Affinity of Fatty Acid for rRat Intestinal Fatty Acid Binding Protein: Further Examination†

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ABSTRACT: The enhancement of the fluorescence quantum yield of 1,8-anilinonaphthalenesulfonic acid (ANS) upon binding to intestinal fatty acid protein (I-FABP) was exploited to devise an assay for free I-FABP. With this assay, we monitored the competition for free I-FABP between ANS and fatty acids and thereby extracted values for the dissociation constants (K_{FA}) of fatty acids for I-FABP. We obtained these constants for the I-FABP ligands oleic acid, arachidonic acid, and palmitic acid. In addition, we measured the dependence of K_{FA} for oleic acid upon temperature and at two pH values. From these data, we calculate the van't Hoff enthalpy of oleic acid binding. This enthalpy is compared with the enthalpies of binding obtained directly from titration calorimetry. Our experiments with the fluorescence-based assay generate values of K_{FA}which disagree with older values obtained from calorimetry and other methods. Our own calorimetric data were analyzed with a view to improving the technique involved in subtraction of a "reference" dilution of the ligand into solution in the absence of the protein. By this maneuver, we obtained "corrected" titrations which could be fitted to values of K_{FA} more in agreement with the values we determined via the fluorescence-based assay than were the older literature values. Our new values for K_{FA} also agree substantially with values derived using a complementary assay technique, one measuring the concentration of free fatty acid, that has recently been developed by Richieri et al. [Richieri et al. (1995) *J. Biol. Chem. 270,* 15076−15084]. We compare the values of $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ for fatty acid binding we have obtained in this work with those we found in earlier work with ANS binding to I-FABP [Kirk et al. (1996) *Biophys. J. 70,* 69−83]. Our interpretation of the origin of the thermodynamic changes for ANS binding in our earlier work is here substantiated and extended to include an evaluation in physical terms of the interaction of I-FABP with fatty acids.

The binding of ligands to macromolecules such as proteins is an essential feature in all molecular biology. The strengths of these interactions can be determined by various assays [cf. Cantor and Schimmel (1980)] based on (1) radiolabel-counting techniques, such as equilibrium dialysis or filter binding assays; (2) spectroscopic signals generated by the free or bound species, e.g. fluorescence quenching or enhancement upon interaction, changes in absorbance, etc.; and (3) chemical methods, e.g. monitoring a reaction which indicates the presence of the ligand species in solution or by following the kinetics of some enzymatic reaction as altered by the presence of the ligand, etc. If the macromolecule of interest is known only to bind the ligand, without chemical reaction, then assays based on enzyme or other reaction kinetics, while they may still be cleverly exploited, are generally of limited value. Additionally, useful assays exist which are based upon the possible release or uptake of heat or protons attending the binding reaction, i.e. isothermal titration calorimetry (ITC) and pH-stat titrations, respectively.

Fatty acid and lipid binding proteins seem to be ubiquitous both within a single organism among various tissues and between several taxa in the animal kingdom (chordates, insects, etc). These proteins bind long chain free fatty acids and lipids (C12−C24) specifically. X-ray crystallographic structures for several of these proteins have been solved [for a review, see Banaszak et al. (1994)]. The physiological role of both extracellular and cytoplasmic forms of these proteins is unclear, although a protective role against deleterious effects of e.g. free fatty acids has been postulated. The binding of naturally occurring lipids to lipid binding proteins has been studied to a considerable extent by the ITC technique (Jakoby et al., 1993; Miller & Cistola, 1993; Lalonde et al., 1994), because of the large amount of heat evolved when these lipids bind to the appropriate binding protein. Calorimetry data such as these allow, in principle, the extraction of values for the ligand dissociation constant, as well as the direct measurement of the enthalpy of binding.

As we shall discuss in this paper, *reliable* values for the dissociation constant of ligand to macromolecule are likely to be obtained in a ligand/macromolecule concentration regime different from that required to obtain the best value for the enthalpy of binding. Furthermore, the requisite concentration regime for yielding the most useful values for affinity constants may be experimentally inaccessible to the ITC technique.

In a previous contribution (Kirk et al., 1996), we presented data on the binding of the fluorophore 1,8-anilinonaphthalenesulfonic acid (ANS) to recombinant rat intestinal fatty acid binding protein (I-FABP). We investigated this binding by means of both the enhancement of ANS fluorescence upon binding to I-FABP and the heat evolved in ITC experiments upon ANS binding. One conclusion from that

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study was that the calorimetrically determined enthalpy of reaction, $\Delta H°$, was more reliable than the calorimetrically determined binding affinity, or $\Delta G°$; the situation was reversed for data obtained from spectrofluorimetry, namely that the $\Delta G°$ was reliable and the value of $\Delta H°$ from a van't Hoff plot of the binding constants at a number of temperatures, though including within its standard error values consistent with the calorimetry, was nonetheless inferior, because the van't Hoff slope was not itself temperature dependent (within the resolution of the data). This distinction was demonstrated by the internal consistency of *derived* values of the entropy of binding at two temperatures T_1 and T_2, i.e. $\Delta\Delta S°_{bind} \equiv [\Delta H°(\text{calorimetry}) - \Delta G°(\text{fluorimetry})]_{T2}/T_2 - [\Delta H°(\text{calorimetry}) - \Delta G°(\text{fluorimetry})]_{T2}/T_1$ with the calorimetrically derived change in heat capacity, $\Delta C_{p,bind}\ln(T_2/T_1)$ (Kirk et al., 1996). A similar consistency with the heat capacity integral is not obtained if one employs instead the optimal (fit) values of $\Delta G°$(calorimetry) or $\Delta H°$(fluorimetry) to generate $\Delta\Delta S°_{bind}$.

Knowledge about binding affinities for various ligands together with the dissection of these affinities into entropic and enthalpic contributions may help to identify the conditions under which particular molecular forces may serve to strengthen, and when they weaken, the overall binding interaction. In light of the importance attached to obtaining accurate values for $\Delta G°$, therefore, we sought to exploit the spectroscopic technique involved in the ANS-binding assay to obtain reliable values of *fatty acid*-binding affinity. This we accomplished by devising an assay based on the competition for free I-FABP between ANS (of known binding affinity) and added fatty acid (of unknown affinity). A recent contribution from the Kleinfeld group (Richieri et al., 1995) provides an excellent example of a different kind of competitive assay which can be used for this system. Their assay is based on an assay of free fatty acid employing an I-FABP covalently labeled with the fluorescent Acrylodan moiety (Richieri et al., 1992), whereas ours is essentially an assay for free I-FABP. The results we present below are similar to theirs but systematically different from other earlier literature values [compare Lalonde et al. (1994) and Miller and Cistola (1993)], which suggests that there may be systematic errors in the determination of lipid binding affinities from ITC experiments.

In this work, we have re-examined the binding of fatty acids to I-FABP by using both our ANS-based fluorescence assay and ITC measurements. In the process of employing the ITC methodology, we identify a possible source of error in this measurement when subtraction of a reference, ligand-into-buffer, titration is undertaken. Finally, with the methods employed here, we construct an energy "balance sheet" as we did in the ANS instance, describing the changes in $\Delta H°$ and ΔC_p for the process of binding fatty acid (sodium oleate) to I-FABP.

