, Glu70, Lys80. The correlation between the average value for protein...the program O; 0.87 is the coefficients and is contoured at 1

boxyls, but they were not included in the coordinates until the adequate to determine the positioning of the two oleate car-

The positions of the methionine and the oleate's α terminus were thoroughly investigated for possible alternate conformations and discrete disorder, particularly regarding the adjoining electron density marked by the modeling of a single carbon atom.

explanation for these B factor results but believe that the use of low resolution x-ray data aided significantly in the early stages of interpreting the electron density.

The stereochemistry of the final crystal coordinates was compared with a protein data base of 118 high resolution structures using PROCHECK. Assessments of the Ramachandran plot, bad contacts, bond geometry, and side chain geometry are quantified and summarized in the G factor. For the LFABP-oleate complex, the value is 0.29. This result is better than the expected value for equivalent resolution structures and is indicative of the strong geometric restraints applied during the refinement.

All of the non-glycine amino acids in LFABP have ϕ and ψ angles that fall within the “most favored” (93.5%) or “additional allowed” (6.5%) regions. No residues have angles that lie in either the “generously allowed” or “disallowed” sections. Even the glycine angles are within or near energetically stable conformations. Only Asn14 and Lys96 show clear left-handed α-helical character (ϕ,ψ: 59.9°, 36.0° and 49.9°, 37.1°, respectively). These two residues occur in turns between elements of secondary structure connecting the first β-strand to the following α-helix and the seventh and eighth β-strands.

The electron density for the polypeptide main chain exhibits no breaks at the level of 1 σ. However, [2|Fo| - |Fc|] density is incomplete (<1 σ) for at least one side chain atom of the following solvent-exposed residues: formyl-Met1, Glu26, Asp27, Lys47, Lys37, Glu29, Glu30, Lys38. The correlation between the model electron density and the [2|Fo| - |Fc|] electron density was calculated for each residue with the program O; 0.87 is the average value for protein. The equivalent computation for oleate 128 is 0.81, while for oleate 129 it is 0.84. Low values are only found for Glu38 (0.68) and Asp37 (0.50). These two residues are located on the solvent-accessible side of the second α-helix. Although significant enough to roughly place both side chains, the disordered density fit poorly within the geometric limits of acceptable rotamer positions.

The difference Fourier map produced before refinement was adequate to determine the positioning of the two oleate carboxyls, but they were not included in the coordinates until the R factor was less than 0.25. In addition, to ensure that the fatty acids were placed in the correct orientation through the contiguous density, four possibilities were initially tested. Thus, all combinations for placing the carboxylates of the model fatty acids at both ends of the two “sausage-like” segments of electron density were tried. The current carboxyl locations result in the lowest Rfree and agree with the positions implied by the original difference Fourier.

In the later stages of refinement, some ambiguity developed regarding the placement of the side chain of Met74 and the α-terminal carbon atoms for oleate 129 as shown in Fig. 1. The SD atom belonging to Met74 is only 3.0 Å from the α terminus of the oleic acid in the final model. The side chain of Met74 is far from a typical rotamer conformation; the r.m.s. deviation from the closest rotamer is 4.2 Å. Moreover, both the SD and CE atoms have very high temperature factors. Nonetheless, during the refinement, the side chain position of residue Met74 and the last few carbons of the bound oleic acid were thoroughly investigated for possible alternate conformations and discrete disorder. Although many alternative interpretations were tried, the correlation of the model and map shown in Fig. 1 appeared to be the best.

During the evaluation, particularly close attention was paid to the bulge jutting from electron density around the oleate at the position labeled X in Fig. 1. This density consistently reappeared in omit maps and in difference maps calculated during refinement. It does not fit any criteria normally attached to a bound water molecule. The stereodiagram in Fig. 1 reveals the ease by which this density could be alternatively modeled by rotation about the C13–C14 bond of the fatty acid, but then the density would only accommodate a C16 fatty acid. Furthermore, no other satisfactory conformation for this oleate could be found without generating significant negative [2|Fo| - |Fc|] density features surrounding the α terminus atoms. No conformer of Met74 was large enough to fully model the [2|Fo| - |Fc|] density remaining in the region of the bad contact. The dilemma was not solved by refining both configurations simultaneously assuming discrete disorder, although the simultaneous refinement with discrete disorder of both C18 and C16 fatty acids was not attempted. Finally, the coordinates as shown in Fig. 1 result in the lowest Rfree values. As we were unable to resolve the difficulties in interpretation, we have included the crystallographic coordinates of a pseudoatom with a residue.
name of C16 and an atom name of CX. Its purpose is to remind anyone studying the crystallographic model that there was difficulty in interpreting the electron density map at this point.

