Interaction of Chicken Liver Basic Fatty Acid-Binding Protein with Fatty Acids: A ^{13}C NMR and Fluorescence Study†

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Received May 17, 2001; Revised Manuscript Received August 1, 2001

ABSTRACT: Two different groups of liver fatty acid-binding proteins (L-FABPs) are known: the mammalian type and the basic type. Very few members of this second group of L-FABPs have been characterized and studied, whereas most of the past studies were concerned with the mammalian type. The interactions of chicken liver basic fatty acid-binding protein (Lb-FABP) with 1-^{13}C-enriched palmitic acid (PA) and oleic acid (OA) were investigated by ^{13}C NMR spectroscopy. Samples containing fatty acids (FA) and Lb-FABP at different molar ratios exhibited only a single carboxylate resonance corresponding to bound FA, and showed a binding stoichiometry of 1:1 both for PA and for OA. Fluorescence spectroscopy measurements yielded the same binding stoichiometry for the interaction with *cis*-parinaric acid [K_d = 0.38(4) μM]. Competition studies between *cis*-parinaric acid and the natural ligands indicated a decreasing affinity of chicken Lb-FABP for PA, OA, and retinoic acid (RA). ^{13}C NMR proved that pH and ionic strength affect complex stability. The carboxyl signal intensity reversibly decreased upon lowering the pH up to 5. The pH dependence of the bound carboxyl chemical shift yielded an apparent pK_a of 4.8. A decrease of the integrated intensity of the bound carboxylic signal in the NMR spectra was observed while increasing the chloride ion concentration up to 200 mM. This body of evidence indicates that the bound FA is completely ionized at pH 7.4, that its polar head is positioned in a solvent-accessible region, that a FA−protein strong ionic bond is not present, and that high ionic strength causes the release of the bound FA. The reported results show that, insofar as the number of bound ligands and its relative affinity for different FAs are concerned, chicken Lb-FABP is remarkably different from the mammalian liver FABPs, and, within its subfamily, that it is more similar to catfish Lb-FABP while it behaves quite differently from shark or axolotl Lb-FABPs.

The fatty acid-binding proteins (FABPs)[1] are a family of intracellular, low molecular weight, nonenzymatic proteins isolated from many different sources, that exhibit high-affinity binding constants for small endogenous and exogenous lipophilic ligands (*1*, *2*). Although the specific function of each member of this large family has not yet been established, FABPs are assumed to carry out the function of solubilization, storage, and transport of fatty acids and of their CoA derivatives. Characteristic of this family of macromolecules is a common fold comprising a 10-stranded, antiparallel β-barrel that includes 2 short antiparallel α-helices, inserted between the first and the second strand of the β-structure and positioned over 1 of the ends of the barrel. The cavity in the interior of the barrel has been shown to be the lipid-binding site. Despite these general common structural features, the FABPs isolated from different tissues in a given species show a rather low degree of sequence homology; however, when the FABPs from the same tissue belonging to different species, sometimes quite distant in evolution, are considered, sequence homology can reach much higher values, up to 70−80%. The FABPs have been classified and described on the basis of the organ that they were initially isolated from, but several instances are known in which more than one FABP type has been shown to be produced by a single tissue. Indeed, different FABPs purified from the same source can show variability concerning both the types of preferred ligands and the number of ligands bound, and also their preferred binding mode (*3*, *4*). While this variability is most probably related to their individual role in the organ under examination, the nomenclature used to describe the proteins in this family as simply "tissue" FABP is insufficient and can be rather confusing. On the other hand, the detailed study of the structure and of the binding properties of all of the FABPs purified from a single type of tissue should be of relevance in furthering our

† This work was supported by grants from the Ministry of the Universities and Scientific Research, MURST (COFIN 98 & 00).

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[1] Abbreviations: BSA, bovine serum albumin; *cis*-PAR, *cis*-parinaric acid (18:4); FA, fatty acid; FABP, fatty acid-binding protein; L-FABP, liver fatty acid-binding protein; Lb-FABP, liver basic fatty acid-binding protein; OA, oleic acid (18:1); PA, palmitic acid (16:0); RA, retinoic acid.

10.1021/bi011009w CCC: $20.00
Published on Web 09/29/2001

understanding of the molecular details of the specificity of the FABPs in general.

The most thoroughly characterized FABPs isolated from the liver are mammalian liver FABPs, also called L-FABPs, but these are not the only FABPs characteristic of the organ since another type of FABP is also present in the liver of some vertebrates although so far their presence has not been shown in the liver of mammals. This second group of liver FABPs are called liver (basic) FABPs because the first member of the family that was discovered and characterized has a very high isoelectric point (*5*−*9*). The proteins in this group have a rather low level of sequence homology with mammalian L-FABP, but the sequence similarity within this group is very high (70%) (*9*). The first member of the subfamily to be purified and described is chicken liver basic fatty acid-binding protein (Lb-FABP) (molecular mass ca. 14 kDa, pI = 9.0) (*5*) and, at present, the only lipid-binding protein thoroughly characterized from chicken liver. Its structural features, as established from the X-ray crystal structure (*6*), CD (*10*), fluorescence (*10*), and NMR spectroscopy investigations (*11*), indicate that it belongs to the FABP family, although there are many details of the protein structure and specificity of binding that still deserve more detailed studies.

