Modification of the fatty acid binding profile of liver fatty acid binding protein (L-FABP)

A.C. DeMarco, P.P. Patterson, R.C. Cantrill, and D.F. Horrobin

Efamol Research Institute, Kentville, Nova Scotia, Canada

Low molecular-weight fatty acid binding protein (L-FABP) was purified from rat liver by a combination of gel filtration and affinity chromatography. The purified protein had a molecular weight of 14,000 Daltons as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fast protein liquid chromatography (FPLC) gel filtration chromatography. Isoelectric focusing of delipidated preparations gave a major protein band with a pI of 7.5. Delipidated L-FABP was used to determine binding constants for individual saturated, monounsaturated, and polyunsaturated fatty acids. For fatty acids of chain length greater than C14 there was no apparent selectivity based on chain length or degree of unsaturation. When delipidated L-FABP was incubated with an equimolar (0.3 mmol/L) mixture of fatty acids; 16:0, 18:1(n-9), 18:2(n-6), 20:3(n-6), 20:4(n-6), and 22:6(n-3) were bound at equivalent levels (0.18 mol/mol L-FABP). However, stearic acid was bound to a greater extent (approximately two fold) and 18:3(n-6) and 18:3(n-3) were bound to a lesser extent (50%). 12:0, 14:0, and 20:5(n-3) bound poorly to L-FABP. Thus, under these conditions fatty acid binding protein exhibits a selectivity that is not apparent from individual binding constants. When rats were maintained on different diets for 6 weeks, the concentration of L-FABP was reduced only in animals maintained on a fat-free diet. The apparent binding capacity of L-FABP (0.71 mol fatty acid per mol L-FABP) was the same for all diets. However, the endogenous fatty acid composition of L-FABP was strongly influenced by dietary fatty acid composition.

Keywords: liver; fatty acid; binding proteins; FABP

Introduction

Liver fatty acid binding proteins (L-FABP) are small (12–15 kDa) cytosolic proteins responsible for the intracellular storage and transport of fatty acids and other lipophilic molecules.[1] The physiological role of these proteins remains unclear. However, purified L-FABP binds long-chain saturated and unsaturated fatty acids, acyl-coA esters, retinol, and some prostaglandins.[1–3] Direct comparison of the binding constants for different ligands has been complicated by the various assay methods employed and the degree of delipidation of the FABP sample.[4] Also, the multiple bands of purified L-FABP seen on isoelectric focusing gels are thought to arise either from multiple isoforms or as a result of ligand binding.[5,6] The concentration of L-FABP is known to be affected by low fat diets and peroxisome proliferators.[7,8] It is not known, however, how the pattern of endogenous fatty acids may respond to short-term dietary manipulation.

The purpose of the present study was to investigate the specificity of purified delipidated L-FABP for a range of saturated (C12:0, C14:0, C16:0, C18:0), monounsaturated (C18:1), and polyunsaturated fatty acids of the n-3 (C18:3, C20:5, C22:6) and n-6 (C18:2, C18:3, C20:3, C20:4) series in competitive binding assays. We also investigated the pattern of fatty acids bound to delipidated L-FABP after incubation with an equimolar mixture of free fatty acids. These results were compared with the endogenous pattern of fatty acids bound to L-FABP isolated from rats fed diets of different fatty acid composition.

Methods and materials

Chemicals and reagents

[^{14}C]-fatty acids (18:0, 58.0 mCi/mmol; 18:1(n-9), 58.0 mCi/mmol; 18:2(n-6), 51.7 mCi/mmol; 18:3(n-6), 52.0 mCi/mmol; 18:3(n-3), 54.8 mCi/mmol; 20:3(n-6), 54.9 mCi/mmol; 20:4

Address reprint requests to A.C. DeMarco, Efamol Research Institute, P.O. Box 818, Kentville, Nova Scotia, Canada, B4N 4H8.
Received July 8, 1992; accepted February 8, 1993.

(n-6), 52.8 mCi/mmol; and 20:5(n-3), 52.2 mCi/mmol were purchased from DuPont Canada (Mississauga, Ontario, Canada) and unlabeled fatty acids were from Nu-Chek Prep (Elysian, MN USA). Lipidex-1000 was obtained from Packard Instrument Co. (Downers Grove, IL USA). Sephadex G-50 resin, the Superdex 75 HR 10/30 prepacked column, PhastGel IEF, homogenous gels, and sodium dodecyl sulfate (SDS) buffer strips were purchased from Pharmacia Canada (Montreal, Quebec Canada). Ultrafiltration membranes and microseparation devices were from Amicon (Danvers, MA USA), and ω-aminodecylagarose and naphthylchloride were obtained from Sigma Chemical Co. (St. Louis, MO USA). All other chemicals were of analar (AR) purity or better.

Buffer composition The various buffers used in this study were as follows. Buffer A – 0.10 M Na^+phosphate pH 7.4. Buffer B – 0.05 M Na^+phosphate pH 7.4, containing 0.25 M sucrose, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 5 mmol/L ethylene-diaminetetraacetic acid (EDTA). Buffer C – 0.05 M Na^+phosphate pH 6.0 containing 5 mmol/L EDTA. Buffer D – 0.075 M Na^+phosphate pH 6.0, 25 % ethanol.

Diets and animals Adult female Sprague-Dawley rats (200–250 g) were obtained from Charles River (St. Constant, Quebec Canada) and maintained on standard lab rodent chow from Scotia Farm Services (Steam Mill, Nova Scotia Canada). Diets, with the exception of fat free, were prepared by the addition of 5 g of fat per 95 g of regular chow (final fat content 9.5%). The fat sources used were evening primrose oil, fish oil, and hydrogenated coconut oil. The final fatty acid composition of the diets is shown in *Table 1*. Fat-deficient basal mix powder was obtained from Teklad Diets (Madison, WI USA).

