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Interaction of Fatty Acids with Recombinant Rat Intestinal and Liver Fatty Acid-Binding Proteins

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Intestinal enterocytes contain two homologous fatty acid-binding proteins, intestinal fatty acid-binding protein (I-FABP)[2] and liver fatty acid-binding protein (L-FABP). Since the functional basis for this multiplicity is not known, the fatty acid-binding specificity of recombinant forms of both rat I-FABP and rat L-FABP was examined. A systematic comparative analysis of the 18 carbon chain length fatty acid binding parameters, using both radiolabeled (stearic, oleic, and linoleic) and fluorescent (*trans*-parinaric and *cis*-parinaric) fatty acids, was undertaken. Results obtained with a classical Lipidex-1000 binding assay, which requires separation of bound from free fatty acid, were confirmed with a fluorescent fatty acid-binding assay not requiring separation of bound and unbound ligand. Depending on the nature of the fatty acid ligand, I-FABP bound fatty acid had dissociation constants between 0.2 and 3.1 μM and a consistent 1:1 molar ratio. The dissociation constants for L-FABP bound fatty acids ranged between 0.9 and 2.6 μM and the protein bound up to 2 mol fatty acid per mole of protein. Both fatty acid-binding proteins exhibited relatively higher affinity for unsaturated fatty acids as compared to saturated fatty acids of the same chain length. *cis*-Parinaric acid or *trans*-parinaric acid (each containing four double bonds) bound to L-FABP and I-FABP were displaced in a competitive manner by nonfluorescent fatty acid. Hill plots of the binding of *cis*- and *trans*- parinaric acid to L-FABP showed that the binding affinities of the two sites were very similar and did not exhibit cooperativity. The lack of fluorescence self-quenching upon binding 2 mol of either *trans*- or *cis*-parinaric acid/mol L-FABP is consistent with the presence of two binding sites with dissimilar orientation in the L-FABP. Thus, the difference in binding capacity between I-FABP and L-FABP predicts a structurally different binding site or sites.

The fatty acid-binding proteins represent a class of hydrophobic ligand binding proteins whose role in intracellular lipid trafficking (1) and in affecting gene expression (2–6) is becoming an exciting area of focus. Their molecular biology (1, 7, 8), structure (9), and postulated function(s) (2, 10–12) have been extensively reviewed. These abundant proteins represent 3–6% of cell cytosol. However, their tissue distribution is not exclusive and, as shown for the intestinal enterocyte, multiple forms of fatty acid-binding proteins may be present in the same cell. The physiological significance of this multiplicity is unknown. Knowledge of the ligand specificity and of the structure of the fatty acid-binding site(s) of these proteins is important to understanding the role of the fatty acid-binding proteins in regulating lipid metabolism. Nevertheless, the tertiary structure of only one of these proteins, I-FABP, has been resolved (13) and comparative ligand-binding studies are few (14).

A variety of techniques have been used to examine the fatty acid-binding properties of the FABP's, possibly explaining the variation in reported affinities and stoichiometries. For example, it is generally agreed that I-FABP has only one binding site (11, 13, 14). In contrast, the stoichiometry of fatty acid binding to L-FABP has been variously reported as 1:1 (11, 15–19) or 2:1 (9, 14, 20) and depends upon the physical state of the unbound fatty acid and the sample pH (21).

This paper presents the results of a comparative study of the fatty acid-binding characteristics of the liver and intestinal FABPs using a series of 18 carbon chain length fatty acids. Recombinant fatty acid binding proteins were

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[2] Abbreviations used: L-FABP, liver fatty acid binding protein; I-FABP, intestinal fatty acid binding protein; *trans*-parinaric acid, 9*E*, 11*E*, 13*E*, 15*E*-octadecatetraenoic acid; *cis*-parinaric acid, 9*Z*, 11*E*, 13*E*, 15*Z*-octadecatetraenoic acid; SDS, sodium dodecyl sulfate.

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used to avoid possible contamination with other mammalian proteins. Two different fatty acid-binding methods were applied: (i) a classic method using ^{14}C-radiolabeled fatty acid (Lipidex method), and (ii) a new fluorescent fatty acid-binding assay using naturally fluorescent fatty acids, *cis*- and *trans*-parinaric acid, that do not contain bulky side chain constituents. The binding affinity was further characterized by displacing the fluorescent fatty acids with nonfluorescent fatty acids to obtain more information about the structural specificity of fatty acid binding.

MATERIALS AND METHODS

Materials. Lipidex-1000 was purchased from Packard Instrument Co. Inc. (Downers Grove, IL) and from Sigma Chemical Co. (St. Louis, MO). *trans*-Parinaric and *cis*-parinaric acids were obtained from Molecular Probes (Eugene, OR). [1-^{14}C]stearic acid (55.3 mCi/mmol) was obtained from New England Nuclear, (Boston, MA). [9,10-^{3}H(N)]-oleic acid (10 Ci/mmol), and [1-^{14}C]-linoleic acid (56 mCi/mmol) were obtained from Amersham Co. (Arlington Heights, IL). Stearic, oleic, and linoleic acid were obtained from Sigma Chemical Co. (St. Louis, MO).

Protein purification. The recombinant fatty acid binding proteins were purified from *E. coli* host strains carrying plasmid pJBL2 encoding L-FABP or plasmid pIFABPexp6 encoding I-FABP using procedures identical to those described earlier by Lowe *et al.* (14). Purified FABPs were delipidated as described (14, 15) prior to use in binding studies. Purity of the proteins was determined by SDS–polyacrylamide gel electrophoresis also as described earlier (14). Protein concentration was determined by Lowry (22) or Bradford (23) protein assays and corrected according to amino acid analysis. The Bradford protein assay overestimates L-FABP 1.69-fold (14) and I-FABP 1.07-fold while the method of Lowry overestimates L-FABP and I-FABP 1.70-fold and 1.16-fold, respectively.