Some of the FABPs have been cloned and expressed in suitable media and enriched in stable isotopes such as ^{15}N, ^{13}C, thus opening the road for very detailed NMR structural studies. The availability of adequate amounts of isotope-enriched proteins has therefore lead to the determination, through NMR spectroscopy, of the solution structure of apo- or holo-forms and of mutants of several members of the FABP protein family (see, for instance, *12*−*14*). These studies confirmed the common structural features present among the members of this family and gave new information on the degree of variability in the flexibility of the protein backbone.

In the absence of enriched chicken Lb-FABP, we have addressed the issue of its interaction with ligands and report here the results of our studies, using samples of native delipidated protein and ligands selectively enriched in ^{13}C. Indeed, ^{13}C NMR spectroscopy, exploiting the sensitivity of the chemical shift of the carboxylic ^{13}C to the environment and the ionization state, has been shown to be an excellent technique in the study of the number of binding sites, of the amount of bound ligand, of the solvent accessibility of its polar head, and of the stability of the protein−ligand complexes (*3*, *15*−*17*). Our NMR results, together with additional binding experiments we have performed using fluorescence spectroscopy, indicate that chicken Lb-FABP has a single binding site for fatty acids, that the polar head of the fatty acid is solvent-accessible, and that the stability of the complexes is affected by changes in pH and ionic strength.

MATERIALS AND METHODS

Materials. Palmitic acid (PA) and oleic acid (OA), isotopically enriched with ^{13}C at the C_1 position, were purchased from Cambridge Isotope Laboratories Inc. and from Isotec Inc., respectively. *cis*-Parinaric acid was purchased from Molecular Probes and retinoic acid from Aldrich Chemicals. Lipidex-1000 was from Packard Instruments. All other chemicals were reagent grade or better. All the buffer solutions were prepared with deionized Milli-Q water with 0.02% NaN_3. pH measurements were performed before and after each NMR experiment using a Mettler microelectrode. The reported pHs are uncorrected for isotope effects.

Protein Purification. FABP was purified from chicken liver following the procedures described in the literature (*5*). A preparative isoelectric focusing with isoelectric membranes was added as final step of the purification (*18*). The purity of the protein was checked with SDS−PAGE and isoelectric focusing. After delipidation through Lipidex treatment, aliquots of protein solutions in phosphate buffer (10 mM, pH 7.34) were concentrated using a Centricon YM-3 typically up to ca. 1 mM. The final protein concentration was determined by UV measurements (ϵ_{280} = 9423 M^{-1} cm^{-1}).

Ligand Interaction. The solution of the delipidated protein was transferred into a NMR tube where the fatty acid (FA) had been deposited. The deposition occurred by slow evaporation of appropriate volumes of a solution of the FA in freshly distilled diethylic ether. Unless otherwise specified, FAs selectively enriched at C_1 were employed. To establish the binding properties of Lb-FABP, solutions of the protein were incubated stepwise with increasing amounts of the FA (up to a FA:protein molar ratio of 3:1).

NMR Measurements. The samples were incubated at 300 K typically for 7−8 h, and then a ^{13}C NMR spectrum was recorded overnight. ^{13}C{^{1}H} NMR spectra of solutions in 10 mM phosphate buffer (20% of D_2O) were recorded at 300 K on a Bruker DRX-300 or a Bruker AC-300 NMR spectrometer. Typically, a 90° pulse was employed for 20 000 scans with 2.0 s of pulse interval. Series of spectra on the same sample were recorded on the same spectrometer. The same experimental protocol, however, was always applied for allowing the comparison of data recorded on different spectrometers. For quantitative analysis, the resonance (39.7 ppm) from the mobile protein ϵ-Lys/β-Leu carbons was used as an internal reference (*19*). The accuracy of the measured chemical shifts is ±0.1 ppm. ^{1}H NMR spectra of FA/Lb-FABP complexes were recorded on a Bruker DRX-300 NMR spectrometer at 300 K (D1 = 2.0 s, 128 scans) with presaturation of the water signal.

pH and Chloride Titrations. For pH or chloride titrations, the complexes prepared with an excess of FA (3 equiv) or stoichiometric ratios were used. Typically, solutions (500 μL) of the complex FA/Lb-FABP (1.6 mM for PA and 1.12 mM for OA) were titrated with 0.1 M phosphoric acid (pH from 7.22 to 5.05 for the PA complex and from 7.21 to 5.53 for the OA complex). The FA/Lb-FABP complexes were then back-titrated with 0.098 M NaOH up to pH 7.44 for PA and 7.57 for OA. After each addition, the samples were allowed to equilibrate at room temperature before measuring the pH and recording a ^{13}C NMR spectrum overnight in the experimental conditions described above.

