Fatty Acid Binding Proteins from Different Tissues Show Distinct Patterns of Fatty Acid Interactions†

Gary V. Richieri,‡ Ronald T. Ogata,‡ Aukje W. Zimmerman,§ Jacques H. Veerkamp,§ and Alan M. Kleinfeld*,‡

Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, California 92121, and Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands

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ABSTRACT: Fatty acid binding proteins (FABP) form a family of proteins displaying tissue-specific expression. These proteins are involved in fatty acid (FA) transport and metabolism by mechanisms that also appear to be tissue-specific. Cellular retinoid binding proteins are related proteins with unknown roles in FA transport and metabolism. To better understand the origin of these tissue-specific differences we report new measurements, using the acrylodated intestinal fatty acid binding protein (ADIFAB) method, of the binding of fatty acids (FA) to human fatty acid binding proteins (FABP) from brain, heart, intestine, liver, and myelin. We also measured binding of FA to a retinoic acid (CRABP-I) and a retinol (CRBP-II) binding protein and we have extended to 19 different FA our characterization of the FA−ADIFAB and FA−rat intestinal FABP interactions. These studies extend our previous analyses of human FABP from adipocyte and rat FABPs from heart, intestine, and liver. Binding affinities varied according to the order brain ≈ myelin ≈ heart > liver > intestine > CRABP > CRBP. In contrast to previous studies, no protein revealed a high degree of selectivity for particular FA. The results indicate that FA solubility (hydrophobicity) plays a major role in governing binding affinities; affinities tend to increase with increasing hydrophobicity (decreasing solubility) of the FA. However, our results also reveal that, with the exception of the intestinal protein, FABPs exhibit an additional attractive interaction for unsaturated FA that partially compensates for their trend toward lower affinities due to their higher aqueous solubilities. Thermodynamic potentials were determined for oleate and arachidonate binding to a subset of the FABP and retinoid binding proteins. FA binding to all FABPs was enthalpically driven. The $\Delta H°$ values for paralogous FABPs, proteins from the same species but different tissues, reveal an exceptionally wide range of values, from −22 kcal/mol (myelin) to −7 kcal/mol (adipocyte). For orthologous FABPs from the same tissue but different species, $\Delta H°$ values were similar. In contrast to the enthalpic dominance of FA binding to FABP, binding of FA to CRABP-I was entropically driven. This is consistent with the notion that FA specificity for FABP is determined by the enthalpy of binding. Proteins from different tissues also revealed considerable heterogeneity in heat capacity changes upon FA binding, ΔC_p values ranged between 0 and −1.3 kcal mol^{-1} K^{-1}. The results demonstrate that thermodynamic parameters are quite different for paralogous but are quite similar for orthologous FABP, suggesting tissue-specific differences in FABP function that may be conserved across species.

Fatty acid binding proteins (FABP)[1] are 14−15 kDa cytosolic proteins found in different cell types (*1*−*8*) and their amino acid sequences exhibit considerable tissue-specific variation (*9*−*12*). High-resolution structures determined for a number of different FABP reveal well-defined binding sites, generally for single fatty acids (FA), deep within the proteins (*6*, *7*, *13*). Binding studies indicate a high affinity (K_d < 500 nM) for long-chain FA but generally much lower (>500 nM) affinities for other hydrophobic ligands (*12*, *14*−*16*). Affinities, kinetics, and thermodynamics of FA interactions with FABP from different tissues are quite different but these characteristics are quite similar for FABPs from the same tissues of different species (*9*−*12*, *17*−*20*). Their well-defined and high-affinity binding sites together with the tissue-specific nature of their interactions with FA suggest that FABPs may be involved in fatty acid transport and/or metabolism. This function has now been demonstrated directly by a heart-FABP knock-out mouse that reveals lower rates of FA uptake and metabolism as compared to the wild-type mouse (*21*, *22*). The adipocyte FABP, in contrast, does not affect uptake but has a range of effects on FA metabolism (*23*−*26*).

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* Address correspondence to this author: tel 858-455-3724; fax 858-455-3792.

‡ Torrey Pines Institute for Molecular Studies.

§ University of Nijmegen.

[1] Abbreviations: AA, arachidonate (20:4); ADIFAB, acrylodated intestinal fatty acid binding protein; A-FABP, adipocyte fatty acid binding protein; B-FABP, brain fatty acid binding protein; DHA, docosahexaenoate (22:6); FA, fatty acid; FABP, fatty acid binding protein; FFA, free fatty acids; H-FABP, heart fatty acid binding protein; I-FABP, intestinal fatty acid binding protein; LA, linoleate (18:3); L-FABP, liver fatty acid binding protein; LNA, linolenate (18:3); M-FABP, myelin fatty acid binding protein; OA, oleate (18:1); PA, palmitate (16:0); SA, stearate (18:0).