Procedures

Fatty acid binding protein isolation Preparation of high speed supernatant. L-FABP was isolated at 4° C as reported previously.[9] Livers were homogenized in two volumes (wt/vol) of Buffer B and the homogenate was centrifuged at 20,000*g* for 30 min. The supernatant was carefully removed from the pellet and recentrifuged at 105,000*g* for 90 min. The clear high speed supernatant (cytosol) was assayed for L-FABP activity.

Concentration and sephadex chromatography. The cytosol extract was concentrated in an Amicon centrifugal ultrafiltration filter unit with a 10,000 molecular weight cutoff. When the volume was less than 5 mL, the extract was dialyzed against several changes of Buffer C. The dialyzed concentrate was centrifuged at 300*g* for 10 min to remove any insoluble material before being applied to a Sephadex G-50 column (2.5 × 100 cm) equilibrated in Buffer C. Fractions (2.5 mL) were eluted at 1 mL/min and tested for oleate binding activity (see below). Fractions showing oleate binding were combined and concentrated as before.

Lipidex 1000 chromatography. Lipidex 1000 has been reported to bind only free fatty acids at 4° C and to bind both free and protein dissociated fatty acids at 37° C.[10] Concentrated samples of oleate binding material from Sephadex chromatography were passed through a Lipidex 1000 column (1.5 × 30 cm) equilibrated in Buffer C at 37° C. The eluate was monitored at 280 nm and fractions containing delipidated L-FABP were collected and concentrated as above.

Affinity chromatography. An affinity column (1.5 × 30 cm) of naphthoyl aminodecyl-agarose was prepared as described by Wilton[11] and equilibrated in Buffer A. The delipidated L-FABP was applied to the column and washed with Buffer A at 0.5 mL/min until all unbound material was removed. The bound L-FABP was eluted with Buffer D, concentrated as before, and samples stored at −20° C until use.

Fast protein liquid chromatography (FPLC) Quantitation of L-FABP in liver cytosol was accomplished by separating the protein by FPLC gel filtration chromatography and performing an oleate binding assay. A 100 μL sample of cytosol, approximately 1–2 mg of protein, was separated on a Pharmacia Superdex 75 HR 10/30 column with Buffer A as the

Table 1 Diet fatty acid composition*

Fatty acid[a]	Diet[b]				
	RC	FF	EPO	FO	HCO
12:0	tr	tr[c]	tr	tr	24.3
14:0	1.8	tr	0.9	4.4	10.9
16:0	19.6	tr	12.5	17.5	15.5
16:1n-7	2.7	tr	1.5	5.7	1.5
18:0	7.9	4.2	4.7	5.6	11.6
18:1n-9	30.5	2.1	19.3	21.0	16.8
18:2n-6	29.3	4.1	51.8	15.5	15.3
18:3n-6	tr	tr	4.7	0.1	tr
18:3n-3	2.8	3.2	1.6	1.9	1.5
20.3n-9	0.7	2.5	0.5	1.9	0.4
20:3n-6	0.3	2.7	0.2	1.1	0.2
20:4n-6	0.5	76.2	0.2	1.9	0.1
20:5n-3	1.2	tr	0.6	9.5	0.7
22:4n-6	0.2	tr	0.1	0.5	0.1
22:5n-6	0.1	tr	0.1	0.6	tr
22:5n-3	0.2	tr	0.1	1.3	0.1
22:6n-3	0.9	tr	0.4	6.7	0.5

*Results are mean percentage of fatty acids extracted in triplicate from diet.
[a]Number of carbon atoms:number of double bonds (omega notation).
[b]Regular chow (RC), fat-free (FF), RC + evening primrose oil (EPO), RC + fish oil (FO), RC + hydrogenated coconut oil (HCO).
[c]tr (trace) ≤ 1 μg of a fatty acid.

eluant at a flow rate of 0.5 mL/min. The fraction containing L-FABP was identified by SDS-polyacrylamide gel electrophoresis (PAGE) using affinity purified L-FABP as a standard, and was assayed using the oleate binding assay (see below).

Electrophoresis SDS-PAGE (20% gels) and isoelectric focusing (IEF) (pI range 3–9) were performed on precast gels in a Pharmacia PhastGel system. Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (0.1 % Coomassie blue in 45 % methanol and 10 % acetic acid). Gels were destained in the same solution without Coomassie blue and preserved prior to drying in 5% glycerol in 10% acetic acid.

Lipidex 1000 fatty acid binding assay Standard assay. Oleate binding to L-FABP was determined using the method of Glatz and Veerkamp[10] with slight modification. To reduce nonspecific binding of fatty acids, Triton X-100 (0.03 %) was used to maintain the ligand in solution and reduce protein binding to the assay tubes.[12] For the standard fatty acid binding assay, samples were incubated in 1.5 mL polyallomer tubes in 0.01 M Na^+phosphate pH 7.4 containing 3 mmol/L $MgCl_2$ and 0.03 % Triton with 10 μmol/L [1-^{14}C]oleate in a total volume of 0.4 mL. After a 15 min incubation at 30° C, the tubes were placed on ice for 10 min and then mixed with 200 μL of cold Lipidex 1000 suspension in 0.01 M Na^+phosphate buffer pH 7.4 (1:1). The tubes were then centrifuged at 12,000*g* for 10 min, and a 100 μL sample of supernatant was taken for liquid scintillation counting. The assay was standardized by comparing oleate binding by bovine serum albumin (BSA) under our experimental conditions. Oleate binding by BSA was reported previously by Glatz.[10]

Unlabeled fatty acid binding. To determine if L-FABP preferentially bound certain fatty acids, delipidated L-FABP (1 mg) was incubated with an equimolar mixture of cold fatty acids (0.3 μmol of each fatty acid listed in *Table 2*) in 1 mL of the standard assay buffer and incubated for 1 hour at 30° C. To separate free and bound fatty acids, the mixture was cooled to 4° C then passed through a 2-mL column of Lipidex 1000 previously equilibrated in 0.01 M Na^+phosphate pH 7.4. Free ligand remained bound to the Lipidex 1000 while fatty acid-bound L-FABP was eluted. L-FABP bound fatty acids were extracted and analyzed by gas liquid chromatography (GLC).

