Isoforms of Rat Liver Fatty Acid Binding Protein Differ in Structure and Affinity for Fatty Acids and Fatty Acyl CoAs†

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ABSTRACT: Although native rat liver fatty acid binding protein (L-FABP) is composed of isoforms differing in isoelectric point, their comparative structure and function are unknown. These properties of apo- and holo-L-FABP isoforms were resolved by circular dichroism, time-resolved fluorescence spectroscopy, and binding/displacement of fluorescent ligands. Both apo-isoforms had similar hydrodynamic radii of 18.5 Å, but apo-isoform **I** had a greater α-helical content and exhibited a longer Tyr lifetime, indicative of secondary and tertiary structural differences from isoform **II**. Isoforms **I** and **II** both had two fatty acid or fatty acyl CoA binding sites. Ligand binding decreased the isoform hydrodynamic radii by 3−4 Å and increased Tyr rotational motions in a more restricted range. Fatty acyl CoAs were more effective than fatty acids in altering the isoform structures. Scatchard analysis showed that both isoforms bound *cis*- parinaric acid with high affinity (K_d values 41 and 60 nM, respectively) and bound *trans*-parinaric acid with 2- and 7-fold, respectively, higher affinity than for *cis*-parinaric acid. In contrast, isoform **I** had higher affinity for *cis*- and *trans*-parinaroyl CoAs (K_d values of 33 and 14 nM) than did isoform **II** (K_d values of 110 and 97 nM), thereby resulting in biphasic plots of parinaroyl-CoA binding to native L-FABP. Finally, displacement studies indicated that each isoform displayed distinct specificities for fatty acid/fatty acyl CoA chain length and unsaturation. Thus, rat L-FABP isoforms differ markedly in both structure and ligand binding function.

Liver fatty acid binding protein (L-FABP) is one of nearly twenty 14−15 kDa fatty acid binding proteins [reviewed in Veerkamp and Maatman (1995), Banaszak et al. (1994), Borchers and Spener (1994), and Matarese et al. (1989)]. Many of these FABPs have been identified as distinct types in a variety of tissues including liver, heart, intestine [reviewed in Borchers and Spener (1994), and Paulussen and Veerkamp (1990)], adipose tissue (Dutta-Roy et al., 1993; Matarese et al., 1989), and brain (Myers-Payne et al., 1996; Kurtz et al., 1994; Feng et al., 1994). Despite the absence of conclusive evidence for the physiological role of FABPs, much information has been reported over the last 30 years suggesting potential FABP functions [reviewed in Clarke and Armstrong (1989), Bass (1988), Glatz et al. (1993), Ockner et al. (1992), Veerkamp et al. (1993), Issemann et al. (1992), Veerkamp (1995), Borchers and Spener (1994), and Paulussen and Veerkamp (1990)]. Postulated roles include the uptake and metabolism of fatty acids and fatty acyl CoAs, intracellular trafficking of these substrates, peroxisome proliferation, and regulation of lipid synthetic and oxidative enzymes through the binding of substrates, effectors, and potential inhibitors. Interestingly, it has also been suggested that FABPs may modulate cell growth and differentiation [reviewed in Ockner et al. (1993), Carroll et al. (1990), Davidson et al. (1993), Sorof (1994), Bass (1993), Clarke and Armstrong (1989), and Glatz et al. (1993)]. FABP abnormalities have been implicated in cardiac hypertropy (Burton et al., 1994), myocardial ischemia (Das et al., 1991), chronic and acute alcoholism (Pignon et al., 1987; Myers-Payne et al., 1996a,b), diabetes (Baier et al. 1995, 1996), and cancer (Davidson et al., 1993; Sorof, 1994).

An intriguing feature of most FABPs, except adipocyte FABP (Matarese et al., 1989), is additional heterogeneity demonstrated by the presence of isoforms such as those observed with fatty acid binding proteins from liver (Dormann et al., 1993; Das et al., 1989; Haunerland et al., 1984; Ketterer et al., 1976; Matarese et al., 1989), heart (Bartetzko et al. 1993; Unterberg et al., 1990), adipocyte (Matarese et al., 1989), myelin [reviewed in Matarese et al. (1989)], fetal lung (Sa et al., 1993), and placenta (Das et al., 1988). However, the molecular basis of this heterogeneity (bound ligand, multiple mRNAs, deamidation, decarboxylation, tyrosine phosphorylation or cysteine modification, etc.) does not appear to be resolved conclusively in the literature [reviewed in Matarese et al. (1989)]. There is evidence suggesting that L-FABP isoforms were not due to deamidation, multiple mRNAs, decarboxylation, or tyrosine phosphorylation but instead may result from the presence of noncovalently bound ligands (fatty acid) (Spener et al., 1990; Bass, 1985; Trulzsch & Arias, 1981), interactions with isoelectric focusing ampholytes (Ketterer et al., 1976), or cysteine modification (Sato et al., 1996; Dormann et al., 1993; Hitomi et al., 1990).

The secondary and tertiary structures of apo- and holo-FABPs (e.g., those from intestine, heart, and adipocyte) have been extensively investigated by NMR, X-ray crystallography, fluorescence, and circular dichroism [reviewed in Sacchettini and Gordon (1993) and Matarese et al. (1989)]. In contrast, while the primary structure of apo-FABP

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[1] Abbreviations: L-FABP, liver fatty acid binding protein; CoASH, coenzyme A; 9*Z*,11*E*,13*E*,15*Z*-octadecatetraenoic acid, *cis*-parinaric acid; 9*Z*,11*E*,13*E*,15*Z*-octadecatetraenoyl coenzyme A, *cis*-parinaroyl CoA.

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isoforms has been obtained by protein amino acid sequencing (Sato et al., 1996; Hitomi et al., 1990; Dormann et al., 1993; Haunerland et al., 1984) or cDNA characterization (Bartetzko et al., 1993), very little is known about the secondary and the tertiary structure of either FABPs resolved into isoforms (Li & Ishibashi, 1992; Schulenberg-Schell et al., 1988).

Only recently have differential functions of some FABP isoforms begun to be examined. Although differences in types of ligands bound have been reported, detailed comparisons of FABP isoforms specificity in binding fatty acids of different chain length and unsaturation have not been reported. While fatty acyl CoAs were recognized as FABP isoform ligands more than twenty years ago [reviewed in Gossett et al. (1996b)], detailed comparisons of FABP isoform affinities for fatty acyl CoAs differing in carbon number or unsaturation have not been reported. Fatty acyl CoAs are extremely important in view of their involvement not only in the intermediary metabolism of fatty acids, but also in the regulation (at nM levels) of many intracellular functions including signal transduction and gene transcription [reviewed in Raman and DiRusso (1995), Dedukhova et al. (1991), Nikawa et al. (1979), Oram et al. (1975), and Gossett et al. (1996)]. Finally, there is evidence suggesting that FABP isoforms may have physiological roles related to the redox status of the cell (Sato et al., 1996; Dormann et al., 1993), intracellular targeting (Unterberg et al., 1990), ligand binding (Sha et al., 1993), and regulation of enzyme activity (Miyozawa & Hashimoto, 1979; Buhr et al., 1994; Sa et al., 1989; Das et al., 1989).

The present study was designed to structurally and functionally characterize two native liver L-FABP isoforms. The secondary and tertiary structures of both the apo- and holo-L-FABP isoforms were examined by circular dichroic and time resolved fluorescence spectroscopy. Direct binding and displacement assays were used to resolve L-FABP isoform specificity for fatty acid and fatty acyl CoA chain length and unsaturation. In summary, we report for the first time both structural and functional differences between rat apo-L-FABP isoforms.

MATERIALS AND METHODS

Materials. The *cis*- and *trans*-parinaric acids were from Molecular Probes, Eugene, OR, while the *cis*- and *trans*-parinaroyl CoAs were synthesized and purified as described earlier (Hubbell et al., 1994). Coenzyme A (CoASH), acyl-coenzyme A synthase, adenosine 5′-triphosphate (ATP), and *p*-terphenyl as well as saturated and unsaturated fatty acyl CoAs were from Sigma Chemical Co., St. Louis, MO. Saturated and unsaturated fatty acids were obtained from NuChek Prep Inc., Elysian, MN. All other chemicals were reagent grade or better.

Methods. Native rat liver L-FABP, isoform **I** (p*I* 7.55), and isoform **II** (p*I* 7.35) were provided by Dr. Eric Murphy. These proteins were isolated using established methods (Das et al., 1988, 1989; Sa et al., 1993; Matarese et al., 1989; Bartetzko et al., 1993; Unterberg et al., 1990; Dormann et al., 1993; Haunerland et al., 1984; Ketterer et al., 1976) with sequential standard column chromatographic techniques [anion exchange with QAE-Sephadex (Pharmacia, Piscataway, NJ); gel filtration by G-50 Sephadex; cation exchange with Macro-S (Bio-Rad, Hercules, CA); and gel filtration with G-50 Sephadex]. Protein purities of isoforms **I** and **II** were >98% and >99%, respectively. Protein purity was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis with silver staining. Protein identity was confirmed by Western blotting and by mass spectral analysis. Purified isoforms **I** and **II** were delipidated as described previously (Nemecz et al. 1991a,b; Glatz & Veerkamp, 1983b). L-FABP protein concentration was determined by Bradford assay (Bradford, 1976) and corrected according to amino acid analysis (Lowe et al., 1984). The Bradford assay overestimates the L-FABP concentration 1.69-fold.