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Although the evidence supports the notion that FABP function involves FA, a surprising feature of the binding measurements is the lack of a high degree of discrimination among FA types (*15*). This is surprising because in metabolism and biosynthesis, cellular systems display considerable discrimination. For example, the distribution of FA acyl chains in cellular membrane phospholipids can differ by more than 10-fold among different lipid classes and with the serum distribution of FA (*27*). FABP might be expected to contribute to this FA asymmetry, for example, by selectively transporting specific FA between intracellular sites. Selectivity might be achieved by other proteins involved in lipid biosynthesis, but at some step they too would need to selectively bind specific FA. However, the level of discrimination needed to achieve the distribution asymmetries in cell systems is not reflected in the binding affinity differences observed in most previous studies of FABP or in studies of albumin, whose FA binding sites are chemically and structurally distinct from FABPs but whose FA binding characteristics are similar to FABPs (*12*, *15*, *16*, *28*−*33*). Most striking, differences in FA binding affinities for rat intestinal FABP (rI-FABP) are virtually identical to values expected from aqueous solubility of the different FA or equivalently from aqueous/membrane partition (*15*).

An exception to the lack of binding specificity observed in most studies is the result for mouse brain FABP, which showed no binding of palmitate, but docosahexaenoate (DHA) bound with a K_d of 10 nM (*34*). Brain FABP (B-FABP) might be expected to exhibit this pattern because a high degree of DHA enrichment is required in the developing brain (*34*). These results raise the possibility that certain FA−amino acid interactions might generate the same type of selectivity that occurs in cellular systems.

Previous studies from this and other laboratories have revealed differences in thermodynamic potentials for FABPs from different tissues (human adipocyte and heart, intestine, and liver from rat) (*14*, *19*, *35*, *36*). These differences are, however, relatively modest in comparison to those generated by single amino acid replacements in particular FABPs (*15*). For example, while $\Delta H°$ for adipocyte, heart, intestine, and liver for oleate binding range from about −7 to −12 kcal/mol, single Ala substitutions in intestine generate proteins with $\Delta H°$ ranging from −4 to −17 kcal/mol. The modest variation in thermodynamic potentials for these wild-type FABPs may reflect functional requirements that restrict the range of interactions even for quite extensive differences in amino acid sequences, in comparison to those observed for single amino acid replacements. Alternatively, this limited range of thermodynamic potentials may simply reflect the limited number of tissue-specific FABPs sampled.

In this study we extend measurements using the ADIFAB method (*17*, *37*) to the FA binding properties of human FABPs from brain (hB-FABP), heart (hH-FABP), intestine (hI-FABP), liver (hL-FABP), and myelin (hM-FABP) as well as the retinoic acid and retinol binding proteins CRABP-I and CRBP-II. This extension allows a wide-ranging comparison of the FA binding thermodynamics to a series of orthologous and paralogous proteins. For each of these FABPs as well as FABPs that were investigated previously with other FA (*12*), we measured the binding affinity of docosahexaenoate. We have also extended the previous systematic study of rI-FABP binding affinities for different FA, to include the complete series of saturated FA between 12 and 20 carbons as well as two series of unsaturated FA. Our results for human brain FABP do not confirm the high degree of FA selectivity found previously for mouse brain FABP. The results do reveal, however, that, with the exception of the intestinal protein, FABPs possess an additional contribution to the binding free energy for unsaturated relative to saturated fatty acids. This preference is manifested by a trend toward increasing affinity with double bond number, relative to that expected if binding were governed solely by the aqueous solubility of the FA. Moreover, our results reveal considerable heterogeneity in the values of the thermodynamic potentials for the binding of FA to paralogous proteins; binding enthalpies range between −1 and −22 kcal/mol and heat capacities between 0 and −1.3 kcal/mol but quite similar values are found for orthologous proteins.

EXPERIMENTAL PROCEDURES

Materials. Sodium salts of FA were purchased from Nu Chek Prep, Elysian, MN. Stock solutions of FA were prepared at 20−50 mM in water with 25 μM butylated hydroxytoluene, pH ≥ 9.0, and stored under argon at −20 °C. The buffer used to measure FA binding to FABPs consisted of 20 mM HEPES, 150 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4, at pH 7.4. The pET11a and pET11d expression vectors and *Escherichia coli* strain BL21 (DE3) were purchased from Novagen, Madison, WI. Lipidex-5000 was purchased from Packard Instruments and Sigma. Gifts of cDNA clones were obtained as follows: rat CRBP-II and mouse CRABP-I from Dr. E. Li (Washington University), mouse and human A-FABP from Dr. D. Bernlohr (University of Minnesota), human M-FABP from Dr. K. Hayasaka (Yamagota University, Japan), human B-FABP from Dr. H. Shinomiya (Otsuka Gen Research Institute, Tokushima, Japan), and rat H-FABP from Dr. P. Brecher (Boston University). An *E. coli* strain expressing human intestinal FABP was obtained from Dr. J. C. Sacchettini (Texas A&M University, College Station, TX). Bovine H-FABP protein was a gift from Dr. A. Lezius (University of Marburg, Germany) and rat I- and L-FABPs were generated as described (*12*). The fluorescent I-FABP, ADIFAB, is from acrylodan-derivatized recombinant rat intestinal fatty acid binding protein (rI-FABP) prepared as described (*17*) and is available from FFA Sciences LLC, San Diego, CA, and from Molecular Probes, Eugene, OR.

