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Thermodynamics of Fatty Acid Binding to Fatty Acid-binding Proteins and Fatty Acid Partition between Water and Membranes Measured Using the Fluorescent Probe ADIFAB*

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Using the fluorescent probe ADIFAB (acrylodan-derivatized intestinal fatty acid-binding protein) to determine the equilibrium concentration of the free (unbound) fatty acid (FFA), dissociation constants were measured between 10 and 50 °C for the interaction of five different long chain fatty acids (FA) with fatty acid-binding proteins (FABP) from adipocyte, intestine, and heart. Gibbs free energies (ΔG) determined from the dissociation constants were between about −9 and −11 kcal/mol at 25 °C. Thermodynamic parameters for binding were determined using van't Hoff plots of the dissociation constants, which range, over the entire temperature region, between 2 and 3000 nM. For all the unlabeled FABPs, free energies of binding were dominated by large negative enthalpies that ranged from −7 to −12 kcal/mol, and the enthalpies tended to decrease with increasing FA unsaturation. The entropic contributions ($-T\Delta S$) at 25 °C ranged between −4 and +2 kcal/mol and tended to increase with increasing FA unsaturation. To assess the role of FA aqueous solubility in FABP binding, measurements of the partition of FA between unilamellar lipid vesicles and water were also done using ADIFAB; the lipid/water partition coefficients (K_p) determined from these measurements were found to be independent of temperature. The binding of FA to FABP is governed by the sum of contributions of various interactions between FA, water, and FABP. An analysis of the individual contributions suggests that the net free energy of binding results from the canceling in part of a number of separate quite large contributions. The entropic contributions sum almost to zero for most FA and FABPs as a result of the canceling of a large increase in bulk solvent entropy by decreases in configurational entropy upon FA binding to FABP. The net, approximately −10 kcal/mol enthalpy of binding, probably results from an increase in FA configurational enthalpy upon binding to FABP plus a large negative enthalpy from the interaction between the FA and the FABP. This large enthalpy of the FA-FABP interaction suggests that in addition to previously identified specific interactions between the carboxylate portion of the FA and charged amino acids within the binding cavity, other significantly larger enthalpic interactions, presumably involving the hydrocarbon portion of the FA, must contribute to the binding energy.

Fatty acid-binding proteins (FABP)[1] are 14–15-kDa proteins found in the cytosols of a variety of tissues (1–8). Considerable binding and structural evidence indicates that fatty acids (FA) are the natural ligands for these proteins (9–12). Most studies of the binding of FA to FABP have revealed relatively little variation in affinity either as a function of the type of FA or FABP and are generally consistent with a K_d of about 1 μM (Ref. 13; see reviews in Refs. 6 and 12). These previous results, therefore, suggested that the interactions that govern binding are similar for all FA and all FABPs.

As discussed previously (12), the uniformity and magnitude of binding affinities are surprising for several reasons. 1) FABPs from different tissues exhibit considerable differences in amino acid sequence (6). 2) The conformation of the bound ligand as revealed by x-ray crystallography differs with FABP type and to some extent with FA type (6, 7, 14). 3) Different FA have very different aqueous solubilities (12, 15). 4) Rates of transfer of the anthroyloxy FA from FABP to membranes are quite different for different FABPs (16). 5) FABPs with K_d values on the order of 1 μM would not be expected to interact appreciably with FA under physiologic conditions because serum FFA is about 7 nM (17).

We have recently reinvestigated the binding of FA to FABP using a fluorescent probe, composed of acrylodan-derivatized intestinal FABP and denoted ADIFAB (12). In contrast to most previous methods for estimating binding affinities, the ADIFAB method allows one to determine the concentration of FFA in equilibrium with FABP without separating the reaction products. The equilibrium dissociation constants (K_d) measured at 37 °C by this method range, for different FABPs and different FA, between 2 and 1000 nM, indicating much greater binding affinities as well as greater heterogeneity than previous studies.

This ability to measure accurately the binding affinities makes it possible to investigate the specific interactions that contribute to the free energies of binding. Interactions that are likely to contribute significantly include those, as indicated by x-ray crystallography, that involve the interaction of the FA carboxylate through a network of hydrogen bonds with specific Arg residues in the FA binding cavity of the FABP (9, 18, 19). X-ray crystallography also reveals that large numbers of both ordered and disordered water molecules within the FA binding cavity are displaced upon FA binding, and this might also contribute to the free energy of binding. In addition to these interactions that involve the FABP directly, our previous results from equilibrium binding measurements using ADIFAB

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[1] The abbreviations used in this paper: FABP, fatty acid-binding protein; ADIFAB, acrylodated I-FABP; FA, fatty acid; FAH, uncharged FA; FFA, free fatty acids also sometimes referred to as unbound FFA; A-FABP, human adipocyte FABP; H-FABP, rat heart FABP; I-FABP, rat intestinal FABP.

at 37 °C indicate that FA solubility in water plays an important role in determining the relative binding affinities of different types of FA (12). Moreover, these binding measurements also suggest that the magnitude of the free energy change upon transferring a FA from the bulk solvent to the FABP cavity depends on the overall hydrophobicity of the amino acids within the cavity.

In general, the binding of FA to FABP can be described in terms of several discrete events, each of which contribute to the overall free energy. The free energy corresponding to the observed equilibrium constants can be represented as a sum of the difference of free energies of the two states defined as: 1) FA in water + FABP without FA (apoFABP) and 2) water without FA + FA bound to FABP (holoFABP). Thus, the change in the free energy of binding should be a sum of contributions from the changes in the free energy of 1) the bulk solvent, 2) the FA, 3) the protein, and 4) the protein-bound water. In the present study each of these contributions has been estimated using both experimental and theoretical information. The change in the free energy, enthalpy, and entropy of FA binding to FABP was determined by measuring binding at temperatures between 10 and 50 °C. The temperature dependence of the partition of FA between membranes and water was also measured, and these values were used to estimate the change in thermodynamic parameters of bulk solvent upon removal of FA. These results help to elucidate the nature of the binding interaction between FA and FABP.

EXPERIMENTAL PROCEDURES

Materials—Sodium salts of FA were purchased from NuChek 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 10 mM HEPES, 150 mM NaCl, 5 mM KCl, and 1 mM $NaHPO_4$, at pH 7.4. The pET11a and pET11d expression vectors and the BL21 (DE3) *Escherichia coli* strain were purchased from Novagen, Madison, WI. Lipidex-5000 was purchased from Packard Instruments and Sigma. The fluorescent I-FABP, ADIFAB, was prepared from acrylodan-derivatized recombinant rat intestinal fatty acid-binding protein (rI-FABP) as described (15) and is available from Molecular Probes, Eugene, OR.

Lipid Vesicles—Large unilamellar vesicles composed of egg phosphatidylcholine were prepared by extruding the hydrated lipid 10 times through two stacked 0.1-μm polycarbonate filters (Nucleopore) at room temperature, essentially as described (20, 21). Lipid was dissolved in chloroform, evaporated to dryness, and lyophilized overnight, and the dried lipid film was hydrated in buffer.