Radiolabeled fatty acid binding. Purified delipidated FABPs were used in fatty acid-binding assays. The fatty acid-binding assay with Lipidex-1000 (15) was carried out as described by Lowe *et al.* (14) except that the incubation temperature of the samples was lowered to 25°C. In a total assay volume of 1 ml 10 mM potassium phosphate buffer (pH 7.4) the FABP concentration was varied between 0.26 and 0.40 μM. The assay mixture contained 40 μl (5 μCi/ml in 25% EtOH) labeled fatty acid, 960 μl buffer, with or without protein. Fatty acids were added to the assay tubes as 50% ethanol solutions such that the final concentration ranged from 0.1 to 10 μM. The final ethanol concentration was maintained the same in each tube and did not exceed 1%. This level of ethanol was similar to that used in previous studies. After the incubation at 25°C for 20 min the tubes were chilled on ice for 10 min. Continuously stirred Lipidex-1000 50% (v/v) buffer suspension (150 μl) was added to the sample followed by vigorous stirring and incubation for 10 min at 4°C. The assay tubes were centrifuged at 10,000*g* for 4 min at 4°C. A 400-μl aliquot of the supernatant was removed and subjected to scintillation counting using a LS 7000 (Beckman Inc, Fullerton, CA) scintillation counter. Blank assays (no added FABP) were performed in parallel for each concentration of each fatty acid. Radioactivity in a 400-μl aliquot of blank supernatant was subtracted from the amount of radioactivity present in a 400-μl aliquot of FABP-containing supernatant.

$$\text{Net \% bound cpm} = \frac{2.875*\text{FABP spn cpm}}{\text{total assay cpm}} - \frac{2.875*\text{no FABP blank spn cpm}}{\text{total assay cpm}}, \quad [1]$$

where spn refers to supernatant and the constant 2.875 reflects the aliquot volume correction. The data were plotted as a Scatchard plot, with fatty acid concentration on the *X* axis, and "bound" over "free" on the *Y* axis. Bound is represented by net percentage bound cpm multiplied by total picomoles of fatty acid in the assay. Free is total picomoles fatty acid in the assay minus bound picomoles fatty acid.

Fluorescent fatty acid binding. Fluorescent ligand-binding measurements were carried out in a manner analogous to that described earlier for retinol binding to retinol-binding protein (24, 25) with the following modifications. In a final volume of 2 ml the incubation mixture contained 0.26–0.40 μM FABP and 0.1–10 μM *cis*- or *trans*-parinaric acid in 10 mM potassium phosphate buffer (pH 7.4). Each fatty acid concentration had an appropriate control without protein, the fluorescence intensity of which was subtracted from the total fluorescence. Fluorescence intensity was determined after a 10-min equilibration time. It was observed that all fluorescence changes were complete within this time period. Excitation was at 324 nm, using a 450-W Xe-Arc lamp as a light source in a SLM 4800 spectrofluorometer (SLM Instruments, Champaign, IL). Fluorescence emission was recorded through a GG-375 sharp cutoff filter (Janos Technology Inc., Townshend, VT) in order to eliminate highly polarized scattered light. The instrument sensitivity was adjusted to the maximal intensity of the sample at the equilibrium condition. The ethanol concentration in the cuvette did not exceed 1%.

Calculation of protein–ligand complex [EL]. The concentration of protein–ligand complex, free FABP, and free ligand were calculated from the fluorescence intensity changes (F) upon fluorescent fatty acid binding to FABP (25) applied as follows: In the concentration range 0–3 μM absorbance at 324 nm of the samples was <0.15 O.D. At 5 and 10 μM fluorescent fatty acid, fluorescence intensity values were corrected for the inner filter effect. The total fluorescence for the cuvette with *cis*- or *trans*-parinaric acid and FABP is equal to

$$F = f_E[E] + f_L[L] + f_{EL}[EL] \quad [2]$$

and

$$\Delta F = -f_E[E_0 - E] - f_L[L_0 - L] + f_{EL}[EL]. \quad [3]$$

Considering that

$$[E_0 - E] = [L_0 - L] = [EL] \quad [4]$$

then

$$\Delta F = [EL](f_{EL} - f_E - f_L), \quad [5]$$

where $[E_0]$ and $[L_0]$ are the total concentration of FABP and ligand, respectively; $[E]$, $[L]$, and $[EL]$ are concentration of free protein, free ligand, and FABP–ligand complex, respectively; f_E, f_L, and f_{EL} are characteristic fluorescence signals for each parameter, respectively. Since f_E at 324 nm is negligible ($f_E \ll f_L < f_{EL}$),

$$[EL] = \Delta F/(f_{EL} - f_L). \quad [6]$$

The dissociation constant K_d can be expressed for a protein with one binding site (26)

$$\frac{1}{K_d} = [L_0]\left(\frac{[E_0]}{[EL]} - 1\right) - [E_0]\left(1 - \frac{[EL]}{[E_0]}\right). \quad [7]$$

The saturated fraction, defined as $[EL]/[E_0]$, is equal to $\Delta F/\Delta F_{max}$. Upon substituting and rearranging, the following expression is obtained

$$K_d/(1 - (\Delta F/\Delta F_{max})) = ([L_0]/(\Delta F/\Delta F_{max})) - n[E_0]. \quad [8]$$

A plot of $1/(1-(\Delta F/\Delta F_{max}))$ versus $[L_0]/(\Delta F/\Delta F_{max})$ is linear, where the abscissa intercept is $[E_0]$ and the slope is equal to $1/K_d$. Equations [7] and [8] were applied to the I-FABP binding equilibria which involves only one binding site. In the case of L-FABP, the presence of two fatty acid-binding sites were indicated by the radiolabeled competition binding studies, and so we applied an alternative, more general set of equations to examine the question of cooperativity. In the case of two binding sites, the total ligand concentration in the system is

$$[L_0] = [EL_1] + 2[EL_2] + [L] \quad [9]$$

and for the bound ligand, we can write

$$[L_0] - [L] = [EL_1] + 2[EL_2], \quad [10]$$

where $[EL_1]$ is the protein bound with one ligand, $[EL_2]$ the protein with 2 ligands, and $[E_0]$ and $[L_0]$ are the total protein and ligand concentrations, respectively. The fractional saturation (ϕ) in the case of multiple binding sites can be described by the ratio of occupied sites to the total possible sites with the following assumptions: (i) The binding sites have similar K_d's. (ii) The quantum yield (F_b) is equal for each binding site. (iii) The fluorescence is additive when more than one binding site is occupied (i.e., no self-quenching between ligands).