Other analytical procedures Protein was determined using the modified Lowry procedure.[13] Other methods of protein determination viz. 280/260 nm, 280 nm, Biorad modified Coomassie blue assay gave similar results to the Lowry procedure. Other work[14] suggests that the Lowry technique may overestimate the L-FABP content by almost twofold. Data presented in *Table 2* were calculated using the value from the Lowry estimation and were also corrected for this apparent overestimation. Unless otherwise stated in the text, L-FABP protein concentration was determined by the Lowry method and is uncorrected. Fatty acids were extracted from samples of liver and L-FABP by the method of Folch et al.[15] Fatty acid methyl esters (FAME) were prepared by transmethylation with BF_3 in methanol[16] and separated by GLC on either a glass column packed with SP2330 on Chromasorb WAW 100/120 or a Supelcowax 10 capillary column in a Hewlett Packard 5880 gas chromatograph equipped with a flame ionisation detector. Samples were quantified by comparison with heptadecanoic acid (17:0) used as an internal standard.

Statistical methods Statistical comparisons were made by Student *t* test. Data from binding studies were analysed using the Elsevier Enzfitter program. All data were analysed as both "one-site" or "two-site" models and results presented in *Table 2* are taken from the better of the two "fits."

Table 2 L-FABP binding constants*

Fatty acid[a]	Kd, μmol/L	B_{max}, mol/mol
18:0	2.4 ± 1.5[b]	1.5 ± 0.6 (0.82)[c]
18:1n-9	3.2 ± 0.6	2.9 ± 0.1 (1.59)
18:2n-6	7.6 ± 0.9	3.1 ± 0.1 (1.70)
18:3n-6	2.7 ± 0.7[b]	1.8 ± 0.2 (0.98)
18:3n-3	6.0 ± 1.6	2.2 ± 0.2 (1.20)
20:3n-6	2.1 ± 0.5	2.0 ± 0.1 (1.09)
20:4n-6	10.4 ± 1.4	4.1 ± 0.2 (2.24)
20:5n-3	7.5 ± 3.3	1.2 ± 0.1 (0.66)

*Values are mean ± sd (n = 3).
[a]Number of carbon atoms:number of double bonds (omega notation).
[b]Data fitted to two-site model, only high affinity site presented.
[c]Calculated binding capacities assuming overestimation of actual protein concentration.[14] See Methods and materials.

Results

Binding experiments with purified L-FABP

The presence of L-FABP in rat liver extracts and at different stages of preparation was confirmed using the oleate binding assay and by SDS-PAGE. Delipidation by Lipidex chromatography was judged by GLC analysis of FAME from chloroform/methanol extracts of L-FABP. The delipidated rat liver FABP was then used to determine the binding constants for other fatty acids and the effect of the binding of these fatty acids on its electrophoretic mobility.

Electrophoretic mobility of L-FABP Purity of L-FABP at various stages of isolation was analyzed by SDS-PAGE in 20% homogeneous gels (*Figure 1A*). High molecular-weight protein bands are reduced as the sample is passed through different stages of the procedure (*Figure 1A* lanes B–E). Lipidex 1000 chromatography of the Sephadex G-50 samples (lane E) did not substantially alter their protein composition (lane G). Following FPLC gel filtration (lane F), or affinity chromatography (lane H), only a single low molecular weight band of 14,000 Daltons was seen. L-FABP, following delipidation by Lipidex chromatography at 37° C, gave a major band at pI 7–7.5 and minor bands at pI 5.9, 5.2, and 5.0 (*Figure 1B* lane B). Following incubation of the same delipidated L-FABP with oleate (lane C), the major band had a pI of 6.8–7.4 and appeared broader, the minor band at pI 5.9 was more obvious, and two new bands appeared near pI 5.5.