Preparation of FABP Protein—All FABPs (rat intestine, human and mouse adipocyte, and rat heart) were recombinant proteins that were expressed in the BL21 (DE3)/pET11 host/vector expression system as described previously (12, 15). All of the recombinant FABPs were purified from cell lysates by a modification of the method of Lowe *et al.* (22), as described previously (12, 15). 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 and isoelectric focusing polyacrylamide gel electrophoresis. FABP concentration was determined principally by UV absorbance using the consensus protein molar extinction coefficients (12).

[FFA] Determination with ADIFAB—ADIFAB responds to FA binding by undergoing a shift in fluorescence emission from 432 nm in the apo, to 505 nm in the holo form (15). As described previously (12, 15, 21), the concentrations of FFA and FA bound to ADIFAB can be determined according to,

$$[\mathrm{FFA}] = K_d\ 19.5\ (R - R_o)/(11.5 - R) \qquad \text{(Eq. 1)}$$

and

$$[\mathrm{ADIFAB_b}] = [\mathrm{ADIFAB_{total}}]\ 19.5\ (R - R_o)/(11.5 - R + 19.5\ (R - R_o)) \qquad \text{(Eq. 2)}$$

where R is the measured ratio of 505 to 432 nm intensities (with blank intensities subtracted), R_o is this ratio with no FFA present, and K_d is the equilibrium constant. The concentration of FFA in equilibrium with ADIFAB was computed as the total FA added to the cuvette minus the amount bound to ADIFAB minus the amount bound to the cuvette walls. The amount bound to the walls, for each FA and at temperatures between 10 and 50 °C, was determined by using ADIFAB to measure the change in [FFA] upon transfer of the sample between cuvettes as described in Ref. 15. Binding of polyunsaturated FA was temperature-independent and less than 14%. For palmitate and oleate, wall binding was as much as 35%; an amount that decreased with decreasing temperature. Measurements were done either with an SLM 8000C or 4800 fluorometer, and standard deviations of the measured R values were typically <0.3%.

Measurement of FA-FABP Equilibrium Binding—The method used to determine FA binding affinities for FABP has been described previously (12). Briefly, the binding of FA to a given FABP was determined by using ADIFAB, in the presence of fixed concentrations of FABP, to monitor FFA levels in equilibrium with ADIFAB and FABP. Thus FA was added in discrete aliquots to a mixture of ADIFAB and FABP, and after each aliquot the ADIFAB fluorescence was measured to determine FFA and $\mathrm{ADIFAB_b}$. FA titrations were done with an ADIFAB concentration of 0.2 μM (3 μg/ml) and FABP concentrations between 1 and 20 μM. Each aliquot of FA, allowing about 10 min for equilibration between aliquots, was added to the cuvette from FA stocks warmed to temperatures above the FA phase transition, and mixed thoroughly. Although a fraction, proportional to [FFA], of the added FA binds to the walls of the cuvette (15), this value was negligible in the presence of FABP or membranes at the concentrations used in the binding or partition measurements, respectively.

The concentration of bound FABP can be expressed in terms obtained from ADIFAB fluorescence (Equations 1 and 2) as shown by Equation 3.

$$[\mathrm{FABP_b}] = [\mathrm{FA_{total}}] - [\mathrm{ADIFAB_b}] - [\mathrm{FFA}] \qquad \text{(Eq. 3)}$$

Using Equations 1–3, a single-site Scatchard analysis was done in which, for each binding isotherm, the fraction (ν) of FABP that has FA bound relative to the total FABP was determined as

$$\nu = [\mathrm{FABP_b}]/[\mathrm{FABP_{total}}], \qquad \text{(Eq. 4)}$$

and this quantity, divided by [FFA], was fitted to

$$\frac{\nu}{[\mathrm{FFA}]} = \frac{n}{K_d} - \frac{\nu}{K_d}, \qquad \text{(Eq. 5)}$$

in which n is the stoichiometry and K_d the dissociation constant (23). Experimental values of ν/[FFA] were fitted to Equation 5 using a weighted linear regression with standard deviations of about 5% (Origin, MicroCal Software, Northampton, MA).

Determination of Equilibrium Constants from Binding Kinetics—Equilibrium constants for FA binding to ADIFAB were also determined using the on and off rate constants ($K_d = k_{off}/k_{on}$) measured by stopped-flow fluorescence as described previously (12). An SLM Milliflow stopped-flow device coupled to an SLM fluorometer was used to measure the time course of the 505/432 R value. The off rate constant was measured by mixing FA-ADIFAB complexes with fatty acid-free bovine serum albumin, and the on rate was measured by mixing FA and ADIFAB.

Partition Coefficients—Partition coefficients were measured as described previously (24). The coefficient describing partition of FA between membrane and aqueous phase is defined as: $K_p = [\mathrm{FA}]_m/[\mathrm{FFA}]$, where $[\mathrm{FA}]_m$ is the concentration of FA in the membrane phase. Expressing these concentrations in terms of the total sample volume,

$$K_p = \frac{V_a}{V_m} \cdot \frac{[\mathrm{FA_m}]}{[\mathrm{FFA}]} \qquad \text{(Eq. 6)}$$

where V_a and V_m are the volumes of the aqueous and membrane phases, respectively, and $[\mathrm{FA_m}]$ is the concentration of membrane-bound FA relative to the total sample volume ($\approx V_a$). In this equation the value of V_m/V_a for lipid vesicles was estimated as 10^{-6} per molar phospholipid ($V_m/V_a = [\mathrm{L}]$, where [L] is the unit-less value of the molar lipid concentration), using an area per lipid molecule of 70 Å^2 and a bilayer width of 40 Å (25).

Miyazaki *et al.* (26) and Peitzsch and McLaughlin (27) have found that the membrane/water partition coefficients for the uncharged FA (FAH) are about 200-fold greater than for the charged species (FA^-). The partition coefficient determined using Equation 6 is a combination of the partition coefficients for FAH and FA^-. To determine the thermodynamic parameters that apply to a single species, the partition coefficients for individual species must be evaluated from the measured

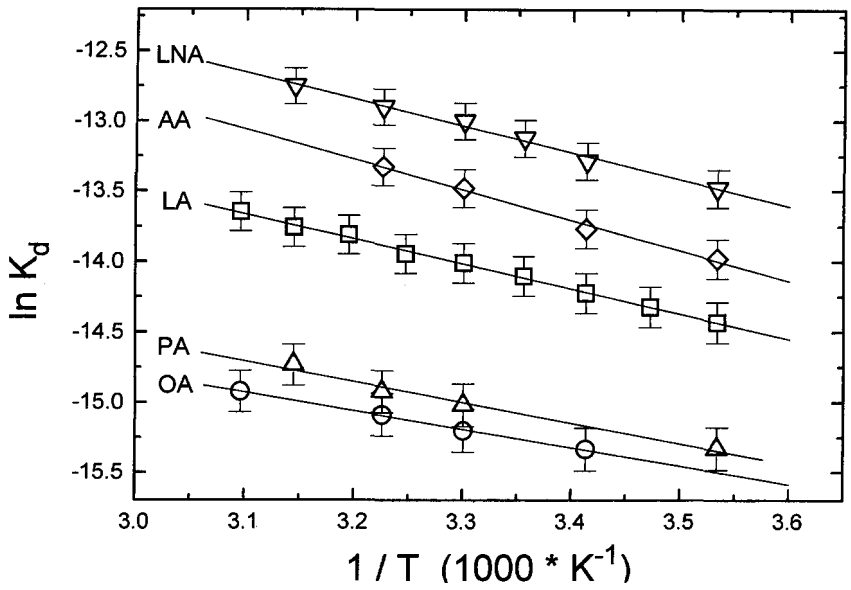

FIG. 1. **Temperature dependence of the binding of FA to ADIFAB.** The *open symbols* in these van't Hoff plots represent K_d values for each FA (see legend of Table I for FA code) determined by the equilibrium measurements described by Equations 1–5 under "Experimental Procedures," and the *lines* drawn through these symbols are the linear fits to these data, whose slopes were used to determine ΔH.

