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Interactions of Oleic Acid with Liver Fatty Acid Binding Protein: A Carbon-13 NMR Study†

David P. Cistola,* Mary T. Walsh, Ronald P. Corey, James A. Hamilton, and Peter Brecher
Biophysics Institute, Housman Medical Research Center, Departments of Biochemistry and Medicine, Boston University School of Medicine, 80 East Concord Street, Boston, Massachusetts 02118
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ABSTRACT: ^{13}C NMR spectroscopy was used to probe the structural interactions between carboxyl-^{13}C-enriched oleic acid (18:1) and rat liver fatty acid binding protein (FABP) and the partitioning of 18:1 between FABP and unilamellar phosphatidylcholine (PC) vesicles. Spectra of systems containing 2–8 mol of 18:1/mol of FABP (but no PC) exhibited one carboxyl resonance (182.2 ppm) corresponding to FABP-bound 18:1. At pH values <8.0, an additional carboxyl resonance, corresponding to unbound 18:1 in a lamellar phase, was observed. Both resonances exhibited ionization shifts with estimated apparent pK_a values of <5 (bound 18:1) and >7 (unbound 18:1). The intensity of the resonance corresponding to FABP-bound 18:1 increased with increasing 18:1/FABP mole ratio and at 8/1 mole ratio indicated that at least 2 and 6 mol of 18:1/mol of FABP were FABP-bound at pH 7.4 and 8.6, respectively. NMR spectra of systems containing equal concentrations (w/v) of FABP and PC and from 1 to 4 mol of total fatty acid (FA)/mol of FABP exhibited two 18:1 carboxyl resonances (182.2 and 178.5 ppm, pH 7.4). The downfield resonance corresponded to FABP-bound 18:1 and the upfield resonance to PC vesicle bound 18:1. At 1/1 mole ratio (FA/FABP), the intensities of both resonances were approximately equal, but at 4/1 mole ratio the resonance for PC vesicle bound 18:1 was 3-fold more intense than that for FABP-bound 18:1. The following conclusions are reached: (i) The carboxyl groups of 18:1 bound to liver FABP experience only one type of binding environment (the aqueous milieu adjacent to the protein surface). (ii) 18:1 bound to FABP is fully ionized at neutral pH, and the anionic 18:1 carboxyl groups are not involved in electrostatic interactions with cationic residues on FABP. (iii) Each mole of FABP bound at least 2 mol of 18:1 at physiological pH. However, in the presence of phospholipid vesicles, FABP bound up to 1 mol of 18:1/mol of FABP.

Fatty acid binding proteins (FABP)[1] are a class of abundant, low molecular weight cytosolic proteins that presumably function in the solubilization and transport of nonesterified fatty acids and their CoA derivatives within cells (Ockner et al., 1972). To aid in defining the functional role of FABP in vivo, the ligand binding properties of FABP have been exam-

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[1] Abbreviations: FABP, fatty acid binding protein(s); FA, fatty acid(s); 18:1, oleic acid and/or potassium oleate; TLC, thin-layer chromatography; PC, egg yolk phosphatidylcholine; P1, FABP preparation 1; P2, FABP preparation 2; T_1, spin–lattice relaxation time; NOE, nuclear Overhauser enhancement; CD, circular dichroic; UV, ultraviolet; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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ined in vitro. Using a variety of binding assays, studies to date have suggested that liver FABP contains one or two binding sites for long-chain FA (Glatz & Veerkamp, 1983; Offner et al., 1986). However, the number and ligand specificity of these sites remains controversial, and the structural aspects of the binding interactions are unclear.

We previously used ^{13}C NMR spectroscopy and ^{13}C-enriched fatty acids to investigate the interactions of FA with bovine albumin (Parks et al., 1983; Hamilton et al., 1984; Cistola et al., 1987a,b). The chemical shifts of FA carboxyl, methylene, and methyl carbons were highly sensitive to FA binding environments on albumin, and ^{13}C NMR spectra revealed multiple resonances corresponding to distinct FA binding sites. In addition, ^{13}C NMR has been used to monitor the partitioning of FA between albumin and phospholipid membranes (Hamilton & Cistola, 1986).

In the present study, ^{13}C NMR spectroscopy was used to investigate the interactions of carboxyl-^{13}C-enriched oleic acid with rat liver FABP and the partitioning of oleic acid between FABP and phospholipid membranes. The results indicate substantial differences between liver FABP and serum albumin with regard to fatty acid binding properties and provide insights into the possible functional role of FABP in vivo.

Materials and Methods

Materials. [90%, 1-^{13}C]Oleic acid was purchased from Cambridge Isotopes (Lot AB-137; Cambridge, MA) and contained methyl ester impurities as detected by TLC. Further purification to >98% was achieved by solvent partitioning of sodium oleate from methyl oleate as described by Burrier and Brecher (1983). Egg yolk phosphatidylcholine was purchased from Lipid Products (Nutley, England) and was >99% pure by TLC.

Isolation of Rat Liver FABP. The method used was that described by Burrier and Brecher (1986) using CM-52 ion-exchange chromatography as a final purification step.

