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Titration calorimetry as a binding assay for lipid-binding proteins

Katherine R. Miller and David P. Cistola
Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, USA

Abstract

Titration calorimetry has been evaluated as a method for obtaining binding constants and thermodynamic parameters for the cytosolic fatty acid- and lipid-binding proteins. An important feature of this method was its ability to accurately determine binding constants in a non-perturbing manner. The equilibrium was not perturbed, since there was no requirement to separate bound and free ligand in order to obtain binding parameters. Also, the structure of the lipid-protein complex was not perturbed, since native ligands were used rather than non-native analogues. As illustrated for liver fatty acid-binding protein, the method distinguished affinity classes whose dissociation constants differed by an order of magnitude or less. It also distinguished endothermic from exothermic binding reactions, as illustrated for the binding of two closely related bile salts to ileal lipid-binding protein. The main limitations of the method were its relatively low sensitivity and the difficulty working with highly insoluble ligands, such as cholesterol or saturated long-chain fatty acids. However, the signal-to-noise ratio was improved by manipulating the buffer conditions, as illustrated for oleate binding to rat intestinal fatty acid binding protein. Binding parameters are reported for oleate interactions with several wild-type and mutant lipid-binding proteins from intestine. Where possible, the binding parameters obtained from calorimetry were compared with results obtained from fluorescence and Lipidex binding assays of comparable systems. (Mol Cell Biochem **123:** 29–37, 1993)

Key words: calorimetry, carrier proteins, lipid-protein interactions, fatty acids, bile salts

Abbreviations: I-FABP – recombinant (*E. coli*-derived) rat Intestinal Fatty Acid-Binding Protein, I–FABP (R106Q) – a mutant form of I-FABP in which arginine-106 has been replaced by glutamine, CRBP-II – recombinant rat Cellular Retinol-Binding Protein, CRBP-II (Q109R) – a mutant form of CRBP-II in which glutamine-109 has been replaced by arginine, L–FABP – recombinant rat Liver Fatty Acid-Binding Protein, I–LBP – recombinant rat Ileal Lipid-Binding Protein, n, lipid : protein molar binding stoichiometry; Kd and Ka, dissociation and association constants for ligand binding, respectively; $\triangle H$ – enthalpy of binding, $\triangle G$ – Gibbs free energy of binding; $\triangle S$, enthalpy of binding; T – Temperature in °K

Address for offprints: D.P. Cistola, Campus Box 8231, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, Missouri, 63110, USA.

Introduction

The cellular functions of lipid-transport proteins cannot be fully realized without a comprehensive knowledge of their binding properties. In particular, it is important to identify physiologically important ligands and establish their binding affinities, stoichiometries and specificities. Since many lipid-binding proteins exhibit no enzymatic activity, binding parameters provide an important quantitative measure for comparing the 'activity' of various wild-type and mutant forms. For this purpose, binding assays that are quantitative, accurate and robust are desirable.

For the intracellular fatty acid- and lipid-binding proteins, a variety of biochemical and biophysical binding assays have been used. The biochemical assays include those based on gel-filtration [1–3], equilibrium dialysis [4], Lipidex[1] [5, 6] and liposomes [7]. Although useful when employed with care, biochemical assays have one common disadvantage: bound ligand must be separated from unbound in order to measure binding parameters. This separation perturbs the binding equilibrium and may give rise to artifacts. A second issue is the manner in which the separation is achieved. With the Lipidex assay, for example, separation of bound from free ligand occurs when the ligand-protein complex is incubated with Lipidex at 0° C. If the Lipidex matrix effectively competes with protein for ligand binding, artifically low binding affinities will be obtained. The potential for such competition is underscored by the fact that Lipidex is also used to delipidate proteins at 37° C [5]. A third disadvantage is that lipids often adhere to glassware, plastic, and dialysis membranes at the micromolar and sub-micromolar concentrations used in most biochemical assays. This phenomenon can introduce significant errors in binding affinities [6].

The problem of separating bound from free ligand is circumvented with biophysical assays such as those based on fluorescence [8–13], electron paramagnetic resonance [14], and nuclear magnetic resonance [15–18]. These assays distinguish bound and free ligand by spectroscopic means, without the need to perturb equilibrium or use competing materials. Binding parameters can be obtained, as well as structural information regarding the local environment of the spectroscopic probe. However, changes in protein fluorescence upon ligand binding are sometimes too small to quantitate. This is particularly true for L-FABP, which has no tryptophan residues. Also, some lipids lack intrinsic fluorescence or paramagnetic properties, including many lipids of physiological interest. In such cases, lipid analogues containing structure-perturbing anthroylxoxy or doxyl probes are required. Lipids that do have intrinsic fluorescence, such as parinaric acid, are labile and prone to oxidation. The binding of native ligands can be monitored by isotope-directed NMR techniques, provided that enrichment with ^{13}C or another suitable isotope is feasible. Although such NMR methods are useful for determining binding stoichiometries and monitoring lipid-protein interactions [17, 18], they are not usually sensitive enough to quantitate binding affinities.