K_p values. To do this, we follow Peitzsch and McLaughlin (27) and note that K_p of Equation 6 can be expressed in terms of the single species partition coefficients as $K_p = K_{FA^-} + (K_{FAH} + 1)$ [FFAH]/[FFA$^-$], where K_{FA^-} and K_{FAH} are, respectively, the partition coefficients of the charged and uncharged species of FA and [FFAH]/[FFA$^-$] is the ratio of the free concentrations of these species. Using the Henderson-Hasselbach equation, [FFAH]/[FFA$^-$] = $10^{pK - pH}$, where the pK of the FA is 4.8 (Ref. 28; page 290), $K_p = K_{FA^-} + (K_{FAH} + 1)\ 10^{4.8 - pH}$. Taking the average of the results of Miyazaki *et al.* (26) and Peitzsch and McLaughlin (27), $K_{FAH} = 220\ K_{FA^-}$ and therefore $K_p \approx K_{FA^-}(1 + 220\ 10^{4.8 - pH})$. Thus at pH 7.4 where the measurements in the present study were done, $K_{FA^-} = 0.64\ K_p$.

Thermodynamic Parameters—To obtain the thermodynamic parameters for the partitioning of FA into membranes, we followed the approach of Peitzsch and McLaughlin (27), who expressed the free energy for transfer of the FA from water into membranes ($\Delta G_{w \rightarrow MEMB}$) in terms of the mole fraction ratio X_m/X_w. The mole fraction of FA within the membrane is $X_m \approx [FA_m]/[L]$, where $[FA_m]$ and [L] are the concentration in the total sample volume of FA and lipid, respectively. Similarly, the mole fraction of FA within the aqueous phase is $X_w \approx [FFA]/55.6$. In terms of the partition coefficient of the charged species of FA ($K_{FA^-} = 0.64\ K_p = 0.64 \cdot ([FA_m]/([FFA] \cdot [L]))$, the mole fraction ratio $X_m/X_w = 36 \cdot K_p$. The free energy for transfer of the charged species of FA from water to membrane is therefore given by Equation 7.

$$\Delta G_{w \rightarrow MEMB} = -RT \ln(X_m/X_w) = -RT \ln(36 \cdot K_p) \quad \text{(Eq. 7)}$$

Several studies have suggested that a term that accounts for solute-solvent differences must be added to this expression for $\Delta G_{w \rightarrow MEMB}$ (29–31). This additional term is significant and would contribute an approximately −10 kcal/mol to the values calculated using Equation 7. This approach, however, remains controversial (see, for example, Refs. 32 and 33), and because the generality of its application is uncertain (34), we have not included this term in the present study.

Free energies for the dissociation of FA from FABP were evaluated from Equation 8.

$$\Delta G_d = RT \ln K_d \quad \text{(Eq. 8)}$$

The corresponding enthalpy changes were determined from the slope of van't Hoff plots and entropy changes were determined from $\Delta G = \Delta H - T\Delta S$.

RESULTS

Temperature Dependence of FA Binding to ADIFAB—Because the probe ADIFAB was used to measure FA binding to unlabeled FABPs and membranes as a function of temperature, it was necessary first to determine the temperature dependence of binding to ADIFAB. This was done for five different FA (palmitate, oleate, linoleate, linolenate, and arachidonate) at temperatures between 10 and 50 °C using the same methods described previously for studies done at 37 °C (Ref. 15 and "Experimental Procedures"). Binding isotherms were determined at each temperature and for each FA. Each isotherm was analyzed by single-site binding to determine the dissociation constant at each temperature.

The dissociation constants obtained from this analysis, which are displayed as van't Hoff plots in Fig. 1, illustrate that the affinities for ADIFAB decrease with increasing temperature for all FA. Fig. 1 also shows that the van't Hoff plots are linear, and fits to these plots were used to obtain the thermodynamic parameters for FA binding to ADIFAB as shown in Table I. These results show that $-T\Delta S$ increases, from −6 to −3.7 kcal/mol, and ΔH decreases, from about −2.9 to −4.4 kcal/mol, with double bond number.

Dissociation constants were also determined kinetically for selected temperatures and FA using the methods described previously (12, 15). On rate constants were determined by measuring the increase in R value as FA binds to ADIFAB, following the stopped-flow mixing of apoADIFAB and FA. Off rate constants were determined by measuring the decrease in R value as FA dissociates from ADIFAB following the stopped-flow mixing of holoADIFAB with fatty acid-free albumin. Measured off and on rate constants for oleate, together with dissociation constants calculated from these kinetic measurements, are listed in Table II. Also listed in Table II are the corresponding K_d values obtained from the linear fit to the equilibrium binding measurements of Fig. 1. As is apparent, dissociation constants determined from equilibrium binding and kinetic measurements are in excellent agreement.

Temperature Dependence of FA Binding to Native FABPs—Binding isotherms were measured for FABPs from human adipocyte (A-FABP), rat intestine (I-FABP), and rat heart (H-FABP), using ADIFAB to measure the FFA concentration. Each isotherm was analyzed using a single-site binding model to determine binding constants and stoichiometries for each of five different FA, at temperatures between 10 and 50 °C. The results of these measurements are shown as van't Hoff plots of the measured K_d values in Fig. 2 and indicate, as in the case of ADIFAB, that for all native FABPs and all FA, the binding affinities decrease with increasing temperature. ΔH were determined from the van't Hoff plots, and these values, together with ΔG and $-T\Delta S$, are listed in Table III, parts a–c. These results indicate that in most cases binding enthalpies are between −9 and −11 kcal/mol, and the $-T\Delta S$ values are gener-

TABLE I
ADIFAB thermodynamic parameters of FA binding

All values are in kcal/mol, and ΔG and $-T\Delta S$ were determined at 25 °C.

Fatty acid[a]	ΔG	ΔH	$-T\Delta S$
PA	−8.9 ± 0.1	−2.9 ± 0.3	−6.0 ± 0.3
OA	−9.0 ± 0.1	−2.6 ± 0.1	−6.4 ± 0.1
LA	−8.4 ± 0.1	−3.5 ± 0.1	−4.9 ± 0.1
LNA	−7.8 ± 0.1	−3.5 ± 0.2	−4.3 ± 0.2
AA	−8.1 ± 0.1	−4.4 ± 0.2	−3.7 ± 0.2

[a] The fatty acid code is: PA, palmitate (16:0), OA, oleate (18:1), LA, linoleate (18:2), LNA, linolenate (18:3), AA, arachidonate (20:4).