Analysis of Liver FABP. Two different preparations of liver FABP were employed: the first (P1) was used for 18:1/FABP binding studies and the second (P2) for 18:1/FABP/PC partitioning studies. SDS–PAGE was performed according to the method of Laemmli (1970) using 3–24% gradient gels. Both preparations demonstrated a major FABP band at 14 kDa and a minor unidentified band (<5%) at 10 kDa. Folch extraction and TLC of P1 (~8 μg of lipid spotted) exhibited a major FA band and a minor triglyceride band (5%) and of P2 a major FA band and a minor diglyceride band (5–10%). No other lipids were detected in P1 or P2.

Attempts to delipidate liver FABP using the charcoal method of Chen (1967) resulted in protein aggregation and precipitation. Therefore, nondefatted liver FABP preparations were used in this study, and the "endogenous" bound fatty acid was quantitated and included in total fatty acid/FABP mole ratios. In previous experiments using a liposome assay (Brecher et al., 1984; Burrier & Brecher, 1986), it was determined that [^{14}C]oleate exchanges reversibly between FABP and phospholipid liposomes. Therefore, in the present experiments, we assume (but have not directly demonstrated) that added ^{13}C-enriched 18:1 equilibrates with the endogenous fatty acids.

Quantitation of bound FFA was performed by gas–liquid chromatography (using an internal standard) following transesterification with BF_3-methanol (Morrison & Smith, 1964). P1 yielded a total of 1.3 mol of FA/mol of FABP with the following distribution: arachidonic (31%), oleic (13%), linoleic (13%), eicosapentenoic (12%), palmitic (8%), erucic (6%), stearic (5%), palmitoleic (4%), and myristic (1%), acids and others (7%, total). Analysis of P2 yielded a total of 0.63 mol of FA/mol of FABP with the following distribution: palmitic (20%), oleic (19%), arachidonic (15%), docosahexaenoic (11%), linoleic (8%), palmitoleic (4%), eicosapentenoic (4%), eicosadienoic (4%), myristic (2%), and heptadecanoic (1%) acids.

FABP was quantitated by measuring its absorbance at 280 nm, using an extinction coefficient of 0.68 calibrated against amino acid analyses of liver FABP (Offner et al., 1986).

Preparation of 18:1/FABP Complexes for NMR. FABP (P1: 37 mg, 1 mg/mL, in 30 mM Tris-HCl, 50 mM KCl) was concentrated by ultrafiltration to 20 mg/mL. ^{13}C NMR spectra were recorded at this concentration prior to addition of 18:1. 18:1/FABP complexes were prepared as previously described in studies using albumin (Parks et al., 1983). An FABP solution (1.6 mL, 20 mg/mL, pH 8.5) was added to an NMR tube containing a stoichiometric amount of aqueous potassium oleate (0.1 mL).

Preparation of the 18:1/FABP/PC Partitioning System. Sonicated unilamellar PC (75 mg/mL in 30 mM Tris-HCl, 50 mM KCl, pH 8.5) vesicles containing no added fatty acid were prepared essentially as described (Hamilton & Small, 1981), but the long ultracentrifugation step was omitted. FA and lysolecithin were not detected by TLC in the sonicated vesicle preparation. Aliquots of PC vesicles (0.44 mL) and liver FABP (31 mg in 1.1 mL; P2) were combined in an NMR tube, and the pH was adjusted to 7.4. The sample containing FABP and PC vesicles (1.54 mL) was transferred into a second NMR tube containing ^{13}C-enriched potassium oleate (0.1 mL), vortexed for several minutes, and adjusted to pH 7.4. The final composition of the sample was total FA/FABP mole ratio 1/1 (included unenriched and enriched FA) and total FABP/PC weight ratio 1/1. An NMR spectrum was obtained for this sample, and then additional ^{13}C-enriched 18:1 was added to give total FA/FABP mole ratios of 2/1, 3/1, and 4/1. Negative-stain electron micrographs (not shown) of the 4/1 sample following NMR analysis revealed a relatively homogeneous population of unilamellar vesicles with a 200–500-Å size distribution.

Carbon-13 NMR Spectroscopy. ^{13}C NMR spectra were recorded on a Bruker WP-200 NMR spectrometer at 50.3 MHz as described elsewhere (Hamilton & Small, 1981). In samples containing no PC vesicles, the chemical shift of the narrow resonance from protein ε-Lys/β-Leu carbons (Gurd & Keim, 1973) was used as an internal reference after this resonance (39.45 ppm) was calibrated against external tetramethylsilane. In samples containing PC vesicles, the PC fatty acyl methyl resonance at 14.10 ppm was used as an internal reference (Hamilton & Cistola, 1986). To make a direct quantitative comparison of chemical shift values for samples containing no PC versus samples containing PC, 0.2 ppm must be added to the former values. The estimated uncertainties in chemical shift values were ±0.1 ppm. Peak intensities were measured by using the integration routines provided within the Bruker DISNMR software package. In selected cases, Lorentzian spectral simulations were generated by using the Bruker GLINFIT program in order to deconvolute overlapping resonances and measure individual peak intensities. NMR sample temperatures were controlled at 31 ± 1 °C. Spectral pulse intervals (2.0 s; $4 \times T_1$) were long enough so that equilibrium 18:1 carboxyl intensities were obtained, and 90° pulses (14 μs) were used. T_1 and NOE values were measured as previously described (Parks et al., 1983).