We have recently investigated the use of titration calorimetry as a non-perturbing method to quantitate the ligand-binding affinities for cytosolic fatty acid- and lipid-binding proteins. The purpose of this paper is to illustrate the application of titration calorimetry to these proteins, to discuss its advantages and limitations for such studies, and to report binding parameters for several wild-type and mutant lipid-binding proteins from intestine.

[1] Lipidex is a trademark of Pharmacia AB, Sweden, for hydroxyalkoxy-propyl dextran, a lipophilic derivative of Sephadex used for various liquid chromatography applications. The binding assay that uses this material to study fatty acid- and lipid-binding proteins [5, 6] is commonly referred to as the 'Lipidex assay'.

Materials and methods

Protein biosynthesis and purification

All of the proteins used in this study were recombinant rat proteins. These proteins were biosynthesized in *E. coli* strain MG1655 and purified as follows. Bacteria harboring the appropriate pMON plasmids [19–22] were grown at pH 7.2 in a New Brunswick Bioflo III High-Density Fermentor using a nutrient-rich medium containing tryptone (10.8 g/L), yeast extract (22.6 g/L), potassium phosphate (0.1 M), MgSO4 (1–5 mM), and CaCl2 (0.1–0.5 mM), as well as trace amounts of FeSO4 and thiamine. Cells were grown to a final density of 15–55, as monitored by O.D. at 600 nm. Plasmid expression was induced during mid log-phase by adding nalidixic acid to 100 μg/ml. After harvesting, the cells were lysed using a French pressure cell, and the proteins purified using a protocol modified from that of Lowe *et al.* [23].

In short, it involved (i) ammonium sulfate fractionation to 60%, saving the supernatant, (ii) dialysis against 20 mM potassium phosphate, pH 7.4, (iii) titration with protamine sulfate to 0.04–0.07% (w/v) to preciptate nucleic acids, (iv) ion-exchange chromatography using quaternary aminoethyl Zeta Prep cartridges, and (v) gel-filtration chromatography using a 140 × 5 cm column of Sephadex G-50. Fatty acids bound to the purified protein were removed using lipophilic Sephadex at 37° C according to Glatz and Veerkamp [5]. The yields of purified protein from individual 4-liter fermentations varied between 200 and 5000 mg, depending on the nutrient content and protein used. The final buffer was 20 mM potassium phosphate, 50 mM KCl, 0.05% NaN3, pH 7.2. Protein concentrations were determined spectrophotometrically as described elsewhere [17].

Preparation of oleate solutions

Oleic acid was first dissolved in chloroform, and its concentration determined by measuring dry weights on a Perkin-Elmer AD-4 microbalance. An appropriate amount of oleate was aliquoted into a separate vial and the solvent evaporated under a stream of nitrogen. A small volume of H_2O (50 µl) and 1.2 equivalents of potassium hydroxide were added, and the sample was thoroughly mixed until a clear solution was obtained. Finally, buffer was added to bring the oleate concentration to 1–7 mM, depending on the experiment. In some experiments, the ligand buffer contained 1% methanol; its heat of dilution upon injection served as a carrier signal to increase the signal-to-noise ratio (see Results and Discussion and Fig. 2).

Titration calorimetry

Titration calorimetry was performed using a Microcal OMEGA differential titrating calorimeter [24]. Up to 50 aliquots (7–10 µL, each) of the ligand solution were automatically injected into the sample cell containing the protein sample. The protein concentration was 0.07–0.15 mM in a volume of 1.5 ml. All titration experiments were performed at 25° C or 37° C. The data were processed using the software package ORIGIN. A function that modeled one or two classes of independent binding sites was used to fit the data [24]. The association and dissociation constants (Ka, Kd), molar binding stoichiometry (n), and enthalpy ($\triangle H$) were determined from the fitted curve. The Gibbs free energy and entropy of binding were calculated using the equations $\triangle G = -RT \ln K_a$ and $\triangle S = (\triangle H - \triangle G)/T$, respectively, where R is the gas constant and T is absolute temperature in ° K.