Preparation of FABP Proteins. All FABPs were recombinant proteins that were expressed in the BL21 (DE3)/pET11 host/vector expression system as described previously (*12*, *16*, *17*). All of the recombinant FABPs were purified from cell lysates by a modification of the method of Lowe et al. (*38*), as described previously (*12*, *17*). The preparation of human heart, liver, intestine, and myelin FABPs is described in ref *16*. Human brain FABP was prepared in a similar way as human heart FABP. All FABP protein was delipidated to remove fatty acids by slow (3−5 h) Lipidex-5000 chromatography at 37 °C, and FABP purity was assessed by SDS−polyacrylamide gel electrophoresis, and HPLC. HPLC was done on a Beckman Gold system with a Biobasic C-4 reverse-phase column (4.6 mm × 100 mm) (Western Analytical Products, Inc., Murrieta, CA). About 20 μg of protein was loaded on the column at an initial

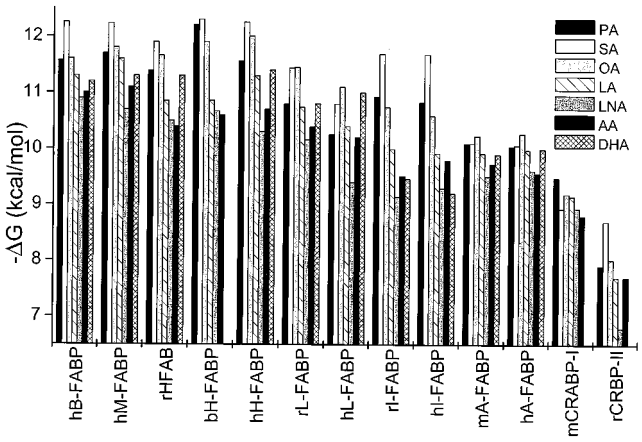

FIGURE 1: Binding free energies for FABPs and retinoid binding proteins from different tissues and species. Binding free energies were determined at 37 °C for each protein and up to seven different FA. Abbreviations are given in Table 1. Values for rH-FABP, bH-FABP, rL-FABP, rI-FABP, mA-FABP, and hA-FABP are from ref *12*.

acetonitrile concentration of 5% and a flow rate of 1 mL/min. The acetonitrile concentration was increased to 35% in 5 min and then to 65% in 30 min, and both 214 and 280 nm ODs were monitored. FABP concentration was determined principally by UV absorbance using the consensus protein molar extinction coefficients (*12*). After isolation the proteins were stored at 4 °C in buffer consisting of 50 mM Tris, 1mM EDTA, 0.5 mM PMSF, and 0.05% Na azide at pH = 8.0.

Fatty Acid Binding to FABP. Measurements of the binding of FA to FABP were done by using ADIFAB fluorescence to monitor the binding of the sodium salts of the FA to each FABP at temperatures between 10 and 45 °C as described (*19*). ADIFAB binding affinities for FA not reported previously were determined as described (*17*). FA titrations were done under conditions in which FA was monomeric, in the stock FA solutions as well as in the cuvette where the reaction with FABP was monitored, by the methods described in refs *17* and *37* to determine the FA solubility. Binding isotherms for FABPs that exhibited linear van't Hoff behavior were used to determine $\Delta H°$ values from the slopes of each of the van't Hoff plots. $\Delta G°$ values were evaluated from the K_ds measured at 25 °C and $-T\Delta S°$ was calculated as $\Delta G° - \Delta H°$. For FABPs that did not reveal linear van't Hoff behavior, thermodynamic potentials $\Delta H°$, $-T\Delta S°$, and $\Delta C_p°$ were determined from the integral form of the van't Hoff expression (*39*).

RESULTS

Comparison of FA Binding Affinities among Proteins from Different Tissues and Species. Affinities for FA binding to seven distinct proteins (human brain, heart, intestine, liver, and myelin FABPs and the mouse CRABP-I and rat CRBP-II) were determined in the present study by the ADIFAB method (see Experimental Procedures). Results of these measurements at 37 °C, together with previous results for six other FABPs, reveal a number of general features (Figure 1 and Table 1). First, K_ds range between 2 and 10 000 nM and the corresponding $\Delta G°$ values range between −12.2 and −6.8 kcal/mol, for all FA and proteins. For the same FA and different proteins K_ds differ by 300−500-fold, while for the same protein and different FA they differ by 3−60-fold. FA affinities for the heart, brain, and myelin FABPs are similar and have the highest affinities, while the retinoid binding proteins bind FA with significantly lower affinities.

Table 1: Fatty Acid Binding Affinities[a]

	fatty acid[c]						
protein[b]	SA	PA	OA	DHA	LA	AA	LNA
hB-FABP[d]	2.3	7	7	13	11	18	21
hM-FABP[d]	2.4	6	5	10	7	15	29
rH-FABP[e]	4	9	6	10	22	45	38
bH-FABP[e]	2	2.4	4.4		22	34	30
hH-FABP[d]	2.3	7	4	9	11	27	55
rL-FABP[e]	9	23	9	23	27	48	69
hL-FABP[d]	23	60	15	19	57	100	240
rI-FABP[e]	6	20	27	213	89	197	367
hI-FABP[d]	6	23	35	309	101	125	317
mA-FABP[e]	76	77	61	107	101	139	196
hA-FABP[e]	80	83	57	84	92	182	167
mCRABP-I[d]	500	223	360	n.d.	380	670	540
mCRBP-II[d]	750	2600	2200	n.d.	3600	2900	10 000

[a] K_d values were measured at 37 °C and are given in nanomolar units. [b] Proteins are abbreviated with b, h, m, and r to indicate bovine, human, mouse, and rat, respectively, and A, B, H, I, L, and M to indicate adipocyte, brain, heart, intestine, liver, and myelin, respectively. [c] FA are abbreviated as follows: PA, palmitate (16:0); SA, stearate (18:0); OA, oleate (18:1); DHA, docosahexaenoate (22:6); LA, linoleate (18:2); AA, arachidonate (20:4); LNA, linolenate (18:3). [d] This study; only the high-affinity site results for liver FABP are shown. [e] From ref *12*.