THEORY AND METHODS

I-FABP binding to its "natural" ligands, i.e. free fatty acids C16−C24, apparently has submicromolar K_d values so that there is a considerable advantage in using ANS with an accurately known K_d to bind to I-FABP and compete with free fatty acid binding, and thus extract the binding constant of I-FABP to fatty acid by means of fatty acid displacement. One can write the dissociation constant of ANS (in the presence of fatty acid) for I-FABP as

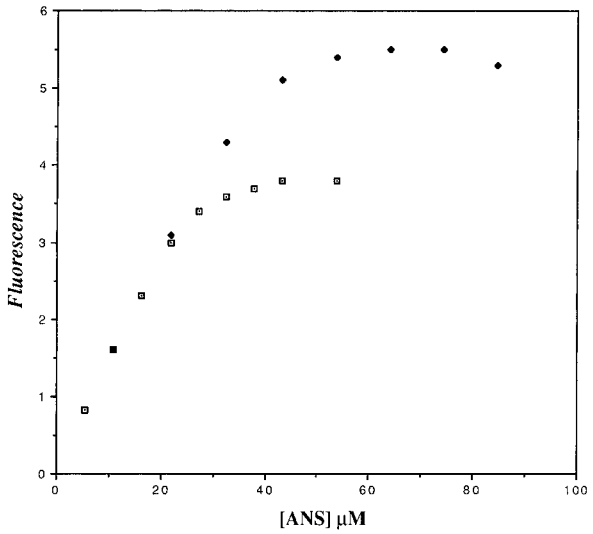

FIGURE 1: Fluorescence of ANS added to 5 mM Tris buffer containing 56 μM I-FABP with the following additions: (⊡) 28 μM sodium oleate and (◆) 14 μM sodium oleate. No background subtraction is made for these data. Cuvettes with 5 mm optical path length were employed, and inner filter corrections were applied as discussed in the text. The extrapolated equivalence point of both graphs represents the amount of free I-FABP into which ANS titrates. Thus, for the 28 μM oleate, there appears 28 μM free I-FABP while for 14 μM oleate, there appears to be nearly 42 μM free I-FABP. This establishes the 1:1 stoichiometry of ANS:I-FABP, and oleate:I-FABP in the complex. For all figures, fluorescence intensity is in arbitrary units.

$$K_{ANS} = P(1 - x - y)(A - xP)/Px \qquad (1a)$$

where x is the fraction of I-FABP (at a total concentration given by P) bound to ANS (at a total concentration given by A) and y is the fraction of I-FABP bound to fatty acid (at a total concentration given by F). We assume a 1:1 stoichiometry for both ANS and fatty acid binding to I-FABP (cf. Figure 1). The dissociation constant for fatty acid is then given by

$$K_{FA} = P(1 - x - y)(F - Py)/Py \qquad (1b)$$

Our previous report showed how one could obtain the value of x:

$$F(P,A) = F(P{-}FA,0) + qA(1 - xP/A) + xP\varphi q \qquad (2a)$$

where $F(P,A)$ is the fluorescence obtained from the solution at a given protein and ANS concentration and $F(P{-}FA,0)$ is the fluorescence background with the protein and fatty acid at given concentrations but without any added ANS. The parameter q is proportional to the solution quantum yield of free ANS and represents the linear factor by which the fluorescence of the solution depends directly on the concentration of added ANS. This necessitates a demonstration that the relationship *is* linear in the chosen concentration regime (cf. Figure 2). Φ is the factor by which the fluorescence of ANS is enhanced upon binding to the protein, a quantity that can be determined directly.

We find the following from two separate experiments: first, the value of K_{ANS}, as in our previous study, whereby a nonlinear least squares analysis is utilized to fit x as a function of P at a fixed A and in the absence of added fatty acid, giving the parameters K_A and Φ; and second, the value

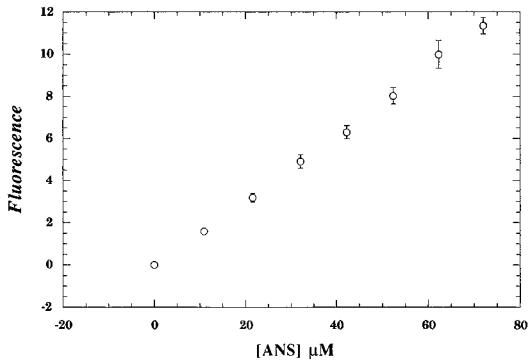

FIGURE 2: Plot of ANS fluorescence *vs* ANS concentration. Inner filter effect corrections were applied as discussed in the text. The linear slope corresponds to the parameter *q* required in the analysis of data for the competition assay, as described. Here, this slope is 0.159.

of *q*, that is, the slope of the fluorescence of a solution containing the given concentration of fatty acid (but no I-FABP) as a function of the concentration of ANS added. The procedure we then employed for the competitive assay was to add ANS in various increments to a given fixed concentration of I-FABP *and* fatty acid. As a simplification, our experiments were conducted with *equal* protein and fatty acid concentrations (cf. Materials). Rearranging eq 2a, we obtain

$$x_i = [F\ (P_i - F_i, A_i) - F\ (P_i - F_i, 0) - A_i q]/P_i q(\Phi - 1) \quad (2b)$$

for the value of the fraction of I-FABP bound by ANS at each *i*th ANS addition. From the values of Φ and *q* obtained from the two preliminary experiments, one finds x_i for each addition of ANS (correcting the protein concentration P_i for each volume addition, etc.) from the above formula. Solving for *y* in terms of *x* in eq **1** yields

$$K_{FA} = K_{ANS}x[F_i - P_i[1 - x - K_{ANS}x]]/[(A - P_i\,x)]/[(A - P_i\,x)(1 - x) - K_{ANS}x] \quad (3a)$$

This last expression provides the basis for a nonlinear least squares determination of K_{FA}. If K_{FA} is written as a function of *x*, *P*, *A*, and K_{ANS}, namely $f(x,P,A,K_{ANS})$, then the variance with respect to K_{FA} is minimized by setting $\partial/\partial K_{FA}[\Sigma_i[K_{FA} - f(x,P,A,K_{ANS})]^2] = 0$. We obtain the simple expression

$$K_{FA} = (1/N)\sum_i C_i[F_i - P_i + P_i(x_i + C_i/B_i)]/[B_i(1 - x_i) - C_i] \quad (3b)$$

where *N* is the number of additions of ANS in any run, $C_i = K_{ANS}x_i$, and $B_i = A_i - P_i x_i$. All the terms in the above expression are either experimentally known or derived from observables. In the conditions of our experiments, where the concentrations *A*, *F*, and *P* are always much greater than the dissociation constants K_{ANS} and K_{FA}, we find that the above formula (**3**b) is well approximated by

$$K_{FA} = (1/N)\sum_i K_A x_i(F_i - P_i + P_i\,x)/[(A_i - P_i\,x_i)(1 - x_i)] \quad (3c)$$

which is the same formula that would result in formula 3b if we had assumed that $1 - x \approx y$. However, as it stands, formula 3b is completely general. We recall that in our experimental conditions we also ensure that $F = P$, which simplifies both expressions.[1]

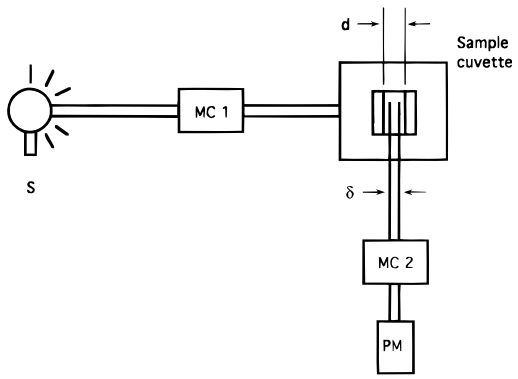

FIGURE 3: Experimental arrangement of spectrofluorimetry measurements displaying the origin of parameters involved in inner filter effect corrections, from Brand and Witholt (1975). S is the light source, MC 1 is the excitation monochrometer, d is the cuvette path length, δ is the emission slit width, MC 2 is the emission monochromator, and PM is the photomultiplier.