Results and discussion

An example of titration calorimetry data for oleate binding to a lipid-binding protein, CRBP-II (Q109R), is illustrated in Fig. 1. This mutant form of CRBP-II, unlike the wild-type protein, binds fatty acids in a manner nearly identical to I-FABP [18]. The upper panel shows the raw data: a plot of heat absorbed or emitted as a function of time. Each downward spike represents the automatic injection of a small amount of ligand solution from the syringe into the protein solution in the sample cell, which is kept at constant temperature. The area under each spike represents the amount of heat absorbed or emitted during the ligand binding and mixing process. A plot of the area under each peak as a function of the injection number, or ligand: protein mole ratio, is shown in the lower panel of Fig. 1. The data points were fitted to a mathematical model that assumed one affinity class of independent, non-cooperative sites [24]. From this fitted curve, the molar binding stoichiometry, association constant (Ka), and enthalpy of binding were obtained. To assess reproducibility and obtain standard deviations, a total of four identical repeat experiments were performed on this sample. The binding parameters are summarized in Table 1.

The signal-to-noise ratio of the raw data and the quality of the results obtained depended on the magnitude of the enthalpy change for a given binding reaction, as well as instrument stability. The total observed enthalpy change (area under each injection peak) included enthalpy changes for ligand binding as well as any other processes that occured upon mixing, such as the dilution of a solute. This heat of dilution increased the intensity of each injection signal and was easily corrected for by control experiments where the ligand solution is injected into buffer without protein. We found that the signal-to-noise ratio for the experiment could be improved by adding a non-interacting solute[2], such as 1% methanol, to the ligand buffer only and not the protein buffer. Examples of oleate titrations of wild-type I-FABP, with and

[2] Control experiments indicated that the presence of 1% methanol in the ligand buffer (corresponding to a final concentration of < 0.2% in the ligand-protein solution) did not give rise to enthapy changes resulting from changes in protein conformation or methanol binding, at least for the proteins examined here. However, this concentration of methanol may not be suitable for other proteins.

