Fatty acid-binding protein from rat liver (L-FABP) binds 2 fatty acids (FA) per protein, in contrast to FABPs from adipocyte, heart, and intestine, for which binding and structural studies are consistent with a single FA binding site. To understand better the unique characteristics of L-FABP, we have carried out equilibrium binding and kinetic measurements of long chain FA using the fluorescent probes of free fatty acids (FFA), ADIFAB and ADIFAB2, to monitor the concentration of FFA in the reaction of FA with L-FABP. We found that the dissociation constants (Kd) ranged from about 1 nM to 4 μM, being largest for myristate at 45 °C and smallest for oleate at 10 °C, and that 2 FA were bound per L-FABP for all temperatures and FA. The binding measurements also revealed that at temperatures below 37 °C, affinities for the two binding sites differ by between 5- and 20-fold but as the temperature was increased, the affinities converge toward equal values. Off-rate constants (koff) were similar for all FA and for temperatures between 10 and 45 °C, ranged from about 0.1 s⁻¹ to 50 s⁻¹. Moreover, for all FA, koff values for dissociation from both the high and low affinity sites were similar, indicating that binding affinity differences at the lower temperatures reflect lower on-rates for binding to the low affinity site. The temperature at which the affinities of the two sites become equivalent depends upon the FA; higher temperatures (45–50 °C) are required for the unsaturated FA and myristate than for the longer chain saturated FA (<37 °C). This transition from different to equivalent affinity binding sites at specific temperatures reflects a nonlinear van’t Hoff behavior of the high affinity site, which in turn is a reflection of large heat capacity changes (between −0.6 and −1.2 kcal K⁻¹ mol⁻¹) that accompany FA binding to the high affinity site. These heat capacity changes, which are unique to L-FABP, do not appear to be correlated with a significant conformational change upon ligand binding. The differences between long chain saturated and unsaturated FA suggest that the conformation of FA bound to L-FABP may differ with both FA type and temperature, and that, in comparison to other FABPs, L-FABP may have distinctly different effects on saturated and unsaturated FA metabolism.

Thermodynamic and Kinetic Properties of Fatty Acid Interactions with Rat Liver Fatty Acid-binding Protein

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Fatty acid-binding proteins (FABP) are a family of 14–15-kDa proteins found in the cytosols of a variety of tissues (1–8). Although FABP from different tissues have different amino acid sequences, x-ray diffraction studies of the adipocyte, heart, and intestinal FABPs reveal similar three-dimensional structures and indicate a single fatty acid (FA) binding site (9–12). Recent studies using fluorescent methods to detect binding of native FA to these FABPs are consistent with a single high affinity binding site (12–15) and thermodynamic studies indicate that the free energy of binding is predominantly enthalpic (14–17). Kinetic measurements for these three proteins reveal that FA dissociation occurs within seconds, that rate constants for binding are more than an order of magnitude less than diffusion limited rates, and that the activation barriers for binding are predominantly enthalpic (18). These kinetic results also indicate that most of the differences in binding affinities for different FA and different FABPs are due to differences in off-rate constant (koff), activation free energies (average of 6.9 kcal/mol) were within 0.2 kcal/mol for all three FABPs.

In contrast with the similarities of these three FABPs, the properties of rat liver FABP (1,19) are distinct. Rat liver FABP (l-FABP) has the lowest amino acid sequence identity among the family of FABPs (6,20,21) and its structure, solved at high resolution, has yet to be reported. In contrast to the other FABPs, studies at 37 °C reveal that l-FABP binds two fatty acids per protein molecule (22–24). Furthermore, at 37 °C, binding affinities of both sites for the long chain saturated FA are similar while those for the unsaturated FA differ by 20-fold (12). Fluorescence and NMR studies indicate that in l-FABP the FA carboxylate groups are exposed to the aqueous environment, in contrast to other FABPs in which the carboxylate portion of the FA is buried relatively deeply within the binding cavity (22,25). Finally, a number of reports have suggested that l-FABP, but not other FABPs, is capable of binding a variety of hydrophobic molecules with high affinity (12,24,26,27).