As we note above, in our procedure, fatty acid and I-FABP are added at the same concentration, typically at 21 μM (and at 15 μM) each. Duplicate runs were used for each concentration of oleic acid, for a total of four determinations. ANS concentration is typically increased from 0 to 70 μM; exciting the sample at 330 nm, we collected the entire emission spectrum from 390 to 640 nm and integrated as described in Kirk et al. (1996). Such high concentrations require both the employment of restricted excitation path length cuvettes ($\leq$5 mm) and inner filter corrections. We applied the inner filter effect correction by the method on Brand and Witholt, (1967). Figure 3 shows the experimental arrangement, providing the definitions required for the terms in the following formula:

$$\kappa = \ln 10\ \alpha\delta/[10^{-\alpha(d/2-\delta/2)} - 10^{-\alpha(d/2+\delta/2)}] \quad (4)$$

where κ is the inner filter effect correction factor, by which the raw fluorescence (integrated) intensities must be multiplied to obtain corrected intensities, and α is equal to the molar decadic extinction coefficient times the concentration of ANS, i.e. α equals the term ϵc of Beer's law.

Errors were propagated by summing the squares of the fractional errors, i.e.

[1] x_i *could* alternatively be solved in terms of K_{FA}, K_{ANS}, and the other parameters so that a nonlinear least squares algorithm could be used to curve-fit the data. But this requires solution of a cubic equation from formula 3b, which as the calculation proceeds involves an excursion into the complex domain (even if the solution set consists solely of real numbers), and hence defeats most implementations of the Marquardt algorithm. The full cubic equation reads $x^3 + x^2[K_{FA}P + 2AK_{FA} + K_{FA}K_{ANS} - K_{ANS}(A - K_{ANS} - F + P)]/P(K_{ANS} - K_{FA}) - xA[AK_{FA} + 2K_{FA}P + K_{FA}K_{ANS} + K_{ANS}(F - P)]/P2(K_{ANS} - K_{FA}) + K_{FA}A^2/P^2(K_{ANS} - K_{FA}) = 0$ (formula N1) which we can rewrite as $x^3 + 3\alpha x^2 - 2\beta x + 2\gamma = 0$ (formula N2), and hence, with K_{FA} much smaller than K_{ANS}, $\alpha \approx -A/3P$, $\beta = (A/2P - 1)AK_{FA}/PK_{ANS}$, and $\gamma \approx A^2K_{FA}/2P^2K_{ANS}$. Then the term $\omega = [(\alpha^3 + \gamma + \alpha\beta)^2 - (\alpha^2 + 2\beta/3)^3]^{1/2}$ is approximately equal to $i[(A/P)^5K_{FA}/K_{ANS}]^{1/2}$. The roots of eq N2 would be found as follows. $x = -\alpha + (\alpha^3 + \gamma + \alpha\beta + \omega)^{1/3} + (\alpha^3 + \gamma + \alpha\beta - \omega)^{1/3}$ (formula N3). Given the complex nature of ω, it is possible for the term $(\partial x/\partial K_{FA})^2$ to be <0, which converts the steepest descent subroutine of the Marquardt algorithm into a steepest ascent algorithm (Press et al., 1992). In addition, as K_{FA} approaches K_{ANS}, the terms β and γ diverge. Lastly, an attempt to "linearize" the above formula N3 by expanding as a Taylor series about $K_{FA} = 0$ fails because $\omega_0 = 0$, and derivatives of all orders of formula N3 contain ω^{-1}.

$$\sigma(K_{FA})_{true}/K_{FA} = \sigma^2[K_{FA}(\phi,K_{ANS})]/K_{FA}{}^2 + [\sigma^2(K_{ANS})/K_{ANS}{}^2]^{1/2} \quad (5)$$

where $K_{FA}(\phi,K_{ANS})$ is the result of formula 3b, $\sigma^2[K_{FA}(\phi,K_{ANS})]$ is the mean variance of these values, and $\sigma^2(K_{ANS})$ is the mean variance for K_{ANS}.

To summarize our assay procedure, (1) we titrate I-FABP into a fixed concentration of ANS, as in Kirk et al. (1996), obtaining thereby from nonlinear least squares fit of the ANS fluorescence *vs* I-FABP concentration values for Φ and K_{ANS} for use in eqs 2 and 3; (2) we titrate ANS into buffer in the presence of a given concentration of fatty acid but in the absence of I-FABP, obtaining from the slope of ANS fluorescence *vs* added ANS concentration the value of the parameter q (eq 2); and (3) we titrate ANS into buffer containing both I-FABP and fatty acid at the same given concentration as in step 2. The integrated fluorescence intensities from this experiment are converted into values of x by applying formula 2b. The x values are applied together with the other known parameters P, K_{ANS}, and the concentrations A_i to find K_{FA} from the least squares formula 3b. We apply inner filter corrections to all the fluorescence intensities in steps 2 and 3 and subtract a "background" from the integrated intensity of a spectrum of buffer + fatty acid (+I-FABP, for step 3) alone.

Calorimetry. Isothermal titration calorimetry was performed as described (Kirk et al., 1996). The procedure adopted there, and in the majority of other studies, is to conduct two experiments. In one, ligand is titrated into solution containing protein (experiment 1), and in the other, ligand is diluted into solution in the absence of protein (experiment 2). When the data from these two experiments are subtracted point for point in the fatty acid−I-FABP system, satisfactory fits (*vide infra*) are not obtained, and the dissociation constants differ very widely from those obtained by the competition assay. A more sophisticated treatment of the background subtraction seems to be required. Let us compare what we require from a titration calorimetry experiment with what it is we operationally obtain. We write the partial molar enthalpy of a species (e.g. the fatty acid) in some state as $h^{\circ species}{}_{state}$, which can be a function of other variables. In particular, let C_1 be the concentration of fatty acid ligand in the syringe and C_2 the concentration in the experimental ITC cell. C_2 is then itself a function of how far the titration has progressed, as well as the protein concentration and the binding constants. For the purposes of determination of $\Delta H^\circ$and ΔG° of the binding process, we fit

$$dH^\circ{}_i = [h^{\circ P-FA} - h^\circ{}_{sol}(C_2 \rightarrow 0)]\, dL_i \quad (6)$$

where dL_i is the number of moles of ligand added at the ith injection (essentially constant over the titration), $h^{\circ P-FA}$ is the partial molar enthalpy of the protein−fatty acid complex, and $h^\circ{}_{sol}$ is the *sum* of the partial molar enthalpy of the fatty acid free in solution and that of the protein in solution. The Riemann sum of $dH^\circ{}_i$ can be taken to be the enthalpy of the binding reaction, naturally in the limit as $dL \rightarrow 0$. Thus, the observed heats (since pressure is constant for the experimental arrangement, and all work done by the system is PV work, so the heat evolved or absorbed in the reaction is just the enthalpy) of each kind of reaction are

