Kinetics of Fatty Acid Interactions with Fatty Acid Binding Proteins from Adipocyte, Heart, and Intestine*

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Rate constants for the interaction of fatty acids (FA) with fatty acid binding proteins (FABP) from adipocyte (A-FABP), heart (H-FABP), and intestine (I-FABP) were determined by using stopped-flow fluorometry and ADIFAB, the fluorescent probe of free fatty acids (FFA), or a new FFA probe, ADIFAB2, constructed by derivatizing with acrylodan the Leu72 — Ala mutant of I-FABP. ADIFAB2, because its binding affinities are about 10-fold greater than ADIFAB, was found to be more accurate for monitoring the kinetics of the higher affinity reactions. On-(κ_on) and off-(κ_off) rate constants were determined as a function of temperature. Our results reveal that in all cases the FA-FABP equilibrium is achieved within 2 s at 37 °C and within 20 s at 10 °C. Off-rate constants varied by about 10-fold among the different underivatized FABPs; κ_on values were smallest for H-FABP and largest for A-FABP, while κ_off values for these proteins generally varied by less than 2-fold. The results show that the previously reported larger affinities of I- and H-FABPs as compared to A-FABP are primarily a reflection of larger κ_on values for I-FABP and smaller κ_off values for H-FABP. Eyring transition state theory was used to evaluate the activation thermodynamic parameters for both on- and off-reactions and the results show that in virtually all cases the rate-limiting steps are predominantly enthalpic. Activation free energies for binding to ADIFAB are generally composed of about 8 kcal/mol unfavorable enthalpy and about 1 kcal/mol favorable entropic contribution. For the underivatized FABPs the activation free energies are all about 7 ± 0.3 kcal/mol, suggesting that the transition state for entering or leaving the binding site involves a common protein structural change. We suggest that entering or leaving the FABP binding cavity involves similar mechanisms for all 3 FABPs and may involve amino acid residues located within the portal regions of these proteins.

Fatty acid binding proteins (FABP) are a family of 14–15-kDa proteins found in the cytosols of various cells (1–8). Although the three-dimensional structures of these proteins are similar, individual members of this family exhibit considerable variation in their amino acid sequences (9–12). In addition, the conformation of the fatty acid (FA) within the binding site differs for different FABPs and, to varying degrees, for different FA within the same protein (9–12). Consistent with these amino acid sequence and FA conformational differences, we have recently found considerable differences in the binding affinities of FA to FABPs from adipocyte, heart, and intestine (12–14). Using the fluorescent probe ADIFAB, we found that equilibrium binding constants differ by about 3 orders of magnitude, depending upon FA and FABP type. This heterogeneity was also reflected in the equilibrium thermodynamic parameters for binding; for adipocyte and heart FABP, the enthalpy of binding becomes more favorable with increasing FA unsaturation and, correspondingly, the entropy becomes less favorable, while for intestinal FABP the enthalpy is roughly constant for all FA, but the entropy term becomes less favorable with increasing unsaturation (14).

Although structural and binding studies provide insight into the interactions of FA and FABP at equilibrium, they leave unanswered questions concerning the kinetic features of these reactions, which are key to understanding a number of functional and structural properties of the FABPs. Determination of the rates of binding and dissociation of FA from FABPs is essential for understanding the kinetic constraints that govern intracellular FA trafficking and metabolism and the mechanism by which FA enter and leave the FABP binding cavity. Measurements of the rate constants and their temperature dependence should provide information about the thermodynamic parameters of the activation barrier for entering and leaving the binding site and the comparison of rate constants for site-specific mutants will provide information about how FA gain access to the binding cavity.

With the exception of our initial results discussed in Refs. 12 and 14, no measurement of the kinetics of FA-FABP binding and dissociation have been reported. However, Storch and colleagues (15–17) have carried out extensive measurements of the transfer of the chemically modified anthroyloxy-FA from FABPs to membranes. These measurements revealed considerable heterogeneity among the FABPs both in the rates and in the transfer mechanisms. These AOFAB results together with our results for unmodified FA and FABPs (12, 14), suggest that rate constants for binding and dissociation of unmodified FA are also sensitive functions of FA and FABP type.

In the present study we have determined the rate constants for the binding of a set of unmodified FA to the fluorescently labeled I-FABP, ADIFAB, ADIFAB, which exhibits distinct fluorescence spectra in the FA-bound and FA-unbound states, was then used to monitor the rate of dissociation of FA from adipocyte, heart, and intestinal FABPs. We also report the development of a new higher affinity FFA probe, ADIFAB2, and describe its use in determining rate constants for higher affinity FA-FABP interactions. The measured dissociation rate constants (κ_off) were used to calculate κ_on values from κ_on = 11291
ADIFAB Determination of Fatty Acid Binding Kinetics

\[
\frac{d[ADIFAB]}{dt} = -k_{on}^f \cdot [ADIFAB]_b + k_{off}^f \cdot [ADIFAB]_b \cdot [FFA] \tag{Eq. 1}
\]

\[
\frac{d[FFA]}{dt} = +k_{on}^f \cdot [ADIFAB]_b + k_{off}^f \cdot [Protein]_b - k_{on}^r \cdot [ADIFAB]_b + k_{off}^r \cdot [Protein]_b \cdot [FFA] \tag{Eq. 2}
\]

\[
\frac{d[Protein]}{dt} = -k_{on}^r \cdot [Protein]_b + k_{on}^r \cdot [Protein]_b \cdot [FFA] \tag{Eq. 3}
\]

where FA binding and dissociation are \(k_{on}^f\) and \(k_{off}^f\) respectively. The off
and on-rate constants for ADIFAB and the FABP or BSA proteins are
designated with superscript AF and Prot, respectively. The subscripts b
and f designate the FA bound and free concentrations, respectively.