Electron density appearing after refinement in omit maps showed clear evidence for an amino-terminal formyl-methionine, which was subsequently included. Also present in omit maps was additional density, which indicated that the side chain of the sole cysteine, residue 69, was chemically modified. The latter density could not be represented by solvent. Its shape and size was not adequate to support cysteinylation or glutathionylation as has been previously reported (42), nor was it sufficient for an oxidized, -SO2 or -SO3, moiety. Instead, a methyl group was added, which best represents the additional volume of electron density.

Clear non-solvent \(|F_o| - |F_c|\) electron density (>5 σ) for an unknown bound molecule was found near the amino-terminal end of the first LFABP helix. Attempts to reinterpret this density with protein atoms, especially with the side chain atoms of N14, were not successful. All known chemical components from the purification and crystallization were modeled into the density, but none fit satisfactorily. Electron density in approximately the same position was observed in the structural studies of ALBP when it was in a complex with oleate, stearate, palmitate, or hexadecanesulfonic acid (43). In the latter two cases, butanoic acid was modeled and refined. The residue name in the coordinate list is C4. Since the shape and volume of this mystery density near LFABP is comparable with that in the ALBP studies, butanoic acid coordinates were again used. Nevertheless, a third oleic acid molecule with a disordered hydrocarbon tail is a second explanation. At very low contouring levels, < 0.5 σ, the difference Fourier exhibits three nearly contiguous segments of electron density, which in toto could encompass this third bound oleate.

**DISCUSSION**

**Overall LFABP Structure and iLBPs Comparison**—Within the iLBPs and LFABPs, LFABP has a uniquely different ligand binding stoichiometry and is more widespread in its tissue expression pattern but has essentially a similar overall conformation. Comparison of the crystal coordinates of other family members fitted to the LFABP model reveals the same basic intracellular hydrophobic ligand binding skeleton. It includes (a) an antiparallel \(\beta\)-barrel with an internalized cavity, (b) a combination of polar and nonpolar residues and bound water within this cavity, (c) a hydrophobic ligand, in this case one of two bound oleic acids, forming an ion pair with an internalized arginine side chain, (d) a gap between the \(\beta\)A and \(\beta\)C strands of the \(\beta\)-barrel, and (e) a lid or portal to the cavity made by the hairpin turns forming the connection between \(\beta\)C and \(\beta\)D, \(\beta\)D and \(\beta\)E, \(\beta\)E and \(\beta\)H, and \(\beta\)E-helix-turn-helix.

For a more analytical comparison to the other family members, the crystal coordinates of other known structures were fitted to those of LFABP by least squares methods. The overall r.m.s. distance differences between LFABP and representatives of the iLBPs are listed in Table III. Based on these average differences, the myelin fatty acid-binding protein P2 appears to be closest in structure. However, the range of differences given in Table III is not very broad (1.3–1.9 Å). The percentage amino acid identities between LFABP and the listed iLBPs range from 22 to 26%. Analogous comparisons of the level of amino acid sequence identities for ALBP, HFABP, and P2 are in the range of 58–67%. In terms of the LFABP linkage to other family members, there appears to be little or no systematic relationship between the level of amino acid sequence identities and the r.m.s. deviation values of the Ca positions among the various crystal structures.

A more detailed comparison with a sampling of the family members is shown in Fig. 2. Overall, the largest conformational differences appear to occur between LFABP and the two retinoid-binding proteins, CRBPI and CRABPI. Starting from the amino terminus, the first conformational difference occurs with residues between 23 and 28. The discrepancy in this region is due to the movement of the first helix and the previous turn toward the cavity interior in the crystal structure of LFABP. However, this inward movement of 1–3 Å fails to fully close off access to the cavity. Many of the other differences occur in the intervening turns between elements of secondary structure. In general, these regions are also indicated by the shaded bar at the bottom of Fig. 2 as the most solvent-accessible. Exceptions to this trend occur at residues between the indicated E, F, and F’ \(\beta\)-strands.

