# Recombinant Liver Fatty Acid Binding Protein Interacts with Fatty Acyl-Coenzyme A†

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ABSTRACT: Rat liver fatty acid binding protein (L-FABP) and rat intestine fatty acid binding protein (I-FABP) are homologous proteins which are both found in intestinal epithelial cells. It was once well accepted that liver fatty acid binding protein bound fatty acyl-CoAs, but the recent finding of a novel acyl-CoA binding protein (ACBP) in preparations of L-FABP has challenged the role of FABPs in acyl-CoA metabolism. Prior to the discovery of ACBP, L-FABP preparations from liver were shown to modulate the rate of fatty acyl-CoA synthesis (Burrier et al., 1987) and their conversion to phospholipids (Bordewick et al., 1989). Studies using FABPs free of ACBP are needed to determine the role of I-FABP and L-FABP in fatty acyl-CoA metabolism. In this study, highly pure recombinant L-FABP and I-FABP were used first to establish binding to fatty acyl-CoAs and then to examine the effects of these FABPs on microsomal phosphatidic acid synthesis. The standard Lipidex-1000 binding assay using [$^{14}$C]oleoyl-CoA and a new fluorescence binding assay using the fluorescent fatty acyl-CoA *cis*-parinaroyl-CoA were used to determine binding. The results of these assays indicate that L-FABP binds fatty acyl-CoAs at two sites with a high-affinity $K_d$ = 3–14 $\mu$M. These binding assays showed that I-FABP has a much lower affinity for fatty acyl-CoAs than does L-FABP. Furthermore, *in vitro* only L-FABP significantly increases the rate of incorporation of oleoyl-CoA into lysophosphatidic acid and phosphatidic acid.

There is considerable evidence that the intracellular fatty acid binding proteins (FABPs)[1] may serve as fatty acid and fatty acyl-CoA binding/transport proteins and as enzyme/membrane modulators/protectors (Burrier et al., 1987; Paulussen & Veerkamp, 1990). The FABPs preferentially bind unsaturated fatty acids (Paulussen & Veerkamp, 1990; Nemecz et al., 1991a; Schroeder et al., 1993). The acyl-CoA specificity of FABPs is less certain (Paulussen & Veerkamp, 1990). The liver fatty acid binding protein (L-FABP)[1] is especially important in this regard since among the FABPs, only the L-FABP and heart FABP (H-FABP) bind both fatty acids and fatty acyl-CoAs (Paulussen & Veerkamp, 1990). Several studies using radiolabeled ligands have shown that L-FABP may bind long-chain acyl-CoAs (Burrier et al., 1987; Bass, 1985), enhance microsomal fatty acyl-CoA synthase (Burrier et al., 1987; Burnett et al., 1979; Jandar, 1979), enhance phosphatidic acid biosynthesis (Bordewick et al., 1989; Burnett et al., 1979; Jandar, 1979; Mishkin & Turcotte, 1974; Miyozawa & Hashimoto, 1979; Hog et al., 1987), and protect the above enzymatic activities from fatty acyl-CoA inhibition (Burrier et al., 1987; Bordewick et al., 1989; Bass, 1985; Burnett et al., 1979; Miyozawa & Hashimoto, 1979; Hog et al., 1987).

Despite the evidence consistent with a role of FABPs in acyl-CoA metabolism and protein modulatory activity, this conclusion may not be straightforward. Contradictory observations showing that H-FABP either stimulates (Samanta et al., 1989) or has no effect (Burrier et al., 1987) on phosphatidic acid biosynthesis have been presented. The effect of L-FABP on fatty acyl-CoA synthesis has been questioned, and inhibition (Noy & Zakim, 1985) rather than stimulation (Burrier et al., 1987; Burnett et al., 1979; Jandar, 1979) has been reported. Even the ability of L-FABP to bind fatty acyl-CoAs (Rasmussen et al., 1990) has been questioned. Most striking is the recent discovery of a new acyl-CoA binding protein (ACBP)[1] completely different from L-FABP (Mogensen et al., 1987). ACBP binds fatty acyl-CoAs but not fatty acids. Some investigators have postulated that all of the earlier acyl-CoA-mediated activities of L-FABP may in fact be due to cross-contamination by ACBP (Knudsen, 1990; Rasmussen et al., 1990; Mogensen et al., 1987). Indeed, it was pointed out that earlier purification procedures for L-FABP may not resolve the 14-kDa L-FABP from the 10-kDa ACBP (Rasmussen et al., 1990; Mogensen et al., 1987).

In order to resolve many of the controversies concerning fatty acyl-CoA binding to FABPs and to investigate further their potential biochemical significance, a series of binding and enzymatic rate assays have been performed. Highly purified recombinant rat L-FABP and recombinant rat I-FABP were used to eliminate possible problems such as cross-contamination by other mammalian proteins such as ACBP.

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[1] Abbreviations: FABP, fatty acid binding protein: L-FABP, liver fatty acid binding protein; I-FABP, intestinal fatty acid binding protein; H-FABP, heart fatty acid binding protein; ACBP, acyl-CoA binding protein; HPLC, high-performance liquid-chromatography; BHT, butylated hydroxytoluene; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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## MATERIALS AND METHODS

### *Materials*

*cis*-Parinaric acid was obtained from Molecular Probes, Eugene, OR. [1-$^{14}$C]Oleoyl-coenzyme A (59.5 mCi/mmol) was obtained from New England Nuclear, Boston, MA. Radiolabeled oleic acid, [9,10-$^{3}$H(N)]oleic acid (10 Ci/mmol), was obtained from Amersham Co., Arlington Heights, IL. Morpholinopropanesulfonic acid (MOPs) was obtained from Serva, New York, NY. Oleoyl-coenzyme A, oleic acid, butylated hydroxytoluene (BHT), "Lipidex" (hydroxyalkoxypropyl dextran, type VI), dithiothreitol (DTT), coenzyme A, acyl-coenzyme A synthetase, ethylenediaminetetraacetic acid (EDTA), adenosine 5′-triphosphate (ATP), dioleoylphosphatidic acid, *sn*-glycerol 3-phosphate, lysophosphatidic acid, NaF, Tricine, and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co., St. Louis, MO. Silicon fluid was purchased from Thomas Scientific, Swedesboro, NJ. ScintiVerse E universal liquid scintillation cocktail was from Fisher, FairLawn, NJ, and Triton X-100 was from Beckman, Fullerton, CA. SEP-PAK C18 columns were from Waters Associates, Milford, MA. All chemicals were reagent grade or better.