Second, for adipocyte, heart, and intestine, affinities for FABPs that originate from different species but from the same tissue are virtually identical. This is consistent with results for adipocyte and heart reported previously for a smaller number of species and FA types (*12*). Results for the liver protein, however, reveal significantly lower (2−4-fold) affinities for the human as compared to the rat protein, with the possible exception of DHA, for which the affinities are quite similar.

Third, for all FABPs affinities generally decrease with decreasing chain length and increasing double bond number. As emphasized previously, this is a reflection of the important role played by the aqueous solubility of the FA in determining binding affinities (*12*). This dependence on solubility is illustrated in Figure 2, where the ratio K_d(FA)/K_d(SA) is plotted for each FABP and the FA are arranged in order of decreasing aqueous/membrane partition coefficient (*40*). The K_d for SA is used to normalize these values because it has the largest partition coefficient or, equivalently, the smallest aqueous solubility of these FA, and SA generally has the largest FABP binding affinity. In this representation FABP ratios increase approximately monotonically, consistent with the important role that FA aqueous solubility plays in binding (*12*, *19*). Although previously results indicated that affinity and solubility were almost perfectly correlated for I-FABP (*15*), these results did not include DHA, and as indicated in Figure 2, DHA appears to have lower affinity than predicted from the aqueous solubility of DHA.

Intestinal FABP Binding Affinities Are Similar to Aqueous/Solvent Partition for a Wide Range of FA. Binding affinities for ADIFAB and rat I-FABP were determined for all members of the series of saturated FA between 12 and 20 carbons and for 10 different unsaturated FA. This extends previous results (*37*, *41*) to the odd-chain saturated FA and to the 16:1, 22:6, and the 20-carbon series of FA. Results

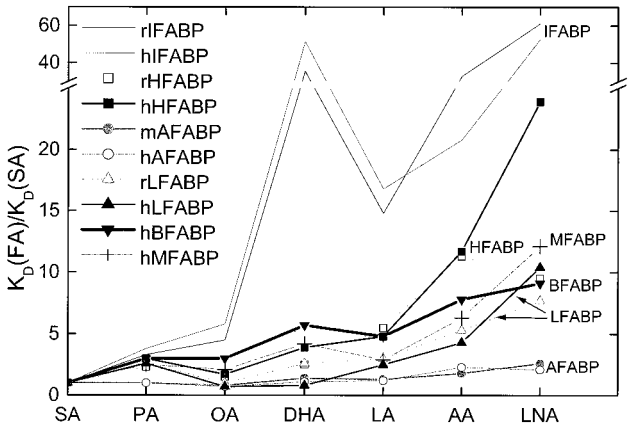

FIGURE 2: Variation of FABP binding affinities with relative solubility of the FA. Values of the K_d for each protein and each FA determined at 37 °C [K_d(FA)] were normalized to the value for stearate binding [K_d(SA)]. This ratio was plotted with increasing FA solubility or equivalently, decreasing aqueous/lipid partition (K_p). Abbreviations are given in Table 1.

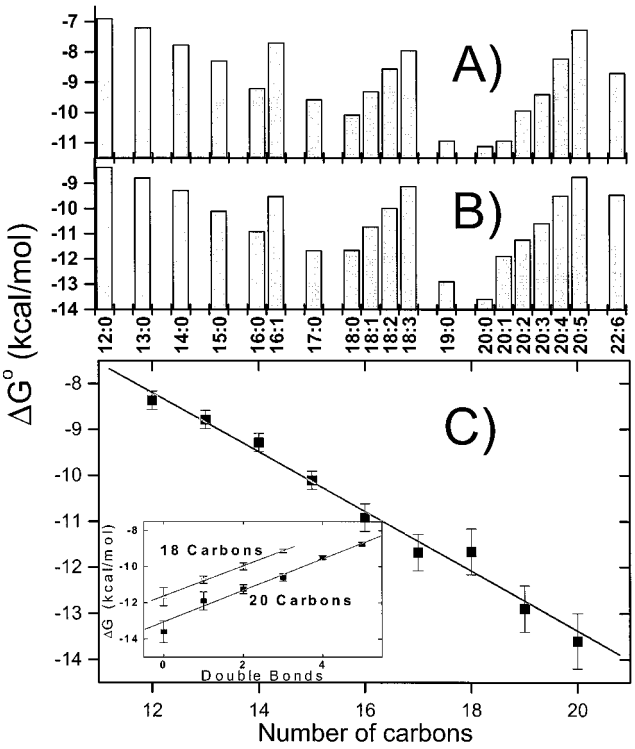

FIGURE 3: ADIFAB and rat intestinal FABP binding free energies for saturated and unsaturated FA with chain lengths between 12 and 22 carbons. Binding free energies were determined at 37 °C. (A) ADIFAB; (B) rI-FABP. (C) Binding free energies to rI-FABP for the saturated FA from 12 to 20 carbons and for the unsaturated series for the 18- and 20-carbon FA (inset). Linear fits yield slopes of −0.65 kcal/mol for the saturated series and 0.82 and 0.87 kcal/mol for the 18- and 20-carbon unsaturated series, respectively. A similar analysis (not shown) for ADIFAB reveals −0.56, 0.74, and 0.78 kcal/mol for the saturated and 18- and 20-carbon unsaturated series, respectively.