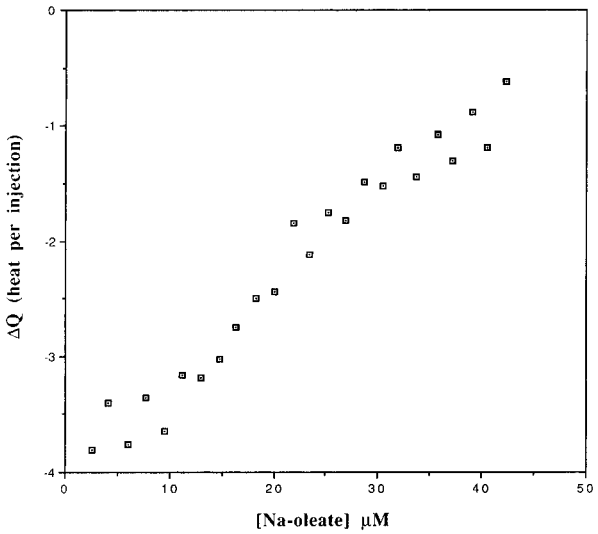

FIGURE 4: Isothermal calorimetry experiment titrating 0.615 mM sodium oleate in 4 μL injections into 5 mM Tris buffer at 22 °C. Heat evolved from each injection is expressed as the time-integral of the feedback power supplied by a heating circuit, a decrease in this power being a negative heat. The functional relationship between free oleate added up to a given injection and the heat evolved during this dilution process is obtained from such a reference experiment, for which the function is used to generate corrections to the heat evolved in the I-FABP titration experiment, as described in the text.

experiment 1

$$Q_i(1) = \{h^\circ{}_{prot}x_i - h^\circ{}_{syringe}(C_1) + h^\circ{}_{sol}(C_2[L_i;(\text{Exp } 1)])(1 - x_i)\}\, dL_i \quad (7a)$$

and

experiment 2

$$Q_i(2) = \{h^\circ{}_{sol}(C_2[L_i;(\text{Exp } 2)]) - h^\circ{}_{syringe}(C_1)\}\, dL_i \quad (7b)$$

where we have included terms for the molar enthalpy of the ligand in the syringe, $h^\circ{}_{syringe}$, as well as for the total number of moles of ligand added up to the ith injection, L_i. The term x_i expresses now the fraction of *ligand* added at the *ith* injection which at equilibrium is bound to the protein. Notice that, since we start the titration runs with $C_2 = 0$, we can subtract the two experimental heats point for point and obtain a result identical to expression 6 multiplied by x_i if $h^\circ{}_{sol}(C_2[L_i;(\text{Exp } 1,2)])$ is *constant*, and therefore equal to $h^\circ{}_{sol}(C_2 \rightarrow 0)$. It is apparent from Figure 4, which shows a titration of fatty acid into solution as per experiment 2, that $h^\circ{}_{sol}(C_2[L_i;(\text{Exp } 2)])$ is not a constant function of L_i (in this case, it seems to be a linear function, but more complicated cases can arise). In fact, because of the affinity binding of fatty acid to protein in our system, $C_2[L_i;(\text{Exp } 2)]$ is never equal to $C_2[L_i;(\text{Exp } 1)]$, that is, never at the *same* injection number i. Thus, we use the data from the second titration to generate the functional form of $h^\circ{}_{sol}(C_2[L_i;(\text{Exp } 2)])$ and determine the function at the (different) concentrations C_2L_i characterizing the ligand additions of experiment 1, i.e. $h^\circ{}_{sol}(C_2[L_i;(\text{Exp } 1)])$. We also generate values for $1 - x_i$ for each injection by utilizing an assumed value for K_{FA}; e.g. one can start by assume the literature value, and if after the analysis one obtains a value for K_{FA} different from this, one can iterate back with the new value until self-consistency is obtained. This is what we have done. We then subtract

$(1 - x)[h°_{sol}(C_2[L_i;(\text{Exp } 1)]) - h°_{syringe}(C_1)]\ dL_i$ from each *i*th data point of experiment 1 to obtain

$$dH_i = (h°_{prot} - h_{syringe}(C_1))x_i\ dL_i \quad (8)$$

and finally, we add a constant term which distinguishes the end of the titration, namely $h°_{syringe}(C_1) - h°_{sol}(C_2 \rightarrow$ end point). This term allows us to reach a zero value at the end of the titration, and it is with this transformed data set we apply the formulas discussed below to curve-fit for the values of $\Delta H°$ and K_{FA}. The addition of the constant quantity $h°_{syringe}(C_1) - h°_{sol}(C_2 \rightarrow$ end point) to every value of dH_i does not affect the fitting of K_{FA} as developed below, but it does mean that the final value of $\Delta H^{0\prime}$ so obtained differs from the thermodynamically interesting quantity that is defined relative to the infinite dilution reference state by the term: $N_A[h°_{sol}(C_2 \rightarrow$ end point$) - h°_{sol}(C_2 \rightarrow 0)]$, where N_A is Avogadro's number. We add this last term (obtained from the difference between the heats at the beginning and end of the experiment 2 titration) to obtain the proper values of $\Delta H°$ at the end of the process. It should also be noted that the K_{FA} so found differs from that defined with fatty acid in its standard state (i.e. relative to an infinite dilution reference state) by the activity coefficient of fatty acid at the titration end point; i.e. our values for the fatty acid dissociation constant for I-FABP are equal to $K_{FA}°\gamma(C_2 \rightarrow$ end), where $K_{FA}°$ is the standard-state dissociation constant. Of course, any thermodynamic measurement not conducted at infinite dilution requires similar corrections. We address this point further in the Results and Discussion.

Our procedure, as we stated above, is to calculate the free fraction of ligand at each L_i using some initial assumption of the effective binding constant to the macromolecule and the known concentration of protein. By assuming some initial value for the K_{FA} (e.g. from the literature), then

$$(1 - x_i)\ dL_i = L_{i,free} - L_{i-1,free} \quad (9a)$$

and

$$L_{i,free} = L_i - {}^1/_2[L_i + P + K_{FA} - [(L_i + P + K_{FA})^2 - 4PL_i]^{1/2}] \quad (9b)$$

After fitting the functional form of $h°_{sol}(C_2[L_i;(\text{Exp } 2)])$ and performing the subtractions discussed above, we then fit dH_i, as a function of dL_i. Adapting a formula from our previous results [Kirk et al. (1996) eqs 4 and 2c], we may write the fraction of I-FABP molecules to which oleic acid is bound as ξ ($\xi_i P_i/L_i = x_i$), and find that

$$\xi = [K_{FA} + P_i + L_i - [(K_{FA} + P_i + L_i)^2 - 4P_iL_i]^{1/2}]/2P \quad (10a)$$

Then, the fractional increase of ξ, $\Delta\xi$, produced upon a small increment in L, dL, would be given by $(\partial\xi/\partial L)\ dL$. Thus

$$\Delta\xi = dL/2P[1 - (K_{FA} + L_i - P_i)/[(K_{FA} + L_i + P_i)^2 - 4P_iL_i]^{1/2}] \quad (10b)$$

Lastly, we translate the measured (and corrected) heats Q_i into the form which is normalized automatically for the user in the Microcal software, as dH_i, and is actually equal to $P_i\Delta\xi\Delta H°'/dL_i$, so that we find the best fit curve to

$$dH_i = \Delta H°'/2[1 - (K_{FA} + L_i - nP)/[(K_{FA} + L_i + nP)^2 - 4nPL_i]^{1/2}] \quad (10c)$$

where $\Delta H°'$, the molar enthalpy of the binding reaction (relative to the final concentration of ligand in the ITC cell), the dissociation constant, and the stoichiometric parameter n (as described in our previous study) are the three parameters extracted from a nonlinear least squares fit.