TABLE II
Kinetic and equilibrium determination of oleate-ADIFAB dissociation constants

Rate constants were measured as described in the text and previously (12).

Temperature	k_{off}	k_{on}	K_d[a]	K_d[b]
°C	s^{-1}	$\times 10^{-7}$ $(sM)^{-1}$	nM	nM
15	0.9 ± 0.1	0.3 ± 0.1	300 ± 90	200
25	1.7 ± 0.2	0.7 ± 0.1	240 ± 50	240
37	4.2 ± 0.3	1.5 ± 0.2	280 ± 40	280

[a] Dissociation constants determined from k_{off}/k_{on}.
[b] Dissociation constants determined from the fit to data in Fig. 1.

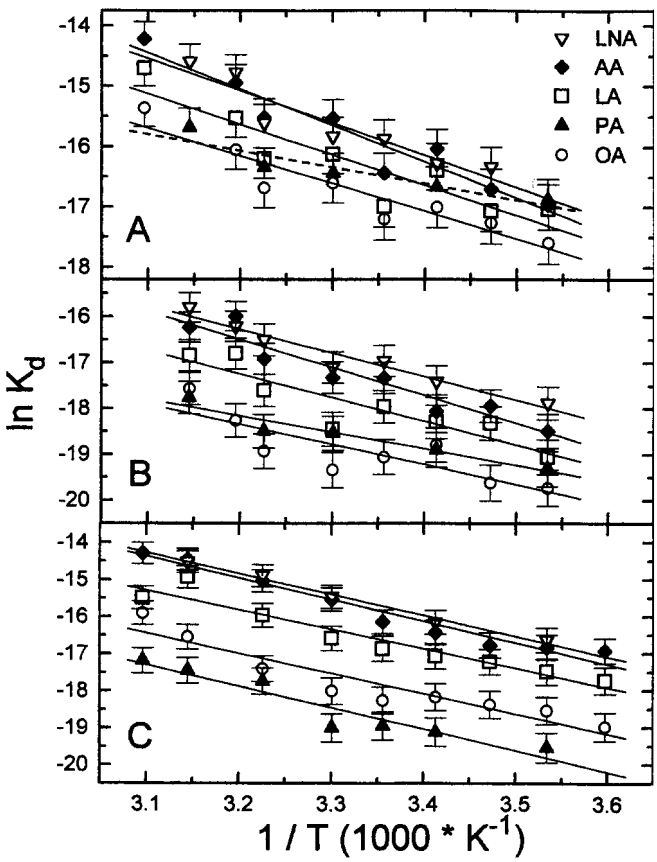

FIG. 2. **Temperature dependence of the binding of FA to FABP.** The symbols in these van't Hoff plots represent K_d values for each FA (see legend of Table I for FA code) determined by the equilibrium measurements described by Equations 1–5 under "Experimental Procedures." *A*, human adipocyte FABP; *B*, rat heart FABP; *C*, rat intestine FABP.

ally near zero. As in the case of ADIFAB, there is a general trend of increasing $-T\Delta S$ and decreasing ΔH with increasing degree of FA unsaturation for all FABPs.

Binding stoichiometries for all FA and FABPs were found to be invariant with temperature (data not shown). As found

TABLE III
Thermodynamic parameters of FA binding

Fatty acid	ΔG	ΔH	$-T\Delta S$
	a. A-FABP[a]		
PA[b]	−9.8 ± 0.1	−5 ± 1	−4 ± 1
OA	−10.0 ± 0.1	−9 ± 1	−1 ± 1
LA	−9.7 ± 0.1	−10 ± 2	+0 ± 2.0
LNA	−9.4 ± 0.1	−11 ± 2	+1 ± 2
AA	−9.5 ± 0.2	−12 ± 1	+3 ± 1
	b. H-FABP[c]		
PA	−11.1 ± 0.1	−7 ± 1	−4 ± 1
OA	−11.3 ± 0.1	−9 ± 2	−3 ± 2
LA	−10.7 ± 0.1	−10 ± 2	−0 ± 2
LNA	−10.1 ± 0.1	−10 ± 1	+0 ± 1
AA	−10.3 ± 0.1	−12 ± 2	+2 ± 2
	c. I-FABP[d]		
PA	−11.1 ± 0.1	−12 ± 2	+0 ± 2
OA	−10.6 ± 0.1	−11 ± 1	+0 ± 1
LA	−9.9 ± 0.1	−11 ± 1	+1 ± 1
LNA	−9.3 ± 0.1	−11.2 ± 0.7	+1.9 ± 0.7
AA	−9.5 ± 0.1	−11.6 ± 0.9	+2.2 ± 0.9

[a] Adipocyte FABP with units and abbreviations as in Table I.
[b] PA results are an average of values determined with human and murine FABP.
[c] Heart FABP with units and abbreviations as in Table I.
[d] Intestinal FABP with units and abbreviations as in Table I.

previously at 37 °C, the stoichiometries were consistent with unity for the adipocyte and intestinal proteins but were significantly less than unity for the heart FABP (12). Specifically, stoichiometries averaged over all temperatures and FA were: 0.90 ± 0.09 for adipocyte, 0.79 ± 0.14 for intestine, and 0.57 ± 0.07 for the heart FABP. As discussed previously, we could find no evidence for loss or degradation of protein that might account for the low stoichiometry value of the heart (muscle) proteins (these low values previously were observed for both bovine and rat heart). Nor could we obtain definitive evidence for dimer formation, which might reduce accessibility to the binding site if the interaction surfaces between the dimers involved the portal region of the protein. Recently, the x-ray crystal structure of FABP has been obtained from desert locust *Schistocerca gregaria* (8). This protein is also a muscle FABP, and its structure reveals a symmetric dimer in which the contact surfaces involve the region analogous to the presumed portal region of the heart protein (35). Thus, the muscle protein may be in the form of a portal to portal dimer, which limits binding to only one of the two sites/dimer, but for reasons unknown dimers of the rat heart FABP, which contains no cysteine residues, cannot be detected by molecular sizing chromatography (12). (Dimers mediated by disulfide linkages can of course be so detected, as found recently for the porcine heart FABP (36).)