Circular Dichroism. CD spectra were recorded on a Cary Model 61 spectropolarimeter calibrated with *d*-10-camphor-

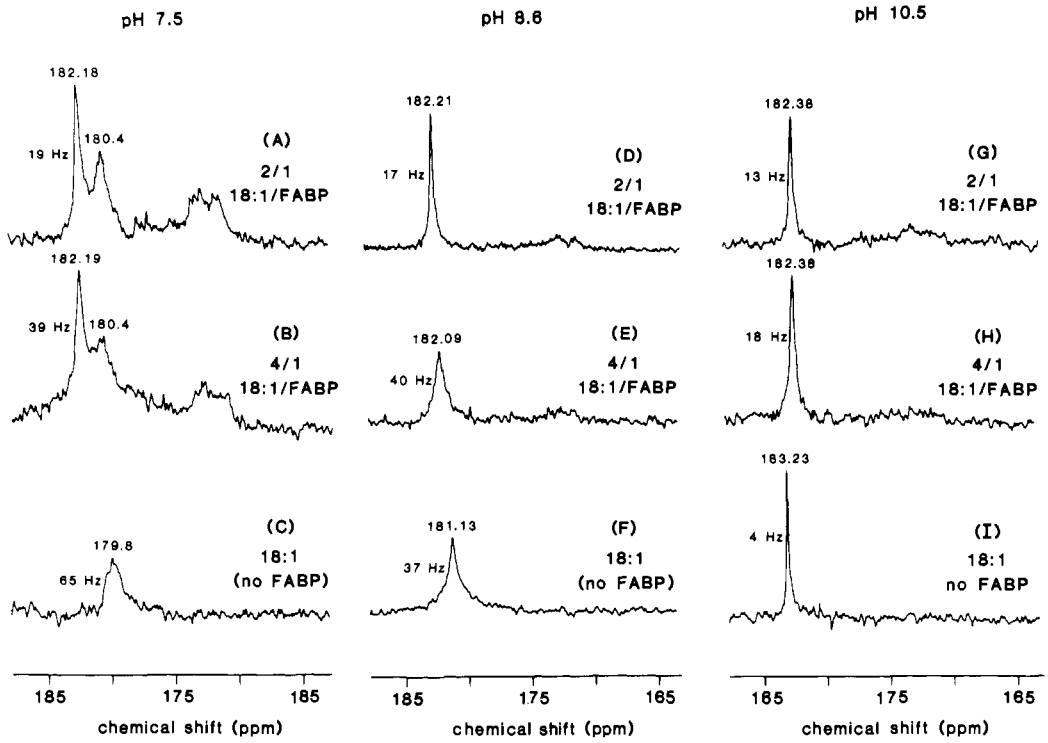

FIGURE 1: Carboxyl/carbonyl region of selected ^{13}C NMR spectra for carboxyl ^{13}C-enriched oleic acid/rat liver FABP complexes at various pH and 18:1/FABP mole ratio values. (Left, middle, and right columns) Spectra at pH 7.5, 8.6, and 10.5, respectively. (A, D, and G) 2/1 18:1/FABP complexes. (B, E, and H) 4/1 18:1/FABP complexes. (C, F, and I): 18:1 in sample buffer under identical conditions except for the omission of FABP. The total 18:1 concentration (2.9 mM) was identical with that in 2/1 18:1/FABP samples (A, D, and G). In addition to ^{13}C-enriched 18:1, each sample contained 1.3 mol/mol of FABP of unenriched endogenous FA (see Materials and Methods). The mole ratio values shown in this figure include only the contribution from ^{13}C-enriched FA. Number of spectra accumulations: (A) 23 156; (B) 29 759; (C) 10 000; (D) 17 276; (E) 4500; (F) 23 744; (G–I) 3000. Vertical scaling is not identical in all spectra.

sulfonic acid. Quartz cells, 1 cm and 1 mm, were used over the wavelength regions 250–215 and 220–190 nm, respectively. FABP samples were filtered prior to CD and UV spectroscopic analysis with 0.2-μm Gelman (Ann Arbor, MI) Acrodisk filters. Reported parameters were calculated from the average of two individual spectra. Molar ellipticities at 222 and 217 nm were used as monitors of relative changes in α and β structure, respectively. For selected spectra, the percentages of α-helix, β-structure, and random coil were estimated by the method of Chang et al. (1978).

Ultraviolet Difference Spectroscopy. UV difference spectra were recorded between 260 and 420 nm with a Perkin-Elmer Lambda 5 spectrophotometer. The samples were identical with those used in CD analyses. Reference samples (0.2 mg/mL FABP at pH 5.1 or 8.6) were placed in the "sample" beam, and samples at varying pH values were placed in the "reference" beam (Sogami & Foster, 1968). All CD and UV spectra were accumulated at ambient temperature (25–28 °C).

Results

The carboxyl and carbonyl regions of ^{13}C NMR spectra of 18:1/FABP complexes at various pH values and 18:1/FABP mole ratios are shown in Figure 1. At pH 7.5, two carboxyl resonances were observed (182.18 and 180.4 ppm) in addition to the broad carbonyl fringe centered at 173 ppm (Figure 1A,B). The latter represents natural abundance carbonyl resonances from the protein polypeptide backbone (Gurd & Keim, 1973). Natural abundance carboxyl resonances corresponding to aspartate and glutamate side-chain residues (Shinodo & Cohen, 1976) were observed at 176.9 and 180.8 ppm, respectively, in spectra (not shown) of samples containing no added ^{13}C-enriched 18:1, but the intensities of these resonances were <5% of the 18:1 carboxyl intensity shown in Figure 1.