With appropriate boundary conditions, Equations 1–3 describe models
used to analyze all three types of kinetic measurements. The initial
conditions (\(t = 0\)) that apply to each of these types of measurements are:
1) for measurements of the off-rate from ADIFAB, \([Protein(0)] = 0\), and
\([ADIFAB(0)]_b \) and \([FFA(0)] \) are both determined from the equilibrium condition that exists before mixing, using the previously measured
off-rate constants (14) for ADIFAB on-rate measurements, \([ADIFAB(0)]_b = 0 \) and \([FFA(0)] = [FFA_{total}] \) and 3) for the FABP
off-rate measurements, \([ADIFAB(0)]_b = 0 \) and \([FABP(0)]_b \) and \([FFA(0)] \) were
determined from equilibrium conditions. The solutions to Equations
1–3 yield the concentrations of FA bound ADIFAB, FA bound Protein,
and FFA as a function of time.

The quantity that is actually measured in these studies is the time
dependent change in the ratio of the fluorescence intensity of ADIFAB
at 505 and 432 nm (R(t)). To obtain rate constants from these measure-
ments, R(t) must be expressed in terms of the solutions to Equations
1–3, \([ADIFAB(t)]_b \) and \([FFA(t)] \). This can be done by expressing the intensities at each wavelength in terms of the contributions of the
bound and free forms of ADIFAB as described previously (13, 21, 22).
Thus the fluorescence intensity at the emission wavelength A is given by:

\[
I(\lambda) = [ADIFAB]_b \cdot I_{AF} + [ADIFAB]_b \cdot I(\lambda) \tag{Eq. 4}
\]

in which \([ADIFAB]_b \) and \([ADIFAB]_b \) are the bound and free concentra-
tions of ADIFAB and \(I_{AF}\) and \(I(\lambda)\) are the specific fluorescence
intensities of these components. The ratio of intensities at 505 and 432 nm
is therefore the following.

\[
R = \frac{[ADIFAB]_b \cdot I_{505} + [ADIFAB]_b \cdot I(505)}{[ADIFAB]_b \cdot I_{432} + [ADIFAB]_b \cdot I(432)} \tag{Eq. 5}
\]

Dividing the numerator and denominator of the right-hand side of
Equation 5 by \([ADIFAB]_b \cdot I_{432}\) we obtain.

\[
R(t) = \frac{R_b + 0.59 \cdot X(t)}{1 + 0.05 \cdot X(t)} \tag{Eq. 6}
\]

where \(R_b\) is the value of R in the absence of FA and the numerical
constants were obtained from the spectral properties of ADIFAB as
described previously (13),

\[
X(t) = \frac{[ADIFAB(t)]_b}{[ADIFAB]_b \cdot e^{-k_{on}^f t}} \tag{Eq. 7}
\]

where \([ADIFAB]_b \) is a solution of Equations 1–3. To
obtain the rate constants it is necessary to fit the measured R(t)
values with the values predicted by Equation 6. The measurements
to determine \(k_{on}^f\) from ADIFAB were done in the presence of excess BSA,
effectively eliminating the reverse reaction, and therefore for this type
of measurement \([ADIFAB(t)]_b \) is simply,

\[
[ADIFAB(t)]_b = [ADIFAB]_b / e^{-k_{off}^f t} \tag{Eq. 8}
\]

Upon substitution of Equation 8 into Equation 6 an expression for R(t)
is obtained that readily can be fitted to measured R(t) values to obtain
\(k_{off}^f\). For measurements of type 2 and type 3, Equations 1–3 were solved
numerically. Thus for type 2 and 3 measurements, values of the rate
constants, initial values, and any other boundary conditions are
selected, Equations 1–3 are solved for these values, R(t) is obtained using
the numerical values of \([ADIFAB(t)]_b \) and these calculated R(t) values
are then compared with the measured ones. This process was facilitated
with the program MLAB (Civilized Software, Bethesda, MD) which
determines the model parameters by using a Marquardt-Levenberg minimization to fit numerical solutions of Equations 1–3 and measured R(t) values. Values obtained by this procedure were verified in selected cases by solving Equations 1–3 using the Runge-Kutta facility of the program Macsyma (Macsyma, Cambridge, MA).