Temperature Dependence of Membrane Partition of FA—The change in free energy upon binding of FA to FABP must involve energy changes in the bulk solvent as well as the FABP. To assess the aqueous contribution to the free energy change, measurements were done to determine the temperature dependence of partition of FA between water and lipid bilayer membranes. The unilamellar lipid bilayer vesicles used in these measurements were prepared from egg phosphatidylcholine by the extrusion method. Membranes were used for these studies because they approximate a simple hydrophobic phase and because values of the partition coefficient (K_p) are relatively insensitive to membrane composition and/or physical state (21, 27). Partition coefficients were determined by using ADIFAB to measure [FFA] in equilibrium with these vesicles as described under "Experimental Procedures" and previously (21). Thus, FA were added to mixtures of ADIFAB (0.2 μM) and

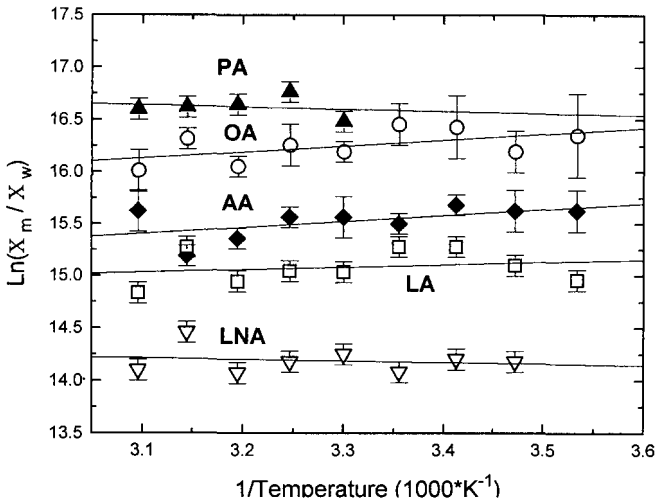

FIG. 3. **Temperature dependence of the partition of FA into lipid vesicles.** The membrane/water mole fractions (X_m/X_w) for each FA were determined from the measured partition coefficients as described under "Experimental Procedures" (see legend of Table I for FA code). Enthalpies (kcal/mol) for each of the FA are: palmitate (*PA*, 16:0), −0.4 ± 0.8; oleate (*OA*, 18:1), 1.1 ± 0.7; linoleate (*LA*, 18:2), 0.5 ± 0.8; linolenate (*LNA*, 18:3), −0.3 ± 0.7; arachidonate (*AA*, 20:4), 1.1 ± 0.7.

lipid vesicles (100 μM in phospholipid), and the measured R values were used to determine [FFA] for each FA, at temperatures between 10 and 50 °C. For these vesicle concentrations, most of the FA is in the membrane, a small fraction binds to ADIFAB, and the rest (FFA) is in the water (under the conditions of these studies, [FFA] is well below the concentration at which FA aggregation occurs). K_p values were determined from the measured [FFA] using Equation 6 and converted to mole fractions as described under "Experimental Procedures." These results are displayed as van't Hoff plots in Fig. 3. They show that for all FA, K_p values are virtually invariant with temperature; therefore, as indicated in the figure legend, no significant net enthalpy is involved in the water-membrane transition. The variation of K_p with FA type, as discussed previously (21), is largely governed by the relative solubility of the different FA; the least soluble saturated FA have the largest K_p.

DISCUSSION

In the present studies we have measured the temperature dependence of the binding of fatty acids to FABPs from adipocyte, intestine, and heart, to probe the chemical nature of the interactions between FA and FABP. Thermodynamic parameters determined from these measurements show that, with the exception of ADIFAB, the free energy of binding is almost totally dominated by enthalpic contributions; for ADIFAB the entropic contribution dominates. In contrast to the substantial temperature dependence of FA binding to FABPs, partition of these same FA into membranes is independent of temperature, suggesting that membrane partition is dominated by entropic contributions. However, the dependence on FA type is similar for both FABP and membranes; binding affinities and K_p values decrease with increasing numbers of double bonds at all temperatures. In the following we will first discuss the specific thermodynamic features and binding characteristics of the individual FABPs and will then address the issue of what interactions in general may be responsible for the overall thermodynamics of FA binding to FABPs.

ADIFAB—Because the measurements in this study were done using ADIFAB, the validity of the results for all FABPs relies on the accuracy of the K_d values for ADIFAB. The fact that K_d values determined by equilibrium binding and kinetics are virtually identical provides assurance of the accuracy of these values. Thermodynamic parameters determined from these K_d measurements display, for all but one FA, predominant $-T\Delta S$ values, which increase monotonically from −6.4 to −3.7 kcal/mol with increasing double bond number from oleate to arachidonate, whereas the enthalpic contributions are all between about −3 and −4 kcal/mol (Table I). The absolute values as well as the relative entropic and enthalpic contributions are significantly different from the values obtained for the unlabeled FABPs, for which the enthalpic and entropic contributions are between about −12 and −5 kcal/mol and −4 and +3, respectively (Table III). In particular, while the ADIFAB enthalpic contribution is between about 7 and 9 kcal/mol more positive than its parent protein I-FABP, its entropic contribution is between about 6 and 7 kcal/mol more negative (Table IV).

TABLE IV
I-FABP-ADIFAB differences

Units and abbreviations are listed in Table I.

Fatty acid	$\Delta\Delta G$	$\Delta\Delta H$	$-T\Delta\Delta S$
PA	−2.2	−9	+6
OA	−1.6	−8	+7
LA	−1.5	−7	+6
LNA	−1.5	−8	+6
AA	−1.3	−7.2	+6

The significantly larger entropy increase upon FA binding to ADIFAB, as compared to I-FABP, is consistent with an increase in rotational freedom of the acrylodan moiety in the holo- as compared to the apoADIFAB (15). Evidence for this increase is the decrease in fluorescence polarization of acrylodan from 0.32 in apoADIFAB to 0.15 in holoADIFAB (15). While the value 0.15 is consistent with considerable local rotational mobility, the value 0.3 is that expected for rotation of the 15-kDa protein as a whole and therefore consistent with virtually no local rotational mobility of the acrylodan moiety. In addition, molecular modeling of the apo and holo crystal structures suggests that the orientation of Lys^{27} changes upon FA binding in a manner that might force the attached acrylodan from a highly restricted position within the portal region to one that is mobile and almost orthogonal to the protein's surface (data not shown). The much smaller ADIFAB net enthalpy (average $\Delta\Delta H_{ADIFAB\text{-}IFAB}$ = 7 kcal/mol) probably results because the strong interactions between the acrylodan and protein, which are required to immobilize the acrylodan, must be disrupted before the FA can enter the cavity. This would suggest, if derivatizing Lys^{27} with acrylodan does not affect the conformation of the cavity, that acrylodan should primarily reduce the on rate of FA binding to apoADIFAB but have little effect on the off rate. Preliminary studies of the binding kinetics are, in fact, consistent with this prediction (Ref. 12 and data not shown).

Natural FABPs—FABPs from adipocyte, intestine, and heart exhibit similar binding thermodynamics (Table III). In almost all instances, the free energy is dominated by an enthalpic term. Previous calorimetry studies also found substantial enthalpic contributions for FA binding to FABP. Oleate binding to I-FABP was found to have a ΔH of −8.1 and a $-T\Delta S$ of +1 kcal/mol (37), and oleate and arachidonate binding to A-FABP were found to have ΔH values of −7.8 and −7.4 kcal/mol and $-T\Delta S$ values of +1.7, and +0.6 kcal/mol, respectively (14). Although the present study, indicating large enthalpic and small entropic changes (Table III), is in qualitative agreement with these calorimetry results, ΔH values in the present study are more negative by about 2–4 kcal. The weaker interaction observed by calorimetry may be a consequence of either the presence of organic solvent added with the FA or the use of FA concentrations that greatly exceed their solubility limits

(12).