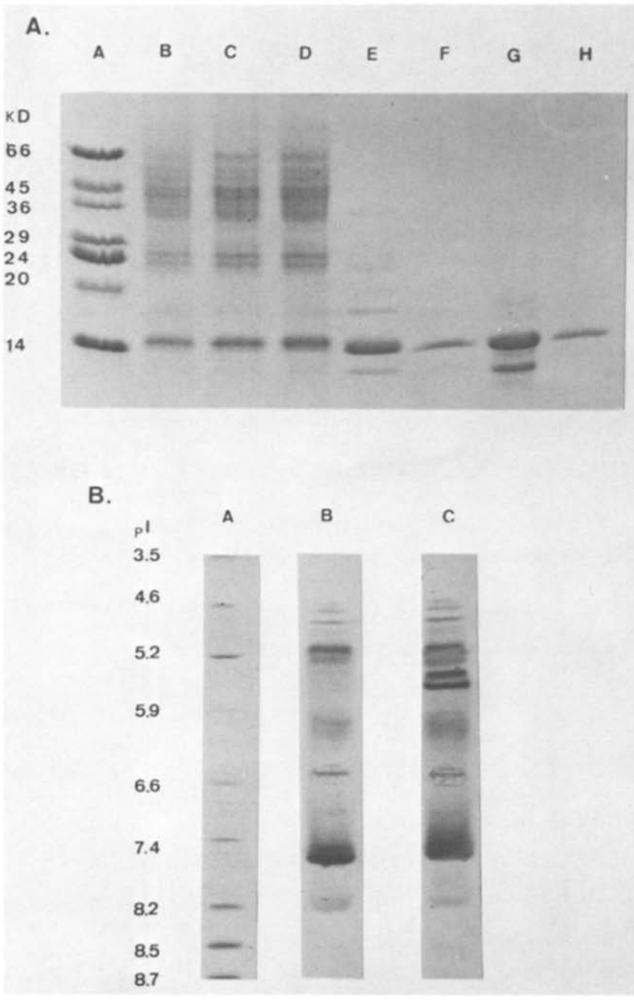

Figure 1 Electrophoretic analysis of L-FABP. **A.** SDS-PAGE of different stages of purification. Lanes are identified as follows: A, molecular weight markers; B, liver extract following low speed centrifugation; C, high speed supernatant cytosol; D, concentrated cytosol; E, combined low molecular fractions from Sephadex G-50 chromatography; F, FPLC fraction containing low molecular weight oleate binding activity; G, L-FABP preparation following Lipidex chromatography of material shown in lane E; H, affinity chromatography purified L-FABP following FPLC separation (lane F). **B.** Isoelectric focusing of Lipidex-stripped FABP before and after incubation with 10 μmol/L oleate. A, pI markers; B, delipidated L-FABP; c, oleate-bound L-FABP.

Lipidex 1000 binding assay with labeled fatty acids Under the conditions employed, the oleate binding was saturable, linear with protein concentration, and equilibrium was reached within 5 min (data not shown). B_{max} and Kd for oleate were 2.9 μmol/μmol and 3.2 μmol/L, respectively.

A comparison of the binding affinity and capacity of L-FABP for a number of fatty acids was undertaken using the Lipidex 1000 binding assay and the same preparation of delipidated FABP. *Figure 2* shows the saturation binding curves for different fatty acids of the n-6 series (18:2, 18:3, 20:3, 20:4). There were substantial differences in both the binding affinity and capacity of FABP for fatty acids with differing chain lengths and unsaturation. *Table 2* gives the binding constants and binding capacity for the fatty acids used in these experiments. Only fatty acids of chain length equal to or greater than 16 carbons were bound. The high affinity binding capacity for stearic acid (1.5 mol/mol L-FABP) was less than that found for any other fatty acid except 20:5(n-3). Binding capacity for mono- and polyunsaturated fatty acids ranged from 1–4 mol/mol L-FABP. The capacity for fatty acids of the n-3 series (1–2 mol/mol L-FABP) was lower than the capacity measured for n-6 fatty acids (1.8–4 mol/mol L-FABP). Binding affinities ranged between 10 μmol/L for 20:4(n-6) and 2 μmol/L for 20:3(n-6). Correcting the binding capacity of L-FABP for the overestimation of the protein content reduced the capacities for different fatty acids to 0.6–1.7 mol/mol L-FABP. There did not appear to be any relationship between binding constant and chain length or degree of unsaturation.

Binding assay with a mixture of unlabeled fatty acids To determine if individual binding constants played a role in determining the composition of the fatty acids bound to L-FABP, delipidated L-FABP was incubated with an equimolar mixture of 12 fatty acids. Following the removal of unbound ligand, on a Lipidex 1000 column at 4° C, the L-FABP pattern of bound fatty acid was analyzed. *Table 3* shows the percent binding and the molar ratio of bound fatty acids. 12:0 and 14:0 were not bound. Individually the saturates, 18:0 and 16:0, and monounsaturated fatty acid 18:1(n-9) were bound to the greatest extent, but polyunsaturated fatty acids accounted for almost 50% of the accumulated fatty acids. Both 18:3 isomers, of the n-3 or n-6 series, were poorly bound, and 20:5(n-3) was bound to the least extent of all the fatty acids tested in this competitive assay. The total fatty acid bound per mol of L-FABP was 1.58 mol. The true value for the total bound fatty acid may be approximately 0.9 mol, applying the previously published correction factor.[14]

L-FABP from dietary manipulation experiments

L-FABP concentration versus diet The concentration of L-FABP was determined in high speed supernatants of liver extracts, following the separation of L-FABP from albumin by FPLC gel filtration chromatography (*Table 4*). Oleate binding to the fraction containing L-FABP was compared with the binding to purified L-FABP, and the concentration was extrapolated. The concentration of L-FABP was 3–5% of the cytosolic protein in all five diets. The dietary fat source did not alter the liver FABP content (*Table 4*), but L-FABP was slightly decreased in animals fed a fat-free diet.

L-FABP fatty acid composition from rats fed various diets Preparative Sephadex G-50 gel filtration was used to isolate enough L-FABP to perform endogenous fatty acid analysis. The fatty acid composition of L-FABP isolated from animals maintained on regular chow is shown in *Table 5*. 18:1(n-9), 18:2(n-6), and 20:4(n-6) were the major constituents bound to L-FABP. Four-