To understand the characteristics of FA-l-FABP interactions we have, in the present study, measured equilibrium binding and the kinetics of FA interactions with l-FABP as a function of temperature for several long chain FA. These measurements were done with the fluorescent probes of FFA, ADIFAB and ADIFAB2, using methods developed previously to determine the interactions of FA with the adipocyte, heart, and intestinal FABPs (12–14,18). This report also extends our previous study (18) of the characteristics of the ADIFAB2 probe whose properties are better suited than ADIFAB to the study of the l-FABP. Thermodynamic parameters were determined using a van’t Hoff analysis of the equilibrium measurements and an Eyring transition state analysis of the kinetic measurements.
Large Heat Capacity Changes Accompany FA Binding to Liver FABP

**EXPERIMENTAL PROCEDURES**

Materials—All measurements were done, as described previously (12–14), using the sodium salts of the FA purchased from Nu Chek Prep, Elysian MN. The buffer used to measure FA binding to L-FABP consisted of 20 mM HEPES, 150 mM NaCl, 5 mM KCl, and 1 mM NaHPO₄, at pH 7.4. Recombinant FABP was expressed in the BL21 (DE3)/pET11 host/vector expression system as described previously (12–14). The pET11a and pET11d expression vectors and the BL21 (DE3)/pET11 host/vector expression system as described previously (12–14). The fluorescent FFA probe, ADIFAB, was prepared from acrylodan-derivatized recombinant rat intestinal fatty acid-binding protein (rI-FABP) as described (13) and is available from Molecular Probes, Eugene, OR. ADIFAB2 was synthesized by derivatizing an L72A mutant of rat intestinal FABP with acrylodan as described (18).

**[FFA] Determination with ADIFAB2**—Preliminary characterization of the ADIFAB2 probe was done previously for palmitate and oleate (18). We have extended this characterization in the present study to linoleate, linolenate, and arachidonate and have provided further spectral properties for all the FA. As discussed previously, with certain modifications the same formalism as used for obtaining [FFA] values from the ADIFAB measurements can be used for ADIFAB2 (18). Needed was analysis with ADIFAB2 of the spectral parameters Rmax, that appear in the expression relating [FFA] and (R), the ratio of intensities at emission wavelengths of 550 and 440 nm (Equation 1 of Ref. 12). These are different than for ADIFAB and depend on FA type and temperature. Measurements to determine these parameters followed the same methods as for ADIFAB (13).

**Measurement of FA-FABP Equilibrium Binding**—The method used to determine FA binding affinities for FABP was described previously (12, 14). Briefly, the binding of FA to a given FABP was determined by using ADIFAB or ADIFAB2, 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 the concentration of bound ADIFAB. FA titrations were done with an ADIFAB concentration of 0.2 μM (3 μg/ml) and FABP concentrations between 2 and 6 μM. The titrations were analyzed using two-site models with equivalent or different affinities as described previously (12). Thermodynamic parameters (ΔG, ΔH, ΔS, ΔCp) were determined by fitting the K values corresponding to the low affinity site with the differential (linear) form of the van’t Hoff equation and for the high affinity site we used the integral form (29),

\[
\ln \left( \frac{F_{on}}{F_{off}} \right) = -\frac{\Delta H}{R} - \frac{\Delta C_p}{R} \left( \frac{T}{T_0} \right) + \Delta C_p \ln \left( \frac{T}{T_0} \right) (\text{Eq. 1})
\]

where T₀ is 25 °C, ΔH₀ and C_p are the corresponding enthalpy change and dissociation constant, and ΔC_p is the heat capacity change.