Consistent with our previous measurements of the K_d values, ΔG values follow the pattern: ΔG (adipocyte) > ΔG (intestine) > ΔG (heart). For a given FABP, ΔG generally increases with the aqueous solubility of the FA; for the same FA chain length, ΔG increases monotonically with double bond number (Table III). The results in Table III also indicate a trend in which, for each FABP, $-T\Delta S$ increases with increasing FA double bond number and ΔH decreases with increasing double bond number. For example in the case of H-FABP, $-T\Delta S$ increases by about 6 kcal/mol and ΔH decreases by 5 kcal/mol from oleate to arachidonate. These results suggest a favorable, perhaps additive, enthalpic contribution involving FA double bonds that is offset by the unfavorable entropic contribution, consistent with increasing aqueous solubility with double bond number.

In addition to these general trends, the results of Table III reveal significant differences among the different FABPs and for a given FABP significant differences among the different FA. For example, although binding of palmitate to I-FABP has virtually no entropic contribution, binding to heart, and especially adipocyte reveal substantial entropic and correspondingly much smaller enthalpic contributions. These results suggest that the palmitate conformation is less constrained within the adipocyte and heart FABP binding cavities and that specific interactions between the FA and the amino acids and bound water within the binding cavities are substantially greater in the intestinal FABP. (To ensure that the adipocyte results were not idiosyncratic for the human form of the protein, measurements were also done using the murine form and virtually identical results were obtained as summarized in Table III, part a.) Moreover, within the adipocyte binding cavity, palmitate and oleate display greater differences in their thermodynamic parameters than in heart and intestine. The almost 4 kcal/mol greater enthalpic contribution for binding of oleate to adipocyte, as compared to palmitate, probably has its origin in different interactions between the hydrocarbon portions of the FA and the amino acid side chains and/or bound water molecules (19). Indeed, analysis of the crystal structure reveals that oleate and palmitate have similar carboxylate conformations within the adipocyte binding cavity, but palmitate has substantially greater numbers of non-polar amino acid contacts along its hydrocarbon chain than does oleate (11, 19). Furthermore, because the average temperature coefficient of stearate is substantially greater than that of oleate (11), the greater conformational flexibility suggested for palmitate may be characteristic of saturated FA.

Overall Thermodynamics of Binding—Although binding of different FA and FABPs reveal substantial differences in affinities, this heterogeneity involves relatively small differences in energy (≤3 kcal/mol), as is apparent from the variation of the thermodynamic parameters shown in Table III. Elucidation of the specific mechanisms responsible for this heterogeneity will require measurements involving site-specific mutants and the development of more accurate theoretical methods for relating energetics and structure. At this stage, however, a rough analysis can be applied to identify the dominant interactions that govern FA binding to FABP.

The observed thermodynamic characteristics of FA binding to FABPs and partition into membranes reflect the sum of contributions of several interactions involving the FABP, membrane, FA, and water. To assess the relative importance of each of the interactions that govern FA binding to FABP, we will, in the following discussion, estimate the magnitude of the individual interactions involved in FA binding to FABP using the measured binding results as well as estimates obtained from theory and model systems. We begin by expressing the free energy of binding or, equivalently, the free energy for transfer of FA from water to FABP ($\Delta G_{w\rightarrow FABP}$) as a sum of free energies corresponding to the individual interactions that contribute to $\Delta G_{w\rightarrow FABP}$, as shown by Equation 9.

TABLE V
Free energies associated with FA partition into membranes
Units and abbreviations are listed in Table I.

Fatty acid	K_p	$X_m(FA^-)/X_w$	$\Delta G_{w\rightarrow MEMB}$
	$\times 10^{-4}$	$\times 10^{-4}$	*kcal/mol*
PA	60	2160	−10.1
OA	50	1800	−10.0
LA	11	396	−9.1
LNA	5	180	−8.6
AA	12	432	−9.2

$$\Delta G_{w\rightarrow FABP} = \Delta G_w + \Delta G_{FA}^{FABP} + \Delta G_{FABP} + \Delta G_{FA\text{-}FABP} + \Delta G_{BW\text{-}FABP} \quad \text{(Eq. 9)}$$

The term ΔG_w represents the free energy difference between bulk solvent and bulk solvent + FA. The free energy difference between the bound FA-FABP state and the one in which both FA and FABP are free is separated into four terms: ΔG_{FA}^{FABP} is the difference between FA bound to FABP and in bulk solvent, ΔG_{FABP} is the difference between holoFABP and apoFABP, $\Delta G_{FA\text{-}FABP}$ is the interaction energy between the FA and FABP, and $\Delta G_{BW\text{-}FABP}$ is the energy difference between specific free water molecules and these molecules bound to FABP. We now consider each of these contributions in turn.

Bulk Solvent Free Energy (ΔG_w)

Free Energy of Transfer of FA between Water and Membrane ($\Delta G_{w\rightarrow MEMB}$)—The free energy change of the bulk solvent (ΔG_w) that appears in Equation 9 is identical to the change in bulk solvent that occurs in the transfer of FA from water to membrane. In what follows, ΔG_w will be estimated from the free energy of FA partition into membranes. The free energy difference for this latter process ($\Delta G_{w\rightarrow MEMB}$) is composed of contributions from: 1) bulk solvent (ΔG_w), 2) the difference between FA in the membrane and in bulk solvent (ΔG_{FA}^{M}), and 3) the difference in the membrane free energy with and without FA bound (ΔG_{MEMB}). Thus, we obtain Equation 10.

$$\Delta G_{w\rightarrow MEMB} = \Delta G_w + \Delta G_{MEMB} + \Delta G_{FA}^{M} \quad \text{(Eq. 10)}$$

$\Delta G_{w\rightarrow MEMB}$ was determined from the measured partition coefficients as described under "Experimental Procedures." The $\Delta G_{w\rightarrow MEMB}$ values (Table V) range from a high of −8.6 kcal/mol for linolenate, the most soluble FA, to −10.1 kcal/mol for palmitate, the least soluble of the FA. These values are in good agreement with the −9.8 kcal/mol value obtained for palmitate transfer into lipid vesicles by Peitzsch and McLaughlin (27) and with the −9 kcal/mol value for palmitate determined for transfer into *n*-heptane by Tanford (38). The lack of temperature dependence of partition (Fig. 4) and, therefore, a partition that is primarily entropy driven is also in agreement with the results of Peitzsch and McLaughlin (27).

Free Energy Change of the Membrane (ΔG_{MEMB})—Several observations suggest that the free energy change in the membrane (ΔG_{MEMB}) due to FA insertion is small. The lipid order of the egg phosphatidylcholine-composed large unilamellar vesicle bilayers is virtually unaffected by the insertion of FA, at least at low FA concentrations (21), suggesting that $\Delta G_{MEMB} \approx 0$. Moreover, studies of the binding of transmembrane peptides, which are probably much more perturbing to membranes than are FA, are also consistent with a small ΔG_{MEMB} (about 2 kcal/mol) because of compensating entropic and enthalpic peptide-lipid interactions (39). Therefore, to a reasonable approx-

imation, $\Delta G_{w\rightarrow MEMB} \approx \Delta G_w + \Delta G^M_{FA}$.