A solution (500 μL) of the FA/Lb-FABP complex (0.98 mM for PA, 0.96 mM for OA) was transferred into an NMR tube and titrated with increasing amounts of NaCl, up to a final chloride concentration of ca. 200 mM for both complexes. After each addition of NaCl, the solution was incubated at 300 K for 7−8 h, and a ^{13}C NMR spectrum was recorded overnight with the parameters described above.

Fluorescence Measurements. Fluorescence intensity measurements were performed at room temperature on a Jasco 777 spectrofluorometer in 1 cm quartz cuvettes. The absorbance at the excitation wavelength of each sample was always <0.1 to avoid inner filter effects. At the final point of the titrations, the ethanol concentration was kept below 1.5%, since up to this concentration no significant changes in the measured fluorescence were observed.

Tryptophan Fluorescence. Samples (2.5 mL) of delipidated Lb-FABP in phosphate buffer solution (10 mM, pH 7.4) (3.5 and 7 μM for the titration with PA and OA, respectively) were titrated with small increments (2 μL) of ethanolic solutions of the appropriate FA up to 3 equiv. After each addition, the solution was mixed and equilibrated for ca. 1 min, and fluorescence intensity measurements of Trp6 (λ excitation 295 $\pm$ 1.5 nm, λ emission 328 $\pm$ 1.5 nm) were performed. At the final point of the titrations, the ethanol concentration was below 1.0%.

cis-Parinaric Acid Binding Assay. Samples (2.5 mL) of 0.5 μM delipidated Lb-FABP in phosphate buffer solution (10 mM, pH 7.4) were titrated with small increments (2 μL) of a 0.37 mM *cis*-parinaric acid solution in degassed ethanol up to ca. 7 equiv. The concentration of the *cis*-parinaric acid (*cis*-PAR) ethanolic stock solution was previously determined spectrophotometrically ($\epsilon_{305} = 7.8 \times 10^4$ M^{-1} cm^{-1}). After each addition, the solution was mixed and equilibrated for ca. 1 min; then fluorescence intensity measurements of the *cis*-parinaric acid (λ excitation 321 $\pm$ 1.5 nm, λ emission 418 $\pm$ 1.5 nm) were performed. In reverse experiments, 0.5 μM *cis*-parinaric acid solutions (2.5 mL) in phosphate buffer (10 mM, pH 7.4) were titrated with 0.24 mM Lb-FABP up to a fatty acid to protein molar ratio of 1:7. Fluorescence measurements were performed in the same conditions described above. Blank titrations were also performed to account for the fluorescence of the ligand or of the protein alone.

cis-Parinaric Acid Displacement Assay. Samples (2.5 mL) of 7 μM Lb-FABP in phosphate buffer (10 mM, pH 7.4) were incubated with 7 μM *cis*-parinaric acid for 5 min to obtain maximum fluorescence. Each solution was then titrated with small increments of the corresponding ligand [palmitic acid, oleic acid, and retinoic acid (RA)] from a 1.7 mM solution in ethanol. The fluorescence measurements were performed under the same conditions as above.

RESULTS

Complex Formation: Stoichiometry. (A) ^{13}C NMR Spectroscopy. When apo-Lb-FABP is incubated for ca. 7 h with 1 equiv of FA (PA = palmitic acid, OA = oleic acid), FA/Lb-FABP complexes are formed as shown by the ^{13}C NMR spectra of the solutions. Indeed, using $^{13}C_1$-enriched ligands, in the carbonyl-carboxylate region a new intense resonance due to the ^{13}C-enriched carboxylic group of the bound fatty acid appears at 182.2 ppm for PA and at 182.3 ppm for OA, respectively (Figure 1). These new signals are sharp ($\Delta\nu_{1/2}$ ca. 3−4 Hz) when compared with those of the protein and also with respect to the resonance at 39.7 ppm due to the mobile ϵ-lysine/β-leucine carbons (*19*), indicating a significant residual mobility of the carboxylic group in the complex.

To investigate the existence of different and/or multiple binding sites, apo-Lb-FABP was incubated with increasing amounts of FA, and ^{13}C NMR spectra were recorded up to a 3:1 FA:Lb-FABP molar ratio. The intensity of the signal of bound FA was found to progressively increase until a molar ratio of 1:1 was reached, and remained constant thereafter. No other sharp signals were seen to grow in the carboxyl-carbonyl region, but at molar ratios higher than 1, in the ^{13}C NMR spectra of the oleate complexes a broad signal at 179.6 ppm ($\Delta\nu_{1/2}$ ca. 80 Hz) was observed, and turbidity of the samples became apparent due to the formation of lamellar aggregates (*20*). The carboxyl of the complexed oleate still gave a sharp signal, indicating that the possible exchange with the unbound fatty acids was slow on the NMR time scale ($k < 5$ s^{-1}) (Figure 1, inset).