for the saturated FA series reveal increases in $|\Delta G°|$ with increasing FA chain length that are well described by linear fits, yielding incremental increases in $|\Delta G°|$ with increasing carbon number of 0.58 and 0.62 kcal/mol for ADIFAB and I-FABP, respectively (Figure 3 and Table 2). Binding of FA with increasing double bond numbers, for the 18- and 20-carbon series, also reveals a linear dependence for $\Delta G°$ on double bond number in which the incremental decrease in

Table 2: K_d Values for ADIFAB and I-FABP[a]

FA[b]	ADIFAB	I-FABP
12:0	14 000	1290
13:0	8500	650
14:0	3400	289
15:0	1450	77
16:0	330	21
16:1	2700	198
17:0	180	6
18:0	80	6
18:1	280	28
18:2	940	92
18:3	2500	376
19:0	20	1
20:0	15	0.3
20:1	20	4
20:2	100	12
20:3	240	35
20:4	1630	205
20:5	7700	693
22:6	750	218

[a] K_d values were measured at 37 °C and are given in nanomolar units. [b] The FAs are designated by chain length:double bond number.

$|\Delta G°|$ for both series and for both ADIFAB and I-FABP is about 0.8 kcal/mol (Figure 3 and Table 2). The linear dependence on chain length and double bond number as well as the magnitude of the incremental changes in $\Delta G°$ are quite similar to the behavior observed for FA partition between the aqueous phase and simple hydrophobic solvents and membranes (*42*, *43*).

Binding Free Energy Is Predominantly Enthalpic for FABPs. Binding to human brain, heart, intestine, liver, and myelin FABPs and to mCRABP-I was measured for temperatures between 10 and 50 °C, for OA and PA or AA. The K_ds from these measurements were used to construct van't Hoff plots, from which the thermodynamic potentials, $\Delta H°$ and $T\Delta S°$, were determined (Experimental Procedures). The results indicate that the free energy of binding to the FABPs is predominantly enthalpic, with enthalpies that range from about −22 to −9 kcal/mol (Table 3). These results are consistent with previous findings that FA binding for WT FABPs is enthalpically driven (*14*, *19*, *35*, *36*). In contrast, the binding enthalpies for mCRABP-I are much less favorable; the binding reaction for oleate is actually entropically driven.

Heat Capacity Changes Accompanying FA Binding Reveal Tissue-Specific Differences. Binding to intestinal, heart, and myelin FABPs is well described by a linear van't Hoff function, whereas adipocyte, brain, and liver FABPs as well as CRABP-I reveal nonlinear plots. This translates into substantial heat capacity changes (between −0.5 and −0.7 kcal mol^{-1} K^{-1}) for the adipocyte, brain, liver, and CRABP-I proteins, and virtually zero (<0.1 kcal mol^{-1} K^{-1}) changes for intestinal, heart, and myelin FABPs (Table 3).

Binding Stoichiometries. Binding stoichiometries of 1.0 were found for most of the proteins (*12*; the present study). For the liver FABP (both human and rat) we found a stoichiometry of 2.0 with the second site having an affinity 100-fold less than the high-affinity site (*39*; the present study). In contrast, for the heart FABPs, significantly smaller stoichiometries of 0.5 were found previously for rat and bovine heart FABP (*12*). Consistent with these results, a stoichiometry of 0.3 for hH-FABP was found in the present

Table 3: Thermodynamics of FA Binding to FABPs and CRABP[a]

protein	FA	$\Delta G°$	$\Delta H°$	$-T\Delta S°$	$\Delta C_p°$
hH-FABP[b]	PA	-11.6 ± 0.1	-11.6 ± 2	0 ± 2	0
	OA	-11.8 ± 0.1	-11.3 ± 1.4	-0.5 ± 1.4	0
rH-FABP	OA	-11.3 ± 0.1	-9 ± 2	-3 ± 2	0
	AA	-10.3 ± 0.1	-12 ± 2	2 ± 2	0
hB-FABP[b]	OA	-11.7 ± 0.1	-12.3 ± 2	0.6 ± 2	-0.6 ± 0.1
	AA	-10.8 ± 0.1	-15.6 ± 2	4.8 ± 2	-0.7 ± 0.1
hM-FABP[b]	OA	-12.5 ± 0.1	-22 ± 5	9.5 ± 5	0
	AA	-11.2 ± 0.1	-14.8 ± 1	3.6 ± 1	0
hL-FABP[b]	OA	-10.9 ± 0.1	-9 ± 1	-1.9 ± 1	0.7 ± 0.3
	AA	-10.3 ± 0.1	-12 ± 2	1.7 ± 2	0
rL-FABP[d]	OA	-11.7 ± 0.1	-11.4 ± 1	-0.3 ± 1	-0.9 ± 0.1
	AA	-10.8 ± 0.1	-13 ± 1	2 ± 1	-0.8 ± 0.2
rI-FABP[c]	OA	-10.6 ± 0.1	-12 ± 2	1.4 ± 2	0
	AA	-9.5 ± 0.1	-11.6 ± 1	2.1 ± 1	0
mA-FABP[b]	OA	-10.3 ± 0.1	-6.7 ± 1.5	-3.6 ± 1.5	-0.5 ± 0.2
	AA	-9.6 ± 0.1	-9.4 ± 1.4	-0.2 ± 1.4	-0.6 ± 0.2
mCRABP-I[b]	OA	-8.9 ± 0.1	-1.4 ± 1	-7.5 ± 1	-0.6 ± 0.1
	AA	-8.7 ± 0.1	-5.1 ± 1	-3.6 ± 1	-0.5 ± 0.1