### *Methods*

*Protein Purification.* The purification methods for recombinant L-FABP from the *Escherichia coli* strain carrying plasmid pJBL2 for L-FABP and for I-FABP from the *E. coli* strain carrying plasmid pIFABPexp6 for I-FABP were identical to that described by Lowe et al. (1987). The purified FABPs were delipidated by the method of Glatz and Veerkamp (1983) unless otherwise noted.

The protein concentration was determined by the Bradford protein assay (Bradford, 1976) and corrected according to amino acid analysis done by Lowe et al. (1987). The Bradford protein assay overestimates the concentration of L-FABP 1.69-fold and I-FABP 1.07-fold.

Because standard sodium dodecyl sulfate–polyacrylamide gel systems (Laemmli, 1970) could not clearly resolve FABPs from ACBP in our hands, the purity of L-FABP and I-FABP was checked by a sodium dodecyl sulfate gel electrophoresis system designed to resolve low molecular weight proteins (Schägger & von Jogow, 1987) and a silver staining (Ansorge, 1985) method to detect trace impurities (see Results).

The purity of L-FABP and I-FABP was also confirmed by HPLC analysis. Protein samples were loaded on a $C_4$ reverse-phase HPLC column (no. 214TP54; Vydac Inc., Hesperia, CA). The HPLC system for the purification of the protein was a Perkin-Elmer (Norwalk, CT) Series 4 liquid chromatograph and a Perkin-Elmer LC-95 UV/visible spectrophotometer detector with absorbance measured at 280 nm. The proteins were analyzed using a gradient of 0.1% phosphoric acid in acetonitrile and 0.1% phosphoric acid in water, with a constant flow rate of 1 mL/min. The column was equilibrated at 100% aqueous buffer. After sample injection, an 80-min linear gradient to 60% acetonitrile was run. A 20-min linear gradient to 90% acetonitrile was run before the column was returned to equilibration conditions by a 5-min linear gradient. The retention time and integrated area of the protein peaks were obtained with a 3390A recorder integrator (Hewlett Packard, Norwalk, CT).

*Radiolabeled Oleoyl-CoA Binding.* The assay to determine the binding of oleoyl-CoA to FABPs was carried out as described earlier for fatty acids (Nemecz et al., 1991a; Lowe et al., 1987) with the following modifications. The alkoxy derivative of Lipophilic Sephadex was obtained from Sigma rather than the "Lipidex-1000" brand from Packard Instrument Co. After the hydroxyalkoxypropyl dextran type VI powder from Sigma was swelled and washed in methanol, it behaved identically to Lipidex-1000 (data not shown). The incubation and assay temperatures were lowered to 25 °C. Instead of a 10 mM phosphate buffer at pH 7.4, the 100 mM Tris/1 mM DTT, pH 7.4, buffer described by Samanta et al. (1989) was used. A thin layer of silicone on the sides of the 1.5-mL microcentrifuge tubes prevented nonspecific binding of oleoyl-CoA to the sides of the tubes used for incubation (unpublished data). The FABP concentrations were 0.24 $\mu$M for L-FABP and 0.38 $\mu$M for I-FABP. Because of the relatively low specific activity of the purchased [$^{14}$C]oleoyl-CoA, the radiolabeled oleoyl-CoA contributed 0.33 $\mu$M to the oleoyl-CoA concentration in each assay tube. Solutions containing 10% ethanol, [$^{14}$C]oleoyl-CoA, nonlabeled oleoyl-CoA, and buffer were prepared for each set of assay tubes (with and without protein). The total concentration of oleoyl-CoA in the sets of assay tubes ranged from 0.33 to 4.0 $\mu$M. The total incubation volume was 1 mL. The final ethanol concentration in each tube did not exceed 1%. After incubation at 25 °C for 20 min, the assay tubes were chilled on ice for 10 min. A continuously stirred Lipidex 50% (v/v) buffer suspension (150 $\mu$L) was added to the sample followed by vigorous stirring and incubation for 10 min at 4 °C. The assay tubes were centrifuged at 10000*g* for 4 min at 4 °C. A 400-$\mu$L aliquot of the supernatant was removed and subjected to scintillation counting using an LS 7000 (Beckman Inc., Fullerton, CA) scintillation counter. Blank assays (no added FABP) were performed in parallel for each concentration of each fatty acid. The radioactivity in a 400-$\mu$L aliquot of blank supernatant was subtracted from the amount of radioactivity present in a 400-$\mu$L aliquot of an FABP-containing supernatant. The percent acyl-CoA bound was calculated as

$$\text{net \% bound cpm} = \frac{2.875(\text{FABP spn cpm}) - 2.875(\text{no FABP spn cpm})}{\text{total assay cpm}}$$

where spn refers to supernatant and the constant 2.875 reflects the aliquot volume correction. The data were plotted using a least-squares linear regression as a Scatchard plot, with moles of ligand bound per mole of protein on the *x* axis and "bound" over "free" on the *y* axis. "Bound" represents net percent bound cpm multiplied by the total picomoles of fatty acyl-CoA in the assay. "Free" is the total picomoles of fatty acyl-CoA in the assay minus the "bound" picomoles of fatty acyl-CoA (Nemecz et al., 1991a).