Steady-State and Time-Resolved Fluorescence Spectroscopy. Steady-state fluorescence spectra were measured on a PC1 photon counting spectrofluorometer (ISS Inc., Champaign, IL) using 1 cm quartz cuvettes. Sample temperature was kept at 25 °C (±0.1 °C) in a thermostated cell holder. Excitation and emission bandwidths were 4 and 8 nm, respectively.

Time-resolved fluorescence measurements were performed as described earlier (Stolowich et al., 1997; Frolov & Schroeder, 1997; Frolov et al., 1996) using a GREG 250 subnanosecond multifrequency cross-correlation and modulation fluorometer equipped with KOALA automatic sample compartment (ISS Inc., Champaign, IL). Excitation was with an Innova-Sabre argon ion laser (Coherent Laser Group, Palo Alto, CA) with automatic wavelength selection of single selectable lines at $\lambda = 275.4$, 300.2, 302.4, and 305.5 nm (power output 340, 630, 800, and 460 mW, respectively). Sample absorbance at the excitation wavelengths was ≤0.05. All data were obtained in 25 mM phosphate buffer, pH 7.4, at 25 °C.

Fluorescence Lifetime Data Analysis. Lifetime data were analyzed by ISS-187 Software (ISS Inc., Champaign, IL) as a sum of exponentials: $I(t) = \sum\alpha_i f_i \exp(-t/\tau_I)$, where I is fluorescence, τ_i is lifetime, and $\sum\alpha_i = 1$. Fractional intensity, f_i, was $f_i = \alpha_i\tau_I/\sum\alpha_i\tau_I$. Mean lifetime was $\sum f_i\tau_I$. The minimized χ^2 parameter was the criterion for goodness of fit with $\chi^2 < 3$ considered acceptable (Parasassi et al. 1984).

Anisotropy Decay Analysis. Anisotropy decay was modeled by a sum of exponentials as $r(t) = r(0)\sum g_i \exp(-t/\theta_i)$, where $r(0)$ is anisotropy of a fluorophore in the absence of rotational diffusion, θ_i is the rotational correlation time, and g_i is fractional anisotropy. The "goodness" of fit to the applied model was determine as described above using ISS-187 Software (ISS Inc., Champaign, IL). The equivalent hydrodynamic radius of the protein was calculated as

$$R = (3kT\theta/4\pi\eta)^{1/3} \quad (1)$$

where η is solvent viscosity. The value of the same parameter can be estimated from the hydrated protein volume:

$$R = [(3/4\pi)(M/N_0)(V_2 + \delta_1 V_1)]^{1/3} \quad (2)$$

where M is molecular weight; N_0 is Avogadro's number; δ_1 is the fraction of hydration; V_1 is the volume of bound water; V_2 is protein specific partial volume. $\delta_1 = 0.4$ g of H_2O/g protein; $V_2 = 0.72$ cm^{-3} g^{-1} for an average protein (Cantor & Schimmel, 1980), and $V_1 = 1$ cm^3 g^{-1} for water in eq 2.

Circular Dichroism. Spectra of L-FABP isoforms (2 μM, 25 mM phosphate buffer) were recorded in a 1 mm circular cuvette with 1 nm resolution on a J-710 spectropolarimeter

(JASCO, Easton, MD) as described (Stolowich et al., 1997; Frolov et al., 1996).

Fluorescent Fatty Acid and Fatty Acyl-CoA Binding. The binding affinities of L-FABP isoforms for *cis*-parinaric acid, *trans*-parinaric acid, *cis*-parinaroyl CoA, and *trans*-parinaroyl CoA were determined as previously described (Stolowich et al., 1997; Schroeder et al., 1995) with the following modifications: a 2 mL sample of 0.05 μM L-FABP in PBS was titrated with small increments of fatty acid (0.1−1.0 μL) dissolved in 10 mM NaOH. The concentrations of the fatty acid stock solutions were 270−300 μM. Alternately, fatty acyl CoAs were added from a 270 μM stock solution in phosphate buffer, pH 7.4. Each sample and blank (without L-FABP) were thoroughly mixed and allowed to equilibrate for 1−2 min before the maximal fluorescence signal was obtained. All measurements were performed at 24 °C.

Displacement of L-FABP-Bound cis-Parinaroyl CoA by Fatty Acyl CoAs or Fatty Acids. L-FABP (0.05 μM) in phosphate buffer (pH 7.4) was incubated with *cis*-parinaroyl CoA (0.15 μM) for 5 min at 24 °C to obtain maximal fluorescence. L-FABP-bound *cis*-parinaroyl CoA was displaced by 1.5 μM CoASH, fatty acyl CoAs with fatty acyl chain length ranging from 2 to 20 carbons, or fatty acids with acyl chain length ranging from 2 to 20 carbons. All displacing fatty acyl CoAs were dissolved in double distilled water. The critical micellar concentration of long chain fatty acids is in the micromolar range (Matarese et al., 1989; Pryde, 1979). In contrast, the critical micellar concentration for the salts of long chain free fatty acids (C10−C18) is 1000-fold higher, approximately several millimolar (Small, 1986). Therefore, all displacing fatty acids were used as their sodium salts which were prepared by dissolving free fatty acids in 10 mM NaOH. By using fatty acid salts we avoided complications due to differential solubility of the displacing fatty acids. Measurements were corrected for the blank (ligand or protein only) and photobleaching.

RESULTS

Secondary Structure of Apo- and Holo-Isoforms: Circular Dichroism. The circular dichroism spectrum of the apo-isoform **I** exhibited a minimum at 220 nm and one maximum at 196 nm, consistent with the presence of α-helical and β-sheet components in the secondary structure (Figure 1). Spectrum analysis using the self-consistent method of Sreerama and Woody (1993) predicted 46% α-helix, 20% β-sheet, 18% β-turns, and 16% unordered structure in apo-isoform **I**. Addition of up to 10 molar equiv of oleic acid to apo-isoform **I** slightly altered the L-FABP secondary structure by increasing the proportions of α-helix from 46% to 49%, increasing the proportions of β-sheet from 20% to 22%, decreasing the proportions of β-turns from 18% to 13%, and leaving random coil proportions unchanged at 16%. Likewise, addition of 10-fold molar excess of oleoyl CoA led to the enhancement of α-helix from 46% to 49% but, in contrast to effects of fatty acids, fatty acyl CoA decreased β-sheet from 20% to 14%, β-turns remained unchanged, and the unordered random fraction increased from 16% to 18%.

The circular dichroism spectrum of apo-isoform **II** differed significantly from that of isoform **I**. The circular dichroism spectrum of apo-isoform **I** and apo-isoform **II**, measured at the same concentration, displayed a maximum at 193 nm and a minimum at 225 nm (Figure 1). However, the circular dichroism spectrum of apo-isoform **II** had significantly lower positive intensity at 193 nm and a smaller negative intensity at 225 nm (Figure 1). Consequent spectrum analysis showed 37% α-helix, 23% β-sheet, 20% β-turns, and 20% unordered structure. Thus, apo-isoform **II** had less α-helix and more proportions of other secondary structure characteristics than did apo-isoform **I**. Ligands such as oleic acid and oleoyl CoA exhibited markedly different effects on apo-isoform **II** structure as compared to apo-isoform **I** structure. Addition of up to 10 molar equiv of oleic acid to apo-isoform **II** decreased the α-helical proportion from 37% to 33% and decreased β-sheet from 23% to 22%, but increased the proportion of β-turns from 20% to 23% and random coil fraction from 20% to 22%. The presence of 10 molar excess of oleoyl CoA elicited almost no effect on isoform **II** secondary structure: α-helix and β-turns remained unchanged, β-sheet decreased from 23% to 21%, and random coil increased from 20% to 22%.

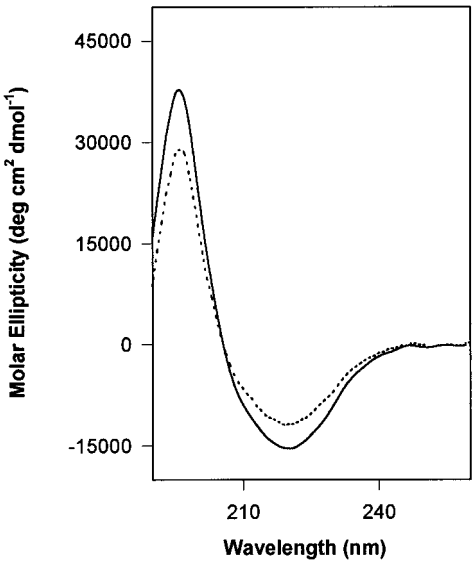

FIGURE 1: Circular dichroism spectra of rat L-FABP isoforms. Solid line, 2 μM isoform **I** in phosphate buffer; dotted line, 2 μM isoform **II** in phosphate buffer, pH 7.4. For more details see Materials and Methods.

In summary, two apo-L-FABP isoforms appeared to differ significantly in secondary structure and in the response of these respective structures to ligand binding. Apo-isoform **I** was primarily characterized as being α-helical (46%) with significant contribution from β-sheet/β-turn (38%). Compared to apo-isoform **I**, apo-isoform **II** was predominantly β-sheet/β-turn (43%) with a slightly smaller α-helical fraction (37%). Despite the general structural similarity between the apo- and holo-isoforms, apo-isoform **I** and apo-isoform **II** responded differently to the presence of the bound ligand. For apo-isoform **I**, binding of fatty acid or fatty acyl CoA modestly increased the α-helical content, while the percentage of α-helix in isoform **II** was substantially decreased after addition of fatty acid and remained unaltered after addition of fatty acyl CoA.