**Stopped-flow Fluorescence**—Rapid mixing fluorescence was done using a KinTek Instrument (State College, PA) to mix equal volumes (about 0.1 ml) of reactants with a dead time of <2 ms. Fluorometric detection was done using two photomultipliers placed on opposite sides of the viewing chamber and intensities were monitored through 20-nm bandwidth filters centered at 435 nm and 550 nm for ADIFAB2 (Omega optical, Brattleboro, VT). A minimum of 5 scans were acquired for each condition of temperature, FA, and FABP. Off-rate constants (k_off) were measured directly by monitoring the time course of FA binding to ADIFAB2 after mixing a solution of FA and FABP in one syringe of the stopped-flow instrument with ADIFAB2 in the second syringe, as described previously (18). The on-rate constants (k_on) were calculated from k_on = h_d/K_c where h_d is the heat capacity change.

**Kinetic Analysis**—As described previously, k_cat values were determined by fitting the measured time courses using a kinetic model describing the transfer of FA from FABP to ADIFAB (18). This model was appropriate for a single binding site and in the present study we have extended this model to account for the two L-FABP binding sites. Thus the kinetic model describing the dissociation of FA from FABP, when both sites are occupied, to the aqueous phase and binding to ADIFAB2 from the aqueous phase is given by the following equations,

\[
\frac{d[ADIFAB2]}{dt} = -k_{on} [FA] \cdot [ADIFAB2]_0 + k_{off} [AA] \cdot [ADIFAB2]_0 \cdot [FA] (\text{Eq. 2})
\]

**RESULTS**

**Characteristics of the Fluorescent FFA Probe ADIFAB2**—We recently described the equilibrium and kinetic properties of palmitate (16:0) and oleate (18:1) binding to ADIFAB2, a FFA probe formed by derivatizing the L72A mutant of I-FABP with acrylodan (18). This probe has 10–20-fold higher affinities and 3-fold larger k_cat values than for ADIFAB, characteristics that are particularly suited for the study of the kinetics of FA binding to liver FABP. In the present study we have extended the characterization of ADIFAB2 for the interactions of linoleate (18:2), linolenate (18:3), and arachidonate (20:4). As mentioned previously (18) and shown in more detail in Fig. 1, the fluorescence emission of ADIFAB2 occurs at longer wavelengths and exhibits different spectral changes upon binding.

**FIG. 1. Fluorescence emission spectra of ADIFAB2 shift to longer wavelengths upon binding FA.** Emission spectra of ADIFAB2 (0.5 μM) was measured at 37 °C, after addition of sodium oleate at the indicated concentrations (nM). Also shown for comparison is an emission spectrum of apo-ADIFAB at 0.2 μM (dashed spectrum).
Large Heat Capacity Changes Accompany FA Binding to Liver FABP

Table I

| Temperature °C | Rmax | Q | Rmax | Q | Rmax | Q |
|---------------|------|---|------|---|------|---|
| 10            | 2.5  | 12.0 | 2.8  | 13.0 | 2.0  | 9.5 |
| 15            | 2.4  | 11.0 | 2.7  | 12.0 | 2.0  | 9.3 |
| 20            | 2.2  | 10.0 | 2.6  | 12.0 | 1.9  | 9.0 |
| 25            | 2.1  | 0.9  | 1.4  | 11.0 | 1.8  | 8.7 |
| 30            | 2.0  | 9.2  | 2.3  | 11.0 | 1.8  | 8.4 |
| 37            | 1.8  | 8.5  | 2.1  | 9.9  | 1.7  | 8.0 |
| 45            | 1.6  | 7.5  | 1.9  | 8.9  | 1.6  | 7.6 |