MATERIALS

Palmitic and arachidonic acid were obtained from Sigma (St. Louis, Mo). While the solid palmitic acid can be accurately weighed out, the arachidonic acid had first to be dissolved in dry tetrahydrofuran (THF) (Fluka, Ronkonkoma, NY) and added to a preweighed vial. The solution was then dried under a stream of high-purity N_2 and reweighed. Oleic acid was obtained from Avanti Polar Liquids (Birmingham, Al). The fatty acids are added to buffer with enough added NaOH from a 1.00 M stock to neutralize the free acid. Buffers and all other reagents were obtained or prepared as described (Kirk et al., 1996). Stock solutions of oleic acid and palmitic acid were stored under Ar at −20 °C. The primary stocks were diluted to prepare experimental stocks which sufficed for one ITC experiment, or one set of ANS titrations. These secondary stocks were degassed with a gentle vacuum for 20 min before use. The final concentration of fatty acid in the prepared secondary stock was checked by addition of a known volume of the stock into a large excess of I-FABP at a known concentration (∼70 μM). After additions of ANS to the end point of fluorescence titration, the concentration of free fatty acid is obtained by subtraction. The stock of arachidonic acid was used immediately, since arachidonic acid decomposes in buffer at 0 °C ∼5% per day at a 1 mM concentration.

The buffer used in these studies (except where noted) was 5 mM tris(hydroxyethyl)aminoethane (*Tris*) (pH 7.7) (22 °C). We avoided excess ionic strength as well as the complications involved when sulfonate buffers are used (Kirk et al., 1996). These conditions give us the highest affinity for ANS (and presumably fatty acid) so that the signal from the competitive assay should be optimal. Since our studies also involved variation of the temperature, it is important that this pH lies in a pH-insensitive region for the affinity of I-FABP and ANS, as we had previously shown, given that the p*K* of Tris decreases by 0.031 pH unit per degree Celsius.

Fluorescence spectroscopy and isothermal titration calorimetry was performed on instrumentation described in our previous publication (Kirk et al., 1996).

RESULTS AND DISCUSSION

The results obtained from titration calorimetry need to be compared with those from the spectroscopically based competitive assay. Table 1 gives the values of K_{FA} for the fatty acids we studied as obtained at 22 °C from the latter assay. In our previous report, we observed that a large decrease in the affinity of I-FABP for ANS occurs between pH 8.3 and 9.3 (i.e. from ∼3 to nearly 50 μM in 50 mM sulfonate buffers; Kirk et al., 1996). That result is mirrored in results with oleic acid, wherein an approximately 60-fold decrease in binding affinity takes place between these two pH values. The value of K_{FA} at the high pH, ∼0.3 μM, is

Table 1: Fatty Acid Dissociation Constants (K_{FA}) at 22 °C[a]

fatty acid	K_{FA} (M)	error	no. of determinations
oleic acid	5.1×10^{-9}	2.8×10^{-9}	4
oleic acid[b]	3.3×10^{-7}	6.0×10^{-8}	2
arachidonic acid	5.0×10^{-8}	1.4×10^{-8}	2
palmitic acid	4.1×10^{-8}	1.1×10^{-8}	2

[a] All determinations were made in 5 mM Tris buffer (pH 7.76) except where noted. The protein concentration was 21 and 15 μM. [b] In CHES buffer, 5 μM, pH 9.3. The protein concentration was 210 μM.

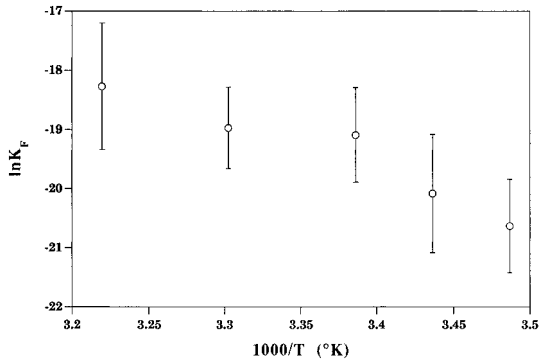

FIGURE 5: van't Hoff plot for the dissociation constant of sodium oleate with I-FABP as a function of temperature. Error bars include the standard errors of the K_{FA} values for each temperature as determined from the competitive assay, as well as standard deviations between several determinations (more than two in each case).

close to the value of 0.2 μM reported earlier [cf. Banaszak et al. (1994)], but at *neutral* pH, our value is significantly different. The recent study by Richieri et al. (1995) gives a value for oleic acid−I-FABP binding affinity much closer to our own, i.e. ∼14 nM for K_{FA} at pH 7.5 and 22 °C. The experimental conditions employed by these authors included buffered solutions with much higher added salt concentrations than ours (155 mM added NaCl and KCl). Our previous study with ANS demonstrated a reduction in affinity with increasing salt concentration so that, if one works backward from our conditions (5 mM ionic strength) to their condition by utilizing our previous data on the salt-effect in ANS (assuming that the salt effect is comparable between ANS and oleic acid), their results agree very well with our own (our value of K_F' at the higher ionic strength would be ∼20 nM). The values obtained for palmitate and arachidonate we present in Table 1 are also broadly similar to those found by Richieri et al. (1995); the values from the earlier literature are 3.6 and 3.7 μM (Lowe et al., 1987), respectively. Specifically, Richieri et al. (1995) found values for arachidonate and palmitate of roughly 110 and 7.2 nM (at 298 K), respectively, so that their values for arachidonate are quite compatible with ours (remembering the higher ionic strength in their study), but their K_{FA} for palmitate is even *lower* than that for oleate, whereas we find arachidonate and palmitate to be fairly similar in affinity.

The temperature dependence of the oleic acid binding was also investigated. Figure 5 shows the results of a van't Hoff analysis of the data from the competitive assay. The best slope of the plot is −8.4 (±2.2) which yields a value of −16.7 (±4.4) kcal/mol for the van't Hoff enthalpy of binding.

Figure 6A shows the isothermal calorimetric titration of oleic acid into 17 μM I-FABP at pH 7.7 and 22 °C; the heat