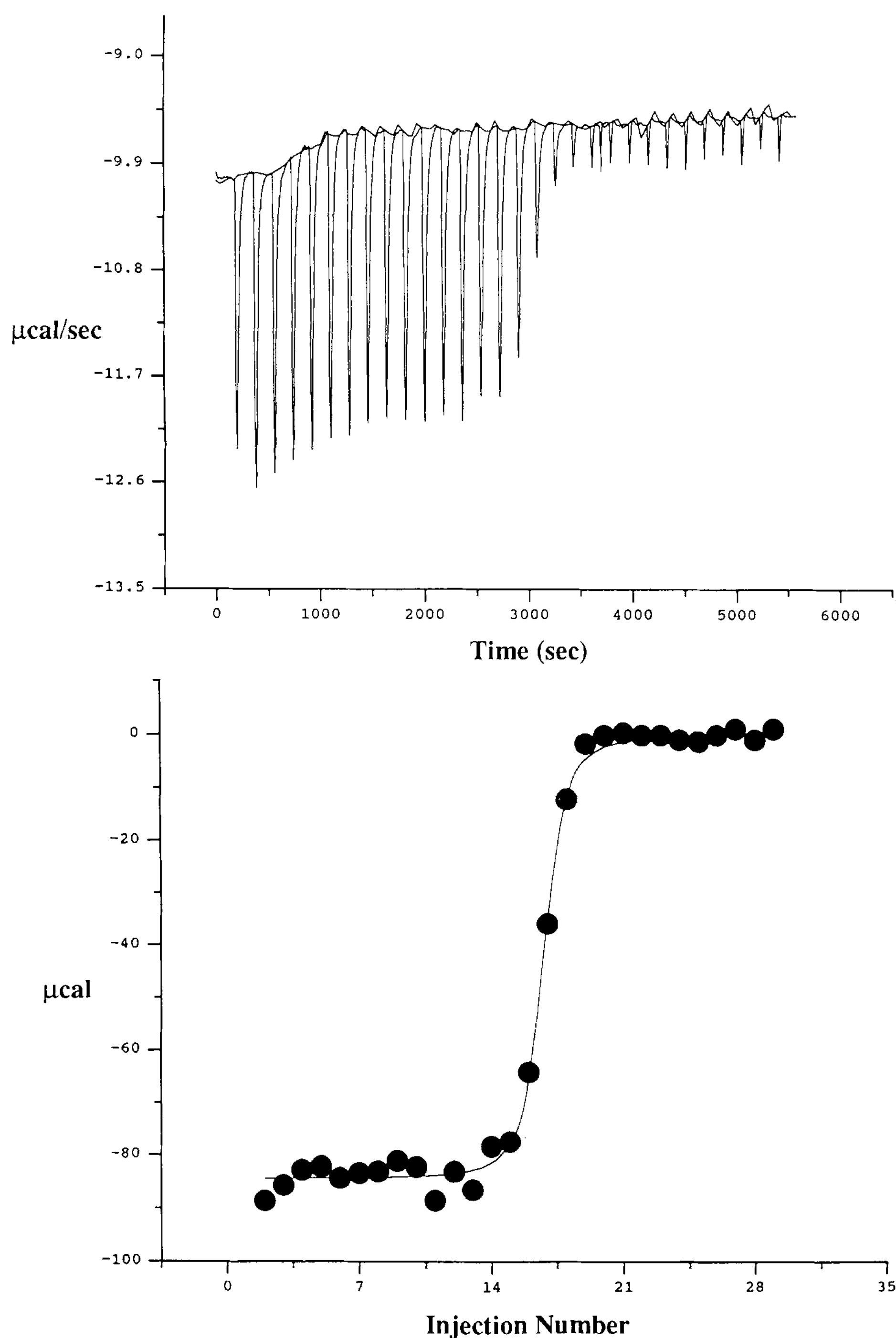

Fig. 1. Titration calorimetry data for the binding of oleate to rat CRBP-II (Q109R) at 25° C. The upper panel illustrates the raw data in the form of an injection diagram. In the lower panel, the filled circles represent the normalized areas under each injection spike from above. The fitted curve was derived from a mathematical model that assumed one affinity class of binding sites. The binding parameters obtained from this experiment are presented in Table 1. The protein concentration in the sample cell was 0.15 mM, and the ligand concentration in the injection syringe, 2.5 mM.

without methanol in the ligand buffer, are shown in Fig. 2. In the absence of methanol (Fig. 2, upper panel), the area under each peak contained enthalpy changes for the binding of oleate to the protein and the dilution of oleate into the protein/buffer solution. With 1% methanol in the ligand buffer (Fig. 2, lower panel), the peaks contained additional area corresponding to the heat of dilution of methanol. The binding parameters obtained

in the absence and presence of methanol were essentially identical, except that the standard deviations were smaller when methanol was present. For example, the Kd values for I-FABP (R106Q) were 4.4 ± 2.0 μM in the absence, and 4.2 ± 0.6 μM in the presence of methanol.

The parameters for oleate binding to several wild-type and mutant lipid-binding proteins from intestine are summarized in Table 1. All of the proteins examined bound one mole of oleate per mole protein, except for L-FABP, which bound 2. The dissociation constants for I-FABP and the first site on L-FABP were the same (0.2 μM). However, the dissociation constants for I-FABP and I-LBP differed by a factor of 140.

Binding curves for L-FABP, obtained without methanol, are illustrated in Fig. 3. The points were fitted to a mathematical model that assumed one (left panel) or two (right panel) affinity classes of binding sites. Based on a χ^2 analysis (Fig. 3 legend), the best fit was obtained with the two-class model. Remarkably, these two affinity classes could be distinguished even though their dissociation constants differed by an order of magnitude or less.

The binding parameters for rat I- and L-FABP obtained from calorimetry could be compared with previously reported values for the same recombinant proteins as obtained from fluorescence and Lipdex assays.

Table 1. Titration calorimetry results for oleate binding to wild-type and mutant lipid-binding proteins at 25° C and 37° C[a]

	I-FABP	L-FABP[b]		I-LBP
Temperature	37° C	37° C		37° C
n	1.1 ± 0.2	1.0	0.9	0.7 ± 0.2
Kd (μM)	0.25 ± 0.15	0.2	0.9	36 ± 17
ΔG (kJ/mol)	− 40 ± 2	− 39.6	− 35.9	− 27 ± 1
ΔH (kJ/mol)	− 36 ± 3	− 16	− 7	− 8 ± 2
TΔS (kJ/mol)	4 ± 4	23	29	19 ± 3
	CRBP-II (Q109R)[c]	I-FABP[c]		I-FABP (R106Q)[c]
Temperature	25° C	25° C		25° C
n	1.1 ± 0.1	1.1 ± 0.1		1.1 ± 0.1
Kd (μM)	0.20 ± 0.03	0.2 ± 0.1		4.2 ± 0.6
ΔG (kJ/mol)	− 38.1 ± 0.4	− 39 ± 2		− 30.7 ± 0.4
ΔH (kJ/mol)	− 25 ± 2	− 34 ± 4		− 4 ± 2
TΔS (kJ/mol)	13 ± 2	4 ± 4		26 ± 2

[a] Each result represents the mean ± standard deviation of at least 3 experiments. [b] Data points from 3 separate experiments were averaged and then analyzed using a model that assumed 2 affinity classes (see Note Added in Proof). Results are reported for two individual binding sites. [c] Results from Jakoby *et al.* [18].