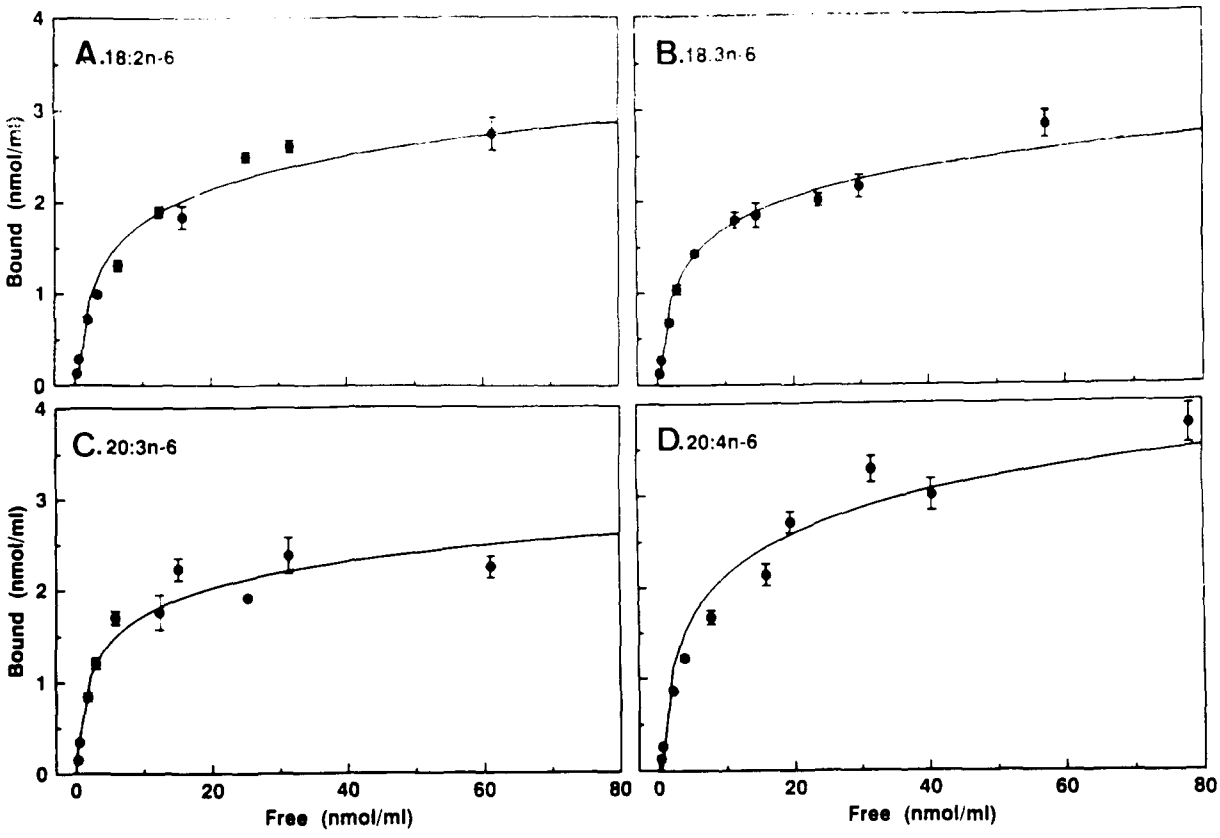

Figure 2 Comparison of in vitro binding of L-FABP to various fatty acids of the n-6 series. The concentration of bound fatty acid (mean ± SE (n = 3)) was determined using the Lipidex binding assay as detailed in Methods and materials.

Table 3 Competitive binding of L-FABP

Fatty acid*	Bound concentration	
12:0	tr[a,b]	tr[c]
14:0	tr	tr
16:0	12.6	0.22
18:0	21.8	0.35
18:1n-9	17.5	0.28
18:2n-6	9.5	0.16
18:3n-6	3.4	0.06
18:3n-3	4.6	0.08
20:3n-6	10.4	0.16
20:4n-6	11.7	0.18
20:5n-3	0.3	0.01
22:6n-3	7.7	0.11
Total	99.5	1.58

*Number of carbon atoms:number of double bonds (omega notation).
[a]Results are expressed as mean percentage of bound fatty acids after incubation with an equimolar mixture of the above free fatty acids (n = 3).
[b]tr (trace) ≤ 0.1%. The difference between the sum of the percentages and 100 represents minor and unidentified fatty acids.
[c]Values are expressed as μmol of a particular fatty acid per μmol L-FABP (n = 3). Values are uncorrected for overestimation of protein content. See Methods and materials.

Table 4 Effect of diet on L-FABP concentration[a]

Diet	L-FABP concentration		
	nmol/mg protein	μmol/liver	% sol protein
Reg chow	2.91 ± 0.37	2.10 ± 0.19	4.08 ± 0.51
Fat free	2.59 ± 0.36*	1.61 ± 0.08**	3.63 ± 0.51
5% EPO	3.15 ± 0.60	1.73 ± 0.23*	4.41 ± 0.84
5% Fish oil	2.86 ± 0.45	2.03 ± 0.17	4.01 ± 0.62
5% HCO	2.85 ± 0.40	2.01 ± 0.28	3.98 ± 0.56

[a]Values are mean ± sd (n = 8). Values are uncorrected for overestimation of protein content. See Methods and materials.
*$P < 0.05$ when compared with reg chow value.
**$P < 0.01$ when compared with reg chow value.

teen percent of the bound fatty acids were saturated, and 86% were unsaturated. The bound fatty acid content of L-FABP from regular chow-fed animals was 0.71 μmol of fatty acid per μmol L-FABP.

The fatty acid content of L-FABP isolated from rats fed different diets is shown in *Table 5*. Diet had no significant effect on the total amount of fatty acid bound to L-FABP (mean fatty acid content of 0.71 mol fatty

Table 5 L-FABP fatty acid composition[a]