Reliable fitting of the measured scans requires constraints in addition to the initial conditions. Measurements of types 2 and 3 involve bimolecular interactions and therefore the reaction kinetics depend upon the concentration of the reactants. Although the nominal concentrations of FA in the reservoir syringes is known, the actual concentration reaching the mixing chamber exhibits variations from scan to scan because of surface absorption of the FA. To help reduce the uncertainties in the rate constants resulting from these variations, the observed values of the initial and equilibrium values of R(t), R∞, and R(∞), in each scan were used to define the actual total FA in the mixing chamber using,

\[
[\text{FA}]_{\text{total}}(\infty) = [\text{FFA}]_{\infty} + [\text{ADIFAB}]_{\infty} + [\text{FABP}]_{\infty} \quad \text{(Eq. 9)}
\]

All three terms on the right-hand side are evaluated using the values of R∞ and R(∞) obtained from the fit, together with the previously measured binding constants (12). Thus from Refs. 12, 13, and 23,

\[
[\text{FFA}]_{\infty} = K_{d} 19.5 (R(\infty) - R_{B})/11.5 - R(\infty) \quad \text{(Eq. 10)}
\]

\[
[\text{ADIFAB}]_{\infty} = [\text{ADIFAB}]_{\text{bound}} 19.5 (R(\infty) - R_{B})/11.5 - R(\infty) + 19.5 (R(\infty) - R_{B}) \quad \text{(Eq. 11)}
\]

\[
[\text{FABP}]_{\infty} = \frac{[\text{FABP}]_{\text{total}}}{K_{d}} \text{R}_{\text{FA}} \quad \text{(Eq. 12)}
\]

With these constraints, fitting of data for the type 2 measurements was done by allowing k_{on}, R_{B}, and R(∞) to vary. For the type 3 measurements the variable parameters were k_{off} and k_{on} with the constraint k_{off}/k_{on} = K_{d}, R_{B}, and R(∞). These procedures resulted in well behaved fitting characteristics in which the fit values of R∞ and R(∞) returned values in agreement with the measured values. The quality of fit was assessed by the sum of squares of the theoretical differences (SSQ), fit residual, and direct observation, and generally gave excellent agreement between theory and experiment, with typical SSQ values < 1 x 10^{-2}.

Limits of Resolution for Determination of Rate Constants for FA Transfer from FABP to ADIFAB—Determination of rate constants relies on the accuracy of the ADIFAB rate constants as well as the characteristics of the kinetic model used to analyze the time course of transfer. To determine how the characteristics of the kinetic model affect the activity with which rate constants can be determined, time courses for the transfer of FA from FABP to ADIFAB were simulated by solving Equations 1–3 with arbitrary rate constants for FABP and ADIFAB. These simulated transfer curves reveal, as seen for the example of oleate dissociation from 1-FABP in Fig. 1, how the concentration of the three reactants, [ADIFAB]_{\infty}, [FFA], and [FABP]_{\infty} are expected to vary with time after mixing. These results show that for small k_{off} values FA dissociation from FABP is tightly correlated with an increase in FA bound to ADIFAB and with a monotonic decrease in the concentration of the free FA. For larger FABP off-rate constants, the rate at which FA dissociates from FABP can exceed the rate at which FA can bind to ADIFAB, thereby uncoupling the two processes. This uncoupling is most evident in the time course for [FFA] where, for large k_{off} values, the concentration at early times actually increases before decaying toward equilibrium. This increase occurs because the FA-FABP solution is perturbed from equilibrium by mixing. Immediately after mixing the FA-FABP solution is diluted 2-fold with the ADIFAB solution, and in this diluted solution [FFA] is initially less than the equilibrium value for the FA/FABP mixture but greater than the equilibrium value of the FA/ADIFAB/FABP mixture. This transient increase is not observed at small k_{off} values because the rate of FA binding to ADIFAB is equal to or greater than the rate of dissociation from FABP. At larger k_{off} values, in contrast, appreciable dissociation from FABP occurs, as the FA-FABP system adjusts to its diluted environment, and this occurs more rapidly than the rate of binding to ADIFAB.

These limits on resolution of FABP off-rates are also apparent in the time course of R(t), the quantity actually measured. As Fig. 1B indicates, the time course approaches a limiting function as k_{off} increases. The ability of ADIFAB to resolve dissociation rate constants depends upon the experimental uncertainty in R(t) and is a function of both the rate constant of dissociation and ADIFAB’s equilibrium constant. In the example of Fig. 1A, ADIFAB would resolve K_{d} values < 30 s^{-1} for FABP (K_{d} = 36 nM). Resolution of off-rate constants from the less tightly binding A-FABP (K_{d} = 60 nM), however, would be < 50 s^{-1} (data not shown). This resolving power of ADIFAB is a direct reflection of the rate of response of ADIFAB to FA binding and therefore is proportional to the on-rate constant as well as ADIFAB’s binding affinity relative to the donor FABP. Thus ADIFAB2, for which values and binding affinities are larger than for ADIFAB (see below), has been used in the present study to help resolve dissociation in those cases where large k_{off} values and/or small K_{d} values limit the response rate of ADIFAB.