$$\phi = \frac{[EL_1] + 2[EL_2]}{2([EL_1] + [EL_2] + [E])} = \frac{[L_0] - [L]}{2[E_0]}. \quad [11]$$

The fraction of ligand bound (f) can described,

$$f = \frac{[EL_1] + 2[EL_2]}{[L_0]}. \quad [12]$$

The relative fluorescence intensity of the sample (F_{rel}) is the sum of the fluorescence yield of free ligand ($F_f[L]$) plus the fluorescence yield of both the partially and fully bound ligands, $F_b([EL_1] + [EL_2])$,

$$(F_{rel} - F_f[L])/F_b = [EL_1] + 2[EL_2]. \quad [13]$$

The saturation of the binding sites can be described by ϕ, the fractional saturation.

$$\phi = \frac{F_{rel} - F_f[L]}{F_b} \Big/ 2[E_0] \quad [14]$$

Replacing Eq. [13] into Eq. [11] and multiplying by $1/[L_0]$ we obtain

$$\phi = \frac{F_{rel}/[L_0] - F_f([L]/[L_0])}{2F_b\frac{[E_0]}{[L_0]}}, \quad [15]$$

which can be reduced to

$$\phi = \frac{f[L_0]}{2[E_0]}. \quad [16]$$

The fractional saturation of the protein binding sites (ϕ) can be related in a straightforward manner (27) to the amount of free ligand [L].

$$\frac{\phi}{(1-\phi)} = \frac{[L]^j}{K_d}. \quad [17]$$

The equation can be rearranged and put into logarithmic form to yield the familiar Hill plot

$$\log\frac{\phi}{1-\phi} = \log\frac{1}{K_d} + j\log[L], \quad [18]$$

where j is the characterization factor of binding [$j = 1$, indicating single binding, $j > 1$, indicating positive cooperativity, and $j < 1$, indicating multiple binding or negative cooperativity, (27)] and K_d is the dissociation constant. In the L-FABP case a mean K_d value was used.

Displacement of fluorescent fatty acids. The displacement of *cis*- and *trans*-parinaric acid by nonfluorescent fatty acids was determined using the percentage of the fluorescent intensity ratio, F/F_{max}, where F represents the fluorescence intensity observed when the competitor (nonfluorescent fatty acid) was present, and F_{max} represents the observed fluorescence intensity in the absence of competing nonfluorescent fatty acid. The inhibitor constants, K_i, were determined from a nonlinear fit to a hyperbolic plot of $F_{max} - F$ versus the competitor concentration $[L_i]$, using the equation; $(F_{max} - F) = C^*[L_i]/(K_i + [L_i])$, where K_i is equal to $[L_i]$ at $F_{max}/2$, and C is a fitting constant equal to the best fit prediction of F_{max}. The fluorescence at each competitor concentration was measured in separate tubes after 10 min equilibration, and it was observed that all fluorescence changes were complete within this time period.

RESULTS

Radiolabeled Fatty Acid Binding

A series of 18 carbon chain length of fatty acids with increasing number of double bonds was used to examine fatty acid binding to L-FABP and I-FABP. Both radiolabeled and fluorescent (see below) fatty acids were used. Radiolabeled fatty acid binding to FABPs was examined using the Lipidex-1000 method. The preference of L-FABP and I-FABP for binding of three radiolabeled fatty acids (stearic, oleic, and linoleic acid) containing 0, 1, and 2 double bonds, respectively, was determined. Multiple assays ($n = 3$–7) were performed with eight or more different fatty acid concentrations for each fatty acid. The results of the binding studies are presented as Scatchard plots in Fig. 1. Scatchard plots of stearic acid (Fig. 1A), oleic acid (Fig. 1B), and linoleic acid (Fig. 1C) binding to L-FABP or I-FABP were linear. Both FABPs exhibited saturable radiolabeled fatty acid binding, with affinities (K_d) between 2.0 and 3.1 μM for I-FABP (Table I) and between 1.4 and 2.6 for L-FABP (Table II). The affinities of the L-FABP as compared to I-FABP for the same fatty acid (0, 1, or 2 double bonded) did not differ significantly (Table I versus Table II). However, L-FABP (Table I) and I-FABP (Table II) both showed a significantly ($p < 0.05$, $n = 3$–7) higher affinity for the unsaturated fatty acids, oleic acid, and linoleic acid, as compared to the saturated fatty acid, stearic acid.

Fluorescent Fatty Acid Binding

A fluorescent fatty acid-binding assay not requiring separation of FABP-bound from unbound fatty acid was developed. *trans*-Parinaric and *cis*-parinaric acid were chosen for this purpose because of the following proper-