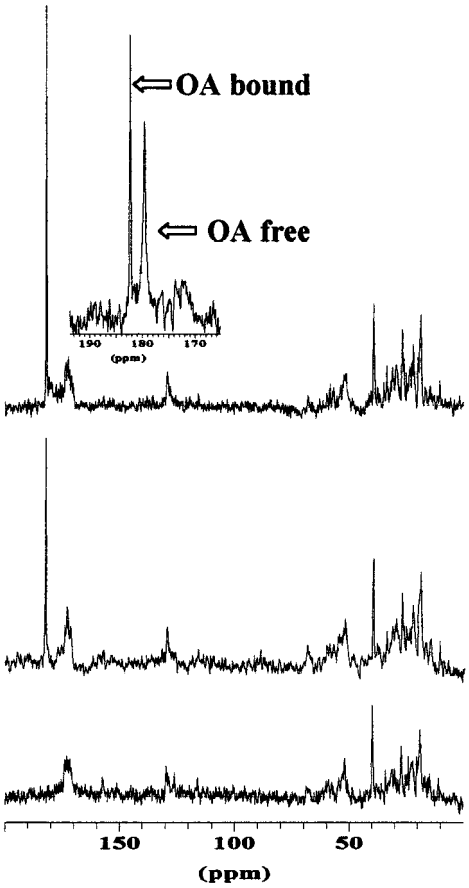

FIGURE 1: Proton-decoupled ^{13}C NMR spectra (300 K, 75.4 MHz) of solutions of natural-abundance Lb-FABP in the apo-form (bottom) and after the interaction with 1 equiv of palmitic acid (middle) and oleic acid (top) selectively enriched in ^{13}C at C_1. The inset shows the carbonyl-carboxyl region of the spectrum after the interaction with 3 equiv of oleic acid.

The amount of complexed FA was estimated following different parameters: (i) the ratio between the integrated intensity of the carboxylic signal and that of the signal at 39.7 ppm; (ii) the absolute intensity of the carboxylic signal as measured in difference spectra, to account for the possible contributions of the resonances of the carboxylate groups of the Glu and/or Asp side chains (the intensity of these signals was found to be negligible); (iii) the ratio between the integrated intensity of the carboxylic signal and that of the protein carbonyl region. In all these experiments, the carboxylic peaks of both fatty acids (palmitic and oleic) increased progressively until saturation was reached at a 1:1 FA:protein ratio (Figure 2).

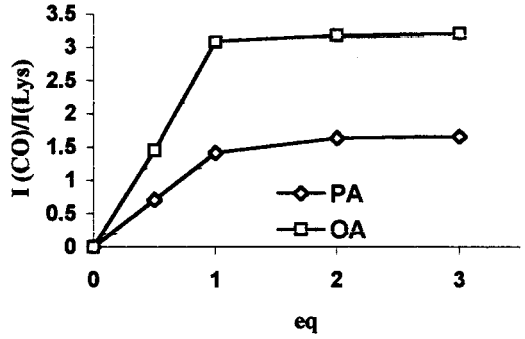

FIGURE 2: Variation of the amount of complexed FA, evaluated according to procedure (i) described in the text, on increasing the amount of deposited FA. The reported results are the mean of at least three independent experiments.

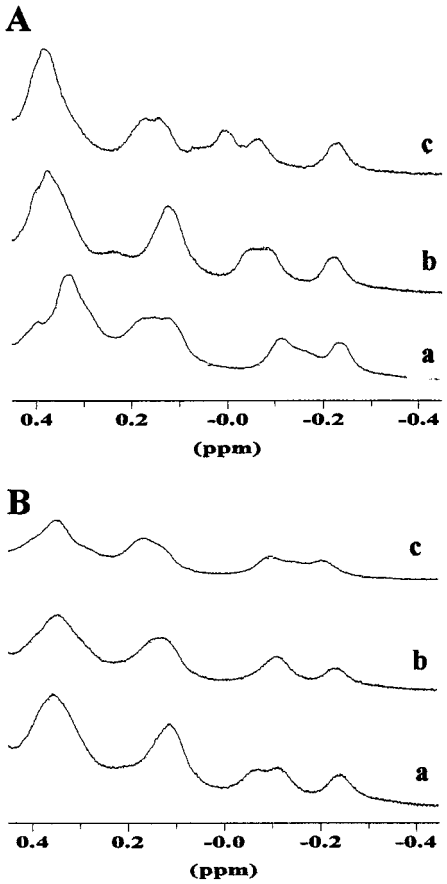

FIGURE 3: (A) High-field region of ^{1}H spectra of solutions of Lb-FABP (300 K, 300.13 MHz): (a) apo; (b) complex with 1 equiv of PA; (c) complex with 1 equiv of OA. (B) High-field region of ^{1}H spectra of solutions of a PA/Lb-FABP complex: (a) pH 7.4; (b) pH 6.2; (c) pH 5.0.

The interaction with the ligands also caused a slight modification of the pH of the protein solution, which decreased, for both complexes, from 7.4 to 7.2 up to the point when the 1:1 ratio was reached and remained constant after the addition of further amounts of PA or decreased slightly (to 7.12) if the fatty acid was OA.