Free Energy Change of the FA (ΔG^M_{FA})—Two terms are expected to contribute to ΔG^M_{FA}: 1) the change in the rotational and translational motion of the FA molecule as a whole due to immobilization by the membrane and 2) the change in FA intramolecular interactions in the membrane as compared to the water. Previous studies have suggested that considerable translational and rotational immobilization might occur when a molecule is transferred from water to membranes, and that this reduction in mobility would be associated with an appreciable (>10 kcal/mol for FA) increase in free energy (39, 40). These estimates appear, however, to be greater than the actual free energy cost of this transfer (41). This is supported by the results of Peitzsch and McLaughlin (27), who compared the membrane partition of the uncharged FA with transfer of FA between water and *n*-heptane (38) and concluded that the free energy cost of FA immobilization by the membrane was negligible. The second potential contribution to ΔG^M_{FA}, due to the change in the internal energy of the FA upon transfer into the membrane, is also likely to be small because the interactions between FA and water and FA and lipid are relatively weak. This speculation is supported by the negligible overall enthalpy change upon transfer between water and membrane. Because ΔH_w is also ≈0 at 25 °C (38), $\Delta H_{w\rightarrow MEMB} \approx 0$ presumably reflects the small magnitude of the FA-membrane interactions, although exactly compensating changes can not be ruled out. We conclude, nevertheless, that both potential contributions are negligible and therefore $\Delta G^M_{FA} \approx 0$ kcal/mol.

(ΔG_w)—Because all terms except ΔG_w in equation (10) are assumed to be negligible, $\Delta G_{w\rightarrow MEMB} \approx \Delta G_w$. As seen in Table V, $\Delta G_{w\rightarrow MEMB}$ and therefore ΔG_w values ranges from −8.6 to −10.1 kcal/mol. Thus a significant free energy gain is obtained by pushing these long chained FA out of the water and into membranes. The same contribution of water (ΔG_w) for membrane transfer must also apply to the transfer of FA from water to the FABP binding sites.

Thermodynamics of FA Binding to FABP

Free Energy Change of the FA (ΔG^{FABP}_{FA})—Having evaluated ΔG_w (Table V), we now consider the remaining terms in Equation 9. The contribution ΔG^{FABP}_{FA} is analogous to the term ΔG^M_{FA} for membranes, and here again this term is composed of two contributions: the first due to the change in the motion of the FA as a whole and the second due to the change in the conformational energy of the FA. As discussed above, the free energy contributions due to immobilization of molecules upon transfer from water to membranes have been overestimated in previous studies. The effect of such contributions for ligand binding to proteins has been investigated recently by Murphy *et al.* (42). Applying experimental and theoretical arguments to the analysis of three separate peptide systems, Murphy *et al.* concluded that $-T\Delta S$ for the translational component of the free energy change, as would apply for FA binding to FABP, is +2.4 kcal/mol. Estimating the contribution to ΔG^{FABP}_{FA} that is due to reduction in the rotational mobility according to Finkelstein and Janin (41) and modeling the FA as a rod-like rotor, we obtain a value for this contribution of about +1 kcal/mol. Thus we estimate that the free energy change due to the decrease in the mobility of the FA as a whole upon binding to FABP is about +3 kcal/mol.

The second contribution to ΔG^{FABP}_{FA}, the change in internal free energy of the FA, is a sum of changes in configurational enthalpy and entropy. Determining these quantities requires information about the average conformation and dynamics of the FA in water and bound within the FABP. While the crystal coordinates and temperature factors of a number of FA bound within FABPs are known, similar information is not available for the FA in water. Recently, Rich (43) has used molecular dynamics to explore FA configurations in vacuum and these results indicate that the maximum energy differences between different FA conformations in vacuum are about +13 kcal/mol. Using the crystal structures of stearate and oleate in adipocyte FABP, Rich[2] has estimated that the enthalpy differences between the minimum energy configurations of these FA in vacuum and their FABP conformations are about +5 kcal/mol. Although similar estimates of the entropy changes are not available, it is likely that rotations about single carbon bonds are more restricted in the protein than in the water. Assuming complete rotational freedom about single C-C bonds in water but none in the protein, $-T\Delta S$ would range between about +12 kcal/mol for stearate (18:0) to 8 kcal/mol for linolenate (18:3), using an entropy contribution of Rln3 for each carbon (41). Certainly without information about the aqueous conformational freedom of the FA and possible contributions from vibrational degrees of freedom, there is considerable uncertainty in these estimates. For the discussion below, however, we assume a configurational enthalpy change of +5 kcal/mol, a configurational entropy change of +10 kcal/mol, and a +3 kcal/mol entropy loss due to the decrease in the overall FA mobility. Together, these contributions yield a total ΔG^{FABP}_{FA} of +18 kcal/mol.

The Free Energy Change of the FABP (ΔG_{FABP})—The term ΔG_{FABP} represents that portion of the free energy difference between the holoFABP and apoFABP that is limited to structural differences of the polypeptides, contributions due to interactions with FA and water are treated separately. Crystallographic studies reveal that the apoFABP structure is virtually unchanged by FA binding; root-mean-square main chain and side chain positions in the apo and holo proteins are generally less than about 0.5Å and 1Å, respectively (7, 14). Although a few residues are discretely disordered in the apo as compared to the holo structure (6, 7), this change is unlikely to contribute significantly to the free energy difference because the apo structure itself is highly ordered. Thus, because FABP structure changes only slightly upon FA binding, ΔG_{FABP} is probably small and we assume for this discussion that $\Delta G_{FABP} \approx 0$.

FA-FABP Interactions ($\Delta G_{FA\text{-}FABP}$)—The term $\Delta G_{FA\text{-}FABP}$ represents interactions between specific amino acid side chains, possibly in complex with bound water molecules, and specific portions of the FA. One of the most clearly defined interaction involves the FA carboxylate and amino acid side chains, most importantly Arg^{106} and Arg^{126}, and ordered water molecules (6, 7). Mutations of the residues involved in this network reduce the binding affinities by about 20–50-fold, suggesting that this interaction contributes between −1.8 and −2.3 kcal/mol to $\Delta G_{FA\text{-}FABP}$ (37, 44). In addition to this interaction, which is localized to the carboxylate portion of the FA, the variation of binding affinities with FA double bond number suggests that an interaction between the hydrocarbon chain of the FA and the hydrophobic residues within the cavity also contributes to $\Delta G_{FA\text{-}FABP}$ (12). Crystallography results also suggest specific interactions between the terminal methyl portion of the FA and residues in the proposed portal region of the protein, as well as specific interactions between bound water molecules and the fatty acid's aliphatic chain (6, 7, 14). Estimates of enthalpic or entropic contributions from these and other interactions that probably play important roles in binding are not yet available. Thus, for the $\Delta G_{FA\text{-}FABP}$ contribution, we assume a −2 kcal/mol enthalpic contribution due to the

[2] M. R. Rich, personal communication.

TABLE VI
Comparison of measured and estimated thermodynamic parameters of FA binding

This table compares measured thermodynamic parameters that are representative of all FA and all FABPs (bottom row) with the sum of terms (row labeled Total) that appear in equation (9). For each term the values of ΔG, ΔH, and $-T\Delta S$ shown in this table were estimated as described in the text. The entries in Total are the sums of the preceding five terms in each of the three columns. Each of the terms represent the following changes upon FA binding: bulk solvent (Water), overall and internal conformations of the FA (FA), changes in the FABP conformation (FABP), FA-FABP interactions (FA-FABP), and water bound within the FABP cavity (BW-FABP). The terms HI and SI represent the as yet unidentified FA-FABP interactions, whose enthalpy (HI) and entropy (SI) contributions are required to balance the measured = total equation; thus HI = −13 and SI = −3 kcal/mol.