A comparison of hydrogen bonds shows that 10 \(\beta\)-strands form the \(\beta\)-barrel in all existing iLBp crystal structures, except in LFABP. Analysis of the crystal structure of LFABP using DSSP (44) suggests 11 \(\beta\)-strands with the additional strand labeled F” in Fig. 2. \(\beta\)’ involves residues 78–79, while \(\beta\)F” comprises residues 84–86. Definitions of \(\beta\)-structure are based...
Liver Fatty Acid-binding Protein with Bound Fatty Acids

Data supporting extraordinary conformational motion in the iLBP secondary elements surrounding the portal, the $\beta E\beta D$, $\beta E\beta F$, and $\beta G\beta H$ turns, and the two helices come from a variety of experiments. First, the cavities of both the apo- and the holocrystal structures of IFABP (61, 63) and CRBPII (58) are sealed by protein atoms, and the atomic coordinates vary little with or without ligand. This means that in these two family members, conformational fluctuations must accompany the binding reaction. Conformation motion in this region is further supported by comparisons of apo- and holo- forms of ALBP where only a slightly uncovered cavity opening is present. In crystallographic studies of other family members, other changes are evident. In the crystal structure of apo-CRABPI, the cavity is completely exposed, while in the holo- form, the cavity is only partially accessible (45). In CRABPII, the differences are due to variations in the $\beta G$ and $\beta D$ strand conformation (45). Finally, the $\beta$ factors for residues in the reverse turns near the helix-turn-helix lid in LFABP are higher than the average. A similar phenomenon is noted in almost all iLBP crystal structures.

Kinetic experiments also support localized conformational flexibility. A rate-limiting step found in the association of oleate with IFABP was interpreted as a requirement for some conformational change allowing the ligand access into the binding cavity (46). After the helical region in IFABP was deleted, the dissociation rate for oleate increased, and although the association rate was mainly unaffected, it was no longer rate-limiting (46). Furthermore, the apo- forms of LFABP (47), CRABP (48), CRBPI (49), and CRBPII (49) are significantly more susceptible to limited proteolysis in these components than the holo- forms, suggestive of greater accessibility at least transiently in the apo-form.

Finally, NMR studies of several family members point to a degree of conformational flexibility in narrowly defined portions of the $\beta$-barrel structure. In NMR structural studies of CRABPI (48), the portal elements of apo- forms generally had fewer nuclear Overhauser effect constraints than that for the holo-form. Similar results were obtained during NMR studies of IFABP (50). The largest conformational differences between the crystal and solution structures of HFABP occurred at the $\beta$-turns between strands $\beta E$ and $\beta F$, between strands $\beta G$ and $\beta H$, and in the region of helix $\alpha II$ (50).

Fig. 3. Two gaps in the LFABP $\beta$-barrel. The stereodiagram depicts a region of the crystal structure of LFABP that lacks the normal hydrogen bonding pattern of $\beta$-barrel proteins. Some distances between main chain heteroatoms are given in Å and represented by the dotted spheres. The first gap to the right is common to all iLBPs. However, shown on the left is another gap found only in LFABP. Intranucleotidic distances are measured for two of four missing hydrogen bonds between the strands $\beta F^\prime$ and $\beta G$. The division of the $\beta F$ strand into two strands is largely a consequence of this gap’s presence rather than a conformational change in $\beta F$. Several bound solvent molecules that reside along this exterior surface and others that partially fill the two gaps are not shown.
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**Fig. 4. The lipid binding cavity in LFABP.** The stereodiagram contains the main chain of LFABP derived from crystallographic analyses. Shown in *semitransparent gray* is the solvent-accessible surface of the cavity. The extent of the cut-off at the portal or top surface of the cavity region was chosen arbitrarily as described in this section. The main chain is represented by the *tube-like structure*, and a few residues are labeled for reference purposes. The two bound fatty acids are depicted by *rods*. Cavity measurements are provided in Table IV. Of the two binding sites, oleate 129 is more buried and is situated in a position similar to bound lipids found in other iLBP crystal and solution structures. Oleate 128 is for reference purposes. The two bound fatty acids are depicted by *tube-like structure*.