(B) ^{1}H NMR Spectroscopy. The protein ^{1}H NMR spectrum shows also modifications following its interaction with fatty acids. In particular, as shown in Figure 3A, the high-field signals undergo similar changes during the formation of the two complexes, which is probably related to conformational adjustments of the side chains of the protein residues

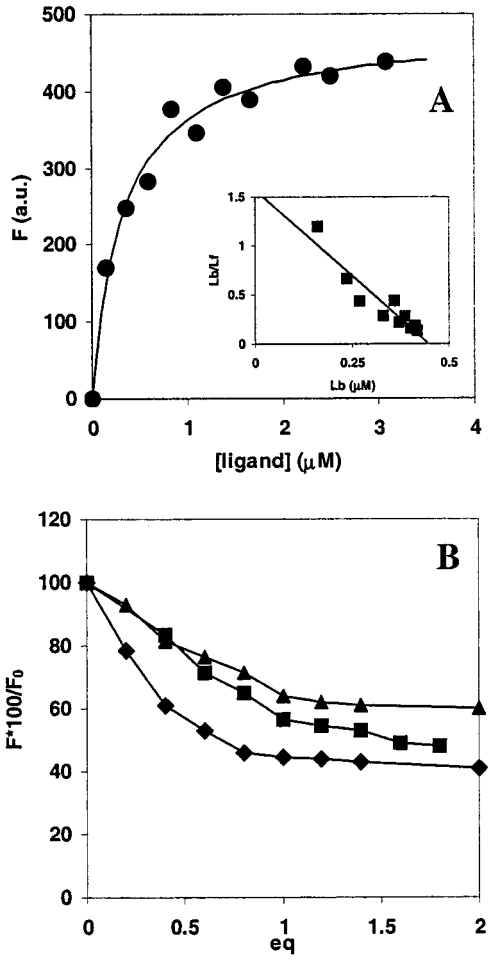

FIGURE 4: (A) Fluorescence titration of a 0.5 μM delipidated chicken Lb-FABP with *cis*-parinaric acid (0.32 mM). The final protein:fluorophore ratio was ca. 1:7. The inset shows the Scatchard plot obtained accordingly. (B) Displacement of *cis*-parinaric acid from chicken Lb-FABP by (◆) palmitic acid, (■) oleic acid, and (▲) retinoic acid.

occurring during complex formation. It is worthwhile noticing that the highest field resonance in the spectrum remains unchanged.

(C) Fluorescence Studies. The emission maximum and the fluorescence intensity of the single tryptophan residue present in chicken Lb-FABP do not significantly change in the presence of increasing amounts of PA or OA (data not shown).

Fluorescence intensities, measured both in the protein and in the ligand titrations, were corrected for the contribution of the ligand or the protein alone, respectively. In both sets of experiments, the data showed saturation behavior, and F_{max} was obtained through nonlinear least-squares fit to a rectangular hyperbola. Good agreement was found between the results from the two sets of experiments. The values of K_d and of B_{max}, determined through Scatchard plots, indicate that chicken Lb-FABP has a single binding site with K_d = 0.38(4) μM. A representative example of titrations of 0.5 μM *cis*-parinaric acid with delipidated Lb-FABP is reported in Figure 4A.

Competition experiments were also performed in order to study the specificity of the binding. Aliquots of ethanolic solutions of PA, OA, and retinoic acid were progressively added to solutions containing the *cis*-PAR/Lb-FABP complex

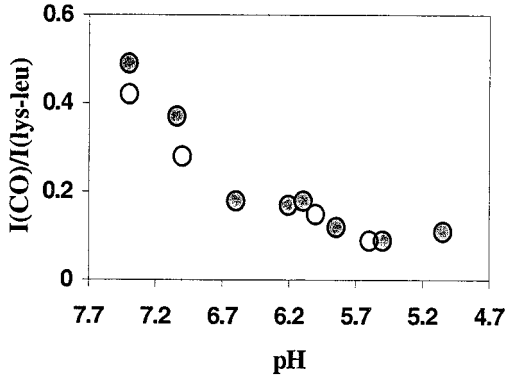

FIGURE 5: pH dependence of the intensity of the PA carboxylic signals (300 K, 75.4 MHz). Closed circles, titration from neutral to acidic pH; open circles, back-titration from acidic to neutral pH.

(7 μM), and the change in fluorescence was followed. The affinity decreases in the order PA > OA > retinoic acid (Figure 4B).

Complex Stability and pH. To check the ionization behavior of the fatty acids in the complexes and their possible involvement in electrostatic interactions with positively charged residues, solutions of FA/Lb-FABP complexes were titrated by addition of 0.1 M phosphoric acid. The pH was thus lowered in 6−8 steps from 7.4 to 5.0, and after each variation, a ^{13}C NMR spectrum was recorded. Both the intensity of the carboxylic signal and its chemical shift were affected by the pH change. The amount of complexed FA, evaluated according to approaches (i) or (iii) described above, decreased progressively (Figure 5), and resulted, in the case of PA, in an almost undetectable signal.