Eyring Transition State Theory—Activation thermodynamic parameters were calculated using Eyring rate theory (24) in which rate constants are related to the transition state activation energy as shown below,

\[
k = \frac{T \pi \kappa_{B}}{h} e^{-\frac{\Delta G^\ddagger}{RT}} \quad \text{(Eq. 13)}
\]

where T is temperature in degrees of Kelvin, \pi is the transmission coefficient and is set to unity in these calculations, \kappa_{B} is Boltzmann’s, and h is Planck’s constant, and \Delta G^\ddagger is the free energy of activation. The activation enthalpy was determined from the slope of Arrhenius plots of the rate constants as the following.
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\[ \Delta H = -R \left( \frac{\Delta G}{T} \right) + T \]  
(Eq. 14)

and the activation entropy was determined as,

\[ T \Delta S = \Delta H - \Delta G \]  
(Eq. 15)

In using this analysis it is assumed that the thermodynamic model provides a reliable representation of the formation of the transition state and that the activation enthalpies and entropies are temperature independent. Recently, studies of equilibrium reactions have called into question the use of the van't Hoff analysis, and therefore the temperature independence of equilibrium enthalpies and entropies, to determine thermodynamic parameters from binding measurements (25, 26). Whether such reservations apply to the activation parameters is unclear. However, two observations suggest that at least for FA-FABP interactions the thermodynamic parameters calculated assuming temperature independence may be accurate. First, as discussed by Weber (25), the errors made in assuming temperature independence may be small when, as is the case in the present study (Table IV), the activation free energies are predominantly enthalpic. Second, the predominance of enthalpy also applies to the equilibrium thermodynamic parameters for FA binding to FABP as determined by both van't Hoff (14) and calorimetry measurements (27, 28), although the van’t Hoff determined \( \Delta H \) values were obtained for all FA (Table I).

Equilibrium and Kinetics of FA Interactions with ADIFAB—In studies in progress we have been examining how Ala substitutions for amino acid residues that are located within the I-FABP binding cavity affect FA-FABP interactions. In the course of these studies we found that the Leu \( ^{72} \rightarrow \) Ala (L72A) mutant possessed FA binding affinities that were 10–20-fold greater than the native I-FABP (data not shown). In order to obtain a new higher affinity FFA probe, ADIFAB2 was constructed by derivatizing the L72A mutant of I-FABP with acrylonitride. Equilibrium binding properties of ADIFAB2 were determined essentially by the same methods as used previously to characterize ADIFAB (Methods 13). FA dissociation constants found for ADIFAB2 (Fig. 5A) ranged between 8 and 50 nM, or 10–20-fold greater affinities than for ADIFAB, consistent with the differences found for the underivatized FABPs. Rate constants for FA interacting with ADIFAB2 were determined by the same methods as for ADIFAB. The results shown in Fig. 5,

**RESULTS**

**ADIFAB Kinetics**—Rate constants for the dissociation of FA from ADIFAB (\( k_{\text{off}} \)) were determined by measuring the dissociation of FA from ADIFAB using fatty acid-free BSA as a sink. In all cases virtually identical rate constants were obtained using BSA concentrations between 6 and 20 \( \mu \)M, indicating saturating levels of BSA (data not shown). Thus Equation 8 can be used to analyze the dissociation time course and as seen in the example shown in Fig. 2, provides an excellent description of this process. Results of this analysis yield \( k_{\text{off}} \) values that range from about 0.8 to 50 s \(^{-1} \) depending upon temperature and FA type (Table I and Fig. 3A). These results show moreover that \( k_{\text{off}} \) for ADIFAB increases with FA type as OA > PA > LA > AA > LNA for each FA, \( k_{\text{off}} \) decreases exponentially with decreasing temperature (Fig. 3A). This variation of \( k_{\text{off}} \) with FA type follows, inversely, the variation of FA affinity for ADIFAB described previously (12, 13).

Time courses for binding of FA to ADIFAB were measured by mixing FA and ADIFAB; our results for oleate binding at temperatures between 15 and 37 °C are shown in Fig. 4. Also shown in this figure are the fits to these measured time courses obtained with the kinetic model represented by Equations 1–3. These fits were obtained by allowing both \( k_{\text{on}} \) and \( k_{\text{off}} \) to vary with the constraint that \( k_{\text{on}}/k_{\text{off}} = K_m \), where \( K_m \) values were those measured previously (12). The results of this analysis as seen in Fig. 3 and Table I, yields \( k_{\text{on}} \) values that range between \( 1 \times 10^3 \) and \( 5 \times 10^6 \) M \(^{-1} \)s \(^{-1} \) and \( k_{\text{off}} \) values that are virtually identical with those obtained directly from measurements of FA transfer from ADIFAB to BSA described above. As was seen for the time course of dissociation, the rate of binding increases with increasing temperature, however, in contrast to the significant variation of \( k_{\text{on}} \) with FA type, virtually identical \( k_{\text{on}} \) values were obtained for all FA (Table I).