[a] Thermodynamic parameters (at 25 °C) were determined from van't Hoff analyses (Experimental Procedures) and are in units of kilocalories per mole for $\Delta G°$, $\Delta H°$, and $T\Delta S°$ and kilocalories per mole per Kelvin for $\Delta C_p°$. Abbreviations for proteins and FA are given in Table 1. A value of 0 for ΔC_p indicates that the van't Hoff plot was linear and that $\Delta C_p° < 0.1$ kcal mol^{-1} K^{-1}. [b] This study. [c] From ref *19*. [d] From ref *39*.

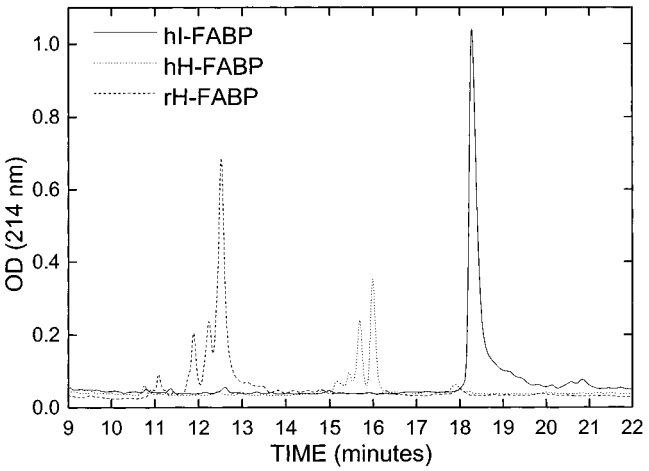

FIGURE 4: HPLC scan of human I-FABP, human H-FABP, and rat H-FABP. Similar amounts of protein (20 μg), as determined from the 280 nm OD, were loaded on a Biobasic-4 column (a C-4 reverse-phase column). The flow rate was 1 mL/min. Acetonitrile was initially 5% in water and was increased to 35% in 5 min and then to 65% in 30 min.

study. Although the origin of these low stoichiometry values was not apparent previously, the HPLC results from the present study (Figure 4) suggest that low stoichiometries reflect the presence of admixtures of proteins that copurify with H-FABP and do not bind FA. HPLC profiles at 214 nm for similar concentrations (determined by the OD at 280 nm) of rat and human heart FABPs and human intestinal FABP reveal multiple peaks for the heart FABPs and essentially a single peak for the intestinal protein (Figure 4). Single peaks were also obtained for the other proteins (adipocyte, brain, liver, and myelin) and these have stoichiometries ≥ 1.0 (data not shown). The height of the largest peaks for each of the rat and human heart FABPs are about 70% and 35%, respectively, that of the intestine, roughly in proportion to the corresponding stoichiometries (0.5 ± 0.1 and 0.3 ± 0.1, respectively) for these two proteins. This suggests that the stoichiometries for the heart proteins appear to be <1.0 because contaminating proteins with relatively large OD (280 nm) are coexpressed in the pET/BL21 system with H-FABP but not other proteins. We do not know why these additional proteins appear only with heart FABP, but as described previously, rat heart FABP runs as a single band on SDS−PAGE, does not appear to be partially folded, and does not form aggregates (*12*). The contaminating proteins do not affect the thermodynamic parameters because the binding isotherms for the heart proteins are well described by a single affinity with a stoichiometry ≤ 0.5 (*12*; data not shown), indicating that FA binding to the contaminant proteins is not significant.

DISCUSSION

In this study we describe FA interactions with five FABPs and two retinoid binding proteins not previously measured with ADIFAB. These results, together with previous measurements of six other FABPs, reveal (1) K_d values that range from 2 to 400 nM, (2) tissue-specific FABP affinities that are roughly the same for different animal species, (3) binding free energies that are primarily enthalpic, and (4) heat capacity changes that are different for FABPs from different tissues. In addition, affinities generally increase with decreasing solubility of the FA and therefore relatively little specificity for particular FA is revealed by any of the proteins. Most striking, for rat I-FABP the affinities for 19 different FA closely parallel those predicted from the partition of FA between aqueous solution and an isotropic hydrocarbon solvent. FABPs do, however, appear to be designed to bind FA (K_ds range from about 2 to 400 nM), rather than other small hydrophobic ligands such as the retinoids, which bind only weakly (K_ds are probably $>1\ \mu$M) to FABPs (*14*, *16*, *44*). Likewise, FA bind only weakly to the retinoid binding proteins, CRABP-I and CRBP-II (K_ds range from about 200 to about 10 000 nM) while retinoids bind to their respective proteins with K_ds that are less than about 100 nM (*45*, *46*). Moreover, FA binding to FABPs is enthalpically driven, while for CRABP-I entropy plays a larger or even dominant role. This is consistent with the notion that interactions embodied in the enthalpic portion of the free energy generates the specificity of FABPs for binding FAs.