The chemical shift of the narrower carboxyl resonance (182.2 ppm; Figure 1A,B) was substantially different from the value obtained for a sample containing 18:1 in buffer (but no FABP) under otherwise identical conditions (179.8 ppm; Figure 1C) but similar to values for several resonances corresponding to 18:1 bound to bovine albumin [182.0–182.3 ppm (Parks et al., 1983)]. Hence, the resonance at 182.2 ppm most likely corresponded to 18:1 bound to FABP. The broader upfield resonance at 180.4 (Figure 1A,B) was similar in line width and chemical shift to the broad resonance obtained for 18:1 in the absence of protein (Figure 1C). All NMR samples at pH 7.5 (including those containing no FABP) were visibly turbid, in contrast to 18:1/FABP samples at pH >8. Aqueous 18:1 near half-ionization (neutral pH) is known to exhibit pH-dependent self-association into lamellar (bilayer) aggregates (Cistola et al., 1986), and such samples are visibly turbid. In addition, the resonance at 180.4 ppm was not observed in spectra of 18:1/FABP complexes at pH values >8.0 (Figure 1D,E,G,H). Taken together, these findings suggested that the carboxyl resonance at 180.4 ppm (Figure 1A,B) represented unbound 18:1 in a lamellar aggregate.

The intensities (areas) of the carboxyl peaks at 182.2 and 180.4 ppm were equal at 2/1 (Figure 1A) and at 4/1 (Figure 1B) mole ratio, as determined by deconvolution of overlapping peaks. In addition, the total carboxyl intensity (sum of both resonances relative to the FABP carbonyl fringe) doubled from 2/1 to 4/1 mole ratio. These results indicated that 50% of the total 18:1 was FABP-bound at 2/1 and 4/1 mole ratios. In addition, because of the well-separated carboxyl resonances ($\sim$2 ppm), FABP-bound and unbound 18:1 must have been in slow exchange (lifetimes $\gg$1 s).

In contrast to spectra at pH 7.5, spectra at pH 8.6 (Figure 1D,E) and 10.5 (Figure 1G,H) exhibited only one carboxyl resonance. At pH 8.6 and 2/1 mole ratio (Figure 1D), the

carboxyl resonance was essentially identical with the downfield carboxyl resonance at pH 7.5 (Figure 1A). At 4/1 mole ratio (Figure 1E), the carboxyl resonance broadened and its chemical shift decreased slightly in the direction of the value for 18:1 in the absence of FABP (Figure 1F). At 8/1 mole ratio (spectrum not shown), the carboxyl resonance exhibited chemical shift and line width values of 181.89 ppm and 52 Hz, respectively. The most likely explanation for these chemical shift and line width changes is an intermediate rate of exchange (lifetimes ~2 s) of 18:1 between FABP-bound and unbound pools at pH 8.6. On the assumption that this explanation is correct, the percentages of FABP-bound 18:1 at 2/1, 4/1, and 8/1 mole ratio were estimated by comparing the observed chemical shifts with values for systems in which 18:1 was essentially 100% FABP-bound and 0% FABP-bound. These percentages were 100%, 89%, and 70%, respectively.

The chemical shift of the sole carboxyl resonance at pH 10.5 (182.38 ppm; Figure 1G,H) was slightly higher than values for 18:1 bound to FABP at pH 7.5 and 8.6 but substantially lower than the value for micellar potassium oleate in the absence of FABP (Figure 1I). At 4/1 mole ratio (Figure 1H), the carboxyl resonance doubled in intensity, remained narrow, and had the same chemical shift value as that at 2/1 mole ratio (Figure 1G). These observations suggested that, at 2/1 and 4/1 mole ratio (pH 10.5), essentially 100% of the 18:1 was bound to FABP. However, at 8/1 mole ratio (spectrum not shown), changes in carboxyl chemical shift and line width values to 182.45 ppm and 23 Hz, respectively, suggested that approximately 92% of the total 18:1 was FABP-bound.

Figure 2 plots several spectroscopic parameters for FA/FABP complexes as a function of pH. As shown in Figure 2A, the chemical shift of the FABP ϵ-Lys resonance increased with increasing pH (above pH 9.0) in a manner consistent with the ionization of Lys side-chain residues (Cistola et al, 1987b). Concomitantly, above pH 9, the chemical shift of the FABP-bound 18:1 carboxyl peak increased slightly. Below pH 7.5, the chemical shifts of the bound and unbound 18:1 peaks decreased with decreasing pH in a manner consistent with the protonation of 18:1 carboxyl groups. Hence, 18:1 bound to FABP at neutral (physiological) pH was fully ionized and negatively charged. The estimated apparent pK_a values for FABP-bound and unbound (lamellar) 18:1 were <5 and >7, respectively. The former value is similar to values for monomeric FA in water in the absence of protein (Cistola et al., 1982, 1987b) and FA bound nonelectrostatically to medium-affinity binding sites on bovine albumin (Cistola et al., 1987b). The latter value is similar to values for self-associated lamellar aggregates of long-chain FA (Cistola, 1985; Cistola et al., 1986) and FA bound to PC vesicles (Hamilton & Cistola, 1986).