Tertiary Structural Properties of Apo- and Holo-L-FABP Isoforms: Tyrosine Fluorescence

Intrinsic Fluorescence Spectral Properties of L-FABP Tyrosine. L-FABP possesses rather unique fluorescence

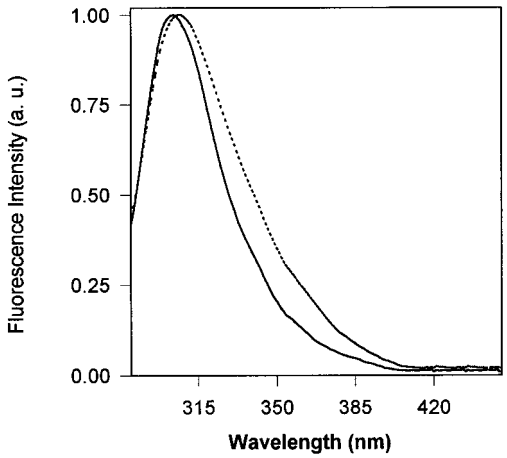

FIGURE 2: Fluorescence emission spectra of rat L-FABP isoforms. Spectra were obtained at 25 °C as in Methods. Solid line, 1 μM isoform **I** in phosphate buffer upon excitation at 275 nm; dotted line, 1 μM isoform **II** in phosphate buffer upon excitation at 275 nm, buffer pH 7.4.

characteristics because it has three tyrosine residues and no tryptophans (Paulussen & Veerkamp, 1990). Upon excitation at 275 nm the corrected fluorescence spectrum of apo-isoform **I** showed a single peak with a maximum at 304 nm and a bandwidth of 44 nm (Figure 2). The fluorescence spectrum of apo-isoform **II** was slightly red-shifted (emission maximum of 306 nm) and broadened (bandwidth of 56 nm) as compared to that of isoform **I** (Figure 2). Since no contaminant proteins were detected in either isoform preparation (see Materials and Methods), the red shift and the broadening of the isoform **II** emission spectrum was consistent with the tyrosines in isoform **II** being exposed to more hydrophilic and heterogeneous environment than that of isoform **I**.

Tyrosine Fluorescence Lifetime in Apo- and Holo-L-FABP Isoforms. The fluorescence decay of tyrosine in apo-isoform **I** exhibited biexponential decay with a long component, $\tau_1 = 3.39$ ns (fraction 0.49), and a short component, $\tau_2 = 1.44$ ns, resulting in an average lifetime of 2.39 ns (Table 1). Addition of 10 molar equiv of oleic acid decreased the long lifetime from 3.39 to 2.29 and the short lifetime from 1.44 to 0.6 ns, while almost doubling the fraction of the long lifetime component, from 0.49 to 0.87. Consequently, the average lifetime decreased by approximately 20%, from 2.39 to 2.07 ns (Table 1). Addition of 10 molar excess of oleoyl CoA exhibited similar, but even more pronounced, effects as compared to addition of fatty acid. Both tyrosine lifetime values in apo-isoform **I** were reduced by almost 60%, from 3.39 to 2.18 ns and from 1.44 to 0.90 ns, respectively (Table 1). This decrease of isoform **I** tyrosine fluorescence lifetimes was accompanied by a significant redistribution of their fractions with the fraction of the long component being increased from 0.49 to 0.69. Consequently, the average lifetime value was reduced ~25% from 2.39 to 1.79 ns (Table 1).

Apo-isoform **II** tyrosine also displayed biexponential fluorescence decay. However, these lifetime values and their fractions differed significantly as compared to isoform **I**: long lifetime component, 2.86 ns (fraction 0.64); short lifetime component, 0.64 ns (Table 1). The average lifetime of apo-isoform tyrosine was calculated as 2.24 ns, significantly shorter than that of apo-isoform **I**. Addition of 10 molar equiv of oleic acid to apo-isoform **II** reduced both tyrosine lifetime components from 2.86 to 2.28 ns (25%) and from 1.19 to 0.56 ns (53%), respectively. The fraction of the long component increased from 0.64 to 0.88. The overall effect of oleic acid binding to isoform **II** was a small decrease in the average tyrosine fluorescence lifetime from 2.24 to 2.08 ns (Table 1). Similar to isoform **I**, the effect of fatty acyl CoA binding to isoform **II** was more dramatic as compared to fatty acid. Addition of 10 molar excess of oleoyl CoA decreased both the long and the short lifetimes from 2.86 to 1.79 ns (60%) and from 1.19 to 0.39 ns (67%) (Table 1), respectively. The fraction of the long lifetime component was raised from 0.64 to 0.79 (~20%). Taken together, oleoyl CoA reduced the average tyrosine fluorescence lifetime of apo-isoform **II** by approximately 33%, from 2.24 to 1.5 ns (Table 1).

In summary, the two L-FABP isoforms differed significantly in their tyrosine fluorescence decay kinetics and in the responsiveness of these decay kinetics to ligand binding, especially oleoyl CoA. This is consistent with significant structural differences between L-FABP isoforms **I** and **II**.

Time-Resolved Anisotropy of Apo- and Holo-L-FABP Isoforms. The measurement of L-FABP time-resolved fluorescence anisotropy provides additional information on protein molecular dynamics in solution, as well as on the local structural mobility of the aromatic amino acid residues inside the protein. The latter was previously reported to be a sensitive indicator of structural changes induced by fatty acid and fatty acyl CoA binding to the recombinant rat L-FABP (Frolov & Schroeder, 1997).

Apo-isoform **I** exhibited a biexponential anisotropy decay kinetics with a long, $\theta_1 = 5.58$ ns, and fast, $\theta_2 = 0.072$ ns, component (Table 2). The long and short components reflected overall protein motion and the average internal motions of L-FABP tyrosine residues, respectively. The hydrodynamic radius of apo-isoform **I**, experimentally determined from the data in Table 2, was 18.5 Å. The theoretical radius derived from eq 2 was the same value, 18.5 Å. These results were consistent with a nearly spherical shape. The estimated "wobbling" cone angle for tyrosine segmental rotations in apo-isoform **I** was $\phi = 27°$ (Table 2). These data suggested a remarkable rotational freedom of tyrosine residues in the apo-isoform **I** protein.

Addition of 10 molar equiv of oleic acid decreased the long correlation time of isoform **I** from 5.58 to 4.51 ns (~20%; Table 2). Under the same conditions, the short component was no longer resolvable, possibly due to even faster rotational motions of tyrosine in the holo-isoform **I**. The decrease of L-FABP tyr maximal anisotropy r_0 (equal to $r_0g_1 + r_0g_2$) from 0.320 to 0.237 (Table 2) was accompanied by the loss of the short rotational component, indicative of fast depolarizing rotational motions. It should be noted, however, that the increased rotational motions of the tyrosine residues was accompanied by spatial restrictions on such motions as indicated by the higher r_0g_1, 0.237 vs 0.224, and lower "wobbling" cone angle, 25 vs 27° (Table 2). The addition of 10 molar excess of oleoyl CoA to the apo-isoform **I** revealed the same pattern as did oleic acid addition: θ_1 decreased from 5.58 to 4.54 ns and the fast component vanished followed by a reduction in r_0 value, from 0.320 to 0.249 (Table 2). Again, oleoyl CoA elicited even more spatial restrictions on isoform **I** tyrosine segmental rotations as indicated by higher r_0g_1, 0.249 vs 0.224, and lower wobbling cone angle, 23 vs 27° (Table 2). Addition

Table 1: Fluorescence Decay of L-FABP Isoforms[a]

protein	ligand	τ_1 (ns)	τ_2 (ns)	f_1	f_2	τ^b (ns)
isoform **I**	none	3.39 ± 0.05	1.44 ± 0.03	0.49 ± 0.01	0.51 ± 0.01	2.39 ± 0.03
	oleic acid	2.29 ± 0.06[c]	0.60 ± 0.09[c]	0.87 ± 0.03[c]	0.13 ± 0.03[c]	2.07 ± 0.03[c]
	oleoyl CoA	2.18 ± 0.12[c]	0.90 ± 0.12[c]	0.69 ± 0.02[c]	0.31 ± 0.02[c]	1.79 ± 0.13[c]
isoform **II**	none	2.86 ± 0.06	1.19 ± 0.03	0.64 ± 0.01	0.35 ± 0.01	2.24 ± 0.03
	oleic acid	2.28 ± 0.03[c]	0.56 ± 0.07[c]	0.88 ± 0.02[c]	0.12 ± 0.02[c]	2.08 ± 0.01[c]
	oleoyl CoA	1.79 ± 0.04[c]	0.39 ± 0.01[c]	0.79 ± 0.01[c]	0.21 ± 0.01[c]	1.50 ± 0.04[c]

[a] The fluorescence of rat L-FABP isoforms (2 μM) with or without 10-fold excess of ligand was analyzed by a sum of exponentials as $I(t) = \sum\alpha_i \exp(-t/\tau_i)$, where $\sum\alpha_i = 1$ and $f_i = \alpha_i\tau_i/\sum\alpha_i\tau_i$. All measurements were made in phosphate buffer (pH 7.4) at 25 °C. The excitation and emission wavelengths were 275 and 313 nm, respectively. Values represent mean ± SE ($n = 3-9$). [b] Mean fluorescence lifetime τ was calculated as $\tau = \sum f_i\ \tau_i$. [c] $P < 0.05$ as compared to no ligand.