**Ligand Binding Cavity**—The stoichiometry of fatty acid binding to LFABP is uniquely different from other iLBP family members in that more than one fatty acid is bound. Two molecules of bound oleate are observed in the holocrystal structure described here. We have tried to account for this difference in stoichiometry by carefully comparing LFABP with the crystal structures of other family members, focusing mainly on the internalized binding cavity. The size and shape of the binding cavity in crystalline LFABP is shown in stereo in Fig. 4. This is a solvent-accessible surface calculated with the GRASP program (51) using a probe radius of 1.4 Å. The cavity in LFABP is essentially a flattened rectangular box with rough dimensions of 13 × 9 × 4 Å. A corner of this interior box is linked to a surface opening by a narrow but contiguous channel of about 10 Å in length and 3–4 Å in width. Approximately 48% of this surface is identified with hydrophobic side chains, while 52% is associated with the side chains of charged or polar residues.

The volume enclosed by the solvent-accessible surface over all of the modeled atoms in the crystal structure is 28,600 Å³, while the area is 7,070 Å² (with a 1.4-Å probe). If only the two bound oleate molecules are stripped away, the volume is reduced by 430 Å³, while the surface area increases by 560 Å². As shown in Fig. 4, the two oleic acid molecules take advantage of nearly all of the cavity space formed by the β-barrel. Table IV contains a surface and volume comparison with other iLBP family members. For LFABP, calculation of the cavity area and volume results in values of 610 Å² and 440 Å³. The cavity surface was generated with GRASP using a probe distance of 1.4 Å (51).

**Table IV.** iLBP lipid cavity area and volume

| Protein | Area  | Volume |
|---------|-------|--------|
| LFABP   | 610   | 440    |
| L-MFABP | 510   | 350    |
| P2      | 420   | 330    |
| HFABP   | 391   | 323    |
| ALBP    | 450   | 310    |
| CRBP1   | 372   | 261    |
| CRBP1I  | 470   | 260    |
| CRBP1I  | 334   | 235    |
| IFABP   | 353   | 234    |
| CRABPI  | 410   | 210    |

* No artificial cut-off was needed to delimit the binding cavity boundary, since it is entirely enclosed by protein atoms (see *Discussion*). Thus, the measurements are more precise than the approximately ±30 Å² or ±20 Å³ found for cavities partially open to the exterior.

The required increase in cavity volume for this binding site is bound to LFABP (52, 65). The conformation of the ligands will be described in a separate section below.

An assessment of the cavity volume and area of holo-LFABP compared with that of other family members is contained in Table IV. Both GRASP and the program VOIDOO (53) were used to delineate the cavity and make measurements. Measurements by VOIDOO were made using all of the provided defaults and atomic radii. Because the binding cavity is not completely enclosed, the results are dependent on where an artificial cut-off is drawn separating the interior from the exterior. While automated in VOIDOO, in GRASP an arbitrary cut-off was chosen, and hypothetical atoms were placed to close off the cavity entrance for all of the iLBPs in a similar manner. This cutoff is indicated by the end of the accessible surface contour at the top of Fig. 4. The LFABP binding cavity is clearly the largest in the iLBP family, exceeding that of the next relative by an additional 26% in volume and 20% in area. A comparison of all cavity surfaces with that of LFABP shows that the majority of this increase is located around the binding site of oleate 129 (not shown).
primarily due to reductions in side chain size or position at seven key residue locations. The LFABP residues at these points are Ser$^{39}$, Asn$^{61}$, Thr$^{93}$, Ser$^{100}$, Thr$^{102}$, Asn$^{111}$, and Ser$^{124}$. A compilation of larger, homologous residues at each spot is found in Table V for several iLBPs. If any one of these residues were introduced into LFABP, it would sterically interfere with binding at the primary site. Except perhaps for the amino acids homologous to Ser$^{100}$ and Thr$^{93}$ on the conformationally divergent $\beta$G$\beta$H loop, the main chain atoms at these locations vary little in position (see Fig. 2). Other nearby residues among the various iLBPs differ only slightly in their dimensions or placement.

The two oleic acids do not completely fill the binding cavity of LFABP. This leaves room for six bound solvent molecules. The residue numbers for these waters are 144, 179–182, and 185. The waters are involved in hydrogen bond networks with the interior hydrophilic residues and the carboxyl oxygen atoms of the oleate molecules (see below). The water molecules presumably affect ligand affinity and probably play an additional structural role. There are also two additional interior buried waters in LFABP that are not located within the ligand binding cavity. These solvent molecules, 167 and 168, reside between the $\beta$D$\beta$E loop and the main chain atoms at these locations vary little in position (see Fig. 2). Other nearby residues among the various iLBPs differ only slightly in their dimensions or placement.