**Abbreviations as in Table I and MA is myristate.**

Table II

| Fatty acid | ADIFAB2 | ADIFAB | \(\Delta G^0\) | \(\Delta H^0\) | \(-T\Delta S^0\) |
|-----------|---------|---------|---------------|---------------|---------------|
| PA        | -10.6 ± 0.2 | -7.6 ± 0.8 | -3.0 ± 0.8 |            |               |
| OA        | -10.2 ± 0.2 | -5.8 ± 0.6 | -4.4 ± 0.7 |            |               |
| LA        | -9.7 ± 0.2  | -5.1 ± 0.4 | -4.6 ± 0.5  |            |               |
| LNA       | -9.1 ± 0.2  | -5.3 ± 0.3  | -3.8 ± 0.3 |            |               |
| AA        | -9.4 ± 0.2  | -7.7 ± 0.5  | -1.7 ± 0.6  |            |               |
| MA        | -7.5 ± 0.2  | -1.5 ± 0.4  | -6 ± 0.4    |            |               |

**Abbreviations as in Table I.**

Kinetics of FA Interactions with l-FABP—Time courses for the dissociation of FA from l-FABP were measured by stopped-flow mixing of FA-l-FABP complexes with ADIFAB2. Rate constants, \(k_{off}\), for FA dissociation from other FABPs were obtained previously (18) by analyzing the time courses of dissociation using a kinetic model appropriate for a single FA binding site. To account for dissociation from 2 FA binding sites in l-FABP we extended our previous single-site kinetic model to a two-site model as described under “Experimental Procedures.” Measurements of the dissociation time courses were done at low and high FA to l-FABP ratios to facilitate the analysis of the more complex time course for two-site dissociation. Thus, dissociation should occur preferentially from the higher affinity site when the FA:FABP mole ratio is less than 1 and from both sites when the ratio is greater than 1. Examples of the dissociation of oleate at mole ratios between 0.5 and 1.6, which are representative of all FA, reveal that the shape of the

FA than ADIFAB. In contrast to ADIFAB, the spectral parameters \(R_{max}\) and \(Q\) vary with FA type and temperature as shown in Table I. Equilibrium binding and kinetic results for linoleate, linolenate, and arachidonate interacting with ADIFAB2, are well described by linear van’t Hoff and Arrhenius behavior, respectively, and their respective thermodynamic parameters are given in Tables II and III. In addition to these results for ADIFAB2, we have also extended to myristate (14:0) our characterization of the binding thermodynamics of ADIFAB. The results of these measurements yielded \(K_d\) values in the range of 2.7–3.6 µM and the thermodynamic parameters shown in Table II.

**Equilibrium Binding of FA and Rat Liver FABP—**Binding of FA was measured using ADIFAB to determine the concentration of FFA in equilibrium with the liver FABP as described previously (12, 14). These measurements were done at temperatures between 10 and 52 °C with the FA: myristate, palmitate, oleate, linoleate, linolenate, and arachidonate. At each temperature and for each FA the binding isotherms were analyzed using a single or two-site equilibrium binding model and as observed previously at 37 °C (12), in all cases satisfactory fits required stoichiometries of 2 FA bound per l-FABP monomer. Equilibrium dissociation constants \((K_d)\) obtained from this analysis are shown for each of the FA as van’t Hoff plots in Fig. 2.

These results show that at most temperatures the two FA binding sites are not equivalent, affinities differ by about 10-fold for most FA. We observed previously, at 37 °C, that palmitate and stearate had equal affinities for both sites while for the unsaturated FA, the two sites had affinities that differed by about 10-fold (12). The present study confirms the previous results at 37 °C, and shows further that differences between the two sites diminish as the temperature increases. As a consequence, above a FA-specific temperature, when the difference in affinity of the two sites is less than about 3-fold, these differences cannot be resolved and the two sites appear to be equivalent. This occurs above 40 °C for all the FA except palmitate where the transition occurs at about 30 °C. Fig. 2 also indicates that the affinities of the two sites converge because the affinity of the tighter binding site decreases nonlinearly with temperature. To a good approximation the low affinity site follows a linear van’t Hoff dependence while the high affinity site reveals a significant positive curvature with increasing temperature. The curved van’t Hoff plot of the high affinity sites indicates that the enthalpy of binding is a sensitive function of temperature and therefore that a large change in heat capacity is associated with FA binding to the high affinity site of l-FABP.
Fatty acid