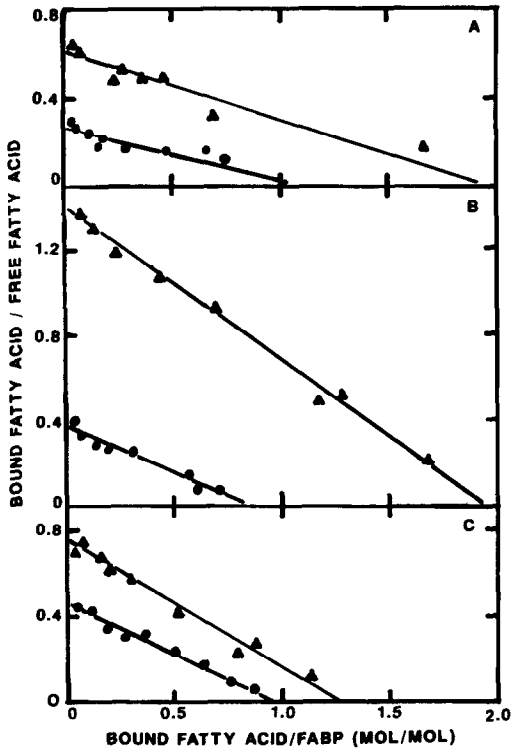

FIG. 1. Scatchard analysis of the binding of radiolabeled fatty acids to I-FABP and L-FABP. Radiolabeled fatty acid binding to I-FABP (circles, lower line) and L-FABP (triangles, upper line) was measured by the Lipidex-1000 method as described under Materials and Methods: A, [1-^{14}C]stearic acid (18:0) binding; B, [9,10-^{3}H(N)]oleic acid (18:1) binding; C, [1-^{14}C]linoleic acid (18:2) binding. Lines were fit to this data using the Graphpad (Institute of Scientific Information, Philadelphia, PA) linear regression program. In each panel values represent the mean (n = 3) at each fatty acid concentration.

ties: (i) like the radiolabeled fatty acids in the preceding section, they are 18 carbon fatty acids, (ii) they are natural products, (iii) they do not contain bulky side chains, and (iv) they can be used as probes for saturated straight chain fatty acids (*trans*-parinaric acid) and unsaturated "kinked" chain fatty acids (*cis*-parinaric acid). The fluorescence intensity of parinaric acids in aqueous solution is very weak. However, when bound to FABP the parinaric acids are excellent fluorophores (Fig. 2). Fluorescence intensity of bound *trans*-parinaric acid (Fig. 2A) and bound *cis*-parinaric acid (Fig. 2B) increases with increasing fluorescent fatty acid concentration for both L-FABP and I-FABP. However, the maximum intensity of *trans*-parinarate fluorescence bound to L-FABP was 2.5 times that bound to I-FABP (Fig. 2A). In the case of *cis*-parinaric acid the maximal fluorescence intensity in L-FABP was 3.4 times that in I-FABP. There was no indication of self-quench between the two parinaric acid molecules bound to L-FABP when either *cis*- or *trans*-parinaric acid was used.

The higher maximal fluorescence intensity of the parinaric acids when bound to L-FABP as compared to I-FABP could be due either to a difference in number and/or affinity of binding sites, cooperativity between the binding sites (in the case of L-FABP), or a difference in binding site environment. Radioligand-binding experiments described above showed that the L-FABP had two binding sites and the I-FABP had one binding site. Binding curves for the fluorescent *trans*-parinaric acid and *cis*-parinaric acid to I-FABP (Fig. 3) and to L-FABP (Fig. 4) were also consistent with two and one fatty acid-binding sites, respectively. Thus a two-fold difference in number of binding sites would account for a 2-fold, but not a 2.5-fold (*trans*-parinaric acid) or a 3.4-fold (*cis*-parinaric acid), difference in fluorescence intensity of the same fluorescent fatty acid bound to L-FABP versus I-FABP. The dissociation constants for the L-FABA bound fatty acids were so similar that separate values could not be determined. Cooperativity between the two binding sites in L-FABP was examined as follows: The data in Fig. 2 support the idea that the maximum observed fluorescence change corresponds to complete occupation of the binding sites. Hill plots of the binding data provided values of (ϕ) plotted in Fig. 4 which shows that the range of saturation shown as the change in (ϕ) was between 0.9 (low occupancy) and 0.1 (close to complete saturation). The slope of these Hill plots (j) is the apparent order of the binding (see methods) which in both the case of *cis*- and *trans*-

TABLE I

Comparative Analysis of the Fatty Acid Binding Affinities of Delipidated, *E. coli*-Derived I-FABP

Fatty acids	Method	K_d (μM)	B_{max} (μM *FA*/μM protein)
		Radiolabeled fatty acids	
Stearic acid (18:0)	Lipidex	3.1 ± 0.34	1.03 ± 0.18 (n = 5)
Oleic acid (18:1)	Lipidex	2.07 ± 0.27[a]	0.88 ± 0.05 (n = 4)
Linoleic acid (18:2)	Lipidex	2.09 ± 0.17[a]	1.07 ± 0.08 (n = 7)
		Fluorescent fatty acids	
cis-Parinaric acid	Fluorescence	0.23 ± 0.03[a,b]	0.96 ± 0.23 (n = 4)
	Lipidex	0.38	0.9
trans-Parinaric acid	Fluorescence	0.66 ± 0.19[a,b]	1.19 ± 0.07 (n = 4)
	Lipidex	1.04	1.05

Note. The Lipidex-1000 assay and the fluorescence assay were used to determine the binding affinity of radiolabeled and fluorescent fatty acids to I-FABP as described under Materials and Methods. The values for B_{max} were derived from the intercepts of the line in abscissa (Fig. 1 for radiolabeled fatty acids; Fig. 3 for fluorescent fatty acids). The slopes of the lines were used to calculate affinities as described under Materials and Methods. Values represent the means ± SEM (n = 3–7).

[a] $p < 0.05$ as compared to stearic acid.

[b] $p < 0.05$ as compared to *cis*-parinaric acid.

TABLE II

Comparative Analysis of the Fatty Acid-Binding Affinities of Delipidated, *E. coli*-Derived L-FABP

Fatty acids	Radiolabeled fatty acids	
	K_d (μM)	B_{max} (μM *FA*/μM protein)
Stearic acid (18:0)	2.59 ± 0.24	1.79 + 0.25
Oleic acid (18:1)	1.45 ± 0.23[a]	2.00 ± 0.06
Linoleic acid (18:2)	1.93 ± 0.13[a]	1.27 ± 0.11
	Fluorescent fatty acids	
	K_d	Hill slope
cis-Parinaric acid	0.72 ± 0.065	0.485
trans-Parinaric acid	1.11 ± 0.15	0.56

Note. The Lipidex-1000 and fluorescence assays were used to determine the binding affinity of radiolabeled and fluorescent fatty acids to L-FABP, respectively. The values for B_{max} were derived from the intercepts of the line in abscissa (Fig. 1 for radiolabeled fatty acids; Fig. 4 for fluorescent fatty acids). The slopes of the lines were used to calculate affinities as described under Materials and Methods. Values represent the means ± SEM (n = 3 or 4).