*Synthesis and Purification of a Fluorescent Fatty Acyl-CoA, cis-Parinaroyl-CoA.* The thioester of *cis*-parinaric acid and coenzyme A (*cis*-parinaroyl-CoA) was prepared via a modification of the method of Taylor et al. (1990). In this procedure, 1 mmol of fatty acid, 0.1% Triton X-100, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM adenosine 5′-triphosphate, and 0.25 unit of acyl-coenzyme A synthetase were incubated for 2 h at 35 °C in 100 mM MOPS buffer, pH 7.5. Every solution containing *cis*-parinaric acid or *cis*-parinaroyl-CoA also contained 0.01% BHT to prevent oxidation; special care was taken to reduce the exposure of these compounds to oxygen and light. Instead of Prep-Sep C18 columns to purify the *cis*-parinaroyl-CoA, disposable SEP-PAK C18 columns were used as an initial purification step. To protect *cis*-parinaroyl-CoA from degradation, its methanol solution from the SEP-PAK column was not dried-down completely *in vacuo* with an R110 rotary evaporator (Buchi Laboratories, Flawel,

Switzerland). The methanol solution containing a partially purified mixture of product, unreacted *cis*-parinaric acid, and free CoA was resolved by HPLC (Taylor et al., 1990; Woldegiorgis et al., 1985).

The HPLC system for the final purification of *cis*-parinaroyl-CoA was identical to that in Taylor et al. (1990), except for the following modifications. A Perkin-Elmer Series 4 liquid chromatograph and a Perkin-Elmer LC-95 UV/visible spectrophotometer detector were used with an Alltech (Deerfield, IL) 150 mm × 4.6 mm, Adsorbosphere (3 μm) reverse-phase $C_{18}$ column. Absorbance was monitored at 254 nm or at 304 nm. The product was separated from contaminants using a series of gradients of acetonitrile and 25 mM $KH_2PO_4$ buffer, pH 5.3. With a constant flow rate of 1 mL/min, the column was equilibrated at 70% aqueous 25 mM $KH_2PO_4$/30% acetonitrile. After sample injection, the column was run at the equilibration conditions for 2 min, followed by a 7-min linear gradient to 52.5% $KH_2PO_4$/47.5% acetonitrile. The 52.5% aqueous $KH_2PO_4$/47.5% acetonitrile solvent was run for 4 min, followed by a 13-min linear gradient to 20% $KH_2PO_4$/80% acetonitrile. This solvent mixture was run for 5 min before the column was returned to equilibration conditions by a 5-min linear gradient. Any unreacted *cis*-parinaric acid adhered to the column throughout this gradient and was removed by washing the column with 100% methanol. The retention time and integrated area of the *cis*-parinaroyl-CoA peak were obtained with a 3390A recorder integrator (Hewlett Packard). The purified *cis*-parinaroyl-CoA eluting from the HPLC column was collected into a ice-cooled test tube which contained enough BHT to bring the total concentration of BHT to 0.01%.

The purity of the HPLC-purified *cis*-parinaroyl-CoA was determined by monitoring the absorbance at 304 nm. The crude methanol extract showed multiple absorbing peaks while reinjection of purified *cis*-parinaroyl-CoA showed only a single peak (see Results).

*Spectroscopy of cis-Parinaroyl-CoA.* In addition to analytical HPLC and comparison to standards, a spectroscopic characterization of *cis*-parinaroyl-CoA was performed. The absorbance spectrum was obtained on a DMS 100 spectrophotometer (Varian Instruments, Palo Alto, CA). Fluorescence excitation and emission spectra for *cis*-parinaroyl-CoA were obtained on an SPF 500 spectrofluorometer (SLM Aminco, Champaign-Urbana, IL) at 25 °C in 1-cm cuvettes. The excitation wavelength was 324 nm; the emission wavelength was 420 nm. The excitation bandwidth was 4 nm; the emission bandwidth was 10 nm.

*Binding of cis-Parinaroyl-CoA to FABP.* The fluorescence assay of Nemecz et al. (1991a) was used to determine the binding parameters of *cis*-parinaroyl-CoA to L-FABP and I-FABP. In these assays, the protein concentration was maintained constant at 0.1 μM for L-FABP and at 0.2 μM for I-FABP while the ligand concentration was varied between 0.025 and 1.5 μM. All tubes were incubated for 10 min in darkness at 25 °C. The fluorescence intensity was measured in 1-mL cuvettes upon excitation at 324 nm, using an SLM 4800 spectrofluorometer with a 450-W Xe-Arc lamp (SLM Instruments). The fluorescence emission was recorded through a GG-375 sharp cutoff filter (Janos Technology Inc., Townsend, VT) in order to eliminate highly polarized scattered light. The inner filter effect could be neglected because the maximum absorbance of the samples was below 0.12 in all cases.

*cis*-Parinaroyl-CoA/FABP binding analysis was performed as follows: Fractional saturation, $R$, was calculated using eq 1 where $\Delta F$ is the change in fluorescence in the presence of

$$\Delta F/\Delta F_{max} = R = EL/E_0 \quad (1)$$

*cis*-parinaroyl-CoA, $\Delta F_{max}$ is the maximal change in fluorescence, $R$ is the fractional saturation, $EL$ is the concentration of L-FABP/*cis*-parinaroyl-CoA complex, and $E_0$ is the initial L-FABP concentration. The saturation factor was inserted into eq 2 where $L_0$ is the initial concentration of *cis*-parinaroyl-

$$1/(1 - R) = K(L_0/R) - KnE_0 \quad (2)$$

CoA and $n$ is the number of binding sites (Gutfreund, 1972). The resulting data were plotted using a least-squares linear regression as $1/(1 - R)$ vs $L_0/R$, with the slope equal to $K$ and the $y$ intercept equal to $-KnE_0$.

*Phosphatidic Acid Biosynthesis.* The effect of L-FABP and I-FABP on the rate of rat liver microsomal phosphatidic acid synthesis from oleoyl-CoA and *sn*-glycerol 3-phosphate or lysophosphatidic acid was examined by two *in vitro* assays described in Bordewick et al. (1989). These assays were modified as follows: The assay mixture final volume was 0.1 mL. The reaction time was lengthened to 15 min. The microsomes used were prepared by the method of Lichtenstein and Brecher (1980) with EDTA[1] treatment. These rat liver microsomes were found to have a higher net acyltransferase activity (likely due to less hydrolysis of product) than those of Bordewick et al. (1989). Consequently, a final microsomal protein concentration of 0.03 mg/mL instead of 0.21 mg/mL was used. The solvent system to resolve the phosphatidic acid on the Whatman (Clifton, NJ) LHP-K TLC plates was $CHCl_3$/MeOH/AcOH/$H_2O$, 82:23:10:2.5 v/v.