Abbreviations as in Tables I and II. Abbreviations for the FA are

FIG. 2. van't Hoff plots for equilibrium binding of FA and L-FABP. Equilibrium binding measurements and analyses were done as described under "Experimental Procedures." The solid circle and triangles are results for the low and high affinities sites, respectively. The solid lines represent fits using the differential (linear) form of the van't Hoff equation for the low affinity results and the integral form (Equation 1) for the high affinity results. Abbreviations for FA are defined in Tables I and II.

TABLE IV
Equilibrium thermodynamic parameters for FA binding to rat liver FABP

| Fatty acid | High affinity site | Low affinity site |
|------------|--------------------|------------------|
|            | \( \Delta G \)     | \( \Delta H \)    | \( T \Delta S \) | \( \Delta C_p \) |
| MA         | -9.7 ± 0.1         | -10 ± 1          | 1 ± 1           | -0.6 ± 0.2 |
| PA         | -11.1 ± 0.1        | -9.6 ± 0.9       | -1.5 ± 0.9      | -0.8 ± 0.1 |
| OA         | -11.7 ± 0.1        | -11.4 ± 0.9      | -0.2 ± 0.9      | -0.9 ± 0.1 |
| LA         | -10.9 ± 0.1        | -10.5 ± 0.8      | -0.4 ± 0.8      | -0.6 ± 0.1 |
| LNA        | -10.3 ± 0.1        | -10 ± 1          | 0 ± 1           | -1.2 ± 0.3 |
| AA         | -10.8 ± 0.1        | -13 ± 1          | 2 ± 1           | -0.8 ± 0.2 |

Abbreviations as in Tables I and II. The results are calculated using the measured \( k_{on} \) and \( k_{off} \) values and the results of this calculation are shown in Table V. Two sets of \( k_{on} \) values are obtained from these calculations because the two FA binding sites have similar \( k_{off} \) values but have \( K_d \) values that generally differ by more than 10-fold. The temperature dependences of each of these \( k_{on} \) values appear to follow linear Arrhenius behavior and we have used this linear behavior to calculate the activation thermodynamic properties for the low affinity site (Table VI).

Neither Tyrosine Fluorescence nor Circular Dichroism Reveal a Conformational Change—Because the high affinity data re-

FIG. 3. Time courses for the dissociation of oleate from L-FABP with different oleate/L-FABP mol ratios. L-FABP (1 \( \mu \)M) complexed with oleate at the indicated oleate to L-FABP mol ratios was stopped-flow mixed with ADIFAB2 (2 \( \mu \)M) and the \( R(t) \) values for ADIFAB2 were measured as a function of time at 25 °C. The solid lines are results obtained by fitting the data with the model described by Equations 2–5. Values for \( k_{off} \) of about 1 s\(^{-1}\) were obtained from this analysis for all oleate/L-FABP mol ratios.

TABLE V
Rate constants for FA interactions with L-FABP and ADIFAB2 at 25 °C

| Fatty acid | High affinity site | Low affinity site | ADIFAB2 |
|------------|--------------------|------------------|---------|
|            | \( k_{on} \)       | \( k_{off} \)     | \( k_{on} \) |
| PA         | 1.0                | 14               | 0.6     | 2.1  | 0.8 | 5.5 |
| OA         | 0.9                | 35               | 0.5     | 0.9  | 1.1 | 3.5 |
| LA         | 1.0                | 9                | 1.0     | 0.5  | 2.3 | 2.7 |
| AA         | 1.3                | 10               | 1.3     | 0.7  | 4.2 | 3.3 |

