Ligand–Protein Electrostatic Interactions Govern the Specificity of Retinol- and Fatty Acid-Binding Proteins†

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ABSTRACT: Cellular retinol-binding protein II (CRBP-II) and intestinal fatty acid-binding protein (I-FABP) are both expressed in small intestinal enterocytes and exhibit 31% sequence identity. I-FABP binds a single molecule of long-chain fatty acid and forms an ion-pair electrostatic interaction between the cationic side chain of arginine-106 and the anionic fatty acid carboxyl group. In contrast, CRBP-II binds *all-trans*-retinol or -retinal and contains a glutamine residue in the corresponding position, residue 109. We have characterized and compared the interactions of fatty acids and retinoids with I-FABP, CRBP-II, and two reciprocal mutant proteins. The mutants were designated CRBP-II(Q109R), where glutamine-109 was replaced by arginine, and I-FABP(R106Q), where arginine-106 was replaced by glutamine. As monitored by titration calorimetry and carbon-13 NMR spectroscopy, the fatty acid-binding properties of CRBP-II(Q109R) were found to be essentially identical to those of wild-type I-FABP. Both proteins bound 1 molecule of fatty acid with identical affinities (K_d = 0.2 μM). The enthalpic contribution to the total free energy of binding was large for both proteins: 66% and 87%, respectively. In addition, the carboxyl groups of fatty acids bound to both proteins were solvent-inaccessible. There was little or no change in the ionization state of the bound fatty acid over a wide pH range, as monitored by the chemical shift of the fatty acid carboxyl ^{13}C resonance. Furthermore, the binding of fatty acid to both proteins was accompanied by a selective perturbation of the guanidino ^{13}C resonance of a single arginine residue. In contrast, the binding of fatty acids to I-FABP(R106Q) was characterized by a 20-fold lower affinity (K_d = 4 μM), a low enthalpic contribution to the free energy of binding (13%), and a solvent-accessible environment for the carboxyl group of the bound fatty acid, as indicated by the complete ionization shift of its carboxyl ^{13}C resonance. Unlike wild-type I-FABP, I-FABP(R106Q) bound one molecule of retinol or retinal with high affinity. In conclusion, the distinct binding properties of I-FABP and CRBP-II result largely from their differing abilities to form an ion-pair interaction between the ligand and one key residue at position 106/109. The ability or inability to form a ligand–protein electrostatic interaction may be a major determinant of the specificites of different members of the intracellular lipid-binding protein family.

Cellular retinol-binding protein II (CRBP-II)[1] is a 134-residue cytosolic protein that binds the vitamin A metabolites *all-trans*-retinol and -retinal (Li et al., 1987). It belongs to a family of abundant intracellular proteins that are thought to facilitate the transport of amphiphilic lipids, including fatty acids, bile salts, and retinoids (Glatz & van der Vusse, 1990; Gordon et al., 1991). There are 10 known members of this protein family, four of which are expressed in the intestinal epithelium: CRBP-II, intestinal fatty acid-binding protein (I-FABP), liver fatty acid-binding protein (L-FABP), and ileal lipid-binding protein (I-LBP).

The structure of I-FABP complexed with palmitic acid has been defined to 2-Å resolution using X-ray crystallography (Sacchettini et al., 1989). The backbone of I-FABP forms a β-clam structure composed of two orthogonal, five-stranded, antiparallel β-sheets and two short α-helices. Fatty acid is bound in the interior cavity of the protein, with the negatively charged carboxylate group of the fatty acid participating in an electrostatic interaction with the positively charged guanidinium moiety of Arg^{106} (Sacchettini et al., 1989; Cistola et al., 1989). CRBP-II, which does not bind fatty acids, has a glutamine residue in the corresponding position, residue 109 (Jones et al., 1988).[2] Another homologous protein, cellular retinoic *acid*-binding protein (CRABP), has an Arg residue at this position.

In a recent attempt to engineer a retinoic acid-binding protein using CRBP-II, glutamine-109 was replaced by arginine using site-directed mutagenesis (Cheng et al., 1991).

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[1] Abbreviations: CRBP-II, *E. coli*-derived rat cellular retinol-binding protein II; CRBP-II(Q109R), a mutant form of CRBP-II in which Gln^{109} has been replaced by Arg; I-FABP, *E. coli*-derived rat intestinal fatty acid-binding protein; I-FABP(R106Q), a mutant form of I-FABP in which Arg^{106} has been replaced by Gln; CRABP, cellular retinoic *acid* binding protein; K_d and K_a, dissociation and association constants for fatty acid binding, respectively; ΔH, enthalpy of binding; ΔG, Gibbs free energy of binding; ΔS, enthalpy of binding; [30%-U-^{13}C]-, protein that has been randomly, fractionally, and uniformly enriched with ^{13}C to a level of 30 atom %.