A fluorescence assay for cis-parinaric acid binding to I-FABP yielded a Kd of 0.23 μM [15], which agrees with the results reported here for oleate binding (Table 1). A different fluorescent assay that employed I-FABP derivitized with acrylodan, a fluorescent dye, yielded essentially the same Kd value, 0.28 μM [25]. However, the Lipidex assay yielded a Kd value of 2.1 μM for oleate binding to I-FABP [13], approximately 10-fold higher than the values obtained by calorimetry and fluorescence. For L-FABP, the fluorescence assay yielded a single affinity class of 2 binding sites with a Kd of 0.72 μM, which is somewhat similar to the two Kd's of 0.2 and 0.9 μM obtained from calorimetry. However, the Lipidex assay results yielded a single Kd value of 1.5 μM [13], also about an order of magnitude higher than the highest affinity class obtained by calorimetry. In summary, the dissociation constants obtained by calorimetry agreed well with those obtained by fluorescence. However, it appears that the Lipidex assay significantly overestimated the Kd value for oleate binding to recombinant rat I- and L-FABP.

A unique feature of titration calorimetry was its ability to directly measure heats of binding and to distinguish exothermic from endothermic reactions. Figure 4 illustrates results for the interactions of two glyco-conjugated bile salts with I-LBP. The only difference between these bile salts, glyco-cholate and glyco-chenodeoxycholate, is the presence or absence of a hydroxyl group at the 12 position of the steroid ring moiety. However, the binding curves for the two bile salts were quite distinct. As shown on the left, glyco-cholate binding to ILBP was characterized by a high-affinity class of sites with endothermic binding, and a lower affinity class with exothermic binding. In contrast, glyco-chenodeoxycholate exhibited exothermic binding for both classes of binding sites. A similar pattern was observed for the corresponding un-conjugated bile salts: cholate and chenodeoxycholate[3]. This result implies that the 12-OH group on glycocholate has a specific effect on the interactions between bile salts and I-LBP.

Another unique feature of the calorimetry assay was its ability to quantitate the enthalpic and entropic contributions to the binding reaction. As shown in Table 1, binding of oleate to I-FABP was characterized by a large enthalpic component and small entropic component. In contrast, binding to the mutant I-FABP (R106Q) resulted in a low enthalpic and high entropic component. The reduction in binding enthalpy upon substitution of Gln

[3] Miller KR, Cistola DP, manuscript in preparation.