[a] $p < 0.05$ as compared to stearic acid.

parinaric acid was close to 0.5, implying that the multiple binding occurs without cooperativity. These data are consistent with the presence of only one fatty acid binding site in I-FABP, and two fatty acid binding sites in L-FABP. In addition, the environment of the fatty acid binding site in L-FABP differed significantly from that of I-FABP.

The fluorescent *cis*- and *trans*-parinaric acids were used as structural model molecules for unsaturated and saturated fatty acids, respectively, in order to examine the fatty acid preference of I-FABP. *cis*-Parinaric and *trans*-parinaric acid bound to I-FABP with K_d's of 0.23 and 0.66 ($p < 0.05$), respectively, thereby confirming the preference of I-FABP for unsaturated (*cis*- double bonds) fatty acids noted above with the radiolabeled fatty acids (Table I). In addition, the fluorescent fatty acids both indicated the presence of only one binding site per I-FABP molecule.

It is noteworthy that both of the fluorescent fatty acids were bound much more tightly to I-FABP (Table I) and L-FABP (Table II) than were any of the radiolabeled fatty acids. Since the chain length of the fluorescent and radiolabeled fatty acids was the same, the tighter binding was due either to a difference in assay procedures (Lipidex-1000 versus fluorescence) or to the presence of four double bonds in the fluorescent fatty acids as compared to zero, one, or two double bonds in the radiolabeled fatty acids. As shown in Table I, when the binding of *cis*- or *trans*-parinaric acid to I-FABP was measured by the Lipidex-1000 method (requiring separation of FABP-bound from unbound fatty acid), essentially similar binding parameters were obtained as with the fluorescence technique (not requiring separation of FABP-bound from unbound fatty acid). Thus, the greater number of double bonds appeared responsible for the tighter binding of the fluorescent as compared to the radiolabeled fatty acids.

Basically similar observations were made with fluorescent fatty acid binding to L-FABP as with I-FABP, with one exception. The L-FABP apparently bound two fluorescent fatty acid molecules per molecule of L-FABP (Table II). Because of the presence of two fatty acid-binding sites in the L-FABP, the interaction of the fluorescent fatty acids with L-FABP would be expected to result in maximal fluorescence intensity about 2-fold higher than binding by I-FABP. However, the measured fluorescence intensity of the *trans*- and *cis*-parinaric acids was 2.5- and 3.4-fold higher in the L-FABP than in the I-FABP (Fig. 2). It is therefore apparent that these proteins differed not only in the number of binding sites but also in the properties of the binding site itself.

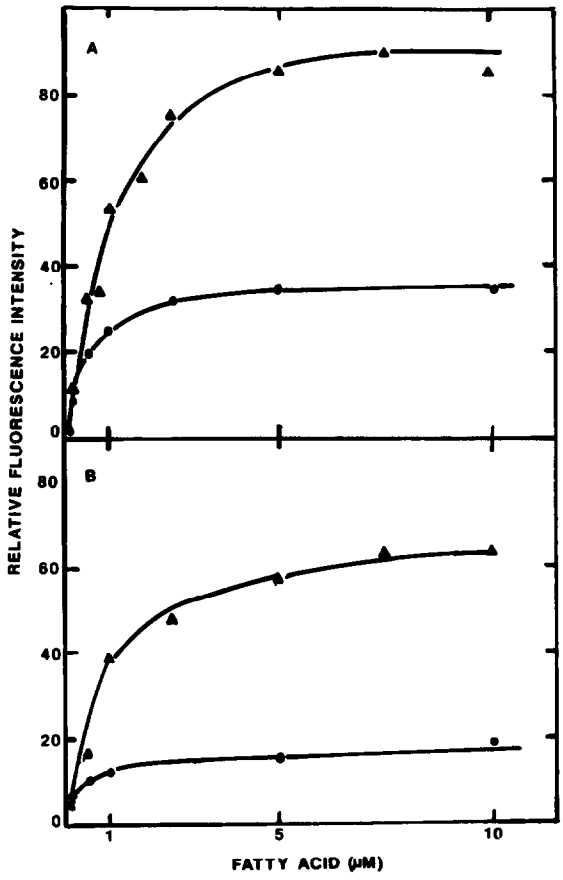

FIG. 2. Interaction of fluorescent fatty acids with I-FABP and L-FABP. Binding of *trans*- (A) and *cis*- (B) parinaric acid to I-FABP (circles, bottom curve) and L-FABP (triangles, top curve) was monitored by increase in fluorescence intensity as described under Materials and Methods.

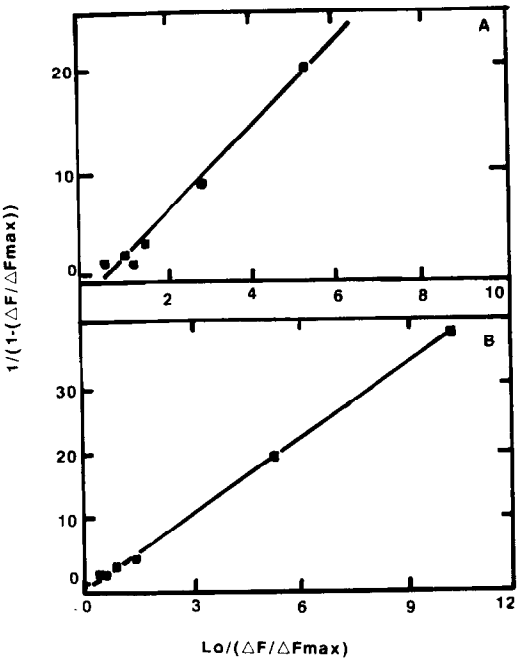

FIG. 3. Binding analysis of fluorescent fatty acids to I-FABP. *trans*-Parinaric acid (A) and *cis*- (B) parinaric acid binding to I-FABP was performed as shown in Fig. 2 and described under Materials and Methods. The data were transformed to linear least-square plots of $1/(1-\Delta F/\Delta F_{max})$ vs $[L_0]/(\Delta F/\Delta F_{max})$. The intercept on the abscissa is $[E_0]$ and the slope is equal to $1/K_d$. Values indicated represent the mean ($n = 3$) at each fatty acid concentration.