Fatty acid[b]	Diet[c]				
	RC	FF	EPO	FO	HCO
16:0	0.08 (9.1)	0.11 (11.1)*	0.08 (12.0)	0.10 (12.8)**	0.07 (9.3)
16:1n-7	0.02 (2.1)	0.13 (12.4)**	0.01 (1.6)	0.03 (3.7)	0.02 (2.0)
18:0	0.02 (3.4)	0.03 (3.9)	0.04 (6.8)**	0.05 (7.5)**	0.04 (5.2)**
18:1n-9	0.21 (27.8)	0.35 (40.3)**	0.10 (16.3)**	0.11 (15.4)**	0.19 (26.9)
18:2n-6	0.13 (16.8)	0.06 (6.6)**	0.18 (28.8)**	0.10 (13.1)*	0.12 (16.6)
18:3n-6	tr[e] (0.1)	tr (0.3)	0.01 (0.8)**	tr (0.1)	tr (0.1)
18:3n-3	0.01 (0.7)	tr (0.5)	tr (0.5)	0.01 (0.9)	0.01 (0.7)
20:3n-9	tr (tr)	0.02 (3.0)	tr (tr)	tr (tr)	tr (tr)
20:3n-6	0.01 (0.8)	0.01 (0.6)	0.01 (1.8)	0.01 (1.1)	0.01 (1.4)
20:4n-6	0.15 (22.3)	0.10 (12.5)**	0.12 (20.7)	0.09 (12.6)**	0.13 (20.4)
20:5n-3	0.02 (2.4)	tr (0.4)	0.01 (0.9)**	0.07 (10.7)**	0.02 (2.5)
22:4n-6	tr (tr)	0.01 (0.7)	tr (0.5)	tr (0.3)	tr (0.4)
22:5n-6	tr (tr)	tr (0.2)	tr (0.1)	tr (0.4)	tr (0.4)
22:5n-3	0.01 (1.2)	tr (tr)	tr (0.3)	0.02 (2.6)**	0.01 (1.2)
22:6n-3	0.06 (10.2)	0.04 (4.9)**	0.02 (3.9)**	0.10 (15.4)**	0.06 (10.8)
Total	0.71	0.87	0.60	0.69	0.68
Saturates[d]	14.4	17.3*	21.3**	22.9**	16.2*
Monounsaturates	31.7	54.8**	19.0**	20.7**	30.2
Polyunsaturates	54.0	28.0**	59.7*	56.4	53.6

[a]Results are expressed as mol fatty acid bound per mol L-FABP. Values in parentheses are mean percentage of fatty acids bound to L-FABP ($n = 8$).
[b]Number of carbon atoms:number of double bonds (omega notation).
[c]Regular chow (RC), fat-free (FF), RC + evening primrose oil (EPO), RC + fish oil (FO), RC + hydrogenated coconut oil (HCO).
[d]Saturates, monounsaturates, and polyunsaturates are calculated from the percent distribution and expressed as percent total.
[e]tr (trace) ≤10 mmol or 0.1% of a fatty acid per mol L-FABP.
*$P < 0.05$ when compared with RC.
**$P < 0.01$ when compared with RC.

acid per mol L-FABP, range 0.60–0.87). The profile of these fatty acids shows that the individual fatty acids bound to L-FABP could be related directly to the dietary fatty acid composition. Animals fed regular chow supplemented with evening primrose oil (EPO) had increased amounts of bound 18:2(n-6), 18:3(n-6), 20:3(n-6), and 20:4(n-6) as compared with L-FABP from regular chow controls. Likewise, the fish oil-supplemented group had increased levels of bound 18:3(n-3), 20:5(n-3), 22:5(n-3) and 22:6(n-3). The animals fed a fat-free diet had increased levels of the monounsaturates and a significant amount of 20:3(n-9). *Table 5* also shows a comparison of the recovery of saturated and unsaturated fatty acids from L-FABP from the various diet groups. In the fat-free group there was an increase in bound monounsaturates relative to polyunsaturated fatty acids when compared with control animals. Both EPO- and fish oil-fed animals showed a decrease in monounsaturates and an increase in saturate content. Polyunsaturate binding remained the same as in controls. The fatty acid profile of L-FABP from the hydrogenated coconut oil (HCO) group was similar to the regular chow-fed animals.

Liver fatty acid composition The total fatty acid composition of livers from animals maintained on prescribed diets was also determined (*Table 6*). The dietary fatty acid composition is reflected in the liver fatty acid profile. The typical fatty acid pattern of the regular chow-fed animals showed 40% saturated fatty acids, 14% monounsaturated, and 46% polyunsaturated fatty acids. This profile was modified by the type of dietary fat supplementation. EPO-fed animals had increased 18:2(n-6) and 18:3(n-6) levels, whereas fish oil-fed animals showed an increased level of n-3 fatty acids. The fat-free group had increased amounts of n-7 and n-9 fatty acids, with a corresponding decrease in 18:2(n-6). The levels of saturates, monounsaturates, and polyunsaturates in livers from the HCO group were identical to those of the regular chow animals.

Discussion

A number of different methods have been used to isolate L-FABP, and it has been characterized by SDS-PAGE, native PAGE, and IEF.[17,18] The appearance of multiple bands may represent isoforms of L-FABP,[5,6] but may also arise from changes in the surface charge of the protein as a result of ligand binding.[19,20] In *Figure 1B* it can be seen that L-FABP, prepared by exclusion and affinity chromatography, may show significant variations in the amount of five protein species, depending on the degree of fatty acid loading.

Binding constants for individual fatty acids have been determined by various methods and are generally in the μmol/L range (0.5–12 μmol/L).[6,18] Slight differences between the values reported (0.5–1.0 μmol/L)[4] and our results (2.1–10.4 μmol/L) probably reflect differences in the degree of delipidation, the different solubilities of the ligands, and associated problems with the determination of free or bound ligand concentration in the

Table 6 Liver fatty acid compostition[a]