Term	ΔG	ΔH	$-T\Delta S$
	kcal/mol	*kcal/mol*	*kcal/mol*
Water	−10	0	−10
FA	+18	+5	+13
FABP	0	0	0
FA-FABP	−2+HI+SI	−2+HI	+SI
BW-FABP	0	0	0
Total	+6+HI+SI	+3+HI	+3+SI
Measured	−10	−10	0

carboxylate interaction, because binding to the Arg mutants is reduced about 50-fold, plus unknown enthalpic (HI) and entropic (SI) contributions due to interactions between the FA and FABP at other regions along the hydrocarbon chain.

Bound Water Interactions ($\Delta G_{BW\text{-}FABP}$)—The term $\Delta G_{\text{BW-FABP}}$ represents the energy change due to the displacement of ordered water molecules by the FA. Two contributions to $\Delta G_{\text{BW-FABP}}$ can be identified: the increase in entropy resulting from the transfer to bulk solvent and the enthalpic cost of breaking hydrogen bonds with amino acid side chains and waters remaining bound within the cavity. Reasonably well defined limits have been suggested for entropy changes due to water binding to proteins. Previous studies have found that hydration entropies for inorganic salts range between 0 and 2 kcal/mol, the largest values corresponding to the most ordered molecules with temperature factors of 5–10 Å^2, and because they are independent of the salt composition, these values have been suggested to apply equally well to proteins (45). Water molecules bound to FABP, however, have significantly larger temperature factors than found in salt crystals; most are greater than 20 Å^2 (46). Using these larger temperature factors and following the approach of Finkelstein and Janin (41), the estimated entropy change per bound water molecule is small (≈0). The range of enthalpic contributions is more difficult to estimate because of the wide range of hydrogen bonding energies between water and charged or polar side chains (47). Moreover, the breaking of hydrogen bonds between water and amino acid side chains may be compensated by the reformation of hydrogen bonds in the bulk solvent. Thus displacement of water from the FABP cavity by FA may contribute relatively little to the free energy of binding, and we assume for this discussion that $\Delta G_{\text{BW-FABP}} \approx 0$.

Comparison of the Measured Thermodynamic Parameters with the Sum of Terms in Equation 9

To compare the measured and estimated thermodynamic parameters, we adopt the following values as representative of the measured values of Table III: $\Delta G_{w\to FABP} = -10$, $\Delta H_{w\to FABP} = -10$, and $-T\Delta S_{w\to FABP} = 0$ kcal/mol. These measured values, together with the estimates of the terms of Equation 9, discussed above, are listed in Table VI. The entries in this table explicitly identify those estimates for which we believe finite but as yet unknown contributions (SI and HI) may be significant. As the results in Table VI suggest, only two terms are likely to contribute significantly to the large net favorable enthalpy of binding (−10 kcal/mol). One of these (ΔG_{FA}^{FABP}) is a substantial unfavorable (positive) contribution resulting from the energy difference of the FA configuration in vacuum and bound within the protein.[2] The second contribution is the enthalpy of FA-FABP interaction, which in addition to the −2 kcal/mol carboxylate-FABP contribution includes a term HI, representing all the other interactions between the FA and protein. It follows from the entries in Table VI that HI is on the order of −13 kcal/mol and, therefore, that binding to FABP involves FA-FABP interactions with magnitudes greatly exceeding those involving the FA carboxylate head group. Summation of the entropic contributions listed in Table VI suggests that the small net entropy of binding results from the sum of the large favorable entropy change caused by the hydrophobic effect (−10 kcal/mol) and substantial reductions in the fatty acid's conformational entropy as well as possible reductions in amino acid side chain configurational entropy as a consequence of interactions with the FA (SI).

The reliability of these conclusions rests on the assumptions made to obtain the estimates for the individual terms in Table VI. In particular, the contributions due to displacement of bound water may not be negligible. This displacement might involve favorable entropy changes that would help balance the FA conformational and any additional unfavorable entropic contributions embodied in the SI term. The displacement of bound water is unlikely, however, to contribute negative enthalpy changes, needed to balance +5 kcal/mol contribution from ΔG_{FA}^{FABP}. The term ΔG_{FA}^{FABP} itself, based on the difference between FA bound to FABP and in the vacuum state rather than in water, may not be accurate. On the other hand, although the magnitude of this term may diminish with improved calculation, it is unlikely that a negative contribution would be obtained; some substantial negative enthalpic interaction is required to account for the measured −10 kcal/mol value. Thus, although the magnitude of the results that follow from Table VI may be altered by improved estimates of the individual interactions, unless other sources of favorable enthalpy and unfavorable entropy can be identified, the HI and SI terms likely contribute significantly to the thermodynamics of binding.

The results presented here suggest that several large thermodynamic terms that characterize FA binding to FABP tend to cancel. The net entropy portion is approximately zero because the large favorable entropy change of the solvent is canceled by an equally large but unfavorable entropy change, most likely due to restraints on the FA configuration. These entropy changes are to a large extent independent of the FABP, and therefore interactions that are FABP-specific are predominantly enthalpic. The large net (~−10 kcal/mol) enthalpic term that dominates the overall free energy of binding ($\Delta G_{w\to FABP}$) also results from the competition of at least two substantial terms: the unfavorable FA configurational enthalpy (~+5 kcal/mol) and the favorable enthalpy of the FA-FABP interaction (2 kcal/mol + HI). At present, only one specific interaction can be identified for which an enthalpy value can be assigned: the carboxylate–hydrogen bonding network, which probably contributes about − 2 kcal/mol to the total approximately −13 kcal/mol expected ΔH value. The comparison of palmitate and oleate binding discussed above suggests that the additional enthalpic interactions involve the hydrocarbon portion of the FA chain and the amino acid side chains and bound water molecules that interact with this part of the FA, a conclusion also reached by LaLonde *et al.* (14) in their study of the adipocyte FABP. Identifying the specific interactions that

account for the remaining approximately −11 kcal/mol will require measurements of the binding energies of a variety of ligands with site-specific FABP mutants coupled with corresponding structural information. Nevertheless, the analysis even at this early stage is significant because it predicts that changes in the amino acid side chains in contact with the hydrocarbon portion of the FA chain should lead to very large changes in the enthalpy of binding.

Although overall enthalpy dominates the binding energy, relatively small entropic contributions play significant roles in determining differences in binding affinities for different FA. As emphasized previously, FA solubility, which contributes to the binding interaction as entropy, plays a major role in determining the differences in FA binding to a given FABP (12). As seen in Table III, net entropy differences for different FA are at least 2 kcal/mol, and this energy difference corresponds to an affinity difference of about 30-fold. Thus, while net enthalpy may contribute the bulk of the net free energy, even relatively small variations in the net entropy contributions can have significant effects on binding affinities.

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