[2] The recently solved crystal structure of CRBP-II indicates that this protein has the same β-clam backbone topology as I-FABP and that Gln^{109} forms a hydrogen bond with the hydroxyl moiety of bound retinol (N. S. Winter, J. Bratt, and L. B. Banaszak, *J. Mol. Biol.*, in press).

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Analysis of the mutant protein, CRBP-II(Q109R), revealed that it did not bind retinoic acid or retinol but bound long-chain fatty acids. The purpose of the present investigation was 2-fold: (i) to determine whether an ion-pair interaction was formed between Arg^{109} of CRBP-II(Q109R) and bound fatty acid and (ii) to evaluate the contribution of such electrostatic interactions to ligand binding properties, particularly specificities and affinities. To this end, we have compared fatty acid and retinoid interactions with wild-type CRBP-II and I-FABP and with the reciprocal mutants CRBP-II(Q109R) and I-FABP(R106Q). These interactions were probed using titration calorimetry, carbon-13 NMR, and fluorescence spectroscopy.

MATERIALS AND METHODS

Materials. The algae-derived amino acid mixture used for the biosynthesis of uniformly ^{13}C-enriched CRBP-II(Q109R), 30 atom% ^{13}C, was purchased from MSD Isotopes. The ^{13}C-enriched algal hydrolysate used for the biosynthesis of uniformly ^{13}C-enriched I-FABP was purchased from Martek, Inc. Palmitic and oleic acids [99%, 1-^{13}C] were purchased from Cambridge Isotope Laboratories, and *all-trans*-retinol, retinal, and retinoic acid were from Sigma Chemical Co.

Protein Biosynthesis and Purification. Unenriched rat I-FABP and I-FABP(R106Q) were biosynthesized in *Escherichia coli* and purified as follows. Bacteria harboring the pMON–I-FABP or –I-FABP(R106Q) plasmid were grown at pH 7.2 in a New Brunswick Bioflo III high-density fermenter using a nutrient-rich medium containing tryptone (10.8 g/L), yeast extract (22.5 g/L), and potassium phosphate (0.1 M), $MgSO_4$ (1-5 mM), and $CaCl_2$ (0.1–0.5 mM), as well as trace amounts of $FeSO_4$ and thiamine. Cells were grown to a final density, as monitored by OD at 600 nm, of 55 and 8 for I-FABP and I-FABP(R106Q), respectively. 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 were purified using a protocol modified from that of Lowe et al. (1987). In short, it involved (i) ammonium sulfate fractionation, saving the 60% 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 (1983). The yields of purified protein from individual 4-L fermentations were approximately 5000 mg and 300 mg, respectively, for I-FABP and I-FABP(R106Q).

Unenriched rat CRBP-II and CRBP-II(Q109R) were biosynthesized and purified from *E. coli* as described by Li et al. (1987) and Cheng et al. (1991).

Uniformly carbon-13 enriched [30%-U-^{13}C]CRBP-II(Q109R) and –I-FABP were biosynthesized in *E. coli* using a two-stage, growth-medium-shift strategy. In the first stage, *E. coli* harboring the appropriate plasmid were grown in a non-isotope-containing tryptone–yeast extract medium at 37 °C using the high density fermenter. During log-phase growth, at a cell density of 13–17 OD units, the bacteria were harvested by centrifugation, resuspended in the ^{13}C-enriched algal hydrolysate or amino acid mixture with a supplemented M-9 medium (Li et al., 1987), and returned to the fermenter. The concentrations of ^{13}C-enriched carbon source in the second-stage media were 0.5% and 1.2% (w/v), respectively, for CRBP-II(Q109R) and I-FABP. During the second growth period, plasmid expression was induced with nalidixic acid, resulting in the biosynthesis of ^{13}C-enriched protein. The proteins were purified as above. The yields of purified protein from individual 1-L fermentations were approximately 100 mg and 200 mg for [30%-U-^{13}C]CRBP-II(Q109R) and –I-FABP, respectively.

Protein concentrations were determined as described elsewhere (Cistola et al., 1989).

Preparation of NMR Samples. Samples containing fatty acid–protein complexes were prepared as in Cistola et al. (1989). Samples containing unenriched *all-trans*-retinol were prepared, in the dark, by adding 4.3 μL of a stock solution of 8.7 mg/mL retinol in 100% ethanol to NMR tubes containing fatty acid–protein complexes.