## RESULTS

*Purity of Recombinant L-FABP and I-FABP.* As pointed out in the introduction, pure preparations of recombinant L-FABP and I-FABP were essential to avoid complication of data interpretation due to cross-contamination by ACBP. The *E. coli* did not produce ACBP, and the recombinant L-FABP and I-FABP did not have any cross-contaminating bacterial proteins. The (SDS)[1] gel electrophoresis was performed using a gel procedure (Schägger & von Jogow, 1987) capable of resolving the 14-kDa L-FABP and 15-kDa I-FABP from smaller proteins, and both Coomassie (Laemmli, 1970) and silver-staining methods (Ansorge, 1985) to detect trace impurities. These gels indicated homogeneity for each protein with only single bands present for 10 and 14 μg, respectively, of both FABPs. There was no detectable band corresponding to a 10-kDa ACBP or any other protein, such as the 8.8-kDa acyl carrier protein of *E. coli*, observed on the electrophoretic gels even though the silver-staining technique is capable of detecting less than 1% of the amount loaded (Ansorge, 1985). HPLC analysis of L-FABP, I-FABP, and ACBP showed relative retention times of 52.9, 56.5, and 49.8 min, respectively. Purified L-FABP and I-FABP did not show any HPLC peaks corresponding to ACBP.

*Radiolabeled Oleoyl-CoA Binding to L-FABP.* At the present time, the standard assay reported to determine fatty acyl-CoA interaction with FABPs or ACBP is the Lipidex competition assay. The assay is a modification of that originally developed to determine fatty acid binding to the FABPs. Therefore, Lipidex and [$^{14}$C]oleoyl-CoA were used to determine the binding of oleoyl-CoA to L-FABP (Figure 1) and I-FABP (Figure 2). The binding curves showed saturable binding for L-FABP (Figure 1A). In contrast, I-FABP did not show saturable binding, but instead showed

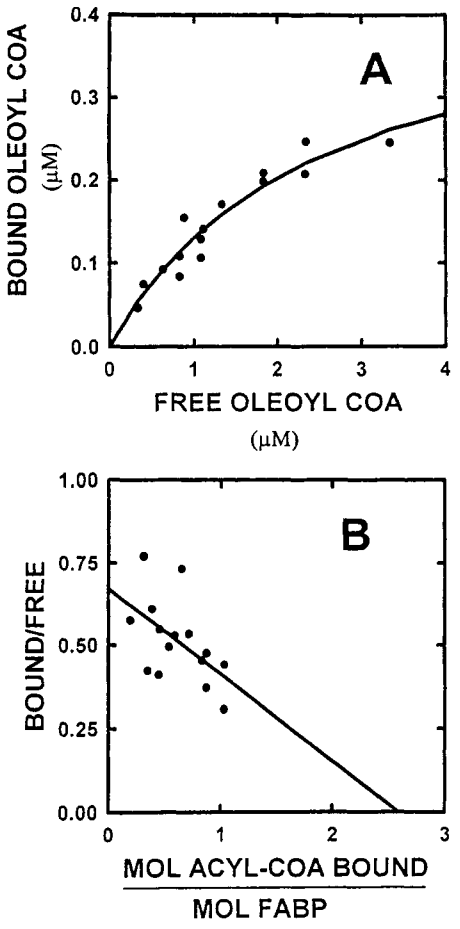


FIGURE 1: Lipidex binding assay. L-FABP (0.24 μM) was incubated with varying concentrations of [$^3$H]oleoyl-coenzyme A (0.33–3.33 μM, with constant total radioactivity) in 100 mM Tris/1 mM DTT, pH 7.4 at 25 °C, for 20 min. The incubation mixture was then treated with Lipidex at 4 °C for 10 min and centrifuged to remove unbound ligand. The radioactivity of the bound ligand was determined and plotted vs free ligand concentration. "Bound" represents net percent bound cpm multiplied by total picomoles of fatty acyl-CoA in the assay. "Free" is total picomoles of fatty acyl-CoA in assay minus "bound" picomoles of fatty acyl-CoA. Each point represents the mean of two measurements. Data represent an $n$ of 3. (A) Binding data for L-FABP (●). Because data for L-FABP showed saturation at higher ligand concentration, a rectangular hyperbola (binding isotherm) was fit to the data by Sigma Plot. (B) Scatchard analysis of L-FABP binding to [$^3$H]oleoyl-coenzyme A. The data were further analyzed via Scatchard analysis and Sigma Plot least-squares linear regression to give a $K_d$ of 3.6 μM and 2.4 binding sites for L-FABP.