Fatty acid[b]	Diet[c]				
	RC	FF	EPO	FO	HCO
14:0	0.4 (0.0)	0.8 (0.0)**	0.2 (0.0)**	0.4 (0.0)	1.2 (0.0)**
16:0	17.7 (0.51)	22.3 (0.50)**	13.7 (0.88)**	16.4 (0.78)	17.1 (0.54)
16:1n-7	1.8 (1.14)	7.4 (1.68)**	0.9 (1.85)**	1.3 (2.97)*	1.5 (1.29)
18:0	21.4 (0.16)	15.6 (0.25)**	20.1 (0.34)	18.8 (0.40)**	22.0 (0.24)
18:1n-9	12.6 (2.21)	28.8 (1.40)**	8.6 (1.90)**	10.2 (1.51)*	12.6 (2.14)
18:2n-6	14.5 (1.16)	2.6 (2.53)**	20.8 (1.38)**	13.6 (0.96)	15.2 (1.09)
18:3n-6	0.2 (0.67)	0.1 (4.29)*	0.6 (1.42)**	tr (0.0)*	0.1 (0.73)*
18:3n-3	0.3 (2.55)	tr (0.0)*	0.3 (1.66)	0.5 (1.69)*	0.4 (1.95)*
20:3n-9	tr[e] (0.0)	3.4 (0.88)**	tr (0.0)	tr (0.0)	tr (0.0)
20:3n-6	1.5 (0.55)	0.9 (0.74)**	1.6 (1.15)	0.7 (1.58)**	1.2 (1.16)*
20:4n-6	17.2 (1.30)	10.7 (1.18)**	22.6 (0.91)**	12.0 (1.05)**	16.3 (1.25)
20:5n-3	1.6 (1.54)	0.3 (1.33)**	0.2 (3.76)**	8.0 (1.35)**	1.3 (1.86)
22:4n-6	0.5 (0.08)	0.3 (2.61)**	1.2 (0.43)**	0.5 (0.55)	0.5 (0.72)
22:5n-6	0.2 (0.48)	1.0 (0.16)**	0.4 (0.22)**	0.2 (2.00)	0.2 (1.91)
22:5n-3	1.3 (0.91)	tr (0.0)**	0.8 (0.39)*	2.9 (0.91)**	1.2 (1.03)
22:6n-3	7.2 (1.40)	5.1 (0.95)**	6.1 (0.64)*	13.5 (1.14)**	7.5 (1.45)
Saturates[d]	39.5	38.7	34.0**	35.6**	40.3
Monounsaturates	14.4	36.2**	9.5**	11.5**	14.1
Polyunsaturates	46.1	25.1**	56.5**	52.9**	45.6

[a]Results are mean percentage of fatty acids extracted in triplicate from rat liver. Values in parentheses are the ratio of the percentage of a particular fatty acid bound to L-FABP to the percentage in liver (n = 8).
[b]Number of carbon atoms:number of double bonds (omega notation).
[c]Regular chow (RC), fat-free (FF), RC + evening primrose oil (EPO), RC + fish oil (FO), RC + hydrogenated coconut oil (HCO).
[d]Saturates, monounsaturates, and polyunsaturates are calculated from the percent distribution and expressed as percent total.
[e]tr (trace) ≤ 0.1% of a fatty acid.
*$P < 0.05$ when compared with RC.
**$P < 0.01$ when compared with RC.

various assays used. Because our binding constants were all determined with a single preparation of delipidated L-FABP, they can be considered to reflect the relative binding affinities.

Fatty acid binding capacities have been reported to be in the range 0.5–2 mol per mol of L-FABP.[21] This variation may result from difficulty in accurately determining the L-FABP protein concentration or bound ligand concentration. Because the measurements presented in *Table 2* are of the same preparation of L-FABP, the relative capacity for individual fatty acids is more apparent (1.2–4.1 mol per mol of L-FABP). If these data are corrected for the overestimation of L-FABP by the Lowry method, then they are similar to previously published data.[21] It is also possible that the higher binding capacities reflect some low affinity binding, although the formation of multimeric forms of L-FABP, as seen for heart-FABP (H-FABP),[22] cannot be ruled out.

The binding by L-FABP of a particular ligand is determined by a combination of its affinity for that ligand and the capacity or number of sites on the L-FABP molecule for that ligand. As reported previously[4] and confirmed in a competitive binding experiment, 12:0 and 14:0 did not bind. L-FABP had a high capacity for binding specific saturate (18:0) and monounsaturate (18:1) fatty acids (21.5 and 17.5%, respectively) compared with individual polyunsaturates (<12%) (*Table 3*). This has been shown to be true for endogenous binding[23] (*Table 5*), although Ockner et al.[14] showed that L-FABP carried a high proportion of saturated fatty acids (47%) in their preparation. These data are, however, not in direct agreement with the Kd values measured with individual fatty acids, suggesting that endogenous fatty acid binding by L-FABP reflects more than the binding affinities of L-FABP.

This phenomenon was further explored in the experiments in which animals were fed diets of different fatty acid content. Although the concentration of L-FABP in the fat-free group was lower than in the other groups (*Table 4*), the concentration of bound ligands was greatest for this group. The range of 0.6–0.9 μmol fatty acid per μmol L-FABP is consistent with reported binding capacities[24] but is lower than the binding capacities determined in vitro (*Table 2*). The increased fatty acid content of L-FABP from animals fed fat-free diets could be the result of the type of fatty acids found in the liver because L-FABP in vitro has a higher affinity for saturated and monounsaturated fatty acids than many of the polyunsaturated fatty acids (*Table 2*).

Table 5 compares the type of fatty acids bound to L-FABP from the animals fed the five diets. It appears that the bound fatty acids of L-FABP may be modulated by dietary fatty acids. However, the ensuing pattern does not directly reflect either the dietary fatty acid pattern or the overall fatty acid pattern of the liver (*Tables 5 and 6*). Polyunsaturated fatty acids were bound by L-FABP to approximately the same extent as their liver content. However, no such relationship existed for saturated and monounsaturated fatty acids. Such a result might be predicted from the competitive binding data presented in *Table 3*. Because L-FABP

may be involved in many facets of the metabolism and intracellular transport of fatty acids, the pattern of fatty acids carried by this protein probably responds to the relative rate of each of these processes and the liver free fatty acid pool.