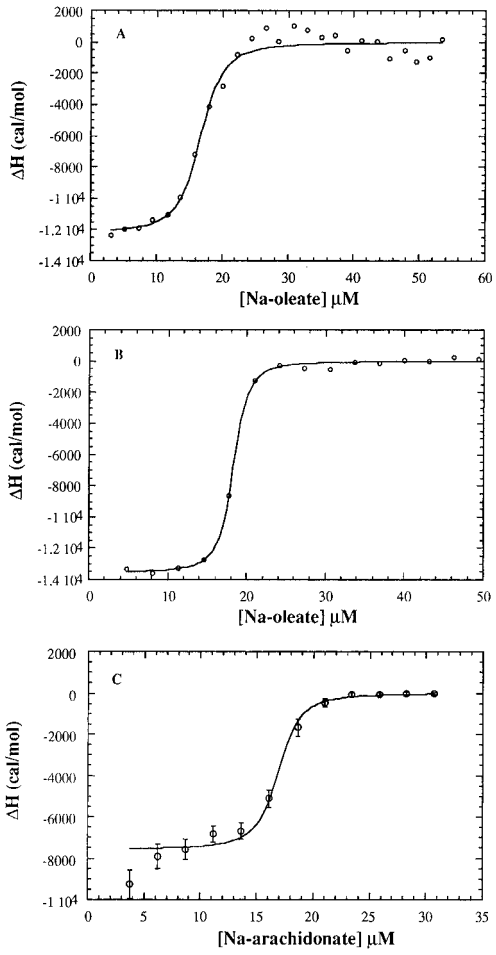

FIGURE 6: (A) ITC titration of sodium oleate into 20 μM I-FABP at 22 °C. The measured heat has been normalized by the concentration of ligand added and has been further corrected by the methods described in the text. The nonlinear least squares fit for this titration yielded $K_{FA} = 0.2 \pm 0.06$ μM, $\Delta H^{\circ\prime} = -12\,300 \pm 390$ cal/mol, and $n = 1.01 \pm 0.02$. The correction for Δh° ($C_2 = 0$; C_2 = end point), as discussed in the text, is +1060 cal/mol, giving a standard-state ΔH° of −11 140 cal/mol. (B) ITC experiment with conditions as in panel A except the temperature, which was 30 °C. The nonlinear least squares fit analysis gives $K_{FA} = 0.052 \pm 0.008$ μM, $\Delta H^{\circ\prime} = -13\,550 \pm 110$ cal/mol, and $n = 0.928 \pm 0.004$, while Δh° ($C_2 = 0$; C_2 = the end point) = +1680 cal/mol. The normalized χ^2 was 2.6. (C) ITC experiment for arachidonic acid titrated into I-FABP (17 μM) at 22 °C. The error displayed represents 6.5% of the experimental heat at each injection; this value of the error is assigned arbitrarily (although consistent with other measurements with arachidonic acid). The values determined from nonlinear least squares analysis were as follows: 0.06 ± 0.016 μM for K_F, -7570 ± 250 cal/mol for $\Delta H^{\circ\prime}$, 1.01 ± 0.02 for n, −220 cal/mol for and Δh° ($C_2 = 0$; C_2 = the end point). The value of χ^2 was 12.7.

of dilution of free oleic acid has been subtracted from this data, as described in Theory and Methods. The enthalpy of the binding process can be read from the figure since the process is complete, and the total change in ΔH_i up to saturation, where $\Delta H_i \approx 0$ (as it should), gives $\Delta H^{\circ\prime}$. The value from the fitting (after corrections as described in Theory and Methods) is −11.2 kcal/mol. The apparent K_{FA} is 0.2 μM. This value is the same as that given in e.g. Banaszak et al. (1994). Figure 6B shows another titration at 30 °C, where ΔH° is found to be −11.9 kcal/mol and K_{FA} is 0.05 μM. Not only is this value considerably smaller than the earlier literature value of K_{FA} (i.e. pre-Richieri et al.), but it is also unreasonable to imagine that the true K_{FA}

would actually be decreasing with increasing temperature when the $\Delta H°$ of binding is by all measures *negative*. Therefore, the ITC value of 0.2 μM for K_{FA} must be in error, an error which is smaller at 30 than at 22 °C. The values obtained here for $\Delta H°$ are also very close to those obtained by Richieri et al. for the van't Hoff enthalpy of oleate binding.

Figure 6C shows a similar titration with arachidonic acid. We obtain a value for $\Delta H°$ of -7.8 ± 0.2 kcal/mol with a K_{FA} of 60 ± 17 nM. Here at least, calorimetry seems to agree with spectroscopy, and it is worthwhile pointing out the apparent trend, namely that agreement between the competitive assay and isothermal calorimetry is better the larger the value of K_{FA} .

The complexity of the functional form of $h°_{sol}(C_2[L_i;$(Exp 2)]) from the reference titration may reflect the complexity of the underlying phase equilibrium with free fatty acid and micelles of various sizes. As Kresheck (1975) pointed out, micelles are "polydisperse...slight impurities exist, and...various empirical plots were used to establish a given CMC." For sodium palmitate, the CMC can be determined from Kresheck's empirical formula to be ~0.9 mM, and one can expect the CMC of sodium oleate to be higher [cf. Tanford (1973)]. This value is far in excess of the concentrations we employ here. But this value for CMC obtained from a particular technique (conductivity) does not imply that association of individual fatty acid molecules into dimers, trimers, etc., does not occur at our observational concentrations. While these *n*-mers do not represent well-defined *phases*, they certainly represent ill-defined, intermediate states between the fully hydrated fatty acid and the micellar phase. The negative enthalpy of dilution from the ligand concentration at the end point of the ITC titration to the infinite dilution reference state for oleate and palmitate—for arachidonate, however, this enthalpy is small and positive (cf. Figure 6 caption)—probably implies that the activity coefficient for fatty acid in the observed solution state is less than 1.0.

Uncertainty in $h°_{sol}(C_2[L_i;$(Exp 2)]) could lead to a great deal of variation in the subtraction, especially in the critical region of the titration for determination of K_{FA}, namely the region where $L_i \sim P$. Since the enthalpies of binding are a few kilocalories per mole at best, and the sensitivity of microcalorimeters is such that a signal of 0.2 μcal/s with a response time of 4 s. is approximately 10 times above the background noise, one can reliably measure single injections corresponding to the formation of 10^{-7} M product, with ~2 μl per each injection (assuming ΔH is roughly -10 kcal/mol). A full titration curve should have a number of points to obtain a good fitting (say ~10 points from zero concentration of added ligand to the flat region). Thus, the lower limit of macromolecule concentration required is about 1 μM. Lalonde et al. (1994) routinely used one 50 times higher than this concentration. For a dissociation constant of 50 nM or lower, calorimetry is simply unable to provide high enough precision to accurately extract values of K_{FA}. Obviously, the more enthalpic the reaction is, the lower this limit becomes, but 1 order of magnitude more enthalpy for most biomolecular reactions is not to be expected.

We illustrate this difficulty in finding precise values of K_{FA} by utilizing *synthetic* data for a hypothetical titration depicted in Figure 7. Here, a assumed value of $\Delta H°'$ (-13.5 kcal/mol) and K_{FA} for FABP with oleic acid (0.02 μM, similar to 38 °C data) were used to generate expected values for the heats evolved upon each injection of an ITC titration. To these values is added noise generated randomly from a Gaussian distribution with a width (σ) corresponding to 6.5% of the maximal reaction heat (which is fairly typical, cf. Figure 6C). These data are then compared with three different curves generated from K_{FA} values of 0.1, 0.05, and 0.01 μM. These curves fit the experimental points with χ^2 values of 8.9, 3.9, and 2.3, respectively. If the program is allowed to find its optimal fit, including the known error, it obtains a value for K_{FA} of 0.034 μM with a χ^2 of 1.4. It will be seen that the most significant data points in the determination of fitted parameters occur as the concentration of added ligand approaches molar equivalence with the macromolecule concentration. If this range is not sampled well, or if significant error (~10%) occurs in this region, the value obtained for K_{FA} will be dramatically affected; e.g. the curve at 0.05 μM or even 0.1 μM could fit best. But the value for $\Delta H°$ obtained in each case is very close (±400 cal) to the true value. Consequently, to obtain reliable values of K_{FA} from calorimetry, the macromolecule concentration should be close to K_{FA} (the ligand concentration being varied from 0 to ~5K_{FA}), while to obtain reliable values of $\Delta H°$, the concentration of macromolecule should be greatly in excess of K_{FA}.

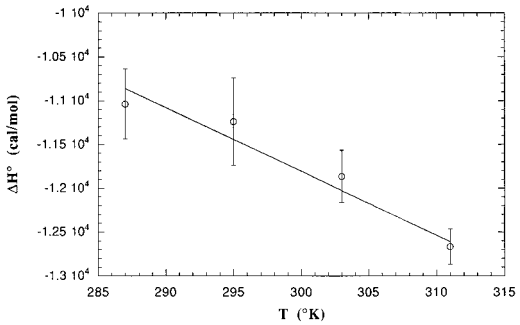

FIGURE 7: Values of $\Delta H°$ of oleate binding to I-FABP determined from nonlinear least squares analysis of ITC experiments conducted at a variety of temperatures, *vs* temperature. Standard errors are from the fittings. The slope gives the value of ΔC_p of ligand binding [-73 ± 17 cal/(deg mol)].