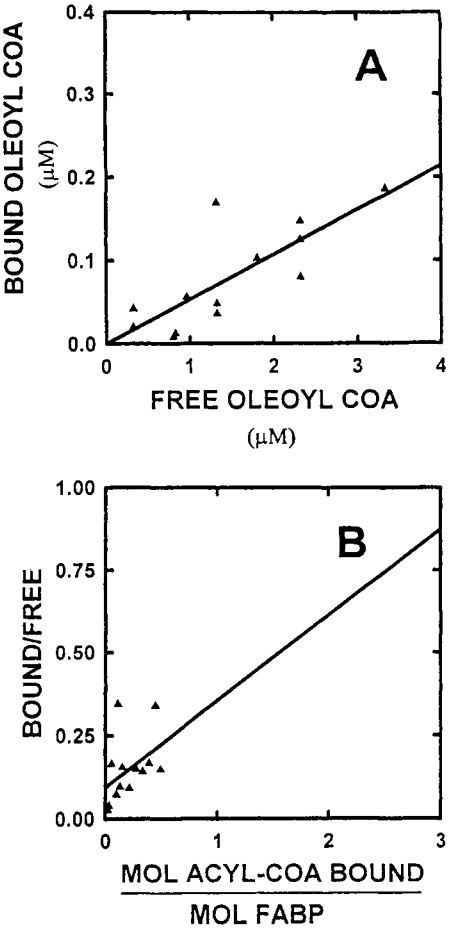


FIGURE 2: Lipidex binding assay. I-FABP (0.38 μM) was incubated with varying concentrations of [$^3$H]oleoyl-coenzyme A (0.33–3.33 μM, with constant total radioactivity) in 100 mM Tris/1 mM DTT, pH 7.4 at 25 °C, for 20 min. The incubation mixture was then treated with Lipidex at 4 °C for 10 min and centrifuged to remove unbound ligand. The radioactivity of the bound ligand was determined and plotted vs free ligand concentration. "Bound" represents net percent bound cpm multiplied by total picomoles of fatty acyl-CoA in the assay. "Free" is total picomoles of fatty acyl-CoA in assay minus "bound" picomoles of fatty acyl-CoA. Each point represents the mean of two measurements. Data represent an $n$ of 3. (A) Binding data for I-FABP (▲). Data for I-FABP could not be fit to a rectangular hyperbola, so a least-squares linear regression was fit to the data. (B) Scatchard Analysis of I-FABP binding to [$^3$H]-oleoyl-coenzyme A. No useful binding data could be inferred from the Scatchard analysis and least-squares linear regression for I-FABP which is included for comparison.

a nonspecific association with oleoyl-CoA (Figure 2A). Transformation of the L-FABP data for L-FABP binding oleoyl-CoA to a Scatchard plot was linear (Figure 1B), consistent with a single $K_d$ = 3.6 ± 0.6 μM and $B_{max}$ = 2.4 ± 0.3 (Table 1). In contrast, Scatchard analysis of the I-FABP binding data with oleoyl-CoA (Figure 2B and Table 1) showed a lack of specific binding of the ligand to the protein. The basis for the "nonspecific" interaction of oleoyl-CoA with I-FABP is not known. However, it may be due to the intrinsic properties of the Lipidex assay. The Lipidex assay is basically a competition assay for oleoyl-CoA binding to Lipidex versus FABP, and results can vary depending on the amount of Lipidex used in the assay. This potential problem was resolved through a spectrofluorometric assay not using Lipidex and not requiring separation of bound from free ligand as shown in the following sections (*vide infra*).

*Synthesis, Purification, and Characterization of cis-Parinaroyl-CoA.* A fluorescence assay that avoids the use of

Table 1: Summary of Acyl-CoA Binding Properties of L-FABP and I-FABP

| protein | assay | ligand | $K_d$ (μM) | $B_{max}$ | $n$ |
|---|---|---|---|---|---|
| L-FABP | Lipidex | [$^3$H]oleoyl-CoA | 3.63 ± 0.64 | 2.44 ± 0.3 | 3 |
| I-FABP | Lipidex | [$^3$H]oleoyl-CoA | none | none | 3 |
| L-FABP | fluorescence | *cis*-parinaroyl-CoA | 13.7 ± 1.71 | 1.80 ± 0.5 | 3 |
| I-FABP | fluorescence | *cis*-parinaroyl-CoA | none | none | 3 |

Lipidex-1000 requires a fluorescent analogue of an acyl-CoA. *cis*-Parinaric acid is a naturally occurring fatty acid (Sklar et al., 1977) that binds to L-FABP and I-FABP similarly to nonfluorescent fatty acids (Nemecz et al., 1991a,b). Therefore, *cis*-parinaroyl-CoA was synthesized and purified as described under Methods. Analytical HPLC showed a sharp peak for *cis*-parinaroyl-CoA at 11.3 min (Figure 3) using either the absorbance at 304 nm (to detect the tetraene fatty acid portion of the molecule) or the absorbance at 254 nm (to

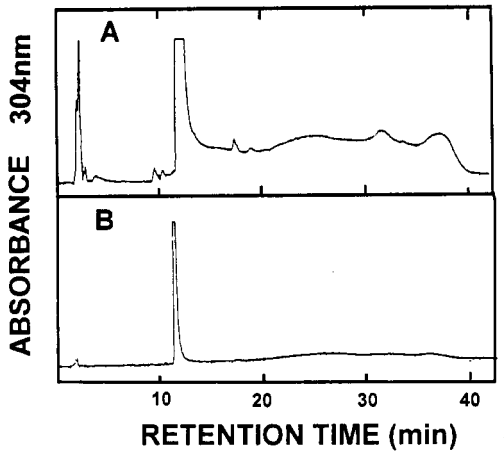


FIGURE 3: HPLC purification of *cis*-parinaroyl-CoA. Both scans show the elution profile of *cis*-parinaroyl-CoA at 304 nm on a gradient of 25 mM $KH_2PO_4$ and acetonitrile. (A) HPLC scan of 3 μg of *cis*-parinaroyl-CoA from methanol extract at 304 nm. (B) HPLC scan of 1 μg of purified *cis*-parinaroyl-CoA at 304 nm.

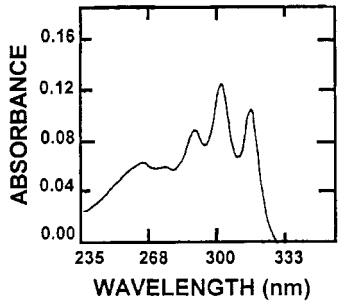


FIGURE 4: Absorption spectra of 2 μM *cis*-parinaroyl-coenzyme A. This scan was performed on a Varian DMS 100 scanning spectrophotometer. The samples were in a 50% ethanol/50% potassium phosphate buffer, pH 7.4, and contained no BHT.

detect the CoA portion). Oleoyl-CoA, which eluted at 14 min, was visible at 254 nm but not at 304 nm. HPLC runs (data not shown) of individual components in the reaction mixture showed that CoA acid was eluted in 3–4 min while *cis*-parinaric acid adhered so tightly to the column that it did not elute during the gradient. *cis*-Parinaric acid was washed from the column with 100% methanol.