Displacement of Bound cis- and trans-Parinaric by Nonfluorescent Fatty Acids

Displacement of *cis*- and *trans*-parinaric acid bound to L-FABP and I-FABP was also used to study relative binding affinities of oleic and linoleic acid (Table III). When present in equimolar ratios (fluorescent/nonfluorescent fatty acids), oleic and linoleic acid were more efficient in displacing both *cis*- and *trans*-parinaric acid from I-FABP than from L-FABP (Table III). This is expected since I-FABP and L-FABP contain one and two bound fluorescent fatty acids, respectively. The competition with the nonfluorescent fatty acids of the binding of *cis*- or *trans*-parinaric acid for the I-FABP-binding site (Table IV) followed a similar trend as that found for L-FABP. Competition studies between the fluorescent fatty acids and stearic acid did not yield reliable data because of the very low solubility of the monomer in the assay conditions (ca. 2.5 μM), and the inability of stearic acid at this concentration to compete with the binding of the fluorescent fatty acid.

DISCUSSION

With few exceptions, comparative ligand binding studies of FABPs have not been reported. Comparisons of relative K_d's and stoichiometries obtained in different laboratories are complicated by differences in assay methodology, source and purity of FABP, and the relative presence of endogenous bound ligand(s), possibly explaining the variation in reported affinities and stoichiometries. In an earlier study the Lipidex-1000 method was used to compare fatty acid binding to L-FABP versus I-FABP, a series of fatty acids varying both in degree of unsaturation and in fatty acid chain length were compared (14). Such comparisons are, however, of limited value due to the dependence of fatty acid binding on chain length as well as unsaturation (11). In addition, the separation process involved in the Lipidex 1000 assay may result in removal of weakly bound fatty acid, thereby lowering the apparent number of fatty acid molecules bound as well as altering the K_d. In this assay, the FABP and fatty acid are incubated at 25°C, resulting in an equilibrium between FABP-bound and unbound fatty acid. The sample containing FABP-bound and unbound fatty acid is subsequently cooled to 0°C followed by addition of Lipidex-1000. Thus, two factors could alter fatty acid binding characteristics in this method: First, at 0°C the fatty acid–FABP equilibrium may be perturbed. Second, the Lipidex-1000 and FABP compete for binding of free fatty acids. The implicit assumption with the Lipidex-1000 assay is that these possibilities are negligible. The Lipidex binding assay thus involves a set of competition equilibria between the Lipidex, the protein, and the free ligand (as monomers

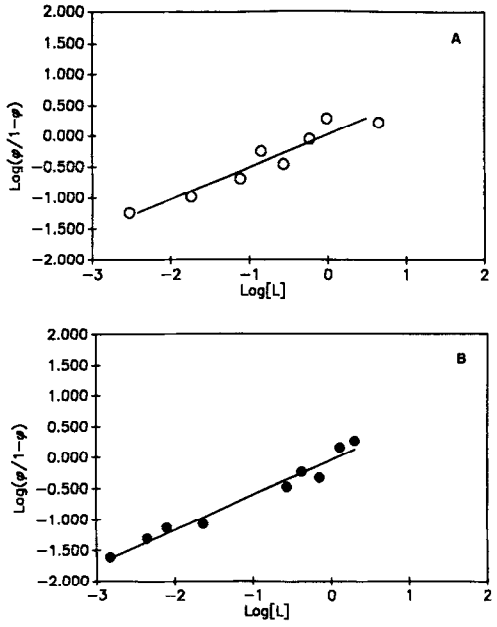

FIG. 4. Binding analysis of fluorescent fatty acids to L-FABP. *trans*-Parinaric acid (A) and *cis*- (B) parinaric acid binding to L-FABP was perfomed as described under Materials and Methods; data were collected as described in the legend to Fig. 2. The data were transformed using the form $\log(\phi/(1-\phi))$ vs $\log[L]$. The slope of the curve, j, is the apparent order of the binding. Values indicated represent the mean ($n = 3$) at each fatty acid concentration.

TABLE III

Displacement of I-FABP or L-FABP Bound Fluorescent *cis*- or *trans*-Parinaric Acid by Nonfluorescent Fatty Acids

	Displacement of fluorescent fatty acids (%)	
Fatty acid	*cis*-Parinaric acid	*trans*-Parinaric acid
	I-FABP	
Oleic acid	63.98 ± 6.53	27.71 ± 2.27
Linoleic acid	29.34 ± 0.69	30.96 ± 2.0
	L-FABP	
Oleic acid	13.46 ± 2.69	22.04 ± 5.63
Linoleic acid	11.82 ± 1.65	11.34 ± 1.38

Note. Displacement of FABP bound *cis*- or *trans*-parinaric acid was determined as described under Materials and Methods. In each assay, concentrations were 0.4 μM FABP, 1 μM *cis*- and *trans*-parinaric acid, and 1 μM fatty acid (ethanol 1%) in 2 ml 10 mM K-phosphate (pH 7.4). Displacement (decrease of fluorescence) is given as a percentage of the control fluorescence. Results are means of three experiments with S.E.

and as micelles). There are also a number of factors such as the solubility of the ligand and its intrinsic affinity for Lipidex which can influence the value of the apparent binding constant. Thus the useful concentration range for this assay is limited. In this regard it is a clear advantage of the fluorescence assay that the equilibria involving the Lipidex are eliminated. Consequently in work presented herein pure recombinant L-FABP and recombinant I-FABP were delipidated and binding of fatty acids was compared using a series of 18 carbon fatty acids and two independent binding assays.