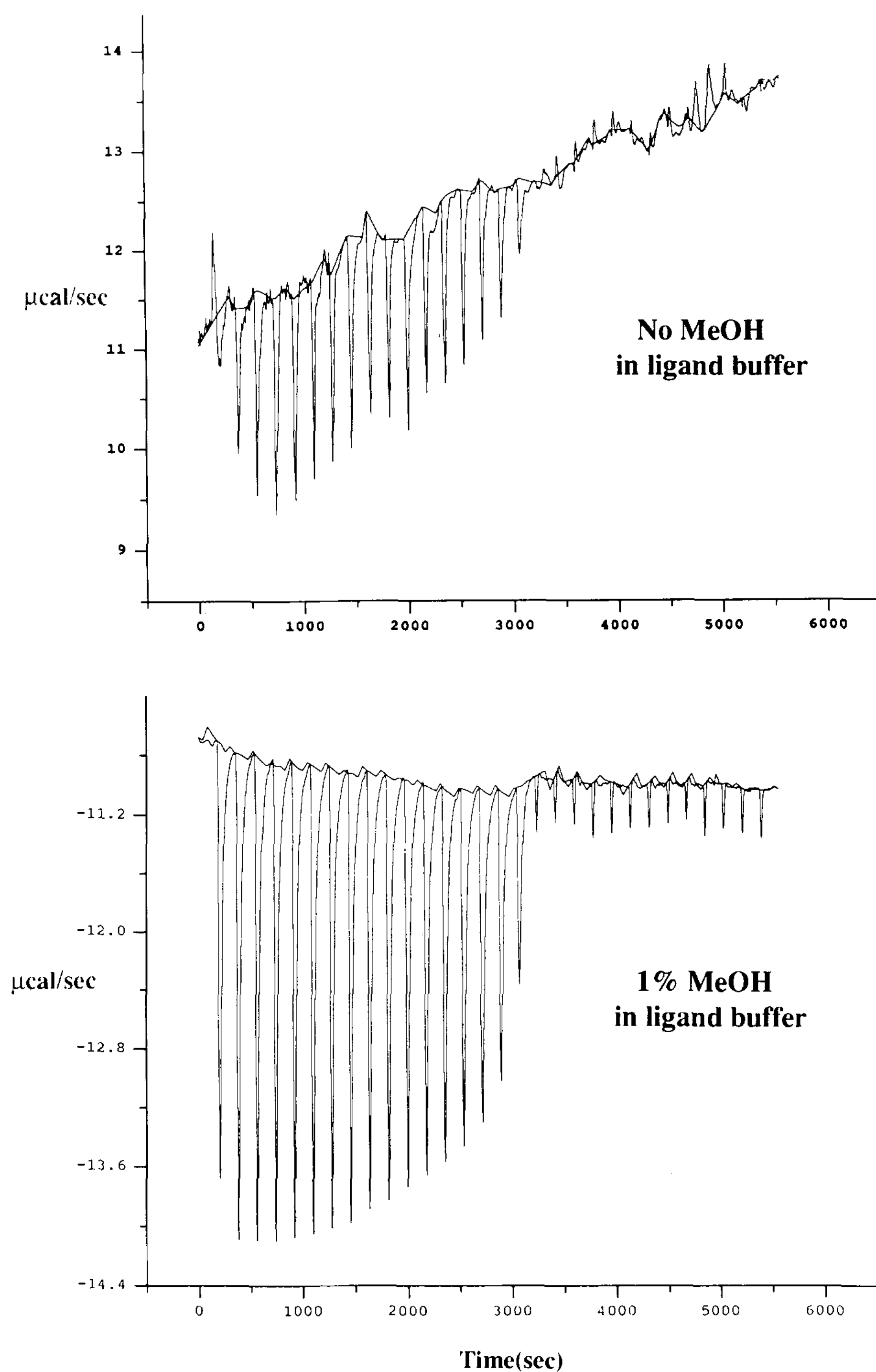

Fig. 2. Titration calorimetry data for the binding of oleate to recombinant rat I-FABP at 37° C. The upper and lower panels represent two essentially identical experiments except for the following. In the upper panel, the ligand buffer was identical to the protein buffer, as described in Materials and methods. In the lower panel, the ligand buffer, unlike the protein buffer, contained 1% methanol in addition to its standard constituents. See Results and discussion for further details.

for Arg-106 correlated well with the loss of a fatty acid-arginine ion-pair electrostatic interaction between ligand and protein, as monitored by NMR [18].

Advantages and limitations of titration calorimetry as applied to lipid-binding proteins