The purity of the HPLC-purified *cis*-parinaroyl-CoA was determined by monitoring the absorbance at 304 nm (Figure 3). The crude methanol extract showed multiple absorbing peaks (Figure 3A) while reinjection of purified *cis*-parinaroyl-CoA showed only a single peak (Figure 3B).

The absorbance spectrum of the HPLC-purified *cis*-parinaroyl-CoA (Figure 4) showed a maximum around 260 nm characteristic of esterified CoA as observed for oleoyl-CoA (data not shown). In addition, the *cis*-parinaroyl-CoA displayed maxima with higher extinction near 277, 289, 304 (the highest extinction), and 317 nm. The latter four absorbance maxima closely resemble those of unesterified *cis*-parinaric acid (Sklar et al., 1977).

Without 0.1% BHT being present, the absorbance at 304 nm decreased approximately 10% per hour at 4 °C, presumably due to free radical oxidation of the tetraene bond of *cis*-parinarate. In the presence of BHT, the stability was maintained for several hours even at room temperature. When stored with BHT at −70 °C, *cis*-parinaroyl-CoA is stable for at least 3–4 months. The extinction coefficient of *cis*-parinaric acid, 78 000 $M^{-1}$ $cm^{-1}$ at 304 nm (Sklar et al., 1977), was used to determine the concentration of *cis*-parinaroyl-CoA because neither the thioester bond nor the CoA moiety

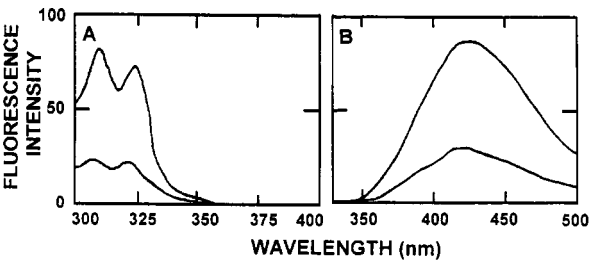


FIGURE 5: Excitation and emission spectra of 1 μM *cis*-parinaroyl-CoA with and without 2 μM L-FABP. Samples were in a 50 mM potassium phosphate buffer, pH 7.4. Spectra were obtained on an SLM 4800 spectrofluorometer. Excitation wavelength was 324 nm, and emission wavelength was 420 nm. Excitation bandwidth was 4 nm, and emission bandwidth was 10 nm. (A) Excitation scan of 1 μM *cis*-parinaroyl-CoA with 2 μM L-FABP (upper line) and without L-FABP (lower line). (B) Emission scan of 1 μM *cis*-parinaroyl-CoA with 2 μM L-FABP (upper line) and without L-FABP (lower line).

significantly contributed to the absorbance of the *cis*-parinaric conjugated tetraene bond system at 304 nm.

Fluorescence excitation and emission spectra of *cis*-parinaroyl-CoA in aqueous buffer with (upper line) and without (lower line) L-FABP are shown in Figure 5. Prominent excitation maxima occurred near 307 and 321 nm (Figure 5A). In aqueous buffer, the single broad fluorescence emission maximum was observed near 420 nm (Figure 5B). The large increase in fluorescence upon binding to L-FABP was the basis for a fluorescence binding assay (*vide infra*).

*Fluorescent cis-Parinaroyl-CoA Binding to FABPs.* As shown previously by Nemecz et al. (1991a), *cis*-parinaric acid binds to L-FABP ($K_d$ = 0.7 μM) and I-FABP ($K_d$ = 0.2 μM) with high affinity. The CoA thioester of this 18:4 fatty acid was used to characterize binding as described under Methods. In the presence of L-FABP *cis*-parinaroyl-CoA, fluorescence excitation (Figure 5A) and emission (Figure 5B) increased 3.9- and 2.7-fold, respectively. There was practically no increase in the fluorescence of *cis*-parinaroyl-CoA when incubated with I-FABP, indicating no such binding.

Therefore, a *cis*-parinaroyl-CoA binding assay based on the aforementioned fluorescence increase in the presence of L-FABP was performed as described earlier under Methods. The results of this assay show saturation binding for *cis*-parinaroyl-CoA binding to L-FABP (Figure 6A). In contrast, *cis*-parinaroyl-CoA did not demonstrate binding to I-FABP (Figure 6B). A Hill plot of the *cis*-parinaroyl-CoA/L-FABP binding data was linear and indicated the presence of two binding sites (Figure 6C). From this plot, a $K_d$ = 13.7 μM and a $B_{max}$ = 1.8 (Table 1) were calculated for *cis*-parinaroyl-CoA binding to L-FABP.

These data for acyl-CoA binding to L-FABP are generally consistent with those obtained by the Lipidex assay (Table 1). However, the $K_d$ for *cis*-parinaroyl-CoA binding was about 3-fold higher than that for oleoyl-CoA binding. This result is somewhat surprising since *cis*-parinaric acid binds with 2-fold higher affinity to L-FABP than does oleic acid (Nemecz et al., 1991a). This observation along with the lack of "non-specific" binding of *cis*-parinaroyl-CoA to I-FABP points out the difficulties associated with determining affinities or ligand specificities on the basis of the Lipidex assay alone. No contribution to fluorescence from an interaction between two *cis*-parinaroyl-CoA molecules bound to the same molecule of L-FABP is expected, because parinarate residues are thought to quench each other's fluorescence when in close proximity (Nemecz et al., 1991a). Because no such quenching of fluorescence is seen when nearing saturation of the binding