In summary, rat liver FABP appears to be a single protein that displays different isoforms depending on the degree or type of fatty acid binding. The range of fatty acids bound in vivo does not appear to be consequent on the binding constants measured with delipidated FABP in vitro. The fatty acid composition of the diet and the liver appear to be important factors in controlling the fatty acid composition but not content of L-FABP.

References

1 Veerkamp, J.H., Peeters, R.A., and Maatman, R.G.H.J. (1991). Structural and functional features of different types of cytoplasmic fatty acid-binding proteins. *Biochim. Biophys. Acta* **1081,** 1–24

2 Sweetser, D.A., Heuckeroth, R.O., and Gordon, J.I. (1987). The metabolic significance of mammalian fatty-acid-binding proteins: abundant proteins in search of a function. *Ann. Rev. Nutr.* **7,** 337–359

3 Dutta-Roy, A.K., Gopalswamy, N., and Trulzsch, D.V. (1987). Prostaglandin E_1 binds to Z protein of rat liver. *Eur. J. Biochem.* **162,** 615–619

4 Bass, N.M. (1988). The cellular fatty acid binding proteins: aspects of structure, regulation, and function. *Int. Rev. Cytol.* **111,** 143–184

5 Haunerland, N., Jagschies, G., Schulenberg, H., and Spener, F. (1984). Fatty acid binding proteins: occurrence of two fatty acid binding proteins in bovine liver cytosol and their binding of fatty acids, cholesterol, and other lipophilic ligands. *Hoppe-Seyler's Z. Physiol. Chem.* **365,** 365–376

6 Schulenberg-Schell, G., Schafer, P., Keuper, H.J.K., Stanislawski, B., Hoffmann, E., Ruterjans, S., and Spener, F. (1988). Interactions of fatty acids with neutral fatty acid binding protein from bovine liver. *Eur. J. Biochem.* **170,** 565–574

7 Matarese, V., Stone, R.L., Waggoner, D.W., and Bernlohr, D.A. (1989). Intracellular fatty acid trafficking and the role of cytosolic lipid binding proteins. *Prog. Lipid Res.* **28,** 245–272

8 Vergani, L., Fanin, M., Martinuzzi, A., Galassi, A., Appi, A., Carrozzo, R., Rosa, M., and Angelini, C. (1990). Liver fatty acid-binding protein in two cases of human lipid storage. *Mol. Cell. Biochem.* **98,** 225–230

9 Dutta-Roy, A.K., DeMarco, A.C., Raha, S.K., Shay, J., Garvey, M., and Horrobin, D.F. (1990). Effects of linoleic and gamma-linolenic acids (efamol evening primrose oil) on fatty acid-binding proteins of rat liver. *Mol. Cell. Biochem.* **98,** 177–182

10 Glatz, J.F.C. and Veerkamp, J.H. (1983). A radiochemical procedure for the assay of fatty acid binding by proteins. *Anal. Biochem.* **132,** 89–95

11 Wilton, D.C. (1989). Studies on fatty acid-binding proteins: the purification of rat liver fatty acid-binding protein and the role of cysteine-69 in fatty acid binding. *Biochem. J.* **261,** 273–276

12 Vork, M.M., Glatz, J.F.C., Surtel, D.A.M., and Van der Vusse, G.J. (1990). Assay of the binding of fatty acids by proteins: evaluation of the Lipidex 1000 procedure. In *Cellular Fatty Acid-Binding Proteins,* (J.F.C. Glatz and G.J. van der Vusse, eds.), p. 113–117, Kluwer Academic Publishers, Norwell, MA, USA

13 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265–275

14 Ockner, R.K., Manning, J.A., and Kane, J.P. (1982). Fatty acid binding protein, isolation from rat liver, characterization, and immunochemical quantification. *J. Biol. Chem.* **257,** 7872–7878

15 Folch, L., Lees, M., and Sloane-Stanley, G.H. (1957). A simple method for isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226,** 497–509

16 Morrison, W.R. and Smith, L.M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron trifluoride-methanol. *J. Lipid Res.* **5,** 600–608

17 Kawashima, Y., Nakagawa, S., Tachibana, Y., and Kozuka, H. (1983). Effects of peroxisome proliferators on fatty acid binding protein in rat liver. *Biochim. Biophys. Acta.* **754,** 21–27

18 Glatz, J.F.C., Janssen, A.M., Baerwaldt, C.C.F., and Veerkamp, J.H. (1985). Purification and characterization of fatty acid-binding proteins from rat heart and liver. *Biochim. Biophys. Acta* **837,** 57–66

19 Storch, J., Bass, N.M., and Kleinfeld, A.M. (1989). Studies of the fatty acid binding site of rat liver fatty acid binding protein using fluorescent fatty acids. *J. Biol. Chem.* **264,** 8708–8713

20 Nemecz, G., Jefferson, J.R., and Schroeder, F. (1991). Polyene fatty acid interactions with recombinant intestinal and liver fatty acid-binding proteins. *J. Biol. Chem.* **266,** 17112–17123

21 Cistola, D.P., Walsh, M.T., Corey, R.P., Hamilton, J.A., and Brecher, P. (1988). Interactions of oleic acid with liver fatty acid binding protein: a carbon-13 NMR study. *Biochemistry* **27,** 711–716

22 Fournier, N.C. and Rahim, M. (1985). Control of energy production in the heart: a new function for fatty acid binding protein. *Biochemistry* **24,** 2387–2396

23 Veerkamp, J.H. and Paulussen, R.J.A. (1987). Fatty acid transport in muscle: the role of fatty acid-binding proteins. *Biochem. Soc. Trans.* **15,** 331–336

24 Paulussen, R.J.A., Jansen, G.P.M., and Veerkamp, J.H. (1986). Fatty acid-binding capacity of cytosolic proteins of various rat tissues: effect of postnatal development, starvation, sex, clofibrate feeding and light cycle. *Biochim. Biophys. Acta* **877,** 342–349