For these reasons, as noted above, the values of $\Delta H°$ from titration calorimetry are more reliable than the calorimetric values of K_{FA}, while the values from our competitive assay for K_{FA} are more reliable than the value of $\Delta H°_{van't\ Hoff}$ we obtained from them. Within the standard error, the van't Hoff enthalpy of oleic acid binding agrees with our calorimetric results (i.e. -11.9 ± 0.4 kcal/mol at 30 °C). Naghibi et al. (1995) have shown that calorimetric and van't Hoff enthalpies are often at variance. The reasons for this small discrepancy remain obscure, though it is likely that a spectroscopically monitored equilibrium process may not be composed of the exact subprocesses which are monitored calorimetrically. We do not have enough data points to employ the integrated van't Hoff formula to find $\Delta H°$ and ΔC_p via nonlinear least squares fitting, as Naghibi et al. (1995) did. However, it should be noted that, because the value of ΔC_p we determined is small, our linear van't Hoff assumption is not greatly in error. At 22 °C, our results further imply that the $\Delta S°$ of binding is ~0.2 cal/(deg mol). The binding is thus essentially enthalpically driven (which was also the conclusion in our study of ANS binding), which

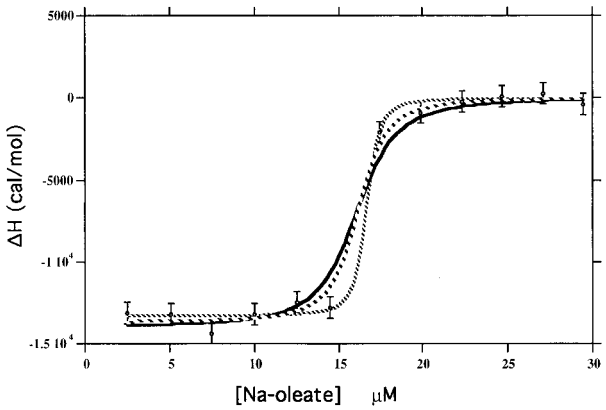

FIGURE 8: Synthetic titration data generated for a hypothetical ITC experiment, as described in the text. A random error is introduced via a Gaussian noise distribution with a standard deviation of 500 cal/mol at each injection. The separate synthetic points are plotted corresponding to injections of 2.5 μM added ligand each. Three alternative curves are shown, which display the consequences of different assumptions of the binding affinity: (\\\) $K_{FA} = 0.01$ μM, (bold \ \ \) $K_{FA} = 0.05$ μM, and (bold line) $K_{FA} = 0.1$ μM.

is essentially the same conclusion drawn by Richieri et al. (1995).

Our calorimetrically determined values for $\Delta H°$ for oleic acid binding at a variety of temperatures are plotted in Figure 8. The slope of this figure gives the change in the constant pressure heat capacity upon ligand association, or ΔC_p, which is -73 cal/(deg mol). This is much smaller than the value for ΔC_p we obtained in the case of ANS binding to I-FABP. We discuss these differences below.

Comparison of ANS and Fatty Acid Binding to I-FABP. Both binding events are enthalpically driven, and partially opposed by the entropy change, with fatty acid binding being slightly more enthalpically favored (approximately -1 kcal/mol) and considerably less entropically opposed [+10 cal/(deg mol) at room temperature] than ANS binding. The $\Delta S°$ values at 14, 22, 30, and 38 °C were -2.5 ± 0.8, $+0.2 \pm 1.2$, $+1.5 \pm 0.9$, and $+4.4 \pm 1$ cal/(deg mol), respectively. The change in heat capacity of the system upon binding is more negative in the case of ANS than for fatty acid. These two facts can be correlated. We can suppose that the differences between binding entropies for two species to the same protein could be described in terms of the differences in the heat capacity of binding [cf. Murphy et al. (1994)]:

$$\Delta\Delta S° = \Delta\Delta S^{ref}_{int} + \Delta\Delta C_p \ln T/T^{ref} \qquad (11a)$$

where $\Delta\Delta C_p$ is the difference in the binding heat capacities between oleate and ANS, or (-72 to -283) $\sim$210 cal/(deg mol); $\Delta\Delta S^{ref}_{int}$ is an intrinsic difference in entropy of binding, due possibly to differential amounts of desolvation entropy upon binding oleic acid *vs* ANS ($\Delta\Delta S^{ref}_{solv}$), or in the degree of lost configurational entropy between the two species ($\Delta\Delta S^{ref}_{config}$); and T^{ref} is a reference temperature at which the intrinsic differences in entropy are measured. The magnitude of ΔC_p for ANS is roughly similar to the ΔC_p for the transfer of aromatic hydrocarbons to the organic phase from water. In fact, a ΔC_p of this order has been called a "signature" of hydrophobic hydration (Privalov & Gill, 1989). On these grounds, the change in C_p accompanying *fatty acid* binding must then be considered anomalously small.

We have argued in our previous work that the binding to ANS may not be stereotypical hydrophobic interaction, and it seems likely that the same might be said for fatty acid binding. Whether or not the fatty acid−I-FABP interaction is driven by the hydrophobic interaction, the sheer magnitude of changes in enthalpy or heat capacity cannot tell us anything quantitative about the nature of the bonds being broken and the number of each kind. Qualitatively speaking, the net exchange of weaker bonds for stronger bonds will generate a negative enthalpy and a relatively large negative change in heat capacity (since a weak vibration contributes R to the constant volume heat capacity at low temperatures yet a strong vibration would be frozen out). The introduction of weak bonds (H bonds) into an originally "nonbonded" configuration will generate a relatively smaller negative enthalpy with a *positive* change in heat capacity. Thus, a linear combination of weak-to-strong bond and 0-to-weak bond changes could generate practically any observed net change in ΔH and ΔC_p. But the exact composition of weak-to-strong and 0-to-weak bonding changes responsible depends on the exact details of each type of bond, so any of a large number of possible combinations could in principle yield the same net change in the enthalpy and heat capacity.

From this qualitative point of view alone, one can suppose that ANS binding is characterized by an exchange of weaker for stronger bonds, e.g. the release of weakly bound water from the protein and ligand into the bulk phase; the case of fatty acid binding to I-FABP would include the same or similar processes, but with an additional contribution due to the formation of weak interactions which were *not* present in the initial state, i.e. ANS binding mimics binding of a significant portion of the free fatty acid but not all of it. For example, it is reasonable to suppose that the fatty acid tail enjoys additional van der Waals interactions with the protein which are not present in the interaction of ANS with the protein, and for which the fatty acid species, as it occurs dissolved in water, has no equivalent either. The van der Waals forces exerted on the lipid by water would be nondirectional and nonspecific compared with those exerted by the protein so that the protein might restrict some alkyl chain librations or rocking motions, etc., more effectively than water does. To the extent that these enthalpy and heat capacity changes are driven and determined by a changed interaction of ligand + macromolecule with water, they can be ascribed to hydrophobic effects, but the identity and nature of those interactions which replace the water−ligand and water−protein interactions during the binding process makes a quantitative difference in the final energy "balance sheet".