Table 2: Fluorescence Anisotropy Decay of L-FABP Isoforms[a]

protein	ligand	θ_1 (ns)[b]	θ_2 (ns)	r_0g_1[d]	r_0g_2	ϕ (deg)[c]
isoform **I**	none	5.58 ± 0.06	0.072 ± 0.012	0.224 ± 0.001	0.096 ± 0.001	27
	oleic acid	4.51 ± 0.11*		0.237 ± 0.001*		25
	oleoyl CoA	4.54 ± 0.23*		0.249 ± 0.002*		23
isoform **II**	none	6.19 ± 0.30	0.070 ± 0.005	0.229 ± 0.001	0.091 ± 0.001	25
	oleic acid	5.24 ± 0.19*	0.049 ± 0.012	0.237 ± 0.002*	0.083 ± 0.002*	25
	oleoyl CoA	4.14 ± 0.03*		0.256 ± 0.004*		21

[a] The anisotropy of tyrosine in L-FABP isoforms (2 μM) with or without 10-fold molar excess of ligand was analyzed by a sum of exponentials as $r(t) = r(0) = \sum g_i \exp(-t/\theta_i)$. [b] The excitation and emission wavelengths were 275 and 313 nm, respectively. [c] The wobbling cone angle was calculated for the L-FABP tyrosine on the basis of time-resolved anisotropy data presented in the table and by using the following equation:

$$r/r(0) = (3\cos^2\phi - 1)/2,$$

where r is the residual (limiting) anisotropy and $r(0)$ is the sum of $r(0)g_1 + r(0)g_2$. The sum of $g_1 + g_2 = 1$. Values represent mean ± SE ($n = 3-5$). [d] An asterisk refers to $P < 0.05$ as compared to no ligand.

of fatty acid and fatty acyl CoA slightly affected the apo-isoform **I** overall tertiary structure by reducing its hydrodynamic radius from 18.5 to 17.0 Å.

Compared to isoform **I**, apo-isoform **II** displayed similar fluorescence anisotropy kinetic: long component, 6.19 ns, and short component, 0.07 ns (Table 2). The value of the long rotational correlation time corresponded to a hydrodynamic radius of 19.0 Å. However, the value of the wobbling cone angle was 25° (Table 2). The presence of oleic acid led to the reduction of the long component, from 6.19 to 5.24 ns (20%), but did not affect the protein hydrodynamic radius, 18.5 vs 18.0 Å. Remarkably, oleic acid did not affect either the value of the short component, nor the amplitude of tyrosine rotational motions (Table 2). In contrast, addition of oleoyl CoA drastically decreased the value of the apo-isoform **II** long rotational component by almost 34%, from 6.19 to 4.14 ns, resulting in reduction of the respective hydrodynamic radius from 18.5 to 16.6 Å. As was observed with isoform **I**, the short rotational component was no longer detectable upon addition of oleoyl CoA. The overall effect on isoform **II** tyrosine segmental rotations was a reduction in rotational freedom, as judged by the decrease in the respective wobbling cone angle, from 25 to 21° (Table 2).

In summary, although both apo isoforms were characterized by similar rotational dynamics in solution, the segmental rotations of tyrosine residues in isoform **II** were more restricted than those of isoform **I**. Moreover, both the overall protein rotation and the internal tyrosine motions were significantly altered when the ligand, especially fatty acyl CoA, was bound to the protein. In general, the two isoforms responded differentially to the binding of both oleic acid and oleoyl CoA. The most striking distinction between isoform **I** and isoform **II** was observed upon their interaction with fatty acyl CoA.

L-FABP Isoform Affinity for Fluorescent Fatty Acid and Fatty Acyl CoA

Fatty Acid Binding. The relative affinity of L-FABP isoforms for kinked chain fatty acids vs straight chain fatty acids was examined using fluorescent *cis*-parinaric acid and *trans*-parinaric acid, respectively. The parinaric acids are naturally occurring fatty acids oriented to form either an 18-carbon, kinked-chain, fluorescent analogue (*cis*-parinaric acid) of oleic acid or an 18-carbon, straight-chain, fluorescent analogue (*trans*-parinaric acid) of stearic acid. Both parinarate conformers fluoresce poorly in aqueous, possibly due to strong electrostatic interactions of these fluorophores with the molecules of water. Upon titration of L-FABP isoform **I** or **II** with *cis*- or *trans*-parinaric acid saturation curves were observed for both *cis*- and *trans*-parinaric acid binding as shown in Figure 3A−D, respectively. The analysis of the binding curves (Stolowich et al., 1997; Schroeder et al., 1995) allowed determination of the respective binding parameters, K_d and B_{max}, as shown by representative Scatchard plots (Figure 3A−D, inset). The results on binding of *cis*- and *trans*-parinaric acids to L-FABP isoforms are summarized in Table 3. Each L-FABP isoform showed the presence of two binding sites with similar affinities for either *cis*- or *trans*-parinaric acid per L-FABP molecule. However, it appears that the binding affinity of a straight (*trans*) fatty acid was a several fold higher ($p < 0.05$) than that of a kinked (*cis*) fatty acid in both isoforms. However, no major variations in affinities were observed when the same fluorescent parinarate was compared between the L-FABP isoforms.

Fatty Acyl CoA Binding. Fatty acyl CoA binding was examined to determine if the structural specificities of the L-FABP isoforms would affect their ability to bind fatty acyl

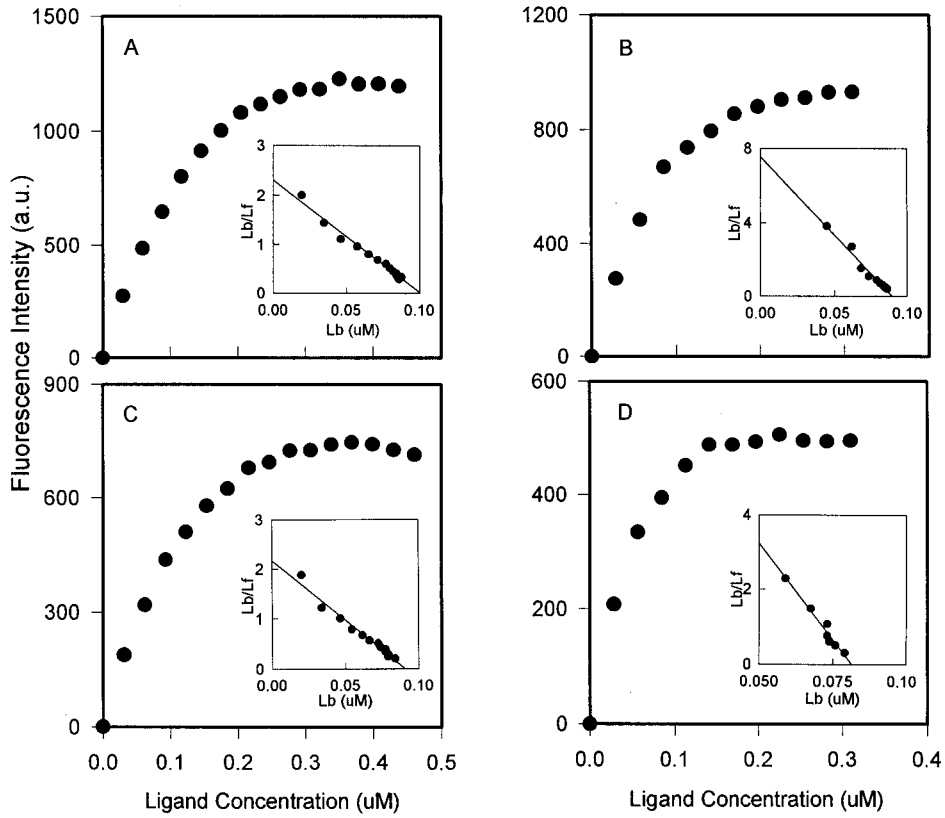

FIGURE 3: Titration of rat L-FABP isoforms with parinaric acids. (A) Titration of isoform **I** (0.18 μM) with *cis*-parinaric acid (0−0.45 μM) as followed by the increase in fluorescence intensity. Excitation at 310, emission at 416 nm. Inset: Scatchard plot of the titration of isoform **I** with *cis*-parinaric acid. (B) Titration of isoform **I** (0.18 μM) with *trans*-parinaric acid (0−0.4 μM). λ_{ex} = 310, λ_{em} = 416 nm. Inset: Scatchard plot of the titration of isoform **I** with *trans*-parinaric acid. (C) Titration of isoform **II** (0.18 μM) with *cis*-parinaric acid (0−0.45 μM). λ_{ex} = 310, λ_{em} = 416 nm. Inset: Scatchard plot of the titration of isoform **II** with *cis*-parinaric acid. (D) titration of isoform **II** (0.18 μM) with *trans*-parinaric acid (0−0.4 μM). λ_{ex} = 310, λ_{em} = 416 nm. Inset: Scatchard plot of the titration of isoform **II** with *trans*-parinaric acid.

Table 3: Binding of Fatty Acids and Fatty Acyl CoAs to L-FABP and Its Isoforms[a]

protein	ligand	K_d (nM)	B_{max}
L-FABP	*cis*-parinaric acid	171 ± 46	1.95 ± 0.07
	trans-parinaric acid	44 ± 8	1.93 ± 0.03
	cis-parinaroyl CoA[b]	8 ± 3	1.0
		97 ± 42	1.0
	trans-parinaroyl CoA[c]	10 ± 1	1.0
		180 ± 23	1.0
isoform **I**	*cis*-parinaric acid	41 ± 10	1.91 ± 0.1
	trans-parinaric acid	18 ± 6	2.1 ± 0.13
	cis-parinaroyl CoA	33 ± 11	1.5 ± 0.12
	trans-parinaroyl CoA	14 ± 5	1.51 ± 0.10
isoform **II**	*cis*-parinaric acid	60 ± 17	1.77 ± 0.18
	trans-parinaric acid	8 ± 2	1.74 ± 0.05
	cis-parinaroyl CoA	110 ± 34	1.77 ± 0.10
	trans-parinaroyl CoA	97 ± 20	1.82 ± 0.04

[a] Values represent mean ± SE (n = 3−5). [b,c] Data were treated for multiple equilibria as described (Schulenberg-Schell et al., 1988). For more details see Methods.