Arrenius plots of the on- and off-rate constants (Fig. 3) were analyzed in terms of Eyring transition state theory which yielded the thermodynamic parameters of activation shown in Table II. As these results indicate, the free energies of activation for dissociation range between about 16 and 17 kcal/mol and show a decrease with increasing double bond number for the 18 carbon length FA. Table II also shows that for all FA the enthalpic portion of the activation energy is significantly larger, between about 12 and 13 kcal/mol, than the entropic component (3–4 kcal/mol). For the binding step the thermodynamic parameters are quite similar for all FA and in particular indicate that the activation barrier is predominantly enthalpic with average \( \Delta H^\ddagger \) values of about 9.5 kcal/mol (Table II).

**Table I**

<table>
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<th>LNA</th>
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**Table II**

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Values for \( k_{\text{on}} \) are in units of \( 10^7 \) M \(^{-1} \)s \(^{-1} \) and for \( k_{\text{off}} \) in units of M \(^{-1} \)s \(^{-1} \). Standard deviations for \( k_{\text{on}} \) values range from about 15 to 40% for the derivatized and underivatized proteins, respectively. Abbreviations for the FA are: PA, palmitate (16:0); OA, oleate (18:1); LA, linoleate (18:2); LNA, linolenate (18:3); and AA, arachidonate (20:4).
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B and solid lines are shown as symbols and the results of linear regressions are shown as solid lines through the data. A, log of \( k_{\text{off}} \); B, log of \( k_{\text{on}} \). Abbreviations are as listed in Table I.

B and C, and Table I indicate that \( k_{\text{off}} \) for ADIFAB2 is about 5-fold smaller than for ADIFAB, while the \( k_{\text{on}} \) rate constants are generally about 2-fold greater. Because of its faster response time and increased sensitivity for low FFA levels, ADIFAB2 was used to resolve off-rate constants for FA-FABP interactions involving rapid dissociation and/or high affinity such as palmitate and oleate transfer from I-FABP and H-FABP, respectively. Equivalent results were obtained with ADIFAB and ADIFAB2 where, as in the cases of palmitate and oleate transfer from I-FABP both probes could be used to determine transfer rates. Equilibrium and transition state thermodynamic parameters for ADIFAB2 are shown in Table III.

The equilibrium thermodynamic parameters are qualitatively similar to those obtained previously for ADIFAB, showing substantial enthalpic and entropic components; in contrast, for the native I-FABP, enthalpies are about 12 kcal/mol and the entropic components are approximately zero (12). Just as for ADIFAB and the underivatized proteins, the activation free energies for binding to ADIFAB2 (−7 kcal/mol) are dominated by enthalpies which are 8.6 and 10.6 kcal/mol for oleate and palmitate, respectively (Table III). Enthalpies also dominate the dissociation of FA from ADIFAB2, with values that are between ADIFAB (Table II) and I-FABP (Table IV).

Adipocyte-FABP—Time courses for dissociation of palmitate from mouse A-FABP are shown in Fig. 6A together with fits to these measurements using the kinetic model represented by Equations 1–3. Time courses for the dissociation of arachidonate from each of the three different native FABPs are shown in Fig. 6B. These results are representative of all FA/FABP combinations and illustrate that dissociation from A- and I-FABP is considerably faster than from H-FABP. The results of Fig. 6A also show that the rate of dissociation increases with temperature and that between 10 and 37 °C dissociation from A-FABP is complete within about 2 s. A-FABP off-rate constants determined from these as well as from measurements with the other FA are shown as Arrhenius plots in Fig. 7 and are listed in Table I. The \( k_{\text{off}} \) values for the 18 carbon FA increase with the degree of FA unsaturation and for all FA \( k_{\text{off}} \) range between about 1 and 20 s\(^{-1}\). Off-rate constants for oleate were also measured using human A-FABP and these values are virtually identical to those found with mouse A-FABP (data not shown). On-rate constants calculated from the \( k_{\text{off}} \) and previously determined \( K_{d} \) values (12), are shown in Fig. 7B and Table I. These results reveal little difference in \( k_{\text{on}} \) among the different FA. Values for \( k_{\text{on}} \) increase with temperature and between 10 and 37 °C range from about 1 to 40 \( \times 10^{6} \) M\(^{-1}\) s\(^{-1}\), about 5-fold greater than for ADIFAB.

The Arrhenius plots for the A-FABP rate constants were analyzed using the Eyring transition state model (Table IV). The results show that the free energy needed to form the transition state for dissociation (about 17 kcal/mol) is composed of a large enthalpic (13–16 kcal/mol) and a smaller entropic (0–4 kcal/mol) component. Within the uncertainties of these results (1–3 kcal/mol), the thermodynamic parameters are similar for all of the FA. Thermodynamic parameters for the binding step are also similar for all FA and reveal that, with the exception of arachidonate, the activation free energies which are about 8 kcal/mol, are predominantly enthalpic (Table IV).