CD and UV difference spectra for FA/FABP complexes were recorded as a function of pH in order to monitor pH-dependent conformational changes in FABP. Large changes in CD molar ellipticities (Figure 2B) and UV difference parameters (Figure 2C) were observed at pH values >11.0 and <5.0. However, little change was observed between pH 7.5 and 10.5, particularly between pH 7.5 and 8.6.

^{13}C NMR spectra for a system containing 18:1, FABP, and PC vesicles at pH 7.4 are shown in Figure 3. The carboxyl resonance at 182.33 ppm corresponded to 18:1 bound to FABP (cf. Figure 1), and the resonance at 178.55 ppm to 18:1 bound to PC vesicles (Hamilton & Cistola, 1986). Hence, FABP-bound and vesicle-bound 18:1 were in slow exchange (lifetimes $\gg$5 s). At 1/1 mole ratio of FA/FABP (Figure 3A), the sample contained 0.63 mol of unenriched endogenous FA (see

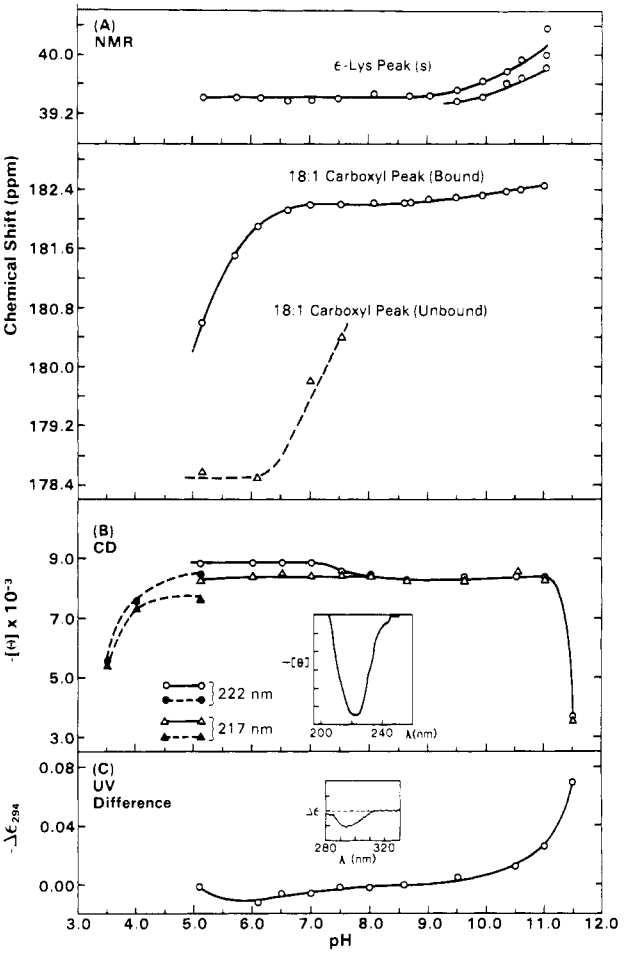

FIGURE 2: Various spectroscopic parameters for fatty acid/rat liver FABP complexes as a function of pH. (A) ^{13}C NMR chemical shifts for FABP ϵ-carbon of lysine (circles), FABP-bound 18:1 (squares), and unbound lamellar 18:1 carboxyl carbons. The NMR sample contained 2 mol of ^{13}C-enriched 18:1 and 1.3 mol of unenriched endogenous FA. (B) Circular dichroism molecular ellipticities at 222 (circles) and 217 nm (triangles). For data shown in open symbols, the sample (0.19 mg/mL FABP) was titrated from pH 5.1 to 11.5 and back to 8.6. For data shown in solid symbols, a separate sample (0.14 mg/mL) was titrated from pH 5.1 to 3.5. Both samples contained 1 mol of endogenous FA/mol of FABP. The inset shows a representative CD spectrum at pH 8.6. The calculated percentages of α-helix and β-structure for this spectrum were 21% and 41%, respectively. (C) UV difference parameters ($\Delta\epsilon_{294nm}$). The sample and protocol were the same as that used for CD measurements (B, open symbols, above). The inset shows a representative UV difference spectrum containing the trough centered at 294 nm used for derivation of the UV difference parameter. This parameter has been used as an experimental marker for conformational changes in bovine serum albumin (Sogami & Foster, 1968).

Materials and Methods) and only 0.37 mol of ^{13}C-enriched 18:1 per mole of FABP; hence, carboxyl peak intensities appeared relatively weak. However, at 2/1, 3/1, and 4/1 mole ratio (Figure 3B–D), both carboxyl resonances were clearly detected. A plot of 18:1 carboxyl intensities (relative to total carbonyl intensity and corrected for differences in ^{13}C-enrichment) is shown in Figure 3 (inset). The relative intensity of the resonance corresponding to FABP-bound 18:1 (squares) increased with increasing FA/FABP mole ratio but appeared to reach a plateau at higher mole ratios. In contrast, the intensity of the resonance corresponding to vesicle-bound 18:1 (triangles) exhibited greater incremental increases and did not plateau at higher mole ratio values. The total 18:1 carboxyl intensity (solid circles) increased in a linear fashion.