The Bound Oleic Acid—In the crystal structure, the more internalized fatty acid, oleate 129, is completely surrounded by protein atoms, structural water, and the nearby atoms of the second bound fatty acid. Because of the location of the ligand within the protein, the binding site for oleate 129 is likely to be the first occupied. Since a portion of the fatty acid atoms in the second site interact with the ligand at the primary site, their binding energies must be linked in some presently unknown manner. As can be seen in Fig. 4, the $\omega$ terminus of oleate 128 is packed against oleate 129. Furthermore, in the absence of the primary fatty acid, much of the hydrophobic character of the second binding site would not exist. In fact, a 185-Å$^2$ area is buried by the acyl chain interactions between the two oleate conformations using a surface calculated with a 1.4-Å probe. The rest of the second binding site occupies the channel leading to the external milieu. Despite the fact that the carboxyl of oleate 129 is exposed to the solvent, it is still involved in a network of hydrogen bonds. From this single crystal structure, it is unknown whether any conformational changes accompany binding at the primary or secondary sites.

The U-shaped conformation of the more internalized fatty acid, oleate 129, appears to be facilitated by the C9–C10 double and C13–C14 single bonds, which both have synclinal dihedral angles. The remaining torsional angles along the aliphatic chain are roughly antiplanar, although the C7–C8 and C8–C9 angles are closer to anticlinal. It is important to remember that the ligand positions are time-averaged over the data collection period and that the resolution of this experiment will not differentiate small positional differences on the order of 0.5 Å.

Fig. 5 shows the omit $|F_o| - |F_c|$ electron density at a 3 σ level along with all protein atoms, ligand atoms, and solvent within 4 Å from any carbon atom of oleate 129. Beginning with the aliphatic end, the final C17–C18 atoms are in close contact with the side chain of Met$^{74}$ located on the $\beta$E$\beta$F turn. As a result, the side chain of Met$^{74}$ deviates greatly from the normal rotamer conformations and has a high B factor. Based on the 8–2.3-Å electron density, the least ordered region of the oleic acid is that for the C14–C16 atoms. The possible existence of an alternative conformation was investigated, but supporting electron density was found to be incomplete. As noted under “Results,” the single atom sphere numbered 131 occupies the region where the alternative conformation was anticipated (see Fig. 5).

Of the two binding sites, oleate 129 is involved in much more extensive hydrogen bonding interactions at the carboxyl group. The residues shown in Fig. 5 that are involved in this network are Ser$^{39}$, Arg$^{122}$, and Ser$^{124}$. Of the six bound waters found inside the cavity, three are members of this network. The waters appear as gray spheres and are numbered 179–181. Additional residues with atoms in contact with oleate 129 are Ile$^{41}$, Phe$^{63}$, Glu$^{72}$, Thr$^{73}$, Thr$^{93}$, and Thr$^{102}$. Last, a fourth solvent molecule, number 185, is within van der Waals distance of the hydrophobic acyl chain. Note that these residues and waters represent only a small portion of the cavity confines. The solvent-accessible surface of LFABP lost due to the interaction of this oleic acid is 400 Å$^2$.

Earlier site-directed mutagenesis (21, 64) and chemical modification (20) data established the importance of Arg$^{122}$ in the binding of at least one ligand. The mutants at this position show a reduced but not a total loss in binding of oleate. This may be explained by the fact that in the crystal structure, Arg$^{122}$ is only one of the polar groups interacting with the carboxylate at the primary binding site. Also, the second binding site may remain fully functional. Furthermore, the remaining hydrophobic interactions may still favor binding. A recently expressed double mutant, T102Q/R122Q, was shown to lack lipid binding ability (21, 64). In the crystal structure, the T102Q mutation would clearly sterically block the primary binding site in LFABP.