Number of Fatty Acid-Binding Sites in I-FABP and L-FABP

The Lipidex-1000 fatty acid binding assay indicated that I-FABP has a single fatty acid-binding site. The fluorescent *trans*- and *cis*-parinaric acid-binding assay confirms this observation. These fatty acids also showed a single fatty acid-binding site in I-FABP. These observations are consistent with results of others using a variety of techniques including NMR and X-ray diffraction (11, 13, 14).

In contrast to the general agreement on the number of fatty acid binding sites in I-FABP, there is considerable uncertainty for the L-FABP. The stoichiometry of fatty acid binding by L-FABP has been variously reported as 1:1 (11, 15–19) or 2:1 (9, 14) and depends upon the physical state of the unbound fatty acid and the sample pH (21). This uncertainty may be due in part to the use of fluorescent fatty acid analogues that contain bulky side chains, e.g., anthroyloxy, pyrene, or dansyl (17–19). It has been reported that fatty acids substituted with such bulky groups prevent binding of a second fatty acid to L-FABP (28). In contrast, as shown in the present investigation, *cis*- and *trans*-parinaric acid used in a fluorescence binding assay both indicate the presence of two fatty acid binding sites in L-FABP. *cis*-Parinaric and *trans*-parinaric acid are fluorescent fatty acids that do not contain bulky reporter groups. Instead, they contain a series of four conjugated double bonds as part of the methylene chain.

The fluorescent fatty acids are also useful in regard to the tertiary orientation of the fatty acid-binding sites in the FABPs. Due to the presence of multiple binding sites in L-FABP the two fluorescent parinaric acid ligands could influence each other's optical signals, either by quench, or by changes in the binding environment of one as a result of the occupancy of the second (38, 39). The data presented herein indicate that the two bound fatty acids do not quench each other. Fluorescence plateaued with increasing parinaric acid concentration. If quenching had occurred upon occupancy of the second binding site, a decrease in fluorescence would have been observed. Moreover, other investigators using anthroyl-labeled fatty acids also did not observe quenching when the L-FABP binding sites were saturated (19). Thus, the two fatty acids bound to L-FABP cannot occupy the same binding pocket of the protein and be arranged in parallel within that pocket. Thus, the two binding sites of L-FABP most likely have an orthogonal orientation and/or are physically separated in the L-FABP. An earlier study using parinaric acid optical activity (circular dichroism) preliminarily concluded that the fatty acids occupy the same binding site (9). However, it is not known if both binding sites were occupied by the fluorescent fatty acids under the conditions of the circular dichroism measurements. Last, there was no indication that the quantum yield differs significantly between the fatty acids bound to the first site and that bound to the second. It is thus assumed (in Eq. [13]) that the two fluorescence fatty acids are reporting independently on the occupancy of their respective binding sites. In support of this, the curve in Fig.

TABLE IV

Competitive Inhibition of Fluorescent Fatty Acid Binding by I-FABP by Nonfluorescent Fatty Acids

	K_i (μM)	
Ligand	*cis*-Parinaric acid	*trans*-Parinaric acid
Oleic acid	0.39 ± 0.09	1.46 ± 0.40
Linoleic acid	1.50 ± 0.41	1.47 ± 0.32

Note. Competitive inhibition assays were performed in 2 ml K-phosphate buffer (pH 7.4) containing 0.4 μM I-FABP, 1 μM *cis*- or *trans*-parinaric acid, and 0.1 to 5 μM competitive inhibitor fatty acid. The inhibition of fluorescence intensity increase was measured. Inhibition constants (K_i) were calculated from the F_{max}–F binding hyperbola. Values represent the means ± SEM (n = 3).

2 shows a smooth concentration-dependent change upon the addition of either *cis*- or *trans*-parinaric acid, which is also consistent with close similarity of the quantum yields between the parinaric acid bound to the first and that bound to the second site.

The possibility of conformational changes in the fatty acid-binding proteins as a result of fatty acid binding cannot be ruled out on the basis of the data reported herein. X-ray crystal data on I-FABP show that I-FABP does not undergo conformational change upon fatty acid binding (13). Similar data on the effect of fatty acid binding on L-FABP conformation are not yet available. Nevertheless, the data presented herein indicate that if conformational changes do occur in L-FABP upon fatty acid binding, they do not appear to influence the binding affinity for the second fatty acid ligand, and there is no indication that the quantum yield of the first bound molecule of parinaric acid is influenced by the binding of the second.

Comparative Fatty Acid-Binding Specificity of L-FABP and I-FABP

As shown in the results section, two different binding assays using a series of 18 carbon radiolabeled (stearic, oleic, linoleic) or fluorescent (*cis*- and *trans*-parinaric) fatty acids indicated that I-FABP preferentially binds unsaturated fatty acids. The fatty acid binding affinities of I-FABP related to the number of double bonds were $4 > 2 > 1 > 0$. Most important, the fluorescence assay and the Lipidex-1000 assay (with either *cis*- or *trans*-parinaric acid) gave nearly identical results: the K_d's and the stoichiometry of binding (0.23 μM and 1:1) were not significantly different. This observation for the first time validates the Lipidex-1000 technique with a fluorescent technique that does not require separation of FABP bound fatty acid from unbound fatty acid. The only other reported study of the relative preference of I-FABP for specific fatty acids also showed a higher K_d for I-FABP binding the unsaturated fatty acid, oleate, as compared to the saturated fatty acid, palmitate. Interestingly, the fluorescent fatty acids, *cis*- and *trans*-parinaric acid, bound more tightly to I-FABP than any of the other fatty acids tested. These data would lead to the prediction that arachidonic acid, a nonfluorescent fatty acid with four double bonds would also bind with very high affinity to I-FABP. Unexpectedly, arachidonic acid bound to I-FABP about as well as the saturated fatty acid palmitate (14). Possibly the conjugated versus nonconjugated nature of the double bonds in parinarate versus arachidonate can account for the difference in expected results.