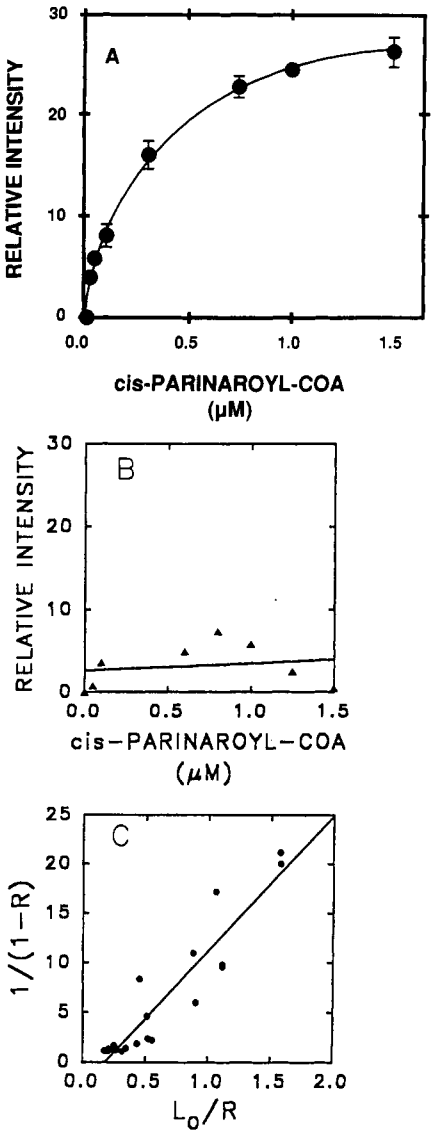


FIGURE 6: L-FABP and I-FABP: fluorescence binding assay. (A) L-FABP (●) (0.1 μM) and (B) I-FABP (▲) (0.2 μM) were incubated with varying concentrations (0.025–1.5 μM) of HPLC purified *cis*-parinaroyl-CoA at 25 °C in 50 mM potassium phosphate buffer for 15 min. The fluorescence of the sample was measured (Ex = 324 nm). Parallel blank samples lacking protein were measured with the results subtracted to yield the relative fluorescence of the bound ligand. Data shown are from one representative assay with the error bars showing the range of two determinations. (C) Data ($n = 3$) for L-FABP were further analyzed using a Hill plot with Sigma Plot least-squares linear regression. Each point is the mean of two determinations. $R$ is the fractional saturation.

sites with ligand (Figure 6A), one may speculate that the binding sites of L-FABP are unlikely to be directly adjacent to each other. In summary, the results with the *cis*-parinaroyl-CoA binding assay suggest that there is significant binding of acyl-CoA to L-FABP but not to I-FABP.

*Modulation of Microsomal Phosphatidic Acid Synthesis.* The microsomal acylation of *sn*-glycerol 3-phosphate occurs via two different enzymes which sequentially incorporate one acyl group each. The first acylation by glycerophosphate acyltransferase, which yields lysophosphatidic acid, is the rate-limiting step of phosphatidic acid biosynthesis. The effect of delipidated L-FABP and delipidated I-FABP on this step of production of phosphatidic acid by rat liver microsomes is shown in Figure 7. The stimulatory effect of L-FABP on microsomal phosphatidic acid synthesis from *sn*-glycerol 3-phosphate (Figure 7) was quite pronounced, with a maximal

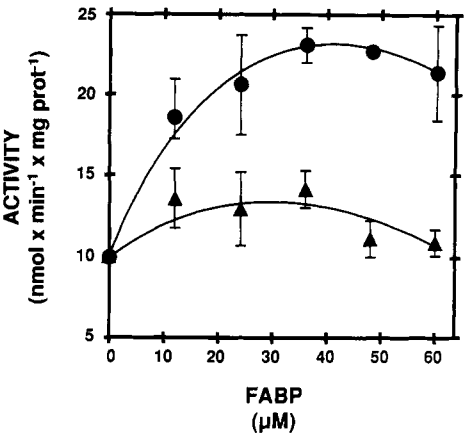


FIGURE 7: Modulation of microsomal phosphatidic acid biosynthesis from *sn*-glycerol 3-phosphate by I-FABP and L-FABP at 37 °C. Final volumes of 0.1 mL contained 735 μM *sn*-glycerol 3-phosphate, 118 μM oleoyl-CoA, 15 mM dithiothreitol, 80 mM NaF, and 3 μg of microsonal protein in a 70 mM Tris, pH 7.5 buffer. Points represent the mean of three determinations. (●) denotes points from L-FABP-containing samples; (▲) denotes points from I-FABP-containing samples.

stimulation 130% over the rate in the absence of added L-FABP. Intestinal-FABP had a much smaller effect on the rate of the first acylation by glycerophosphate acyltransferase. In order to test if the observed modulation by L-FABP of microsomal phosphatidic acid synthesis from *sn*-glycerol 3-phosphate was due to the acyl-CoA binding capabilities of L-FABP, the assay was repeated with a sample of 30 μM L-FABP preincubated with 0.6 mol of oleate per mole of L-FABP, 30 μM delipidated L-FABP, and an FABP-free control. The delipidated L-FABP showed an 82.5% ± 2.6% SEM stimulation of microsomal phosphatidic acid synthesis over the control level, but the L-FABP preincubated with oleate showed only a 51.3% ± 1.7% SEM stimulation over the control level.

Incorporation of a second acyl group into lysophosphatidic acid by monoacylglycerophosphate acyltransferase produces phosphatidic acid. The *in vitro* assay for the second acylation uses [$^{14}$O]oleoyl-CoA and lysophosphatidic acid as substrates. This microsomal assay for the second acylation of phosphatidic acid biosynthesis showed specific activities (nanomoles per minute per milligram of microsomal protein) of 87.0 ± 6.26 SEM for the controls without FABP, 105 ± 2.17 SEM for 30 μM L-FABP, and 70.1 ± 5.44 SEM for 30 μM I-FABP. These data represent a 21.5% stimulation for 30 μM L-FABP and an inhibition of 19.5% for 30 μM I-FABP.

The increased basal activity of our microsomes over the basal activity reported by Bordewick et al. (1989) is probably due to the EDTA-containing buffers used in the initial washes of our microsome preparation. The EDTA removed the $Ca^{2+}$ ions which may stimulate the hydrolysis of phosphatidic acid (the product measured for both of the microsomal acyltransferase assays) to glycerol phosphate by phospholipases of the A-type. The EDTA is also expected to remove the $Mg^{2+}$ ions which stimulate the conversion of phosphatidic acid to diacylglycerol by phosphatidate phosphohydrolase (Brindley, 1985).