Binding Specificity Is Sensitive to Tissue Origin. For any particular FA, binding affinities for the FABPs display a substantial tissue-dependent difference, ranging from about 10-fold for LNA to 40-fold for SA among the FABPs tested (Table 1 and Figure 1). This tissue specificity of binding affinities may reflect tissue-specific differences in FA metabolism. Support for such a relationship and an indication of the importance of the FABP function is the highly conserved affinities displayed by FABPs from the same tissue type but different species, at least for the intestinal, heart, and adipocyte proteins (Table 1). This conservation of binding characteristics across different species, which was observed previously for rat and bovine heart and mouse and human adipocyte FABPs (*12*), has been extended in the present study to the human heart and the rat and human intestinal proteins. Liver FABP, in which the binding affinity to rat is greater than to human, appears to be an exception, although even in this case the average difference is only about a factor of 2. Liver has an especially important and

complex role in lipid trafficking, and we speculate that fine-tuning the L-FABP characteristics may be necessary in generating species-specific differences in lipid metabolism.

As discussed previously (*41*), a tissue-specific function that is consistent with the observed affinities, at least for those tissues in which FA transport between tissues may be important, is one in which FABPs play a significant role in determining intracellular FFA levels. Thus tissues with relatively low-affinity FABPs such as adipocyte and intestine (K_ds for oleate of 60 and 30 nM, respectively) might have intracellular FFA levels that are higher than serum (7 nM) and would thereby be able to export FFA out into the serum. In contrast, the heart, for which FA are the major energy source, and possibly liver have FABPs with higher affinities (4 and 10 nM, respectively) and would be able to extract FFA from the serum.

FABPs from brain and myelin may function in FA transport/metabolism only during development, when transport of FA across the blood brain barrier is significant (*34*). The developing brain has an essential requirement for long-chain polyunsaturated FA such as DHA, which is enriched in brain lipids (*47*). Human brain FABP might, with the appropriate distribution of binding affinities, function in the fetus or neonate to preferentially extract the essential FA from serum, as reported for mouse brain (*34*). Our results, however, show that although the human brain and myelin FABPs have relatively high affinities for polyunsaturated FA, these affinities are weaker than for saturated and monounsaturated FA and in general binding affinities for brain and myelin are not significantly different than for the heart FABPs. Thus our results do not support the notion that specific FA binding characteristics of brain FABP are necessary to generate a lipid composition of brain tissue that is enriched in particular polyunsaturated FAs.

Lack of Pronounced FA Binding Specificity. FA aqueous solubility seems to play an important role in determining the affinity for FABP (*19*). The most striking example of this occurs for I-FABP, where the $\Delta G°$ for binding of six different long-chain FA were virtually identical to the $\Delta G°$ for partition of these FA between aqueous phase and membranes (*15*). With the exception of DHA, which has a smaller than predicted affinity, the results for an additional 11 FA in the present study provide further support for the dominant role that aqueous solubility plays in determining binding affinities for I-FABP. Specifically, the increments in $\Delta G°$ with each additional carbon atom and double bond, -0.62 and -0.82 kcal/mol, respectively (Figure 3), are consistent with values obtained for FA and/or alkane partition between aqueous phase and organic solvents (*42*, *43*).

Although the affinity of FA for I-FABP appears to be dominated by the aqueous solubility of the FA, this does not mean that the binding to I-FABP is equivalent to partition into a simple hydrophobic solvent. On the contrary, although the free energy for FA partition between the aqueous and membrane phases is a predominantly entropic process, the free energy of binding to I-FABP is predominantly enthalpic; $\Delta G° = -10 \pm 1$ kcal/mol and $\Delta H° \cong -11$ kcal/mol (*19*). Although enthalpy dominates the free energy of binding for all FABPs, for I-FABP, in contrast to other FABPs, the enthalpy of binding is virtually identical for all FA. Although a small component of $\Delta G°$ ($|T\Delta S°| \leq 2$ kcal/mol) the entropic portion decreases with increasing aqueous solubility

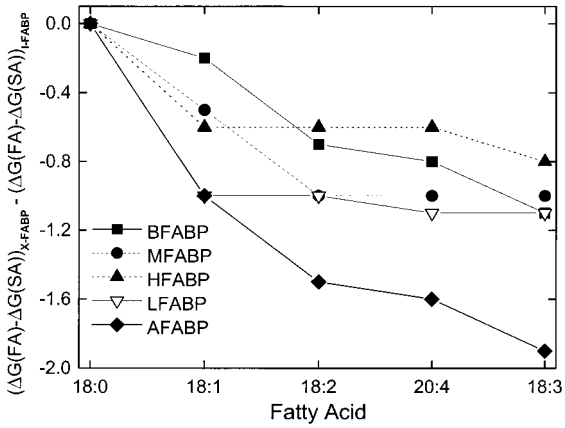

FIGURE 5: Double-bond-mediated increase in binding free energy. The difference in binding free energy, at 37 °C, between each FABP and FA and the value for stearate was computed. The value for this difference for the intestinal FABP was subtracted from the corresponding values for each of the other FABPs listed in the figure. This quantity was plotted for the 18 carbon series of FA, revealing an average -0.4 kcal/mol contribution of each double bond to the binding free energy.