Heart FABP—Dissociation rates from heart FABP are considerably slower than from adipocyte or intestinal FABP, as exemplified by arachidonate at 25 °C shown in Fig. 6B. This result also shows that the change in R value at equilibrium is considerably smaller with H-FABP as compared to A- and I-FABP. This is a direct reflection of the lower equilibrium FFA levels for the higher affinity H-FABP (\( K_{d} = 26 \) nM) than A- and I-FABP (\( K_{d} \) values both about 130 nM). ADIFAB2 was used to measure palmitate and oleate dissociation from H-FABP because of its significantly higher affinity as compared to ADIFAB. The measured off-rate constants for heart FABP range between 0.2 and 3 s\(^{-1}\) for the 5 different FA and are shown as Arrhenius plots in Fig. 8A and Table I. On-rate...
ADIFAB Determination of Fatty Acid Binding Kinetics

**TABLE II**

Eyring transition state analysis of ADIFAB off-rate constants

<table>
<thead>
<tr>
<th></th>
<th>PA</th>
<th>OA</th>
<th>LA</th>
<th>LNA</th>
<th>AA</th>
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<tr>
<td>ΔG‡</td>
<td>7.7 ± 0.1*</td>
<td>7.6 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>8.0 ± 0.1</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>ΔH‡</td>
<td>9.5 ± 0.7</td>
<td>9.8 ± 0.7</td>
<td>9.3 ± 0.6</td>
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<tr>
<td>−TΔS‡</td>
<td>−1.8 ± 0.5</td>
<td>−2.3 ± 0.5</td>
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<td>−1.2 ± 0.4</td>
<td>−0.9 ± 0.5</td>
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<td>ADIFAB off-step parameters</td>
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<tr>
<td>ΔG‡</td>
<td>16.7 ± 0.1</td>
<td>16.7 ± 0.1</td>
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<td>TΔS‡</td>
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<td>4.2 ± 0.5</td>
<td>3.2 ± 0.4</td>
<td>2.9 ± 0.4</td>
<td>2.9 ± 0.5</td>
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</tbody>
</table>

*All ΔG‡ and TΔS‡ values were calculated at 25 °C. Energies are in kcal/mol. Standard deviations of 15 and 20% were used for ADIFAB off- and on-rate constants, respectively. Uncertainties (S.D.) for each of the thermodynamic parameters were calculated, by using standard error propagation rules (34), as 0.6 6ik/ k, where 6ik is the standard deviation of the rate constant k, 2 6slope, and 0.6 6ik/ k + 6slope for ΔG‡; ΔH‡, and TΔS‡, respectively.

**TABLE III**

ADIFAB2 equilibrium and EYRING transition state analysis

<table>
<thead>
<tr>
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<th>OA</th>
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<td>ADIFAB2 equilibrium parameters</td>
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<tr>
<td>ΔG‡</td>
<td>10.4 ± 0.1</td>
<td>10.4 ± 0.1</td>
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<tr>
<td>ΔH‡</td>
<td>6.3 ± 0.6</td>
<td>5.9 ± 0.6</td>
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<tr>
<td>TΔS‡</td>
<td>4.5 ± 0.6</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>ADIFAB2 on-step parameters</td>
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<tr>
<td>ΔG‡</td>
<td>7.0 ± 0.1</td>
<td>7.3 ± 0.1</td>
</tr>
<tr>
<td>ΔH‡</td>
<td>10.6 ± 0.7</td>
<td>8.6 ± 0.4</td>
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<tr>
<td>−TΔS‡</td>
<td>−3.6 ± 0.5</td>
<td>−1.4 ± 0.5</td>
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<tr>
<td>ADIFAB2 off-step parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG‡</td>
<td>17.7 ± 0.1</td>
<td>17.5 ± 0.1</td>
</tr>
<tr>
<td>ΔH‡</td>
<td>17.6 ± 0.6</td>
<td>14.5 ± 0.4</td>
</tr>
<tr>
<td>−TΔS‡</td>
<td>0.1 ± 0.7</td>
<td>2.9 ± 0.5</td>
</tr>
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</table>

**FIG. 5.** van't Hoff plots of the equilibrium and Arrhenius plots of the rate constants for ADIFAB2. Equilibrium measurements were done as described previously for ADIFAB (13) and rate constants were determined by the same methods as for ADIFAB. A, equilibrium dissociation constants for palmitate and oleate. B and C, off- and on-rate constants for palmitate and oleate. Measured values are shown as symbols and the results of linear regressions are shown as solid lines through the data.

The off-rate constants calculated from these off-rate constants and the previously measured k_{off} values range between about 1 and 10⁻⁹ M⁻¹ s⁻¹. These results are also shown as Arrhenius plots in Fig. 8B. The variation of rate constants with FA type generally reflects the behavior expected from the K_d values. For example, off-rate constants for the unsaturated FA increase with K_d. Although much of the variation in K_d can be attributed to variations in k_{off}, especially large k_{on} values result in high affinities for palmitate (Fig. 8 and Table I). Eyring transition state analysis of the results of Fig. 8 are shown in Table IV. These results indicate that the activation free energy of FA dissociation is about 18 kcal/mol and is virtually all entropic. Although the magnitude of the barriers are considerably smaller (ΔG‡ is about 7 kcal/mol), the on-rate activation pathway is also predominantly enthalpic.