Omit $|F_o| - |F_c|$ electron density for residues within 4 Å of the second bound oleate appears in Fig. 6. The fatty acid at this site is nearly fully extended in contrast to oleate 129. The C9–C10 double bond and C11–C17 single bonds show approximately synclinal dihedral angles. The remaining torsional angles are all roughly antiplanar. The electron density for this oleate molecule is weaker at the solvent-exposed end, and the B factors rise as one follows the hydrophobic tail toward the carboxylate. In fact, the $|2F_o| - |F_c|$ electron density was initially suggestive of two conformations for the first five carbons of the oleic acid. However, strong negative difference electron density arose for one configuration.

### Table V

| LFABP | Homologous iLB residues |
|-------|------------------------|
| Ser$^{39}$ | Ile, L-MFABP, Lys, CRBPI, CRBP II; Met, ALBP |
| Asn$^{61}$ | Phe, L-MFABP, Met, CRBP |
| Thr$^{93}$ | His, HFABP, L-MFABP; Glu, ALBP, P2 |
| Ser$^{100}$ | Ile, ALBP, P2; Leu, HFABP; Trp, CRBPI, CRBPII |
| Thr$^{102}$ | Gln, CRBPI, CRBPII, Arg, ALBP, CRABPI, HFABP, L-MFABP, P2 |
| Asn$^{111}$ | Ile, L-MFABP |
| Ser$^{124}$ | Phe, CRBPI, CRBPII; Tyr, ALBP, CRABPI, HFABP, L-MFABP, P2 |

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The current conformation resides in contiguous electron density and results in lower $B$ factors and in the formation of the largest possible number of hydrogen bonds. The carboxylate group is found near residues forming the portal into the cavity in a solvent-exposed position. Nonetheless, this group is still involved in a hydrogen bonding network as implied in Fig. 6. The side chain of Asp88 from another LFABP monomer is of less importance, since it interacts with the ligand because of crystal lattice packing. This location for the carboxylate agrees with results from NMR experimentation (17) and studies combining mutagenesis with fluorescence quenching (54). The total accessible surface buried by interactions with oleate 128 is 420 Å$^2$. From C1 to C14 there are no interactions with atoms of the primary fatty acid. On this basis, the prior presence of fatty acid at the primary site may be required if anything longer than a C14 fatty acid were to bind at the secondary site.

As mentioned under “Results,” the crystals also contained a small amount of electron density of unknown origin. This density is located in a position similar to electron density in ALBP and is shown in stereo in Fig. 7 modeled as a 4-carbon carboxylic acid. The good correlation in the LFABP map between butanoic acid and the electron density is apparent. The unknown substance has the properties of an anionic compound, since the two atoms at the carboxyl end are within hydrogen bonding distance to main chain nitrogen atoms of residues Asn14, Phe15, and Glu16. The formation of four hydrogen bonds is possible. The amount of solvent-accessible area buried by this interaction is 30 Å$^2$. Phe15 and Glu16 are positioned at the N-terminal end of helix $a$1 (Fig. 7, upper right). Hence, the electron density is nearly at the precise position for anionic interactions with the positive dipole of the N terminus of this $a$-helix. Although highly speculative, the appearance of the density might suggest an exterior binding site for fatty acids. Only the first few carbon atoms would be ordered; the remaining part of the ligand would be assumed to be largely disordered.

Conclusions—The structure of holo-LFABP has been deter-
mined from crystals prepared with oleic acid. LFABP has the same overall conformation as other members of the intracellular lipid-binding protein family. However, a few differences in the conformation of the protein lead to dramatically different ligand binding properties. Instead of a single hydrophobic ligand as found with all of the other iLBPs, LFABP binds two molecules of oleic acid. The two binding sites interact in an unusual fashion. We have labeled a location characterized by the conformation of the protein lead to dramatically different ligand binding properties. Instead of a single hydrophobic ligand as found with all of the other iLBPs, LFABP binds two molecules of oleic acid. The two binding sites interact in an unusual fashion. We have labeled a location characterized by 

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**FIG. 7.** Omit [ $P_{	ext{F}}$ ] electron density not accounted for by LFABP or solvent atoms. The stereodiagram was assembled as described in the legend to Fig. 5. The segment of the crystal structure that is shown on the exterior of the molecule and is described more fully in the text. Amino acid residues 13–17 are illustrated, all within 4 Å of this unknown molecule. The shape of the electron density matches that of a carboxylate from a fatty acid. After tests of all known components of the purification and crystallization were exhausted, butanoic acid was found to best fit the density. Again, the map contouring is at 3 $\sigma$, calculated over an 8–2.3-Å range.
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