Abbreviations as in Table I.
Activation thermodynamic parameters for the high and low affinity sites of l-FABP

| Fatty acid | ΔG0 | ΔH0 | −ΔTS0 | ΔCp0 |
|------------|-----|-----|--------|-------|
| PA         | 17.4 ± 0.1 | 27 ± 1 | −9 ± 1 | 0 ± 0.1 |
| OA         | 17.5 ± 0.1 | 35 ± 2 | −20 ± 2 | 0.6 ± 0.5 |
| LA         | 17.4 ± 0.1 | 28 ± 1 | −10 ± 1 | 0.9 ± 0.1 |
| AA         | 17.3 ± 0.1 | 25 ± 1 | −8 ± 1 | 0.2 ± 0.1 |
| PA         | 6.3 ± 0.1 | 14 ± 1 | −7 ± 1 |
| OA         | 5.8 ± 0.1 | 24 ± 1 | −19 ± 1 |
| LA         | 6.6 ± 0.1 | 17 ± 2 | −10 ± 2 |
| AA         | 6.5 ± 0.1 | 12 ± 1 | −5 ± 1 |
| PA         | 17.7 ± 0.1 | 26 ± 2 | −9 ± 2 |
| OA         | 17.9 ± 0.1 | 26 ± 2 | −8 ± 2 |
| LA         | 17.4 ± 0.1 | 16 ± 2 | −1 ± 2 |
| AA         | 17.3 ± 0.1 | 24 ± 1 | −7 ± 1 |
| PA         | 7.5 ± 0.1 | 21 ± 2 | −13 ± 2 |
| OA         | 8.0 ± 0.1 | 18 ± 2 | −10 ± 2 |
| LA         | 8.4 ± 0.1 | 11 ± 1 | −3 ± 1 |
| AA         | 8.1 ± 0.1 | 12 ± 1 | −4 ± 1 |

Abbreviations as in Table I.

DISCUSSION

In the present study we have measured the interaction of long chain FA with rat liver FABP. For all FA two binding sites were found with affinities that differ by ~5–20-fold at lower temperatures and converge toward equal affinities at higher temperatures. For all FA, k_off values for dissociation from both sites are similar, indicating that binding affinity differences at the lower temperatures reflect lower on-rates for binding to the low affinity site. The temperature at which the affinities of the two sites become equivalent depends upon the FA; higher temperatures (45–50 °C) are required for the unsaturated FA and myristate than for the longer chain saturated FA (~37 °C). This transition from different to equivalent affinity binding sites at specific temperatures reflects the nonlinear van’t Hoff behavior of the high affinity site, which in turn is a reflection of large heat capacity changes that accompany FA binding to the high affinity site. These binding characteristics are unique to liver FABP; FA binding to adipocyte, heart, and intestinal FABPs are well characterized by single site interactions with negligible heat capacities (14–17).

Comparison with Previous Studies—Previous measurements (24, 26–28, 33) of FA binding to rat and bovine liver FABP have reported significantly larger K_d values (from 250 to greater than 1000 nM) than in the present study. As discussed previously, these larger apparent K_d values are likely a consequence of the use in these previous studies of methods that attempt to physically separate bound and free FA and/or are of insufficient sensitivity (12, 15). In spite of these differences, binding stoichiometries of two FA per l-FABP monomer, as found in the present study, have also been observed in previous studies (12, 22, 24, 26, 27). In particular, 13C NMR results reveal two palmitate binding sites at 37 °C (22), in agreement with our measurements that reveal 2 high affinity sites for palmitate and stearate at 37 °C (Ref. 12 and the present results). For oleate binding, the NMR results indicate only a single binding site at 37 °C. In the NMR study, however, high concentrations of oleate were used that formed a liquid crystalline phase in equilibrium with l-FABP, and it is possible that binding competition with this phase precluded significant binding to the low affinity oleate site. We found previously that formation of FA aggregates reduces binding to ADIFAB (13).