^{13}C NMR spectra for the system containing 18:1, FABP, and PC vesicles at 4/1 FA/FABP were accumulated as a

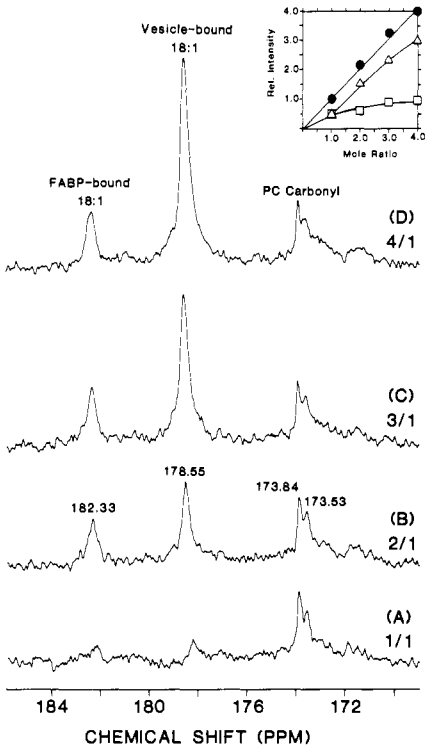

FIGURE 3: Carboxyl/carbonyl region of ^{13}C NMR spectra for the 18:1/FABP/PC vesicle system at pH 7.4: (A) 1 mol total FA/mol of FABP (sample contains 0.63 mol of unenriched endogenous FA plus 0.37 mol of ^{13}C-enriched 18:1 per mole of FABP); (B) 2 mol total FA/mol of FABP (1.37 mol of ^{13}C-enriched 18:1); (C) 3 mol total FA/mol of FABP (2.37 mol of ^{13}C-enriched 18:1); (D) 4 mol total FA/mol of FABP (3.37 mol of ^{13}C-enriched 18:1). Number of spectral accumulations, 8000. The T_1 values (0.5 s) and NOE values (1.3) for the carboxyl peaks corresponding to FABP-bound and PC vesicle bound 18:1 were equal. (Inset) Plot of carboxyl peak intensity (relative to carbonyl intensity and normalized to 100% ^{13}C-enrichment) as a function of total FA/FABP mole ratio. (Solid circles) Total carboxyl (vesicle-bound plus FABP-bound 18:1) intensity; (triangles) carboxyl intensity corresponding to vesicle-bound 18:1; (squares) carboxyl intensity corresponding to FABP-bound 18:1.

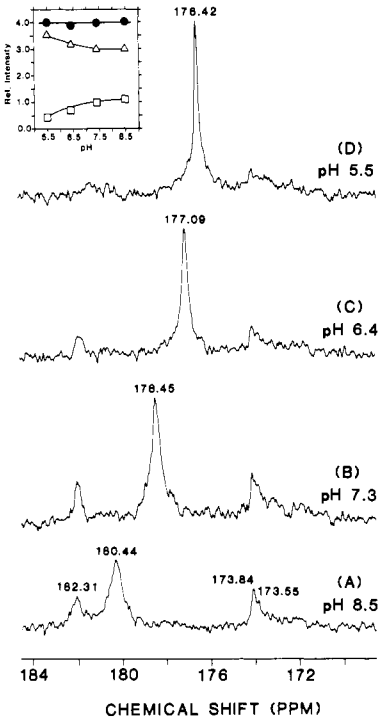

FIGURE 4: Carboxyl/carbonyl region of ^{13}C NMR spectra for the 18:1/FABP/PC vesicle system, 4 mol total FA/mol of FABP, at (A) pH 8.5, (B) pH 7.3, (C) pH 6.4, and (D) pH 5.5. Sample composition same as in Figure 3D. Number of spectral accumulations, 2500. (Inset) Plot of carboxyl peak intensity as a function of pH. All other specifications as in Figure 3 caption.

function of pH (Figure 4). The chemical shift of the carboxyl resonance for vesicle-bound 18:1 decreased with decreasing pH in a manner consistent with the protonation of 18:1 carboxyl groups (Hamilton & Cistola, 1986). At pH 7.3 (Figure 4B), vesicle-bound 18:1 was half-ionized, while FABP-bound 18:1 was fully ionized in the same sample.

The relative intensities of carboxyl peaks for FABP-bound and vesicle-bound 18:1 are shown in Figure 4 (inset). The intensity of the resonance for FABP-bound 18:1 (squares) decreased and the resonance for vesicle-bound 18:1 (triangles) increased from pH 7.4 to 5.5. The total 18:1 carboxyl intensity (solid circles) did not change over this pH range. No changes in individual or total carboxyl intensities were observed beween pH 7.4 and 8.5.