For both complexes, a high-field shift of the carboxylic signal was observed on lowering the pH (Figure 6A,B), as expected for the protonation of anionic carboxyl groups (*16*, *21*). No significant changes in the chemical shifts of the signal at 39.7 ppm were found, suggesting that no lysine side chain was involved in strong electrostatic interactions with the fatty acid polar head.

These chemical shift and intensity changes were reversible, and the sharp low-field resonances could be restored by back-titration with NaOH (Figures 5 and 6). Assuming an ionization $\Delta\delta$ of 4.8 (*3*) (a value observed for the titration of medium-chain carboxylic acids both in aqueous solution and also when bound to bovine serum albumin) (*16*), an apparent pK_a = 4.8(2) could be estimated for the bound fatty acids (Figure 6C and Supporting Information).

The protein ^{1}H spectra also showed modifications following the lowering of the pH. In particular, the highest field region of the spectra changed smoothly and reversibly, displaying a pattern similar to that of the apo-protein at pH 5.0 (Figure 3B).

Complex Stability and Ionic Strength. The effects of ionic strength on complex formation and stability were studied. The different complexes were prepared by incubation of the fatty acids in buffers containing only 10 mM sodium phosphate, pH 7.4. Aliquots of a sodium chloride solution were then added to these samples, and the change in the fatty acid content was evaluated through ^{13}C NMR spectra. Figure 7 shows the decrease in the amount of bound fatty acid observed for both complexes, that approaches 50% of the initial occupancy when [Cl^-] = 100 mM.

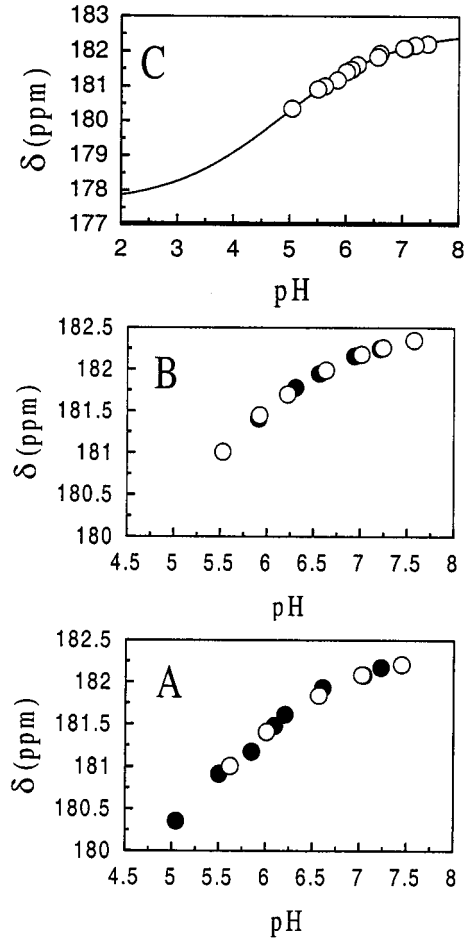

FIGURE 6: pH dependence of the chemical shifts of the FA carboxylic signals (300 K, 75.4 MHz). Closed circles, titration from neutral to acidic pH; open circles, back-titration from acidic to neutral pH. (A) Palmitic acid; (B) oleic acid; (C) Henderson−Hasselbach fitting of the PA/Lb-FABP complex data. The solid line represents the curve fit obtained with the program KyPlot.

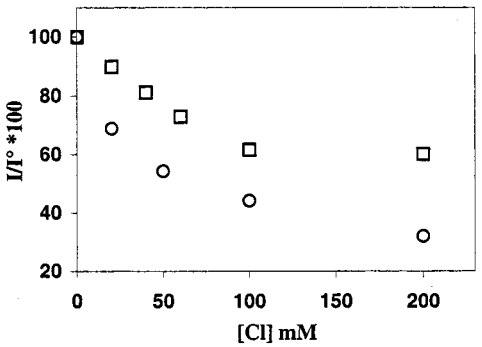

FIGURE 7: Ionic strength dependence of the ^{13}C NMR signal of the bound FA [(□) oleic acid; (○) palmitic acid], evaluated according procedure (i), upon increasing the concentration of chloride ions (300 K, 75.4 MHz).

Equilibration Experiments. The ease of release of the bound fatty acid was studied by exchanging the ^{13}C enriched with the corresponding natural-abundance fatty acid and examining the ^{13}C NMR spectra at fixed periods of time. The complexes, prepared by adding 1 equiv of ^{13}C-enriched PA or OA to the protein, were incubated with 3 equiv of the respective natural-abundance fatty acid. After 7 h of incubation at 300 K, ^{13}C NMR spectra were recorded overnight as described above. The behavior of the two complexes was markedly different. The first spectrum of the

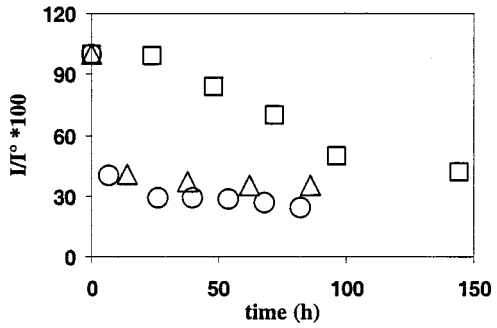

FIGURE 8: ^{13}C NMR data of the time course of equilibration among complexes prepared with ^{13}C-enriched ligands and free natural-abundance ligands. In parentheses are the bound:free ratios of (□) *PA/PA (1:3), (△) *OA/OA (1:3), and (○) *PA/OA (1:2) at 300 K, 75.4 MHz.