CoAs. When L-FABP isoforms were titrated with *cis*- or *trans*-parinaroyl CoA, fluorescence intensity at the emission maximum (420 nm) increased without altering the position of the emission maximum (data not shown). Binding curves for fluorescent fatty acyl CoAs were constructed as described above and both *cis*- and *trans*-parinaroyl CoAs showed clear saturation curves (Figure 4A−D). The data obtained for fluorescent parinaroyl CoAs were consistent with the presence of two binding sites with similar affinities for the same parinaroyl CoA on the same L-FABP isoform (Figure 4A−D, inset). However, unlike the higher affinity for the *trans*- vs *cis*-parinaric acid, neither isoform displayed a significant difference in binding affinity for *trans*- vs *cis*-parinaroyl CoA (Table 3). However, isoform **I** had 3- and 7-fold higher binding affinity than isoform **II** for *cis*- and *trans*-parinaroyl CoAs, respectively (Table 3).

Binding Affinities of Rat L-FABP for Fluorescent Fatty Acids and Fatty Acyl CoAs. The large difference in binding affinity for *cis*- and *trans*-parinaroyl CoAs between the two L-FABP isoforms suggested that for native rat liver L-FABP (comprised of both isoforms **I** and **II**) one might expect multiple equilibria, i.e., the existence of two dissimilar binding sites for fatty acyl CoAs characterized by different K_d values. As expected, binding of either *cis*- or *trans*-parinaroyl CoA to native L-FABP showed highly nonlinear, two-phase Scatchard plots which were consistent with the existence of multiple equilibria (Figure 5A and B, inset). The analysis of binding isotherms (Schulenberg-Schell et al., 1988) resulted in K_d values of 8 ± 3 and 97 ± 42 nM and 10 ± 1 and 180 ± 23 nM for *cis*- and *trans*-parinaroyl CoA, respectively (Table 3). Interestingly, in the case of rat L-FABP individual K_d values for both fatty acyl CoAs displayed a resemblance to those in the individual isoforms, isoform **I** and the isoform **II** (Table 3). In contrast, individual binding sites for fatty acids in native L-FABP were not resolvable, as shown by the data in the Figure 5C and D. This fact could be due to the much more similar affinities for each fatty acid noted for isoforms **I** and **II** (Table 3).

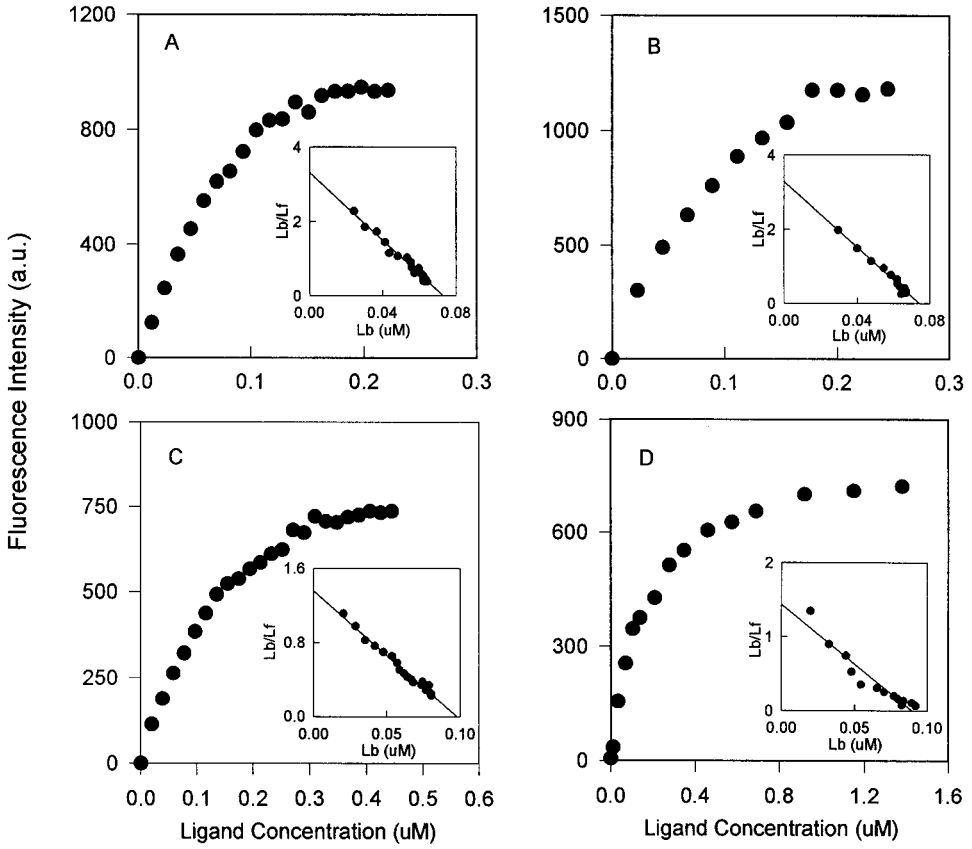

FIGURE 4: Titration of rat L-FABP isoforms with parinaroyl CoAs. (A) titration of isoform **I** (0.18 μM) with *cis*-parinaroyl CoA (0−0.25 μM) as followed by the increase in fluorescence intensity. Excitation at 310, emission at 416 nm. Inset: Scatchard plot of the titration of isoform **I** with *cis*-parinaroyl CoA. (B) titration of isoform **I** (0.18 μM) with *trans*-parinaroyl CoA (0−0.3 μM). $\lambda_{ex} = 310$, $\lambda_{em} = 416$ nm. Inset: Scatchard plot of the titration of isoform **I** with *trans*-parinaroyl CoA. (C) Titration of isoform **II** (0.18 μM) with *cis*-parinaroyl CoA (0−0.5 μM). $\lambda_{ex} = 310$, $\lambda_{em} = 416$ nm. Inset: Scatchard plot of the titration of isoform **II** with *cis*-parinaroyl CoA. (D) Titration of isoform **II** (0.18 μM) with *trans*-parinaroyl CoA (0−1.6 μM). $\lambda_{ex} = 310$, $\lambda_{em} = 416$ nm. Inset: Scatchard plot of the titration of isoform **II** with *trans*-parinaroyl CoA.

Fatty Acyl CoA Binding Site Specificities of L-FABP Isoforms

In order to further investigate the relative specificities of L-FABP isoform fatty acyl CoA binding site(s), two types of displacement experiments were performed.

Displacement of cis-Parinaroyl CoA by Fatty Acids. The two L-FABP isoforms exhibited distinct differences in *cis*-parinaroyl CoA displacement specificity by fatty acids. As shown in Table 4, the C20:0 arachidic acid and C12:0 lauric acid did not displace *cis*-parinaroyl CoA bound to isoform **I** but were able to displace *cis*-parinaroyl CoA bound to isoform **II** by 13% and 16% displacement, respectively. As compared to unsaturated fatty acids, the saturated C18:0 stearic acid and C16:0 palmitic acid were more effective displacers (∼2-fold) of *cis*-parinaroyl CoA from isoform **II** than from isoform **I** (Table 4). Finally, the C20 polyunsaturated arachidonic acid (but not the saturated C20 arachidic acid) was a more efficient diplacer of *cis*-parinaroyl CoA from isoform **II** than from isoform **I**. In summary, of the two L-FABP isoforms, isoform **II** appeared to have a higher overall affinity and range of affinities for saturated (C20−C12) fatty acids.

Displacement of cis-Parinaroyl CoA by Fatty Acyl CoAs. The specificities of L-FABP isoforms for binding fatty acyl CoAs were determined in experiments where *cis*-parinaroyl CoA bound to isoform **I** or to isoform **II** was displaced by a series of saturated and unsaturated fatty acyl CoAs of different chain length. Both saturated (C20−C12) and unsaturated (C20:3−C14:1) fatty acyl CoAs were highly efficient displacers with the efficiency ranging from 90% to 40% (Table 5). Again, several trends were evident. First, this displacement was not due to the presence of the CoA moiety. CoASH did not displace *cis*-parinaroyl CoA. Second, a comparison of Tables 5 and 4 indicates that the fatty acyl CoAs were more efficient displacers of bound *cis*-parinaroyl CoA than were the fatty acids. Third, the fatty acyl CoAs were effective over a broader range of fatty acyl CoA than fatty acid chain lengths. Fourth, in both isoforms the displacement efficiency of saturated fatty acyl CoAs gradually decreased from 90% to 40% as the fatty acyl chain length decreased from 20 (arachidoyl CoA) to 12 (lauroyl CoA) carbon atoms. No such gradual decrease was observed with unsaturated fatty acyl CoAs. The highest displacement efficiency (∼80%) was detected for the C20:4 arachidonoyl CoA, the C20:3 11,14,17-eicosatrienoyl CoA, and the C18:2 linoleoyl CoA. The C18:3 linolenoyl CoA and the C14:1 myristoyl CoA were less potent displacers, ∼60% and ∼40%, respectively (Table 5).