Since equilibrium NMR carboxyl peak intensities were obtained and since NOE values for 18:1 carboxyl resonances were equal (Figure 3, caption), 18:1 carboxyl peak intensities are proportional to the relative mole quantities of 18:1 associated with FABP and PC vesicles. Thus, the NMR data can be expressed as a partition coefficient defined as moles of 18:1 bound to PC per gram of PC to moles of 18:1 bound to FABP per gram of FABP (Table I). At 1/1 FA/FABP mole ratio (pH 7.4), the partition coefficient of 1.0 indicated that FABP and PC vesicles bound equal quantities of 18:1. However, between 2/1 and 4/1 mole ratio, the partition coefficient increased as PC vesicles bound proportionally more 18:1 than did FABP. In addition, the partition coefficient increased with decreasing pH but did not change between pH 7.4 and 8.5 (Table I). At 4/1 mole ratio (pH 7.4), the partition coefficient of 3.1 indicated that ~1 mol of FA was bound to FABP (per mole of FABP) and the remaining 3 mol were bound to PC vesicles.

Table I: Partition Coefficients[a]

	pH 8.5	pH 7.4	pH 6.4	pH 5.5
1/1 FA/FABP		1.0		
2/1 FA/FABP		2.5		
3/1 FA/FABP		2.6		
4/1 FA/FABP	2.7	3.1	4.5	8.1

[a] The equilibrium partition coefficient is defined as the moles of 18:1 bound to PC vesicles per gram of PC divided by the moles of 18:1 bound to FABP per gram of FABP. Relative mole quantities of 18:1 were derived from NMR spectra shown in Figures 3 and 4. The estimated uncertainty in partition coefficients is ±0.3. The far left column indicates the total mole ratio of FA to FABP present in the sample.

Discussion

^{13}C NMR spectroscopy using carboxyl-^{13}C-enriched oleic acid permitted resolution of 18:1 carboxyl resonances from FABP carbonyl resonances and provided good signal-to-noise ratios at physiologically relevant 18:1/FABP ratios. FA carboxyl chemical shifts are highly sensitive to local molecular environment, and 18:1 bound to FABP was readily distinguished from unbound lamellar and micellar 18:1 and 18:1 bound to PC vesicles. FA carboxyl chemical shifts are also sensitive to ionization state, and the ionization behavior of unbound, FABP-bound, and vesicle-bound 18:1 was determined. Finally, equilibrium NMR peak intensities, along with chemical analyses, permitted determination of the relative and absolute molar quantities of 18:1 associated with FABP and non-FABP components under various conditions.

Unlike 18:1/albumin complexes that show several 18:1 carboxyl resonances (Parks et al., 1983), 18:1/FABP com-

plexes (at all mole ratio and pH values studied) exhibited only one FA carboxyl resonance corresponding to protein-bound 18:1. This result does not necessarily imply that the FABP molecule has only one binding site for 18:1. Since the carboxyl resonance corresponding to FABP-bound 18:1 increased with increasing 18:1/FABP mole ratio (Figure 1), this resonance must have represented more than one 18:1 binding site on FABP. Thus, it is likely that the carboxyl groups of bound 18:1 molecules in several different binding sites experienced a similar molecular environment (i.e., the aqueous milieu near the protein surface).[2]

The ionization behavior of 18:1 bound to FABP indicated that the carboxyl groups of bound 18:1 molecules were anionic (at neutral pH), solvent-accessible, and free of specific ion-pair electrostatic interactions with cationic residues on FABP. This finding can be contrasted with the high-affinity binding of FA with bovine albumin, where electrostatic interactions between anionic FA and clusters of cationic protein residues play an important role in the binding process (Cistola et al., 1987b).

The observed changes in NMR spectra between pH 7.5 and 8.6 (for samples containing no PC vesicles; Figure 1) suggested that the intrinsic binding properties of FABP may have changed over this pH range. However, CD and UV difference spectra detected little or no conformational change between pH 7.5 and 8.6 (Figure 2B,C), and the partitioning of 18:1 between FABP and PC vesicles did not change between pH 7.5 and 8.5 (Figure 4, inset). Therefore, we suggest that the physical properties of unbound 18:1, rather than the intrinsic binding properties of FABP, changed between pH 7.5 and 8.6 in samples containing no PC. Unbound 18:1 formed a lamellar phase (large vesicles) at pH 7.5, and this phase competed with FABP for binding of 18:1 monomers. At pH 8.6, much less of the lamellar phase was present, and competition was not observed. The partitioning of 18:1 monomers between FABP and 18:1 vesicles at pH 7.5 was analogous to the partitioning seen in the samples containing PC vesicles.

At pH 7.5, FABP bound at least two molecules of 18:1 per molecule of FABP, but the upper limit of binding (binding capacity) was not clearly defined. At pH 8.6, FABP appeared to bind as many as six molecules of 18:1 per molecule of FABP. This ^{13}C NMR result suggests that liver FABP may contain more than two FA binding sites and appears to contradict results obtained by other binding assays. Using gel filtration (Mishkin et al., 1972; Lunzer et al., 1977), Lipidex-1000 (Glatz & Veerkamp, 1983; Glatz et al., 1984; Scallen et al., 1985; Bass, 1985), and liposome partitioning (Offner et al., 1986), investigators have concluded that liver FABP contains one or two fatty acid binding sites. These assays require separation of bound from unbound ligand using chromatography or centrifugation, and the agents used to separate unbound FA from protein-bound FA may compete with FABP for ligand binding. Hence, the results obtained may not reflect the equilibrium binding properties of FABP in the absence of other fatty acid acceptors and may lead to an underestimation of the number of ligand binding sites on FABP. Since the ^{13}C NMR binding assay does not require separation of bound from unbound ligand, use of other FA acceptors can be avoided and equilibrium binding conditions are preserved. However, precise definition of the maximum number of FA binding sites on liver FABP must await further investigation.