OA complex (10 h) showed that the system was already at equilibrium while that of the PA complex reached only 50% equilibration after 130 h. This phenomenon is probably related to the different state of the outer phase (solid for PA and lamellar for OA). The possible competition between chicken Lb-FABP and other potential fatty acid acceptors was investigated using more concentrated samples of the *PA/Lb-FABP complex. When these complexes were incubated with 2 equiv of n.a. OA and ^{13}C NMR spectra collected at shorter intervals, the availability of an aqueous acceptor phase was found to be crucial for ligand release, as shown in Figure 8. In the case of labeled/unlabeled oleic acid release, the apparent equilibrium distribution is greater that the statistical average (0.36 vs 0.25) whereas for the labeled palmitic/unlabeled oleic acid exchange the same value is lower than the statistical average.

DISCUSSION

The single resonance observed in the ^{13}C spectra of all the interaction experiments proves that there is no heterogeneous binding in chicken Lb-FABP, while the saturation behavior at a molecular ratio of 1:1 indicates that chicken Lb-FABP has a single binding site for the ligands examined. This stoichiometry is also confirmed by the fluorescence interaction experiments with *cis*-parinaric acid. These results, using as probes both potential endogenous ligands and exogenous molecules with a structure similar to a fatty acid, are in line with those reported earlier (*10*) using a fluorescent probe with a much bulkier structure. The possibility that the 1:1 rather 2:1 stoichiometry peculiar of this liver protein could be due to the use of unnaturally bulky probes can therefore be discarded. Therefore, the chicken Lb-FABP binding properties are found to be different from those of the mammalian type liver FABPs for which interaction experiments have been performed. The mammalian liver FABPs have been shown to be able to bind two molecules of fatty acids in their binding site (*3*, *17*, *22*). Indeed, the crystal structure of the rat OA/L-FABP complex has shown that two oleate molecules (*4*) are present in the cavity of the cocrystals. Studies on the interaction of the two isomers of parinaric acid with rat L-FABP (*23, 24*), its two isoforms (*23*), and the axolotl (*9*) mammalian type liver L-FABP showed that the ligand-binding sites could accommodate two molecules of such ligands. When other FABPs belonging to the family of the protein we are discussing here, the liver basic L-FABPs, are examined though, the results depend on the ligand structure. The axolotl (*9*) and the shark (*8*) liver basic L-FABPs bind two molecules of *cis*-parinaric acid but only one molecule of the trans-isomer. In the case of chicken liver basic L-FABP, a stoichiometry of 1:1 is observed notwithstanding the kinked or straight nature and the bulkiness of the ligand (*10*); only catfish, among the studied basic L-FABPs, appears to display similar binding properties (*7*).

The affinity of chicken Lb-FABP for *cis*-parinaric acid is generally smaller than that of other L-FABPs (*7*−*9*, *23*). The competition experiments showed a preference toward saturated ligands as occurs for catfish Lb-FABP (*7*) and at variance from the mammalian liver FABPs, that have a greater affinity for unsaturated fatty acids (*22*, *23*, *25*), and from the other orthologous basic FABPs that seem to bind preferentially retinoic acid (*8*, *9*).

The chemical shifts of the carboxylic groups of PA and OA bound to chicken Lb-FABP are very close, indicating that the binding mode of the two fatty acids is similar. The resonances are in the range typical of deprotonated carboxylic groups, and the small decrease of pH observed after interaction with the FAs supports the idea that the FA binding takes place in the anionic form. Similar chemical shifts in neutral solutions (182.2 ppm), and an analogous behavior upon increasing the acidity of the solution, were observed for site *c* of the FA/BSA complexes (*15*, *19*) and for fatty acids bound to rat L-FABP (*3*, *17*), suggesting that also in the present case the polar head of the fatty acid is bound near the protein/solvent interface and accessible to the solvent. Indeed, in the unrefined crystal structure (*6*) of chicken Lb-FABP, some residual electron density was found in the protein cavity, but its interpretation in terms of a ligand model was not easy because the amino acid sequence of the protein was not known. It is also worth noting that these crystals were grown at a pH at which the ligand occupancy is predicted to be reduced by the results we present here. The fact that the chemical shift signal associated with mobile ϵ-Lys carbon atoms, at 39.7 ppm, is not affected by the lowering of the pH suggests that no strong ion-pair interactions occur with the Lys residues. There are, however, other positive residues (e.g., Arg55) that could provide a microenvironment favorable to the binding of an anionic ligand, even in the absence of specific electrostatic interactions.