With one exception (11,14,17-eicosatrienoyl CoA), both L-FABP isoforms showed very similar affinity for both saturated and unsaturated fatty acyl CoAs.

DISCUSSION

The intracellular fatty acid binding proteins are a diverse group of proteins as exemplified not only by the existence

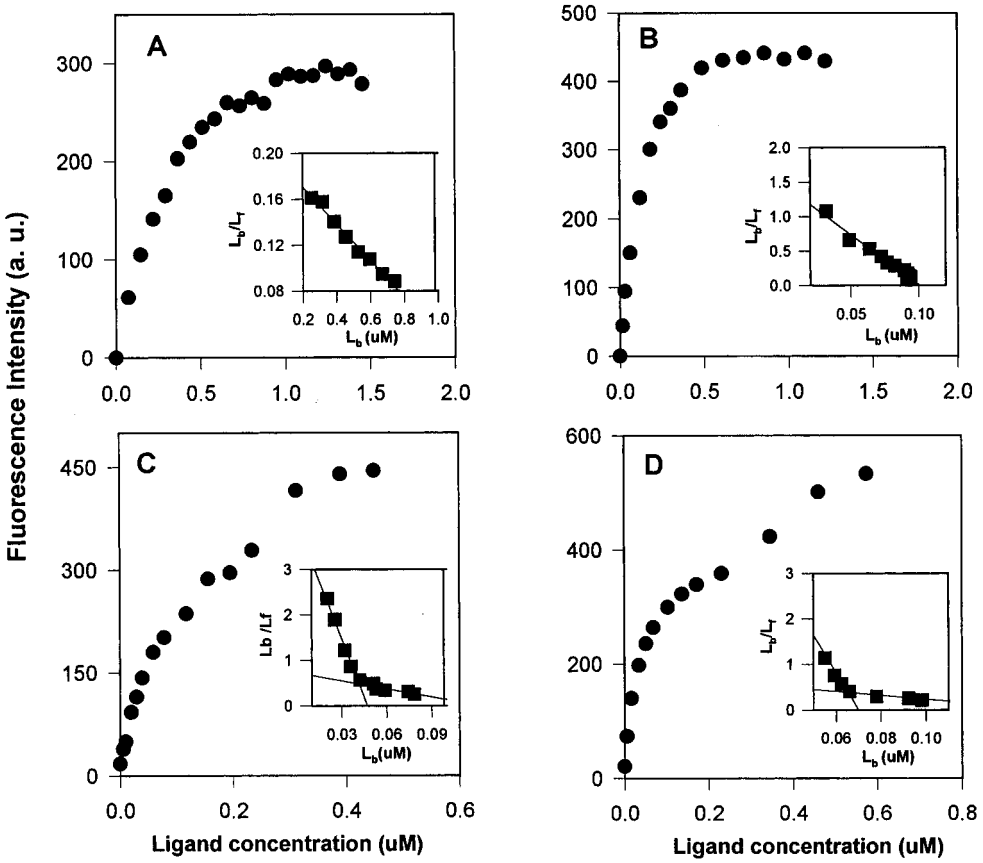

FIGURE 5: Titration of rat L-FABP with parinaric acids and parinaroyl CoAs. (A) Titration rat L-FABP (0.18 μM) with *cis*-parinaric acid (0−1.5 μM) as followed by the increase in fluorescence intensity. Excitation at 310, emission at 416 nm. Inset: Scatchard plot of the titration of rat L-FABP with *cis*-parinaric acid. (B) Titration of rat L-FABP (0.18 μM) with *trans*-parinaric acid (0−1.5 μM). $\lambda_{ex} = 310$, $\lambda_{em} = 416$ nm. Inset: Scatchard plot of the titration of rat L-FABP with *trans*-parinaric acid. (C) Titration of rat L-FABP (0.18 μM) with *cis*-parinaroyl CoA (0−0.5 μM). $\lambda_{ex} = 310$, $\lambda_{em} = 416$ nm. Inset: Scatchard plot of the titration of rat L-FABP with *cis*-parinaroyl CoA. (D) Titration of rat L-FABP (0.18 μM) with *trans*-parinaroyl CoA (0−0.6 μM). $\lambda_{ex} = 310$, $\lambda_{em} = 416$ nm. Inset: Scatchard plot of the titration of rat L-FABP with *trans*-parinaroyl CoA.

Table 4: Displacement of *cis*-Parinaroyl CoA by Fatty Acids[a]

displacing ligand	chain length	double bonds	displacement (%) isoform **I**	isoform **II**
arachidic acid	C20	0	0	13 ± 5
stearic acid	C18	0	16 ± 6	33 ± 8
palmitic acid	C16	0	10 ± 5	23 ± 9
myristic acid	C14	0	22 ± 1	18 ± 7
lauric acid	C12	0	0	16 ± 2
n-decanoic acid	C10	0	0	0
n-octanoic acid	C8	0	0	0
n-hexanoic acid	C6	0	0	0
n-butyric acid	C4	0	0	0
acetic acid	C2	0	0	0
CoA	C0	0	0	0
docosohexanoic acid	C22	6	52±1	42±8
arachidonic acid	C20	4	67 ± 6	49 ± 4
11,14,17-eicosatrienoic acid	C20	3	56 ± 3	50 ± 15
linolenic acid	C18	3	45 ± 8	41 ± 2
linoleic acid	C18	2	60 ± 3	54 ± 8
oleic acid	C18	1	33 ± 2	26 ± 7
pamitoleic acid	C16	1	46 ± 1	47 ± 3
myristoleic acid	C14	1	27 ± 6	21 ± 3

[a] Proteins (0.05 μM) were pre-incubated with *cis*-parinaroyl CoA (0.15 μM), followed by addition of displacing fatty acid (1.5 μM) as described in Methods. Values present the mean ± SE ($n = 3-6$).

Table 5: Displacement of *cis*-Parinaroyl CoA by Fatty Acyl CoAs[a]

displacing ligand	chain length	double bonds	displacement (%) isoform **I**	isoform **II**
arachidoyl CoA	C20	0	91 ± 3	83 ± 3
stearoyl CoA	C18	0	87 ± 3	78 ± 1
palmitoyl CoA	C16	0	80 ± 4	76 ± 1
myristoyl CoA	C14	0	64 ± 2	60 ± 5
lauroyl CoA	C12	0	43 ± 1	44 ± 1
n-decanoyl CoA	C10	0	0	0
n-octanoyl CoA	C8	0	0	0
n-hexanoyl CoA	C6	0	0	0
n-butyryl CoA	C4	0	0	0
acetyl CoA	C2	0	0	0
CoA	C0	0	0	0
arachidonoyl CoA	C20	4	80 ± 1	74 ± 7
11,14,17-eicosatrienoyl CoA	C20	3	83 ± 1	69 ± 3
linolenoyl CoA	C18	3	63 ± 2	59 ± 5
linoleoyl CoA	C18	2	85 ± 1	80 ± 2
myristoleoyl CoA	C14	1	39 ± 5	43 ± 5

[a] Proteins (0.05 μM) were pre-incubated with *cis*-parinaroyl CoA (0.15 μM), followed by addition of displacing fatty acyl CoA (1.5 μM) as described in Methods. Values represent the mean ± SE ($n = 3-6$).

of unique types of FABPs (e.g., liver, heart, intestine, adipose, brain) but also by their expression as multiple isoforms. In contrast, macroheterogeneity as isoforms has not been observed with other cytosolic lipid binding protein families, i.e., sterol carrier protein-2, acyl CoA binding protein, phospholipid exchange proteins, and cellular retinol binding protein. While many significant advances have been made toward both structural and functional characterization of the native FABPs [reviewed in Hubbell et al. (1994), Frolov and Schroeder (1997); Schroeder et al. (1993, 1996, Paulussen and Veerkamp (1990), Matarese et al. (1989), and Sacchettini and Gordon (1993)], almost all of these studies

utilized FABPs that had not been resolved into isoforms. Thus, isoform heterogeneity may complicate interpretation of structural and functional properties of native FABPs. Almost nothing is known regarding the structure, ligand specificity, or other functions of FABP isoforms [reviewed in Matarese et al. (1989) and Sacchettini and Gordon (1993)]. One potential approach to resolving some of these issues is the direct comparison of isoform structures and ligand binding properties.

In the present work, rat liver L-FABP was resolved into two isoforms by standard anion exchange chromatographic techniques: 25% isoform **I** and 75% isoform **II**. As indicated in Methods, both isoforms appeared as single bands on SDS polyacrylamide gels and Western blots. In addition, Western blotting with specific antisera against other cytosolic lipid binding proteins in the same molecular weight range did not detect contamination in the L-FABP isoforms **I** and **II**. While these data were consistent with the isoforms being pure L-FABP, this did not preclude the possibility that the two isoforms might have been cross-contaminated with each other. However, if extensive cross-contamination of the two isoforms had occurred, then little difference would have been observed in isoelectric focusing patterns, structure, or ligand binding specificity between the isoforms. Instead, the two isoforms differed significantly in all three properties.

While the present report does not provide primary sequence data for the two rat liver L-FABP isoforms, recent data of others shed light on this issue. The primary amino acid sequence of the two L-FABP isoforms from bovine (Dormann et al., 1993) and rat (Sato et al., 1996) liver were recently resolved. The bovine L-FABP isoform heterogeneity was due primarily to amino acid exchange (two different mRNAs). In addition, a small portion of the lower p*I* bovine L-FABP was actually due to S-thiolation (glutathionylation) of the higher p*I* L-FABP. In contrast, the rat liver L-FABP isoforms did not display amino acid exchange, but had identical primary amino acid sequence. However, the lower p*I* L-FABP isoform from rat liver was S-thiolated with cysteine. It was suggested that the extent of the S-thiolation might be dependent on the redox state of the liver hepatocytes (Sato et al., 1996).