The fatty acid binding to L-FABP was investigated in a similar manner as for I-FABP. The L-FABP also showed a preferential binding for unsaturated fatty acids in the following order of double bonds: $4 > 2 > 1 > 0$. This preference for unsaturated fatty acids in *in vitro* assays is entirely consistent with results of some (14, 28, 29) but not all previous studies (11, 17, 30). Arachidonic acid (14) and *cis*-parinaric acid were bound with higher affinity to L-FABP than saturated fatty acids. Interestingly, several reports showed the existence of an L-FABP isoform highly enriched in arachidonic acid (11, 31).

Competitive Fatty Acid Binding Specificity to I-FABP or L-FABP

The preference of L-FABP and I-FABP for binding unsaturated fatty acids *in vitro* would lead to the prediction that these fatty acid-binding proteins should preferentially bind unsaturated fatty acids *in vivo*. Surprisingly, the composition of endogenous fatty acids bound to L-FABP and I-FABP expressed in *E. coli* growing at 42°C showed that 70 and 100% of the endogenous fatty acids bound to L-FABP and I-FABP, respectively, were saturated (14). However, *E. coli* grown at 37°C contain only 15–36% *cis*-unsaturated fatty acids (33), and *E. coli* grown at 42°C are expected to contain even less *cis*-unsaturated fatty acids. The endogenous bound fatty acid composition will be determined by both the relative affinities of these proteins for different fatty acids and the fatty acid milieu available for binding. Thus, the apparent discrepancy between these observations *in vitro* and the fatty acids bound endogenously (*in vivo*) to the L-FABP is explained by the much larger availability of saturated fatty acids in the *E. coli*. These findings are thus consistent with an approximately 1.5 to 2 times higher affinity for unsaturated fatty acids. In most cases a preponderance of unsaturated fatty acids is expected in mammalian tissues at 37°C. However, nutrition, endocrine, and other factors can affect this composition.

On the basis of several reports indicating a relative preference of L-FABP and not I-FABP, for unsaturated fatty acids, earlier investigators postulated the I-FABP functions primarily in cytoplasmic transport of intestinal luminally derived fatty acid while L-FABP was involved in the transport of plasma-derived fatty acids (34, 35). However, the data presented herein showed similar preference of L-FABP and I-FABP for unsaturated fatty acids. In addition, immunohistochemical results of others (36) are also not consistent with this hypothesis.

Structure of the Fatty Acid-Binding Site

The tertiary structure of I-FABP and the binding of palmitate within this structure has been investigated extensively (13, 21, 32). These investigators concluded that I-FABP has a clam shell-like structure with a 9×30-Å gap forming the binding site that accommodates only a single fatty acid plus 7 solvent molecules (holo-I-FABP) or 13 solvent molecules in the absence of fatty acid (apo-I-FABP) (13, 32). The fatty acid, palmitate, assumed a bent configuration between carbon 2 and 3 with a left-handed helical conformation for the rest of the acyl chain.

The fluorophore of *cis*- and *trans*-parinaric acids is a rigid conjugated tetraene series of double bonds extending from carbon 9 to carbon 15. Thus, because they can still bend between carbons 2 and 3, the parinaric acids are both easily accommodated into the binding site of I-FABP. Although the binding of fluorescent fatty acids with bulky attached fluorophores to I-FABP has not been reported, the small size of the fatty acid-binding pocket of I-FABP [one saturated fatty acid plus about 7 solvent molecules, (13)] is expected to be insufficient for binding these bulky fatty acids.

The tertiary structure of the fatty acid-binding site of L-FABP has not been reported. However, several observations indicate that the structure of the fatty acid-binding sites in L-FABP may be quite different from that of I-FABP. First, the L-FABP fatty acid-binding site(s) accommodate(s) two rather than one fatty acid. Other data indicate that under some circumstances even a third fatty acid binding site may be accommodated in L-FABP (21). Second, as pointed out earlier (26), the fatty acid-binding site of L-FABP is unable to accommodate more than one fatty acid with a bulky fluorescence side chain. Third, in a recent study a complex induced circular dichroism for *trans*-parinaric acid bound to L-FABP was observed (37). These investigators concluded that L-FABP has two *trans*-parinaric acid molecules oriented in parallel in the same binding site. However, other investigators examining the structure of the phosphatidylcholine transfer protein phospholipid binding site showed that if the *cis*-parinaric acids attached to a phosphatidylcholine molecule were oriented in parallel self-quenching of fluorescence was observed (38, 39). Fourth, the displacement experiments provided additional information that the L-FABP binding site(s) structurally can differ from that of I-FABP. Fifth, structural data indicate that the fatty acid carboxyl terminal is oppositely oriented in I-FABP (13, 21) and L-FABP (19, 21).

In summary, the use of the fluorescent fatty acids, *cis*- and *trans*-parinaric acids, in conjunction with radiolabeled fatty acids and two independent binding assays has provided valuable new information regarding the number of binding site(s), the fatty acid specificity of the binding site(s), and the structure of the binding site(s) in I-FABP and L-FABP. In addition, the simultaneous use of fluorescent fatty acids in the Lipidex-1000 assay (requiring separation of bound from unbound fatty acid) and the fluorescence intensity assay (not requiring separation of bound from unbound fatty acid) and the close agreement between the results for the first time validates the Lipidex-1000 procedure used by many previous investigators. Last, the choice of *cis*- and *trans*-parinaric acid as fluorescent analogues of straight chain saturated and kinked chain unsaturated fatty acids provided data entirely consistent with those of the radiolabeled fatty acid-binding assay. The parinaric acids also proved to be much better fluorescent fatty acid probe molecules than the bulky side chain-substituted fluorescent fatty acids since they gave the same number of binding sites as radiolabeled fatty acids for L-FABP. This was not the case with side chain-substituted fluorescent fatty acids used previously.

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