[2] An alternative explanation is that 18:1 is exchanging rapidly between several FABP binding sites. However, this explanation is less likely because the rates of hydration of FA from proteins are slow [k = 0.1–0.01 s^{-1} (Daniels et al., 1985)].

In the presence of PC vesicles, liver FABP bound up to 1 mol of 18:1/mol of FABP. Taken together, the NMR results indicated FABP has one oleate binding site with an affinity approximately equal to 18:1 "binding sites" on PC vesicles, and one or more 18:1 binding sites with affinities much lower than those of 18:1 binding sites on PC vesicles. It is possible that only the higher affinity oleate binding site on liver FABP plays a role in fatty acid metabolism in vivo. These NMR results differ from findings using fluorescent FA analogues (Storch et al., 1986). The fluorescence results suggest that the affinity of the FA analogue for liver FABP was an order of magnitude greater than that for PC vesicles.

The functional role of liver FABP is not clearly established. A cytoprotective function, analogous to that served by albumin in the circulation, has been proposed for liver FABP (Bass, 1985). Specifically, liver FABP is suggested to protect cell membranes and membrane-bound enzymes from the cytotoxic effects of fatty acids by removing most of the FA from cellular membranes. However, results from this study (Figure 3) demonstrated that ≥50% of the total 18:1 partitioned into PC vesicles in the presence of FABP, even when the mole ratio of FA to FABP was low. Hence, liver FABP is much less effective than albumin in preventing accumulation of FA in phospholipid vesicles. Extrapolating from our results with model systems, we would predict that liver FABP is not capable of serving this cytoprotective function in vivo. However, our NMR results are not inconsistent with the hypothesis that FABP functions to bind FA, increase FA solubility in the cytosol, and thereby increase the rates of transfer of FA between intracellular membranes (Ockner et al., 1972).

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NMR Study of the Complexes between a Synthetic Peptide Derived from the B Subunit of Cholera Toxin and Three Monoclonal Antibodies against It†

Jacob Anglister,*,‡ Chaim Jacob,§,∥ Olga Assulin,‡ Gil Ast,‡ Rachel Pinker,‡ and Ruth Arnon§
Departments of Polymer Research and Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel
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ABSTRACT: The contact interactions between a synthetic peptide and three different anti-peptide monoclonal antibodies have been studied by nuclear magnetic resonance (NMR). The synthetic peptide is CTP3 (residues 50–64 of the B subunit of cholera toxin) suggested as a possible epitope for synthetic vaccine against cholera. The hybridoma cell lines TE33 and TE32 derived after immunization with CTP3 produce antibodies cross-reactive with the native toxin. The cell line TE34 produces anti-CTP3 antibodies that do not bind the toxin. Selective deuteriation of the antibodies has been used to simplify the proton NMR spectra and to assign resonances to specific types of amino acids. The difference spectra between the proton NMR spectrum of the peptide–Fab complex and that of Fab indicate that the combining site structures of TE32 and TE33 are very similar but differ considerably from the combining site structure of TE34. By magnetization transfer experiments with selectively deuteriated Fab fragment of the antibody, we have found that in TE32 and TE33 the histidine residue of the peptide is buried in a hydrophobic pocket of the antibody combining site, formed by a tryptophan and two tyrosine residues. The hydrophobic nature of the pocket is further demonstrated by the lack of any pH titration effect on the chemical shift of the C4H of the bound peptide histidine. In contrast, for TE34 we have found only one tyrosine residue in contact with the histidine of the peptide. No magnetization transfer was observed between the tryptophan residues of TE34 and the histidine residue of the peptide. The involvement of a tryptophan residue in the combining site of TE33 and TE32 is further demonstrated by the considerable quenching of antibody fluorescence observed upon the binding of the peptide. Such quenching is not observed in TE34. The observed differences between these two types of monoclonal antibodies may be relevant to the complementarity of their respective binding sites to the conformation of the CTP3 peptide in the native toxin.

Nuclear magnetic resonance (NMR) spectroscopy is a very useful tool in obtaining information on the tertiary structures of compounds of biochemical interest, such as peptides and proteins, as well as for monitoring structural changes induced by complex formation between biologically active molecules, e.g., enzymes and their substrates, or antibodies and antigens.

Indeed, NMR spectroscopy was previously employed for the study of antibody combining site structure in the pioneering work of Dwek, Richards, and their respective co-workers. Dwek's group demonstrated that the difference between the NMR spectrum of the Fv fragment of the antibody and the NMR spectrum of the Fv complex with dinitrophenyl (DNP) can be used as a fingerprint for the combining site structure (Dower & Dwek, 1979; Dower et al., 1977). Richards and his co-workers used phosphorus and fluorine NMR to study

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* Correspondence should be addressed to this author. He is a Bat Sheva De Rotchild Fellow and Incumbent of the Graham and Rhona Beck Career Development Chair.
‡ Department of Polymer Research.
§ Department of Chemical Immunology.
∥ Present address: Department of Medical Microbiology, Stanford University School of Medicine, Stanford, CA 94305.

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