Circular Dichroism. Circular dichroic spectra were collected from 250 to 190 nm for filtered samples at ambient temperature (23 ± 2 °C) using a Jasco J600 spectropolarimeter. The protein concentrations were 0.1–0.2 mg/mL, and cell path lengths of 0.1 and 0.01 cm were used.

Titration Calorimetry. Titration calorimetry was performed using a Microcal OMEGA differential titrating calorimeter (Wiseman et al., 1989). Twenty to thirty 7–10-μL aliquots of the ligand solution at 1–5 mM were injected into the protein sample (1.5 mL at 0.07–0.15 mM) to a final sample ligand:protein mole ratio >1. 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. The binding parameters obtained in the presence and absence of methanol were essentially identical, except that the standard deviations were smaller when methanol was present. All titrations were executed at 25 °C and pH 7.4. The data were processed using the software package ORIGIN. A function that modeled one class of independent binding sites was used to fit the data (Wiseman et al., 1989). The association and dissociation constants (K_a, K_d), molar binding stoichiometry (n), and enthalpy (ΔH) were determined from the fitted curve. The Gibbs free energy and entropy of binding were calculated using the equations $\Delta G = -RT \ln K_a$ and $\Delta S = (\Delta H - \Delta G)/T$, respectively, where R is the gas constant and T is absolute temperature in Kelvin.

Carbon-13 NMR Spectroscopy. Proton-decoupled ^{13}C NMR spectra were recorded on a Varian Unity 500 three-channel NMR spectrometer (125.697 MHz for ^{13}C) equipped with a Sun SPARC-2 workstation and a Nalorac 10-mm special-select probe. For most spectra, 90° ^{13}C pulses (11.5 μs at 200 W) were used, and pulse intervals were ~1.3 times the spin-lattice relaxation times for the fatty acid carboxyl or arginine guanidino carbons. Spin-lattice relaxation times were determined using the inversion–recovery method, and {^1H}–^{13}C nuclear Overhauser enhancement values were measured by comparing the peak heights from spectra accumulated with inverse-gated decoupling and continuous waltz-16 decoupling. The ^1H decoupling field was centered at 4.7 ppm and its strength, $\gamma B_2/2\pi$, was 1258 Hz at 0.6 W. Chemical shifts were referenced using the ε-carbon resonance of Lys residues at 39.52 ppm, after calibration of this against external tetramethylsilane in chloroform. No susceptibility corrections were made. The estimated uncertainties in chemical shifts were ±0.1 ppm.

Fluorescence Spectroscopy. Steady-state fluorescence measurements were made using instrumentation and methods as detailed elsewhere (Cheng et al., 1991). I-FABP and I-FABP(R106Q) were titrated fluorometrically with retinol, retinal, and retinoic acid. The protein was excited at 290 nm,

Table I: Titration Calorimetry Results for Oleate Binding to Mutant and Wild-Type Lipid-Binding Proteins at 25 °C[a]

Parameter	CRBP-II(Q109R)	I-FABP	I-FABP(R106Q)
n	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
K_d (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
$T\Delta S$ (kJ/mol)	13 ● 2	4 ± 4	26 ± 2

[a] Each result represents the mean ± standard deviation of four experiments, except for I-FABP(R106Q), which represents three experiments. Wild-type CRBP-II exhibited no detectable interaction with oleate (see Figure 1 and Results).

and (tryptophan) fluorescence was monitored at 330 nm. The protein concentrations were 6.4 and 6.3 μM for I-FABP and I-FABP(R106Q), respectively. Single measurements were made, and the estimated uncertainties in relative fluorescence intensities were ±0.02. A solution of *N*-acetyl-L-tryptophanamide having an absorbance at 290 nm equal to that of the apoprotein solutions was used as a blank, in order to correct for nonspecific quenching and inner-filter effects (Cogan et al., 1976). Upper-limit estimates of the dissociation constants for retinoid binding were obtained using linear least-squares plots of the Cogan equation, as detailed elsewhere (Cogan et al., 1976; Li et al., 1987; Cheng et al., 1991).

RESULTS

Circular Dichroism Experiments To Assess Solution Conformation. Spectra were acquired and compared for CRBP-II and CRBP-II(Q109R), and for I-FABP and I-FABP(R106Q), in order to determine whether the single-site mutations had any observable effect on the conformation of these proteins in solution. The circular dichroic spectra of the two CRBP-II variants were indistinguishable, as were the spectra of the I-FABP variants (data not shown). Both sets of spectra contained the features characteristic of a predominately β-sheet protein: a single minimum between 200 and 250 centered at ~217 nm and a single maximum centered at ~198 nm.