## DISCUSSION

In the present investigation, recombinant L-FABP and I-FABP were used to clarify the role of these FABPs in fatty acyl-CoA binding and metabolism. The recombinant proteins were completely devoid of ACBP and contaminating bacterial

proteins as indicated both by a high-resolution SDS–polyacrylamide gel electrophoresis method and by reversed-phase HPLC. The first type of acyl-CoA binding assay used herein, a modification of the Lipidex competition assay of Glatz and Veerkamp (1983), utilized radiolabeled oleoyl-CoA as a ligand. This along with another competition assay (liposomal-bound acyl-CoA) has been used in all previous reports of acyl-CoA binding to FABPs or ACBP. The experiments shown herein indicated significant binding of oleoyl-CoA to L-FABP (2 mol/mol) but not to I-FABP.

Because of its arrangement of double bonds, the naturally occurring *cis*-parinaric acid is fluorescent (when located in a hydrophobic environment) and has a bent configuration similar to oleic acid. *cis*-Parinaric acid has been shown to bind to both I-FABP and L-FABP with affinities similar to oleic acid (Nemecz et al., 1991a). The acyl-CoA derivative of *cis*-parinaric acid, *cis*-parinaroyl-CoA, was synthesized as described under Methods and was used in fluorescence binding assays with L-FABP and I-FABP. These assays provided further evidence for acyl-CoA binding to L-FABP but not to I-FABP and obviated several of the problems associated with the Lipidex assay which include potential nonspecific interaction, competition, displacement, or separation artifacts.

The possible physiological significance for the differential acyl-CoA binding of the FABPs was tested via two *in vitro* biochemical assays: the microsomal acylation of *sn*-glycerol 3-phosphate and lysophosphatidic acid (monoacylglycerol 3-phosphate) to phosphatidic acid (Bordewick et al., 1989). Results from this study indicate that L-FABP, but not I-FABP, markedly stimulates the microsomal incorporation of fatty acyl-CoAs into phosphatidic acid. Specifically, L-FABP stimulated the rate-limiting step in which the first acyl group is incorporated to form the monoacylglycerol 3-phosphate (lysophosphatidic acid). L-FABP also stimulated the microsomal conversion of lysophosphatidic acid to phosphatidic acid, but to a much lesser degree. These observations are consistent with those of reports (Bordewick et al., 1989; Vancura & Haldar, 1992) showing microsomal conversion of glycerol 3-phosphate to lysophosphatidic acid which is stimulated by a 15-kDa cytosolic lysophosphatidic acid binding protein identified as L-FABP. While it may be possible that all of the stimulation of the production of lysophosphatidic acid from *sn*-glycerol 3-phosphate by L-FABP results from the removal of product from the reaction, this mechanism cannot explain L-FABP's stimulation of the production of phosphatidic acid from lysophosphatidic acid because L-FABP does not significantly bind phosphatidic acid (Vancura & Haldar, 1992). Because the production of both lysophosphatidic acid and phosphatidic acid share a common substrate, fatty acyl-CoA, and because subsaturating amounts of oleic acid significantly inhibited the stimulatory effect of L-FABP on the synthesis of phosphatidic acid, this stimulatory effect of L-FABP may be due in part to the acyl-CoA binding capabilities of L-FABP. A mechanism for this stimulation which is consistent with the data must involve the binding of acyl-CoA to L-FABP and its transport to the microsomes. The physiologic significance of L-FABP's modulation of microsomal phospholipid biosynthesis is supported by the association of L-FABP with liver endoplasmic reticulum (Bordewick et al., 1989) and an *in vivo* experiment in which mouse L-cell fibroblasts were transfected with the cDNA for L-FABP (Jefferson et al., 1990). L-Cells expressing large amounts of L-FABP were found to contain 47% more phospholipid per gram of protein than low-expression controls. It is not known whether ACBP also functions to stimulate fatty acyl-group incorporation into phospholipids.

Any proposed metabolic role for the binding of L-FABP with fatty acyl-CoAs may be viewed with skepticism because of the presence of ACBP in liver cytosol. Because the apparent $K_d$s (Lipidex assay) for oleoyl-CoA and palmitoyl-CoA binding to rat liver ACBP are 0.13 and 0.28 $\mu$M, respectively, and because the ACBP concentration is normally 2–4-fold higher than the cytosolic acyl-CoA concentration (Knudsen, 1990), one can make a plausible argument that under normal conditions little of the cytosolic fatty acyl-CoA is bound to L-FABP. Any such argument must assume, however, a uniform distribution of the fatty acyl-CoAs, ACBP, and L-FABP throughout the cytosol. One possible role, which has been proposed by others (Glatz & Veerkamp, 1985), is that L-FABP protects the cell from the deleterious effects of large fluctuations in the concentrations of unbound fatty acyl-CoAs. L-FABP is well suited for this role because it is present in the cytosol at much higher levels than either fatty acyl-CoA or fatty acids (Noy et al., 1986b, Cooper et al., 1987, Glatz & Veerkamp, 1985), and because L-FABP seems to facilitate the conversion of fatty acyl-CoAs to less toxic species such as phosphatidic acid (Bordewick et al., 1989; Vancura & Haldar, 1992) and cholesterol ester (Nemecz & Schroeder, 1991; Grinstead et al., 1983). Differences in subcellular compartmentalization, differences in acyl-CoA targeting or trafficking, and differences in stimulation of specific enzymes utilizing acyl-CoAs may show important roles for both L-FABP and ACBP in lipid metabolism, protein function, and second-messenger pathways.

Thus, we have reported the production and purification to homogeneity of recombinant forms of L-FABP and I-FABP with no contamination with ACBP. Liver FABP has been shown to bind *cis*-parinaroyl-CoA with high affinity and ease of analysis which compares favorably with the more cumbersome Lipidex methods. Perhaps most importantly these now well-defined molecular species show differential stimulation of phosphatidic acid synthesis, suggesting the specific participation of L-FABP in a process that has far-reaching metabolic consequences.

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