Kinetics of native FA interactions with l-FABP have not been reported previously, however, rates of dissociation of the anthroyloxy FA have been measured (34). These rate constants (k_off) are about 100-fold smaller and the activation thermodynamic parameters are different than found in the present work. In contrast, dissociation rates and activation thermodynamic parameters of native and anthroyloxy FA from adipocyte and heart FABPs are similar (18), suggesting that the differences between native and anthroyloxy FA observed for liver may be another reflection of the unique characteristics of l-FABP.
structure and FA-FABP interaction energies have been determined. Specifically we will refer to previous results obtained for FABPs from adipose, heart, and intestine (6, 7, 12, 14, 18, 35). The observations that distinguish L-FABP from these other FABPs and particularly warrant explanation are that: 1) l-FABP has two FA binding sites, which depending upon temperature and FA type, can be equivalent or can have affinities that differ by about 10–20-fold, 2) binding to the high affinity site for all FA involves a large change in heat capacity, and 3) the on-rate for binding to l-FABP is faster than for the other proteins.

FA binding to l-FABP may involve two independent sites with different affinities or two interacting sites. Binding isotherms, however, do not allow one to distinguish between these possibilities (36). For either possibility our results suggest that the entry barrier is different for the two sites and that the FA interactions within the two sites are probably different. Because the crystal structure and binding results indicate that the other FABPs bind only a single FA, the l-FABP binding sites may involve an amino acid arrangement and/or FA conformation that is different than for the other FABPs and therefore that the types of interactions that govern binding to l-FABP might have little in common with these other FABPs. On the other hand, the binding to l-FABP may involve relatively small changes to allow for binding of a second FA. Volumes of the binding cavities of the other FABPs estimated from the crystal structures are, in fact, sufficient to accommodate 2 FAs (6). Moreover, in studies not yet published, x-ray crystallographic results for rat liver FABP complexed with oleate reveal two FA binding sites and a protein structure that is very similar to previously determined FABPs2 and NMR solution results for apo-l-FABP, reveals a secondary structure that is also consistent with other FABPs.3

The thermodynamics of FA interactions with the adipocyte, heart, and intestinal FABPs reveal that binding free energies are predominately enthalpic (14, 16, 17), and as Table IV indicates this is also the case for both the low and high affinity sites of l-FABP. Alterations of the FABPs either by covalent modification, as in the case of ADIFAB, or by amino acid mutations (37) can change the thermodynamic pattern, for example, to one in which entropy plays a much greater role. For the high affinity site of l-FABP the enthalpies (−10–13 kcal/mol) are similar in magnitude to those for the other FABPs and the decrease in entropy with increasing degree of FA unsaturation observed for the other FABPs and ascribed to FA solubility, is also observed for l-FABP. These results suggest that, at least for the high affinity site, the FA interactions giving rise to the predominantly enthalpic character of the binding free energy, are similar in the l-FABP and the other FABPs. Thus, although the specific FA-amino acid interactions are probably different in each of the wild-type FABPs, the sum of these interactions is enthalpic, suggesting that this characteristic may be important in FA physiology. Binding to the low affinity site involves considerably smaller enthalpic interactions and correspondingly larger (more favorable) entropic contributions to the free energy than for the high affinity site and for the other FABPs. Conceivably, this less structured nature of the second binding site reflects a reduced specificity for FA, perhaps indicating that for the lower affinity site binding of other ligands may compete with FA. Given the lower affinities of other ligands such as lyso-PC and the bile salts, such competition would be physiologic only if the intracellular concentrations of the other ligands were significantly greater than FA.

Although FA-FABP interactions in liver and the other FABPs possess a number of similarities, the thermodynamics of binding to l-FABP which indicate large negative heat capacities, point to additional and substantially different interactions contributing in l-FABP. In general one might expect ΔCp changes of this magnitude to reflect substantial changes in the FABP conformation upon FA binding and clearly a definitive resolution of this possibility awaits the solution of the apo and holo forms of the protein. However, x-ray crystallographic structures of the other FABPs indicates virtually no secondary structural and only discrete amino acid residue orientation differences between the bound and free proteins (6). Consistent with this lack of secondary structure changes in the other FABPs, no differences were observed in the CD spectra for the apo and oleate bound l-FABP and the small 2–3% reduction we found in Tyr anisotropy upon FA binding is also inconsistent with a significant conformational change.