Titration Calorimetry Experiments To Determine Fatty Acid-Binding Parameters. Titration calorimetry was employed to quantitate the stoichiometries, affinities, enthalpies, entropies, and free energies for oleate binding to CRBP-II(Q109R), wild-type I-FABP, and I-FABP(R106Q). As shown in Table I, the binding parameters for CRBP-II(Q109R) and I-FABP were very similar. Both proteins exhibited a molar binding stoichiometry of 1 and a dissociation constant of 0.2 μM. Also, the enthalpies of binding were large: −25 kJ/mol and −34 kJ/mol; these ΔH values constituted 66% and 87% of the total free energy of binding for CRBP-II(Q109R) and I-FABP, respectively.

For oleate binding to I-FABP(R106Q), the molar stoichiometry was also 1. However, the binding affinity was 20-fold lower than that observed for wild-type I-FABP and CRBP-II(Q109R). Also, unlike I-FABP and CRBP-II(Q109R), the enthalpy of binding was very small (−4 kJ/mol) and constituted only 13% of the total free energy of binding.

^{13}C NMR Experiments To Monitor Fatty Acid Binding and Ionization. Wild-type CRBP-II and CRBP-II(Q109R) were each titrated with carboxyl ^{13}C-enriched fatty acid and ^{13}C NMR spectra were collected at different mole ratio increments. The carboxyl/carbonyl region of these spectra, all accumulated at pH 7.5 and 25 °C, are shown in Figure 1. Addition of ^{13}C-enriched oleate to the sample containing CRBP-II(Q109R) resulted in the appearance of two resonances corresponding to oleate carboxyl carbons (peaks b and u in Figure 1A–E). Peak b was assigned to oleate bound to CRBP-II(Q109R), on the basis of observation that the chemical shift and line width values of peak b (181.6 ppm, 8 Hz) were very similar to those observed for oleate bound to other members of the cytoplasmic lipid-binding protein family, particularly I-FABP (181.4 ppm, 6–8 Hz; Cistola et al., 1989). In contrast, the chemical shift and line width values of peak u (179.8 ppm, ≫50 Hz) corresponded to unbound oleate in a bilayer phase (Cistola et al., 1988a,b).

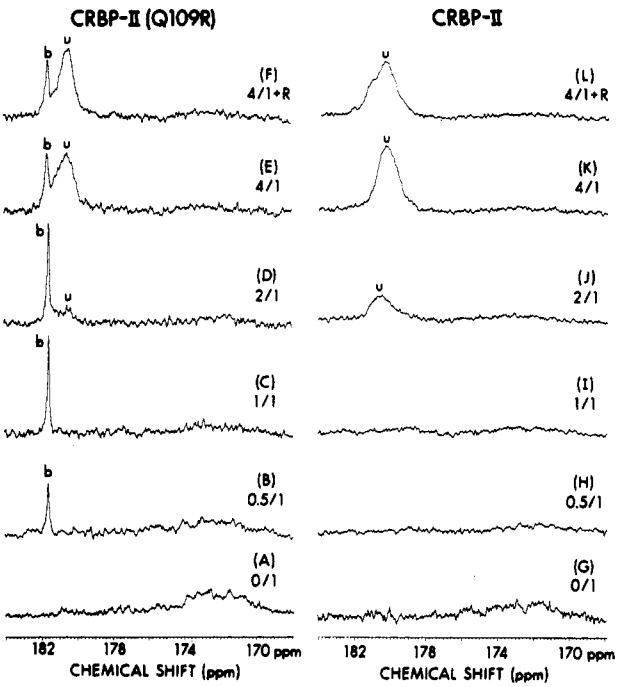

FIGURE 1: Carboxyl/carbonyl region of ^{1}H-decoupled ^{13}C NMR spectra at 125.697 MHz for samples containing [1-^{13}C]oleate and *E. coli*-derived CRBP-II(Q109R) (left column) or wild-type CRBP-II (right column). The fatty acid:protein mole ratio of the sample is indicated above the rightmost portion of each spectrum. In panels F and L, 1 mol of unenriched *all-trans*-retinol (per mole of protein) was added to the samples corresponding to panels E and K, respectively. The spectra were processed with Lorentzian apodization for sensitivity enhancement, using a line-broadening factor of 10 Hz, except for spectrum A, which used 7 Hz. Number of spectral accumulations: 29 120 (A); 4400 (B–F); 17 598 (G); 3600 (H–L). Other sample and spectral conditions: protein concentration, 0.92 mM; temperature, 25 °C; pH, 7.5; pulse interval, 2.0 s; spectral width, 27 000 Hz; time domain points, 32 768, zero-filled once to 65536. Abbreviations: b, carboxyl resonance representing oleate bound to protein; u, carboxyl resonance representing unbound oleate in a bilayer phase; R, *all-trans*-retinol.