With regard to secondary or tertiary structure of the rat liver L-FABP isoforms, the circular dichroism and fluorescence data presented herein show for the first time that isoforms **I** and **II** differ significantly in their secondary and tertiary structure/conformation. Consistent with these structural differences detected by fluorescence and circular dichroism, it was recently observed that the cysteinylated rat liver L-FABP isoform displayed increased susceptibility to proteolysis by cathepsin B and cathepsin D (Sato et al., 1996). Finally, the binding of ligands such as oleic acid or oleoyl CoA to either Isoform led to a significant redistribution of their secondary structure components. This was consistent with both the isoform **I** and the isoform **II** undergoing conformational changes upon the ligand binding. Yet, the two isoforms differed in conformational responsiveness: isoform **I** responded to the presence of a bound fatty acid and fatty acyl CoA by increasing the α-helical content at the expense of the β-structure, while in the isoform **II** the α-helical fraction significantly declined after addition of fatty acid and remained unchanged in the presence of fatty acyl CoA. A similar decrease of α-helical content was also observed upon relipidation of the acidic isoform of native rat L-FABP with oleic acid (Li & Ishibashi, 1992). The conclusion that ligand binding alters L-FABP Isoform conformation is in agreement with the results of two-dimensional ^{1}H-NMR study on the ligand-induced conformational changes in native rat L-FABP (Li & Ishibashi, 1992) and in native bovine L-FABP (Schulenberg-Schell et al., 1988) upon fatty acid binding.

The data presented herein provide a detailed analysis of the ligand specificity of rat liver L-FABP isoforms. These findings are especially important in view of the presence of two fatty acid binding sites in L-FABP. In fact, in some reports of L-FABP binding fatty acids two K_d values have been resolved (Fukai et al., 1989; Miller & Cistola, 1993; Schulenberg-Schell et al., 1988). However, it was not known whether the two K_d values in the native L-FABP protein may be due to the presence of isoforms or to binding site heterogeneity within the same. The present data show that rat liver L-FABP isoforms **I** and **II** differ significantly in affinity for straight chain vs kinked chain fatty acyl CoAs, suggesting that this difference might account for the observation of two K_d values in fatty acyl CoA binding to native rat L-FABP. Limited earlier studies of rat liver isoform fatty acid binding specificity are contradictory. One study suggested that S-thiolation of rat liver L-FABP *in vivo* did not affect fatty acid binding (Sato et al., 1996). In contrast, another study suggested that S-thiolation of rat liver L-FABP *in vitro* decreased the K_d values for unsaturated, but not saturated, fatty acids (Hitomi et al., 1990). The present data show distinct differences in ligand binding by the two isoforms: isoforms **I** and **II** had 2.3- and 7.5-fold higher affinity for kinked chain (unsaturated) vs straight chain (saturated) fatty acids, respectively. The saturated fatty acids were able to displace bound fatty acyl CoA over a wider range of acyl chain length from isoform **II** than isoform **I** (Table 4). Fatty acyl CoAs were better displacers than fatty acids for isoform-bound fatty acyl CoAs. Finally, the two isoforms differed significantly in their affinity for straight chain, but not kinked chain, fatty acyl CoAs. These data suggest that rat liver L-FABP isoforms differ significantly in function with regard to ligand binding specificity.

The very high affinities of L-FABP and its isoforms for fatty acids (K_d values as low as 8 nM) obtained using the fluorescent parinaric acid binding assay were essentially the same as that determined by ADIFAB competition assays for stearic and oleic acids (Richieri et al., 1994). To our knowledge, these are the lowest K_d values ever reported for L-FABPs binding fatty acids (Peeters et al., 1989; Storch et al., 1989; Miller & Cistola, 1993; Rolf et al., 1995; Banaszak et al., 1994; DeMarco et al., 1993; Nemecz et al., 1991a,b; Paulussen & Veerkamp, 1990). Moreover, within the family of fatty acid binding proteins only the brain FABPs showed a comparable affinity (K_d values of ∼10−20 nM) for the NBD-labeled stearic acid (Myers-Payne et al., 1996a,b). It should be noted that in an earlier study with recombinant rat L-FABP more than 10-fold higher K_d values for the fluorescent parinarates were reported, 230−660 nM (Nemecz et al., 1991a), as compared to the present study, 8−60 nM. The reason for this discrepancy is in the former study free fatty acids were used instead of the sodium salts of fatty acids. As pointed out by Materese et al. (1989), the low solubility of the free fatty acids complicates the measurement of FABP affinities for fatty acids. For example, it is well known that the fatty acid salts have much higher solubility

in aqueous solution as compared to the free acids as shown by almost a 1000-fold difference in the respective values of the critical micellar concentration (Small, 1986; Pryde, 1979). In the present study, the maximal fatty acid concentration during the parinarate binding assay never exceeded 2 μM, far below the 2 mM critical micellar concentration for the C18 fatty acid sodium salt (Small, 1986; Pryde, 1979). Therefore, the measured K_d values reflected primarily an equilibrium between a fatty acid bound to a protein and an unbound fatty acid in a solution.

Finally, it should be noted that the absolute K_d values measured for the fatty acyl CoAs and isoform **I** (33 for *cis*-parinaroyl CoA and 14 nM for *trans*-parinaroyl CoA) were the lowest reported for the fatty acid binding proteins (Paulussen & Veerkamp, 1990). Furthermore, these K_d values were in the range of those reported for acyl CoA binding protein, ~6 nM, a protein that binds only fatty acyl CoAs (Faergeman et al. 1996). Isoform **II** also bound fatty acyl CoA at nanomolar affinity. Thus, at the concentrations of fatty acids and fatty acyl CoAs normally found in the cell [reviewed in Gossett et al. (1996)], both of these ligands may effectively compete for L-FABP binding sites. The fact, that the liver fatty acid binding protein and its isoforms have such a high affinity for fatty acyl CoAs is very important since it implicates a potential role of L-FABP in fatty acyl CoA metabolism. Indeed, L-FABP stimulates microsomal glycerol-3-phosphate acyltransferase to form phosphatidic acid as much as 18-fold (McCormack & Brecher, 1987; Burrier et al., 1987; Ockner et al., 1972; Bordewick et al., 1989; Hubbell et al., 1994; Jolly et al., 1997).

In summary, the present study presents for the first time the comparative structural and functional characteristics of rat L-FABP isoforms. One of the new exciting findings made in this study is that along with the high affinity of the isoforms for saturated and unsaturated fatty acids, these isoforms displayed even higher and isoform dependent affinity for the saturated and unsaturated fatty acyl CoAs. The two isoforms differed significantly in their ability to bind fatty acyl CoAs. The latter appeared due, at least partially, to the distinct secondary and tertiary structures of the apo- and holo-L-FABP isoforms. Whether the differential affinity of the two rat L-FABP isoforms for fatty acids translates into differential ability to stimulate microsomal phosphatidic acid formation or other metabolic processes utilizing fatty acyl CoAs remains to be determined.

REFERENCES

Baier, L. J., Sacchettini, J. C., Knowler, W. C., Eads, J., Paolisso, G., Tataranni, P. A., Mochizuki, H., Bennett, P. H., Bogardus, C., & Prochazka, M. (1995) *J. Clin. Invest. 95*, 1281.

Baier, L. J., Bogardus, C., & Sacchettini, J. C. (1996) *J. Biol. Chem. 271*, 10892.

Banaszak, L., Winter, N., Xu, Z., Bernlohr, D. A., Cowan, S., & Jones, T. A. (1994) *Adv. Protein Chem. 45*, 89.

Bartetzko, N., Lezius, A. G., & Spener, F. (1993) *Eur. J. Biochem. 215*, 555.

Bass, N. M. (1985) *Chem. Phys. Lipids 38*, 95.

Bass, N. M. (1988) *Int. Rev. Cytol. 111*, 143.

Bass, N. M. (1993) *Mol. Cell. Biochem. 123*, 191.

Borchers, T., & Spener, F. (1994) in *Current Topics in Membranes* (Hoekstra, D., Ed.) pp 261−294, Academic Press, Inc., Orlando, FL.

Bordewick, U., Heese, M., Borchers, T., Robenek, H., & Spener, F. (1989) *Biol. Chem. Hoppe-Seyler 370*, 229.

Bradford, M. (1976) *Anal. Biochemistry 72*, 248.

Buhr, M. M., Curtis, E. F., & Kakuda, N. S. (1994) *Cryobiology 31*, 224.

Burrier, R. E., Manson, C. R., & Brecher, P. (1987) *Biochim. Biophys. Acta 919*, 221.

Burton, P. B., Hogben, C. E., Joannou, C. L., Clark, A. G., Hsuan, J. J., Totty, N. F., Sorensen, C., Evans, R. W., & Tynan, M. J. (1994) *Biochem. Biophys. Res. Commun. 205*, 1822.

Cantor, C. R., & Schimmel, P. R. (1980) in *Biophysical Chemistry*, W. H. Freeman & Co., San Francisco, CA.

Carroll, S. L., Roth, K. A., & Gordon, J. I. (1990) *Gastroenterology 99*, 1727.

Clarke, S. D., & Armstrong, M. K. (1989) *FASEB J. 3*, 2480.

Das, D. K., Barua, P. K., & Jones, R. M. (1991) *Biochim. Biophys. Acta 1073*, 394.