and accounts for the difference in affinity for different FA. Thus interactions between the bound FA and amino acids within the binding cavity of I-FABP contribute a favorable $\Delta H°$ of about -11 ± 1 kcal/mol and an unfavorable $T\Delta S°$ of about $+10$ kcal/mol, which together with the approximately -10 kcal/mol favorable entropy change of the solvent water upon FA desolvation, yields an overall $\Delta G°$ of about -10 kcal/mol. This average overall $\Delta G°$ is modified by about 2 kcal/mol for different FA by their solubilities in solvent water. That $\Delta G°$ for binding to I-FABP and membranes are so similar may be due in part to the especially hydrophobic amino acid composition of the I-FABP binding cavity (*6*, *7*).

Although affinities for all FABPs tend to decrease with increasing aqueous solubility of the FA, the steep dependence on FA solubility for I-FABP seems to be exceptional (Figure 2). The weaker dependence on solubility for the other FABPs indicates that interactions within the cavity are relatively more favorable for the unsaturated FA as compared to I-FABP. In addition to the FABPs investigated in the present study, bovine epidermal FABP also reveals this preference for unsaturated FA (*28*). As illustrated in Figure 5, this preference for unsaturated FA amounts to an additional $1-2$ kcal/mol favorable free energy relative to I-FABP. This implies that for most FABPs, specific interactions occur between FA double bonds and amino acids/bound water of the binding cavity. Consistent with a specific double-bond interaction, FABP $|\Delta H°|$ values increase (become more favorable) with increasing FA unsaturation except for I-FABP, for which $\Delta H°$ is independent of FA type (*19*; Table 3).

Proteins from Different Tissues Reveal Large Differences in Binding Thermodynamics. Previous studies of FABPs from adipocyte, heart, intestine, and liver revealed $\Delta H°$ values for oleate binding to these proteins that differed by only about 4 kcal/mol (*19*). The results of the present study show that including brain and myelin FABPs extends the differences in $\Delta H°$ values to about 15 kcal/mol for the different FABPs and about 21 kcal/mol if the CRABP-I protein is also included (Table 3). In addition, heat capacity changes generated by FA binding to FABPs from different tissues and the

CRABP-I protein also reveal large tissue-specific differences; ΔC_p values range from 0 to −1.3 kcal mol^{-1} K^{-1} (Table 3; *15*, *39*). In contrast to the differences observed for proteins from different tissues, proteins from the same tissue but different species reveal quite similar thermodynamic potentials.

These tissue-specific differences in thermodynamic potentials reflect differences in the interactions between FA and the protein within the binding cavity. The large magnitude of these differences is not surprising because single amino acid substitutions in these proteins generate changes in thermodynamic potentials of similar magnitude (*15*) and proteins from different tissues have amino sequence identities that are <80% (*16*). Different interactions do not appear to be required solely to generate altered FA binding affinities (Tables 1 and 3). For example, brain, myelin, and heart FABPs have similar FA binding affinities but have $\Delta H°$ values that differ by about 13 kcal/mol and ΔC_p values that are zero for myelin and heart but quite large (−0.6 kcal mol^{-1} K^{-1}) for brain FABP. These different thermodynamic potentials may reflect the need to maintain similar FA binding characteristics for proteins that differ significantly in amino acid sequence. Different tissues may generate specific FA metabolic characteristics with FABPs that have similar FA binding characteristics but, because of their tissue-specific amino acid sequence differences, their FABPs interact with different intracellular components. This would allow tissue-specific regulation of FA metabolism as occurs, for example, through the interaction of hormone-sensitive lipase and FABP in the adipocyte (*25*).

After this work was submitted for publication, a report using isothermal titration calorimetry (ITC) to determine the binding of FA to three liver FABPs, from bovine, human, and mouse, appeared in this journal (*48*). Several features of this study differ from those found in the present and in previous studies (*12*, *39*, *49*). Most importantly, the K_d values for stearate (for example, 1900 nM for the human and 400 nM for the mouse proteins) were much larger (weaker binding) than observed in the present study for human protein (23 nM) or for the rat protein (9 nM) observed previously (*12*). We suggest that the origin of this discrepancy is due to the poor solubility of stearate, estimated to be <1 μM (*17*). This is more than 3000-fold smaller than the 3.1 mM solutions used for ITC injections in ref *48*, making it likely that the heat change measured corresponded to much lower concentrations of stearate in the reaction vesicle than assumed. As a consequence, the conclusion that liver FABPs preferentially bind unsaturated relative to saturated FA by as much 500-fold (*48*) is probably not accurate and contrasts significantly with the present results, which reveal the opposite trend (Table 1). In addition, although the binding constants for oleate and docosahexaenoate found in ref *48* and the present study reveal much closer agreement than for stearate, the free energy values (for example, −45.7 kcal/mol for oleate binding to the human liver FABP) are inconsistent with the K_d values shown in Table 2 of ref *48* and are much smaller than the −11.7 kcal/mol value of Table 3.

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