**DISCUSSION**

In the present study we have determined on- and off-rate constants as a function of temperature for binding of five of the physiologically most important FA to ADIFAB, ADIFAB2, adipocyte, heart, and intestinal FABPs. In all cases the FA-FABP equilibrium is rapid, occurring within about 2 s and 20 s at 37 and 10 °C, respectively. Off-rate constants varied by about 10-fold among the different FABPs, where k_{off} values were smallest for H-FABP and largest for A-FABP. On-rate constants are 10–100-fold smaller than the values predicted for diffusion limited rates, indicating a significant activation barrier for binding and these values also varied by about 10-fold among the different FABPs. The results demonstrate that the kinetic basis for achieving equilibrium is different in different FABPs; the larger affinity of I- and H-FABPs as compared to...
A-FABPs are primarily a reflection of larger reactant concentrations in all measurements were: ADIFAB, 2 μM; FABP, 1 μM; and FA, 1 μM. A, dissociation of palmitate from human A-FABP monitored with ADIFAB at 5 °C intervals for temperatures between 10 and 30 °C and at 37 °C. Solid lines are least squares fits to the data using Equations 1–3 and \( k_{\text{off}} \) values determined from this analysis are plotted in Fig. 7. B, dissociation of arachidonate from A-FABP, H-FABP, and I-FABP at 25 °C. Fits to these data using the kinetic model were used to obtain the \( k_{\text{on}} \) values shown in Table I.

A-FABPs are primarily a reflection of larger \( k_{\text{on}} \) values for I-FABP and smaller \( k_{\text{off}} \) values for H-FABP. For the native FABPs the activation free energies corresponding to these kinetic processes are primarily enthalpic and are of similar magnitude, suggesting that the activation state for entering or leaving the binding site may involve a common protein structural change. In the following we discuss how the results of the present study can be used to provide insight about the nature of the transition state.

**ADIFAB**—The Eyring transition state model yields an activation barrier for binding FA to ADIFAB that is composed of an unfavorable enthalpy change, of about 9 kcal/mol, and a favorable 1–2 kcal/mol entropic contribution (Table II). Several observations suggest that the formation of this transition state involves a change in the orientation of the acrylodan moiety and an interaction between FA and ADIFAB. First, acrylodan is probably involved in the transition because derivatization of I-FABP with acrylodan reduces significantly the rate constant for binding FA (Table I). Second, as discussed previously (13) the transition from the apo to holo state of ADIFAB involves a...
The characteristics of ADIFAB2 are consistent with a qualitatively similar transition state as for ADIFAB. ADIFAB2 does, however, exhibit an approximately 0.5 kcal/mol smaller activation free energy for binding as compared to ADIFAB. This smaller energy of activation is consistent with the observed longer emission wavelength maximum in the apo state; 440 nm for ADIFAB2 as compared to 432 nm for ADIFAB (data not shown), assuming that the acrylodan moiety binds less tightly to regions of higher polarity. The substantially larger activation free energy for dissociation from ADIFAB2 (1 kcal/mol greater than for ADIFAB) is consistent with the greater FA affinities for the undervatized L72A-I-FABP as compared to the wild type protein. The smaller entropies for dissociation may also be a reflection of the weaker binding of acrylodan in ADIFAB2, the holo to transition state decrease in entropy being smaller than in the case of the more tighter binding and therefore more constrained ADIFAB.

These results for ADIFAB and ADIFAB2 are consistent with the suggestion that the acrylodan moiety must be displaced in order for FA to gain entry to the ADIFAB binding cavity. Based upon an examination of the x-ray crystallographic structure of I-FABP, Sacchettini et al. (29) have suggested that a specific region (“portal”) at the surface of the protein serves as the entry port for FA access to the binding cavity. The current study provides support for this suggestion because the acrylodan moiety is attached to I-FABP at position 27, which is one of the residues that forms the orifice defining the portal region, and this attachment reduces the k_{on} values by approximately 5-10 fold relative to the undervatized I-FABP (Table I).

Native-FABPs—Activation energies of binding and dissociation of FA were done using ADIFAB2 and other abbreviations as in Table I.
action for native adipocyte, heart, and intestinal FABPs are quite similar to one another, suggesting that the transition state for these proteins share common features. For example, the activation free energies for binding, averaged over all 5 FA, are, respectively, \( k_{\text{off}} = 0.2 \), \( 6.9 \pm 0.4 \), and \( 6.6 \pm 0.3 \) kcal/mol for adipocyte, heart, and intestine, respectively (Table IV). This similarity among the native FABPs is also apparent from the activation thermodynamic parameters of the off-step where it is seen that the free energy change is primarily an enthalpic one of about 16 kcal/mol, the entropic contributions being, on average, about zero. These results do not identify the common structural features that might be involved in the transition state. However, a portal region has been identified at similar locations in all of these proteins (6) and because attachment of acrylodan in this region significantly reduces \( k_{\text{off}} \) (Table I), we speculate that the transition state might involve the portal region. This speculation is supported by preliminary studies in which site-specific mutations of residues that form the portal region have been found to affect both \( k_{\text{on}} \) and \( k_{\text{off}} \) in I-FABP mutants (data not shown). The diameter of the orifice of the portal region that allows access to the binding cavity is about the same as the FA (6, 29). The transition state might therefore involve a fluctuation in the diameter of the orifice which would alternately open and close access to the binding site. Although these putative fluctuations might occur spontaneously, both heart and intestine reveal a significant dependence of the activation enthalpy on FA type, suggesting that at least for these FABPs, the transition state may also involve a FA-FABP interaction.