A similar magnitude ΔCp has recently been observed in the interaction of Na+ with thrombin (38). Because no significant change in the conformation of thrombin could be detected upon Na+ binding it was suggested that the observed −1.1 kcal K−1 mol−1 ΔCp might be due to the loss of entropy, as a consequence of increased water binding to the protein upon Na+ binding (38). However, as discussed previously (14) unless these waters become highly ordered upon binding (temperature factors <10 Å2), the decrease in entropy upon binding may not be significant. Moreover, results for other FABPs indicate that the binding cavity of the apo proteins contain large numbers of ordered and disordered water molecules and that upon FA binding several of these waters, including some of the ordered molecules in the case of the intestinal FABP, are displaced from the cavity (6, 7). Thus either l-FABP is exceptional in that the number of highly ordered waters within the binding cavity increases upon FA binding or some other mechanism is needed to account for the observed ΔCp. In fact, NMR and fluorescence evidence (22, 25) does indicate that the FA carboxylate is more accessible to bulk solvent in l-FABP than in the other FABPs, raising the possibility that the role of water in FA binding to l-FABP may be different than for the other FABPs and that this liver-specific water interaction is related to the observed ΔCp.

Kinetic characteristics of l-FABP are in many respects similar to those for the other FABPs. For example, activation free energies for the off-step from both the low and high affinity sites of l-FABP are 17.5 ± 0.2 kcal/mol (averaged for all FA) while the average for adipocyte, heart, and intestinal FABPs is 17.1 ± 0.7 kcal/mol (18). Differences do exist, however, perhaps the most significant being the larger on-rate constants (Table V) and, consequently, smaller activation free energies (6.3 ± 0.4 kcal/mol) observed for l-FABP’s high affinity site, as compared to the other proteins for which the average ΔG‡ is 6.8 kcal/mol (18). If the rate-limiting step for entering l-FABP involves a portal region similar to those identified from the crystal structures for the other FABPs (6, 7), the easier access to the l-FABP binding site may reflect more disorder of the portal’s amino acid residues because activation entropies are more favorable for l-FABP (~5 to ~19 kcal/mol for −TAS) than for the other FABPs (all >7 kcal/mol). That the l-FABP portal region provides more accessibility (lower activation barrier) to the binding cavity may also be consistent with the observation that the FA carboxylate is more solvent accessible in l-FABP than in l-FABP (22, 25).

Summary—The results of this study predict a number of

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2 Dr. Leonard Banaszak, University of Minnesota, personal communication.
3 Dr. Ruth E. Stark, CUNY, Staten Island, and Dr. Judith Storch, Rutgers University, private communications.
4 G. V. Richieri, R. T. Ogata, and A. M. Kleinfeld, manuscript in preparation.
Large Heat Capacity Changes Accompany FA Binding to Liver FABP

L-FABP structural features, the most important of which are: 1) that at temperatures of about 37 °C, the holo structure with palmitate should exhibit two FA bound in similar conformations whereas the unsaturated FA would reveal either a single bound FA or 2 FA bound with different conformations, and 2) temperature factors in the portal region may be larger than those found in other FABPs. The results also have implications for the unique role, relative to other FABPs, that L-FABP may play in lipid metabolism. In particular, the ability of l-FABP to bind 2 long chain saturated FA but only 1 cis unsaturated FA with high affinity under physiologic conditions suggests the possibility of generating more discrete changes in lipid distributions, in contrast to other binding proteins that, through differences in binding affinities, would modulate FA distributions more continuously.

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