The pK_a values determined for both PA and OA support further the solvent accessibility of the polar head of the fatty acid (*3*, *16*). As a matter of fact, smaller values of pK_as are found when the fatty acid is bound deep inside the cavity with a strong ion-pair interaction. Indeed, the pK_a value of the complex PA/I-FABP, in which the palmitate is bound with R106, was <4 (*3*), whereas for the same complex of the mutant (R106T) (*26*), where that type of interaction was removed, a higher pK_a value was determined.

The overall changes of the bound fatty acid signals, observed in the ^{13}C spectra on increasing the solution acidity, indicate that the ligand is released. In principle, also a conformational change of the protein, occurring during the titration, could cause the release of the ligands. Previous studies (*10*), however, have proven that CD spectra of holo-Lb-FABP were pH-independent in the range 5−10, and that no major conformational changes were observable. Therefore, it is the ligand that undergoes reversible protonation and release upon decreasing the solution pH.

The binding properties of chicken Lb-FABP are modulated by the ionic strength of the solution environment. Remarkable ionic strength effects have been reported for the binding properties (*27*) and the fatty acid transfer rate to phospholipid vesicles (*28*) of rat L-FABP. The increased solubility of the fatty acid in the 'salted medium' has been invoked to explain these effects (*28*). In the present case, however, the relative decrease of the carboxylic resonances is much more relevant for the PA complex compared to the OA complex, a behavior which is the opposite of what would be expected on the basis of solubility arguments. Competition of the inorganic anions toward the charged residues in the portal region could explain our results.

At present, we do not have definite evidence to propose a solution conformation of the fatty acid within the cavity (stretched or U shaped), but some comments are possible. In the ^{1}H NMR spectra, the resonance at highest field is insensitive to complexation and to pH changes. In the study of the solution secondary structure of chicken holo-Lb-FABP (*11*), the highest field resonance reported (−0.24 ppm, pH 5.5) has been attributed to a γ hydrogen of Gln100 in strand H. The alignment of the sequences of the known Lb-FABP shows that this is a conserved residue with its side chain pointing to the inside of the cavity. In the same position in the mammalian L-FABPs, there is a conserved threonine that the X-ray structure (*4*) of the oleate/rat L-FABP complex has shown to be in contact with the ligand in the primary binding site. Thompson et al. (*29*) argue that the increased steric requirements of Gln compared to Thr would lead to unfavorable contacts with a ligand in the primary binding site. Our findings support this view since, if the highest field resonance in the ^{1}H spectrum is due to Gln100, the side chain of this residue is not affected by complex formation and therefore the ligand is not bound as the oleate in the primary site of rat L-FABP. According to X-ray structure and sequence analysis, His98 lies at a distance short enough to give rise to the unusual high-field chemical shift of the γ hydrogen of Gln100. The protein region comprising these residues as well as that near Trp6, as judged from the fluorescence measurements, does not undergo major conformational changes upon fatty acid binding.

The ^{13}C NMR equilibration experiments reported here have a time scale definitely much longer than that required to study the kinetic aspects of fatty acid exchange (*30*, *31*), and this will deserve a specific investigation. The thermodynamic significance of the nonstatistical distributions of the fatty acids between the protein and the oleic acid lamellar phase should not be stressed since these results could be biased by differences in the relaxation times of the two states. Relaxation time measurements will be needed to verify if the affinity of oleic acid toward the protein is indeed greater than for the lamellar free oleic acid phase.

Despite its intrinsic lower sensitivity that requires the use of significant amounts of protein, ^{13}C NMR spectroscopy is unique in allowing the direct observation of endogenous ligands under equilibrium conditions and in providing information on the binding number of sites and binding mode of the ligands. The results reported here indicate that chicken liver basic FABP distinguishes itself among the other FABPs sharing properties of orthologous and paralogous FABPs. Unlike other liver FABPs, and like FABPs from other tissues different from liver, it binds a single fatty acid whose carboxylate polar head appears to be solvent-accessible. As described for the basic FABP found in catfish liver (*7*), saturated ligands are preferred, but this is at variance with the behavior of the basic L-FABPs from axolotl (*9*) or shark (*8*) liver. The chicken Lb-FABP therefore has characteristics that no other members of the related protein families appear to share completely, and their detailed description will be required to understand the peculiar biological role of the protein.

ACKNOWLEDGMENT

We are indebted to Prof. M. Galliano (University of Pavia) for her help in the delipidation of samples of Lb-FABP. CSSSCMTBO−CNR of the University of Milan is gratefully acknowledged for providing instrumental facilities.

SUPPORTING INFORMATION AVAILABLE

Two figures showing (i) the pH dependence of the amount of OA bound to chicken Lb-FABP and (ii) Henderson−Hasselbach fitting of the pH dependence of the bound oleate chemical shift (2 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI011009W