Although the native FABPs may share a common type of transition state, several aspects of the kinetic and equilibrium results suggest that this state is distinctly different for the acrylodan derivatives of I-FABP and, additionally that acrylodan derivatization perturbs the FA-FABP interactions within the binding cavity. Obviously if acrylodan is involved in the transition state of the derivatized proteins then this state is not the same as for the native proteins. Further evidence that these states are different are: 1) the average activation free energy for FA binding to ADIFAB is 1.1 kcal/mol greater than for the native protein, and 2) the binding activation enthalpy (9.5 kcal/mol) for ADIFAB is, with the exception of arachidonate, independent of FA type while \( \Delta H^\ddagger \) for I-FABP reveals a monotonic decrease from 10 to 4 kcal/mol from palmitate to arachidonate. These results suggest that if the orifice presents a barrier for access to the cavity in the native protein, that derivatization of Lys27, one of the key residues within the portal region that allows access to the binding cavity is about the same as the FA (6, 29). The transition state might therefore involve a fluctuation in the diameter of the orifice which would alternately open and close access to the binding site. Although these putative fluctuations might occur spontaneously, both heart and intestine revealing a significant dependence of the activation enthalpy on FA type, suggesting that at least for these FABPs, the transition state may also involve a FA-FABP interaction.

Comparison with AOFA Kinetics—Storch and colleagues (17) have studied the transfer of the AOFA from adipocyte, heart, and liver FABPs to lipid membranes. These studies reveal that while transfer of the AOFA from liver FABP to membranes likely occurs through the intervening aqueous phase, transfer from the adipocyte and heart FABPs appears to be mediated by processes than involve direct FABP-membrane interactions.

The rate constants determined in these studies might not be comparable directly to those determined in the present study because the AOFA-FABP interactions differ significantly from the unlabeled FA-FABP interactions; equilibrium dissociation constants for example, are about 1–2 orders of magnitude greater for the AOFA (30, 31) than for the unlabeled FA (12). Moreover, the rates for transfer of AOFA from FABP to acceptor membranes might be different than those for the dissociation of the unlabeled FA into the aqueous phase. Nevertheless, rate constants and activation thermodynamic parameters for dissociation of the unlabeled FA are in good agreement with those found for the AOFA by Storch and colleagues (32, 33). The rate constant from these studies for 2:AQ-palmitate or stearate transfer from heart FABP, extrapolated to zero acceptor membrane concentration, is about 0.5 s\(^{-1}\) at 25 °C, similar to the values found for the dissociation of unlabeled FA from H-FABP in the present study (Table I). For similar transfer conditions and AOFA’s, transfer rates from adipocyte FABP were found to be about 10-fold greater than from heart FABP (17), similar to the \( k_{\text{off}} \) results shown in Figs. 7 and 8 for the unlabeled FA. Finally, thermodynamic parameters found for the transfer of 2:AQ-palmitate from adipocyte and heart FABP to lipid vesicles (32, 33) were similar to those found in the present study both in their predominately enthalpic character and in their magnitude. That such different molecules reveal similar dissociation characteristics is surprising, a detailed understanding of this issue will require more information than is currently available about the structure of AOFA bound to FABP (37). Because of their significantly smaller affinities we estimate \( k_{\text{on}} \) values for the AOFA are about 10–100-fold lower than for the unlabeled FA, possibly reflecting the greater difficulty for passage of the AOFA through the relatively small orifice defined by the portal region. How interactions with acceptor membranes might affect dissociation of unlabeled FA is unclear, although results comparing rate constants determined for H-FABP with varying ADIFAB and H-FABP concentrations in the present study indicate that collisions between the ADIFAB acceptor and FABPs do not affect dissociation rates (data not shown).

Summary—This study shows that while FABPs reveal considerable variation in binding affinities and rates of response most of this variability results from changes in the rates of dissociation, suggesting that the (transition) mechanism by which FA gain entry to the binding cavity is similar in each of these three FABPs. More detailed understanding of the nature of this mechanism awaits the results of site-specific mutational studies now in progress. We speculate that the rate constants determined in this study have physiologic significance for FA metabolism because the rate at which adipocyte and intestinal FABPs respond to changes in FFA levels is considerably greater than that for the heart FABP. What makes this result potentially relevant for physiology is that both adipose and intestine are concerned with bi-directional intra- to extracellular transport of FA and might be expected to respond rapidly to alterations of FFA levels, whereas in the heart where FA are the major energy substrate, FA transport is presumably unidirectional.

REFERENCES
ADIFAB Determination of Fatty Acid Binding Kinetics

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Kinetics of Fatty Acid Interactions with Fatty Acid Binding Proteins from Adipocyte, Heart, and Intestine
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