Das, T., Sa, G., & Mukherjea, M. (1988) *Lipids 23*, 528.

Das, T., Sa, G., & Mukherjea, M. (1989) *Biochim. Biophys. Acta 1002*, 164.

Davidson, N. O., Ifkovits, C. A., Skarosi, S. F., Hausman, A. M. L., Llor, X., Sitrin, M. D., Montag, A., & Brasitus, T. A. (1993) *Lab. Invest. 68*, 663.

Dedukhova, V. I., Mokhova, E. N., Skulachev, V. P., Starkov, A. A., Arrigoni-Martelli, E., & Bobyleva, V. A. (1991) *FEBS Lett. 295*, 51.

DeMarco, A. C., Patterson, P. P., Cantrill, R. C., & Horrobin, D. F. (1993) *J. Nutr. Biochem. 4*, 515.

Dormann, P., Borchers, T., Korf, U., Hojrup, P., Roepstorff, P., & Spener, F. (1993) *J. Biol. Chem. 268*, 16286.

Dutta-Roy, A. K., Huang, Y., Dunbar, B., & Trayhurn, P. (1993) *Biochim. Biophys. Acta 1169*, 73.

Faergeman, N. J., Sigurskjold, B. W., Kragelund, B. B., Andersen, K. V., & Knudsen, J. (1996) *J. Biochem. 35*, 14118.

Feng, L., Hatten, M. E., & Heintz, N. (1994) *Neuron 12*, 895.

Frolov, A., & Schroeder, F. (1997) *Biochemistry 36*, 505.

Frolov, A., Cho, T. H., Billheimer, J. T., & Schroeder, F. (1996) *J. Biol. Chem. 271*, 31878.

Fukai, F., Kase, T., Shidotani, T., Nagai, T., & Katayama, T. (1989) *Biochem. Int. 18*, 1101.

Glatz, J. F. C., Vork, M. M., Cistola, D. P., & Van der Vusse, G. J. (1993) *Prostaglandins, Leukotrienes Essent. Fatty Acids 48*, 33.

Glatz, J., & Veerkamp, J. (1983) *Anal. Biochem 132*, 89.

Gossett, R. E., Frolov A. A., Behnke, W. D., Kier, A. B., & Schroeder, F. (1996) *Lipids 31*, 895.

Haunerland, N., Jagschies, G., Schulenberg, H., & Spener, F. (1984) *Hoppe-Seylers Z. Physiolog. Chem. 365*, 365.

Hitomi, M., Odani, S., & Ono, T. (1990) *Eur. J. Biochem. 187*, 713.

Hubbell, T., Behnke, W. D., Woodford, J. K., & Schroeder, F. (1994) *Biochemistry 33*, 3327.

Issemann, I., Prince, R., Tugwood, J., & Green, S. (1992) *Biochem. Soc. Trans. 20*, 824.

Jolly, C. A., Hubbell, T., Behnke, W. D., & Schroeder, F. (1997) *Arch. Biochem. Biophys.* (in press).

Ketterer, B., Tipping, E., Hackney, J. F., & Beale, D. (1976) *Biochem. J. 155*, 511.

Kurtz, A., Zimmer, A., Schnutgen, F., Bruning, G., Spener, F., & Muller, T. (1994) *Development 120*, 2637.

Li, M., & Ishibashi, T. (1992) *Biomed. Res. 13*, 335.

Lowe, J. B., Strauss, A. W., & Gordon, J. I. (1984) *J. Biol. Chem. 259*, 12696.

Matarese, V., Stone, R. L., Waggoner, D. W., & Bernlohr, D. A. (1989) *Prog. Lipid Res. 28*, 245.

McCormack, M., & Brecher, P. (1987) *Biochem. J. 244*, 717.

Miller, K. R., & Cistola, D. P. (1993) *Mol. Cell. Biochem. 123*, 29.

Miyozawa, S., & Hashimoto, T. (1979) *Shimshu Med. J. 27*, 288.

Myers-Payne, S., Fontaine, R. N., Loeffler, A. L., Hubbell, T., Pu, L., Rao, A. M., Kier, A. B., Wood, W. G., & Schroeder, F. (1996a) *J. Neurochem. 66*, 313.

Myers-Payne, S. C., Hubbell, T., Pu, L., Schnütgen, F., Borchers, T., Wood, W. G., Spener, F., & Schroeder, F. (1996b) *J. Neurochem. 66*, 1648.

Nemecz, G., Hubbell, T., Jefferson, J. R., Lowe, J. B., & Schroeder, F. (1991a) *Arch. Biochem. Biophys. 286*, 300.

Nemecz, G., Jefferson, J. R., & Schroeder, F. (1991b) *J. Biol. Chem. 266*, 17112.

Nikawa, J., Tanabe, T., Ogiwara, H., Shiba, T., & Numa, S. (1979) *FEBS Lett. 102*, 223.
Ockner, R. K., Manning, J. A., Poppenhausen, R. B., & Ho, W. K. (1972) *Science 177*, 56.
Ockner, R. K., Kaikaus, R. M., & Bass, N. M. (1992) *Prog. Clin. Biol. Res. 375*, 189.
Ockner, R. K., Kaikaus, R. M., & Bass, N. M. (1993) *Hepatology 18*, 669.
Oram, J. F., Wenger, J. I., & Neely, J. R. (1975) *J. Biol. Chem. 250*, 73.
Parasassi, T., Conti, F., & Gratton, E. (1984) *Biochemistry 23*, 5660.
Paulussen, R. J. A., & Veerkamp, J. H. (1990) in *Subcellular Biochemistry* (Hilderson, H. J., Ed.) pp 175−226, Plenum Press, New York.
Peeters, R. A., in 't Groen, M. A., de Moel, M. P., van Moerkerk, H. T., & Veerkamp, J. H. (1989) *Int. J. Biochem. 21*, 407.
Pignon, J., Bailey, N. C., Baraona, E., & Lieber, C. S. (1987) *Hepatology 7*, 865.
Pryde, E. H. (1979) in *Fatty Acids*, The American Oil Chemists' Society, Champaign, IL.
Raman, N., & DiRusso, C. C. (1995) *J. Biol. Chem. 270*, 1092.
Richieri, G. V., Ogata, R. T., & Kleinfeld, A. M. (1994) *J. Biol. Chem. 269*, 23918.
Rolf, B., Oudenampsen-Kruger, E., Borchers, T., Faergeman, N. J., Knudsen, J., Lezius, A., & Spener, F. (1995) *Biochim Biophys Acta 1259*, 245.
Sa, G., Das, T., & Mukherjea, M. (1989) *Exp. Lung Res. 15*, 619.
Sa, G., Das, T., & Mukherjea, M. (1993) *Mol. Cell. Biochem. 129*, 67.
Sacchettini, J. C., & Gordon, J. I. (1993) *J. Biol. Chem. 268*, 18399.
Sato, T., Baba, K., Takahaski, Y., Uchiumi, T., & Odani, S. (1996) *J. Biochemistry 120*, 908.
Schroeder, F., Jefferson, J. R., Powell, D., Incerpi, S., Woodford, J. K., Colles, S. M., Myers-Payne, S., Emge, T., Hubbell, T., Moncecchi, D., Prows, D. R., & Heyliger, C. E. (1993) *Mol. Cell. Biochem. 123*, 73.
Schroeder, F., Myers-Payne, S. C., Billheimer, J. T., & Wood, W. G. (1995) *Biochemistry 34*, 11919.
Schroeder, F., Frolov, A. A., Murphy, E. J., Atshaves, B. P., Pu, L., Wood, W. G., Foxworth, W. B., & Kier, A. B. (1996) *Proc. Soc. Exp. Biol. Med. 213*, 149.
Schulenberg-Schell, H., Schafer, P., Keuper, H. J., Stanislawski, B., Hoffmann, E., Ruterjans, H., & Spener, F. (1988) *Eur. J. Biochem. 170*, 565.
Sha, R. S., Kane, C. D., Xu, Z., Banaszak, L. J., & Bernlohr, D. A. (1993) *J. Biol. Chem. 268*, 7885.
Small, D. M. (1986) in *The Physical Chemistry of Lipids*, Plenium Press, New York.
Sorof, S. (1994) *Cancer Metastasis Rev. 13*, 317.
Spener, F., Unterberg, C., Borchers, T., & Grosse, R. (1990) *Mol. Cell. Biochem. 98*, 57.
Sreerama, N., & Woody, R. (1993) *Anal. Biochem. 209*, 32.
Stolowich, N. J., Frolov, A., Atshaves, B. P., Murphy, E., Jolly, C. A., Billheimer, J. T., Scott, A. I., & Schroeder, F. (1997) *Biochemistry 36*, 1719.
Storch, J., Bass, N. M., & Kleinfeld, A. M. (1989) *J. Biol. Chem. 264*, 8708.
Trulzsch, D., & Arias, I. M. (1981) *Arch. Biochem. Biophys. 209*, 433.
Unterberg, C., Borchers, T., Hojrup, P., Roepstorff, P., Knudsen, J., & Spener, F. (1990) *J. Biol. Chem. 265*, 16255.
Veerkamp, J. H. (1995) *Proc. Nutr. Soc. 54*, 23.
Veerkamp, J. H., & Maatman, R. G. (1995) *Prog. Lipid Res. 34*, 17.
Veerkamp, J. H., Van Kuppevelt, T. H., Maatman, R. G., & Prinsen, C. F. (1993) *Prostaglandins, Leukotrienes Essent. Fatty Acids 49*, 887.

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