Perhaps the most important feature of this method was

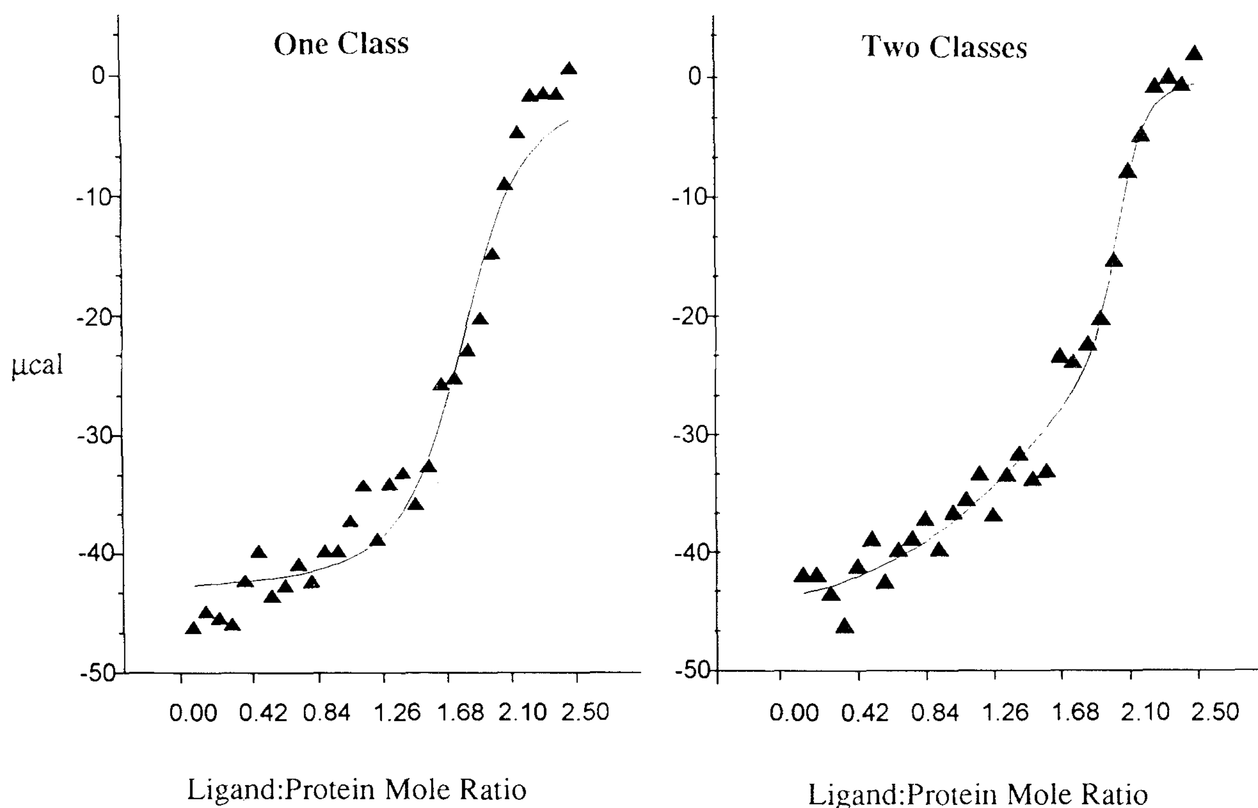

Fig. 3. Titration calorimetry binding curves for oleate binding to recombinant rat L-FABP at 37° C. The data were fitted to a mathematical model that assumed one (left panel) or two (right panel) affinity classes of binding sites. The χ^2 values for the goodness of fit were 0.95 and 0.54, for the one- and two-class models, respectively. The binding parameters for the analysis using the two-class model are presented in Table 1.

its ability to obtain accurate binding constants for a variety of lipid-protein systems in a non-perturbing manner. The binding equilibrium was not perturbed, since there was no need to separate bound from free ligand. Also, the structure of the ligand-protein complex was not perturbed, since there was no need to employ non-native spectroscopic probes. In principle, the calorimetry method can be applied to virtually any ligand-protein combination, since the parameter being measured (ΔH) is a fundamental property of the binding reaction. For the interactions of fatty acids and bile salts with lipid-binding proteins tested so far, the dissociation constants were in a favorable range for this technique: $\sim 10^{-7}$ to 10^{-4} M.

As illustrated above, other advantages of this approach in the study of lipid-binding proteins were the ability to distinguish (i) classes of binding sites that differ in affinity by an order of magnitude or less, (ii) enthalpic and entropic contributions to binding, and (iii) endothermic from exothermic binding interactions. This thermodynamic/energetic view of the binding process complemented the structural view of binding that is obtained by x-ray crystallography, fluorescence and nuclear magnetic resonance.

The greatest limitation of the calorimetry method for studying lipid-protein interactions is the difficulty working with lipid ligands that are highly insoluble, such as cholesterol and saturated long-chain fatty acids. In order to obtain adequate signal-to-noise ratios, the concentration of lipid-binding protein required was approximately 0.05–0.15 mM. The corresponding concentration of ligand in the injection syringe was $\sim$ 1–7 mM. At these concentrations, some lipids such as palmitate exist in crystalline phases in aqueous buffers [26, 27]. Because these insoluble crystals do not equilibrate rapidly on mixing, titration calorimetry cannot be used under these circumstances. However, other lipids exist in 'melted' phases such as bilayers, micelles or monomeric dispersions under these conditions. These lipids include unsaturated fatty acids, bile salts, lysophospho-lipids, and acyl CoA's. Although the lipid may not be in a monomeric state in the syringe, equilibrium between the micellar- or bilayer-phase lipid and the protein is rapid upon mixing. As illustrated above for oleate, which existed in a bilayer