Returning to eq 11, it seems likely that the configurational entropy of fatty acid lost upon binding to I-FABP must be greater than that for ANS, since ANS has only two (coupled) degrees of freedom which can be effectively restricted (about the dihedral angle of the anilino moiety with respect to the napthyl ring and the angle by which the phenyl ring is rotated about the N$-C_{1'}$ axis), whereas oleate has 16 internal rotations. Thus, if we define the reference difference in entropy of binding between the two species as

$$\Delta\Delta S^{ref}_{int} \equiv \Delta S^{ref}(\text{oleic})_{int} - \Delta S^{ref}(\text{ANS})_{int} \approx \Delta\Delta S^{ref}_{config} + \Delta\Delta S^{ref}_{solv} \qquad (11b)$$

then $\Delta\Delta S^{ref}_{config}$ must be very negative, since the conformation of I-FABP changes little upon binding fatty acid (Scapin

et al., 1992) or, presumably, ANS. Our data suggest that, if T^{ref} is taken to be ~15 °C in eq 11a, $\Delta\Delta S°$ approaches zero (indeed both *individual* $\Delta S°$ values for each process approach zero, i.e. at roughly 12 °C for ANS binding and ~ 20 °C for oleate) so that $\Delta\Delta S^{ref}_{config} = -\Delta\Delta S^{ref}_{solv}$ (and the same is true individually if the cratic entropy is included, i.e. $\Delta S^{ref}_{config} + \Delta S_{cratic} \cong -\Delta S^{ref}_{solv}$ for each ligand) near this temperature. Thus, ΔS^{ref}_{solv}(oleate) must be $\gg \Delta S^{ref}_{solv}$(ANS) and positive. The formula for $\Delta S°_{solv}$ given by Murphy et al. (1994, (1990, 1994), namely

$$\Delta S_{solv} = \Delta S\rightarrow_{solv} + \Delta C_p \ln T/T^* \qquad (11c)$$

hypothesized to be true for a "universal" reference temperature T^* of 112 °C at which the hydrophobic contribution to solvation entropy is said to vanish, would seem to be inadequate to explain our results, since the difference ΔS^*_{solv}(oleate) − ΔS^*_{solv}(ANS) must be even greater than the difference between the ΔS^{ref}_{solv} values given that ΔC_p(oleate) is much less negative than ΔC_p(ANS). The hypothetical reference entropy ΔS^*_{solv} is said by Murphy et al. (1995) to depend only on electrostatic and/or protonation effects, which, it would seem, are unlikely to differ significantly between oleate and ANS. That is, the relative change in affinity which occurs at high pH for ANS (Kirk et al., 1996) is mirrored in our results reported here (cf. Table 1) with oleate, so that at, e.g., pH 7.5 there is no differential protonation of I-FABP with bound oleate *vs* that of I-FABP with bound ANS. Similarly, the effects of ionic strength upon the affinity for ANS (Kirk et al., 1996) could be used to predict, via a simple Debye−Hueckel activity coefficient calculation, the expected dissociation constant of I-FABP for oleate at the higher ionic strength employed by Richieri et al. (1995), with reasonable success.

As we mentioned, the effects at higher pH for the binding of oleic acid and for ANS binding are similar and presumably have a corresponding source. If one tabulates all the positively charged residues in the apo I-FABP crystal structure (Sacchettini et al., 1989) which are in or near the binding cavity, only *Arg*-106 does not participate in an ion-pair association with another residue. This *Arg* is buried in the cavity and is in fact intimately coordinated, i.e. probably ion-paired, to the fatty -acid carboxylate in the crystal structure of I-FABP with bound palmitate (Scapin et al., 1992). We have tentatively ascribed the significant decrease in binding affinity for ANS which occurs at this pH to the titration of this residue (Kirk et al, 1996), which would imply a significantly lower p*K* for this residue than is generally found. It is presumably the screening of this charge which is also responsible for the salt effect we observed in our previous study. Taking the results of this study into account, our assumption in the previous report that ANS binds at the same site as fatty acid seems justified.

Interestingly, in a report on ^{13}C NMR of fatty acid binding proteins in the presence of labeled fatty acid (Cistola et al., 1989), while one palmitate carboxylate peak representing I-FABP-bound fatty acid was in fact insensitive to changing pH (up to pH 11.3), there nonetheless appeared a new peak at pH >9, which the authors took to represent some palmitate nonspecifically interacting with I-FABP. Since even at high pH fatty acid binding affinity is submicromolar, and any fatty acid which inserts into the binding cavity probably dissociates very slowly in any case, it is probable that the changes in affinity we observe with pH would be mostly due to slower "on" rates, and these changes might be invisible to their technique. But this new NMR peak might instead represent an electrostatically alterred coordination environment for the palmitate carboxylate as it occurs in its *normal* binding site at higher pH. We should also point out that bound fatty acid will stablilize a countercharge in such a way that the p*K* of the titrating group with fatty acid bound will be more positive than that of the free species by $\Delta pK = \log(K_F^{-H}/K_F)$, where K_F (K_F^{-H}) is the fatty acid dissociation constant for the protonated (deprotonated) I-FABP.

SUMMARY

We have applied the results from our previous work on ANS binding to I-FABP to examine the affinity of this protein for fatty acids. There are discrepancies in the value of the dissociation constants for oleic, arachidonic, and palmitic acids between our results (or other new results which have been based on a competitive assay, from the Kleinfeld laboratory; Richieri et al., 1995) and those of the older literature which were based on data from titration calorimetry as well as other methods. This necessitated a re-examination of the ITC methodology as applied to fatty acids. A significant source of error is introduced in such measurements when, as in the case of fatty acids, there is a considerable heat of dilution of the ligand by itself which is not a constant function of ligand concentration. The critical region of ligand concentration for determination of K_{FA} from titration of ligands with very high affinity for the macromolecule occurs when the ligand concentration approaches that of the macromolecule. In this concentration region, a significant amount of heat may be induced by ligand dilution which can be mistaken for heat of ligand binding and yet is not compensated by the point-for-point subtraction of a reference titration. Applying a correction for these effects, we have extracted values for K_{FA} via ITC measurements which show an improved agreement with our K_{FA} values derived from the fluorescence-based assay, compared with the ITC-based K_F values reported previously. The general picture we constructed to explain the thermodynamic changes characterizing ligand binding to I-FABP in the case of ANS (Kirk et al., 1996) is reinforced by many of the features of fatty acid binding. As shown also by Richieri et al. (1995), the binding is enthalpically driven, with a large decrease in affinity occurring at high pH (pH ~9), which may be due to the loss of a contributory electrostatic interaction with *Arg*-106 of I-FABP. Fatty acid binding is accompanied by a slight positive change in entropy, as opposed to the negative entropic contribution in the case of ANS. ANS also demonstrates a considerably larger negative heat capacity of binding than fatty acid, which we have ascribed to the presence of weak interactions of the protein with the fatty acid alkyl chain that are missing in the ANS−protein interaction. The hydrophobic effect is probably the driving force for ligand binding in both cases, but as modified by the presence of charge−charge effects, this may not represent a conventional hydrophobic interaction.

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Corrections

Evidence Favoring Molybdenum−Carbon Bond Formation in Xanthine Oxidase Action: ^{17}O- and ^{13}C-ENDOR and Kinetic Studies, by Barry D. Howes, Robert C. Bray,* Raymond L. Richards, Nigel A. Turner, Brian Bennett, and David J. Lowe*, Volume 35, Number 5, February 6, 1996, pages 1432−1443.

Page 1436. In Table 2, footnote *c* has been omitted. The footnote should read as follows: See Brower et al. (1986) and Brisdon et al. (1993).

Page 1442. The page numbers for the Burrow et al. (1995) reference should read 2583−2589.

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