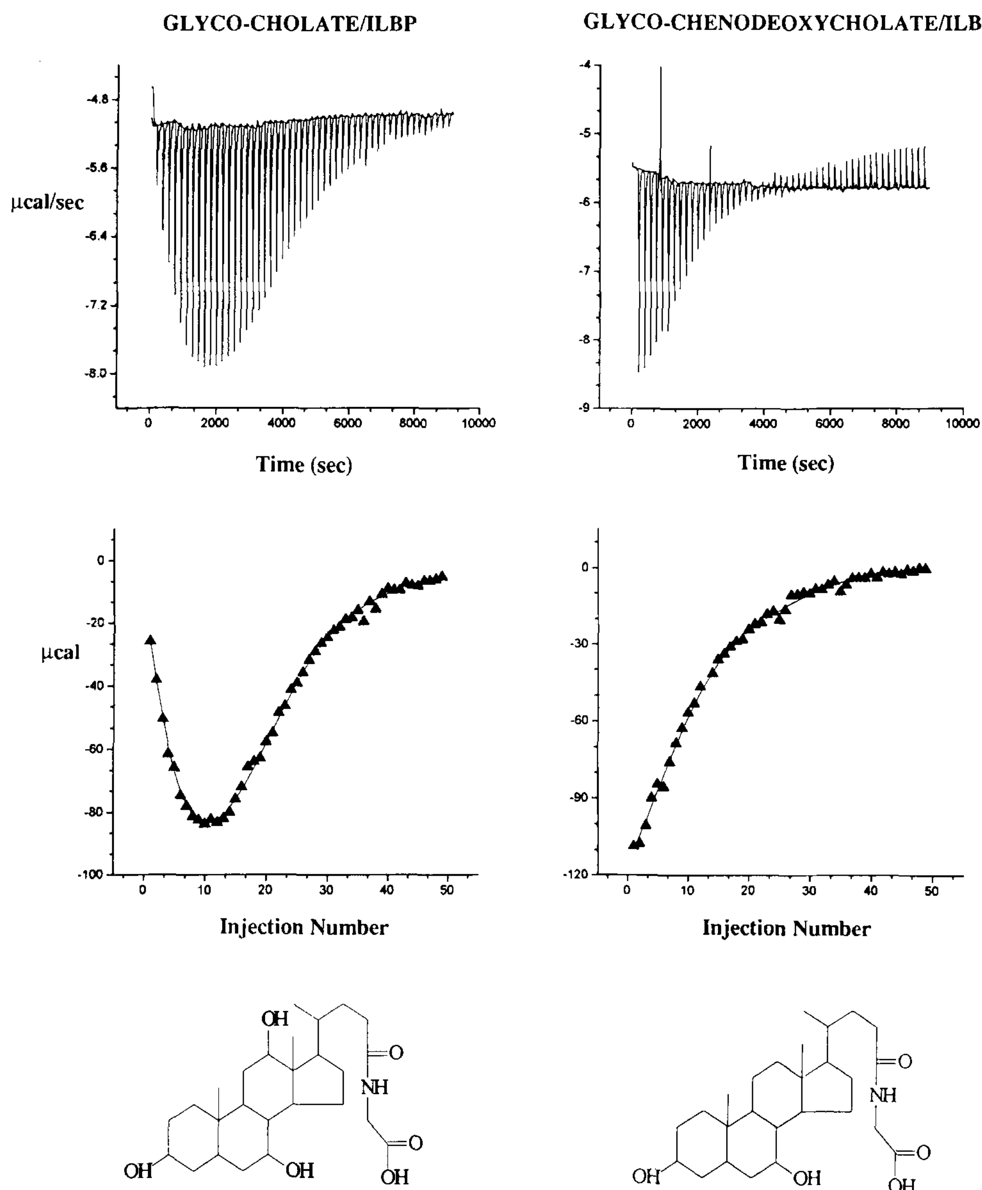

Fig. 4. Titration calorimetry data for the binding of glyco-cholate (left column) or glyco-chenodeoxycholate (right column) to recombinant rat ileal lipid-binding protein at 37° C. The bimodal curves seen for glyco-cholate binding resulted from two classes of affinity sites with opposite signs of ΔH. The high affinity class was endothermic, and the low affinity class, exothermic. In contrast, the results for glyco-chenodeoxycholate indicated exothermic binding for both classes of sites.

phase in the injection syringe [26, 27], the calorimetry method worked very well. Enthalpy changes associated with events other than binding, i.e., phase changes from bilayer to monomer, were accounted for in the heat of 'dilution' that was subtracted from the total heat measured. However, a present challenge for this titration calorimetry method (and other methods as well!) is to devise strategies that circumvent the problems of slow and incomplete equilibration of highly insoluble ligands with binding proteins.

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terological Association. Prof. Cistola is an AGA/Johnson & Johnson/Merck Research Scholar.

Note added in proof

Recent modifications to our calorimetry instrument by the manufacturer have improved the signal-to-noise-ratio, and this improvement had a small, but significant effect on the results for oleate binding to L-FABP. Unlike the earlier data set (Fig. 3), two distinct affinity classes of binding are now unambiguously observed in both the raw data and fitted curves. The revised K_d values for oleate binding to L-FABP (mean ± s.d. for 3 repeats) are 0.31 ± 0.12 μM (site 1) and 4.0 ± 0.44 μM (site 2).

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