For CRBP-II(Q109R), addition of oleate up to a sample mole ratio of 1:1 resulted in an increase in intensity of peak b. Above 1:1, peak u increased in intensity with added oleate. In contrast, for wild-type CRBP-II, peak b was not observed at all (Figure 1G–K). Addition of oleate to this sample resulted only in the appearance and increase of unbound oleate, as monitored by peak u. These NMR data indicated that CRBP-II(Q109R), but not wild-type CRBP-II, bound oleate.

To determine if a natural ligand for CRBP-II, *all-trans*-retinol, would compete with fatty acid for binding to CRBP-II(Q109R), 1 mol of non-isotope-enriched retinol/mol protein was added to the sample depicted in Figure 1E. As shown in Figure 1F, the addition of a stoichiometric amount of retinol had no significant effect on the binding of oleate to CRBP-II(Q109R).

A set of experiments parallel to those in Figure 1 was performed using ^{13}C-enriched palmitate rather than oleate in

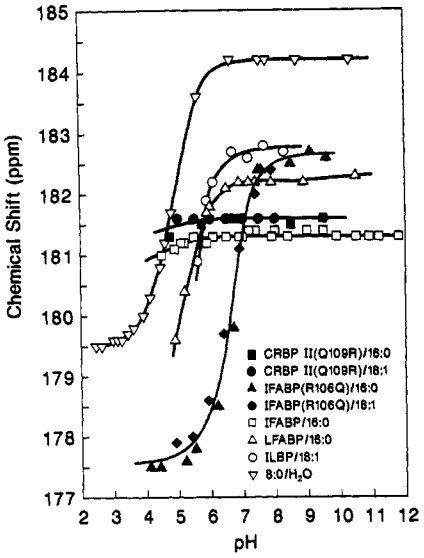

FIGURE 2: ^{13}C NMR ionization curves for [1-^{13}C]oleate and -palmitate bound to *E. coli*-derived CRBP-II(Q109R), I-FABP-(R106Q), and I-FABP at 25 °C. Also shown are curves for fatty acids bound to liver fatty acid-binding protein and ileal-lipid binding protein, as well as monomeric octanoate in water in the absence of protein, taken from Cistola et al. (1989), Sacchettini et al. (1990), and Cistola et al. (1987), respectively. The concentrations of CRBP-II(Q109R) and I-FABP(R106Q) were 0.76 mM and 0.35 mM, while the mole ratio of fatty acid:protein in the sample was 4:1. Number of spectral accumulations: 500 for CRBP-II(Q109R), 2000 for I-FABP(R106Q). All other spectral conditions were as described in Figure 1.

order to evaluate whether the binding properties of CRBP-II(Q109R) and wild-type CRBP-II differed for a saturated vs an unsaturated fatty acid. As in Figure 1, the spectra for CRBP-II(Q109R) with added palmitate exhibited a fatty acid carboxyl resonance with the same chemical shift and line width as that of peak b (data not shown). Peak u was not observed, however, since unbound palmitate froms a crystalline 1:1 acid–soap phase under these conditions (Cistola et al., 1986, 1989). The corresponding spectra for wild-type CRBP-II exhibited no fatty acid carboxyl resonances.

To monitor the ionization behavior of fatty acids bound to CRBP-II(Q109R), samples containing oleate or palmitate complexed with CRBP-II(Q109R) were titrated with 1 N HCl or KOH, and ^{13}C NMR spectra were obtained at ~0.5 pH unit increments. In Figure 2, the chemical shift values of peak b as a function of pH are depicted by the filled circles (oleate) and filled squares (palmitate). This ionization curve indicated that only a slight change in the ionization state of fatty acid bound to CRBP-II(Q109R) occurred over a wide pH range. This relatively featureless ionization curve was very similar to that observed for fatty acids bound to wild-type I-FABP (Figure 2, open squares; Cistola et al., 1989).

The NMR ionization curve for I-FABP(R106Q) was distinctly different from those obtained for wild-type I-FABP and CRBP-II(Q109R). As shown in Figure 2 (filled triangles and diamonds), the chemical shift values for fatty acids bound to I-FABP(R106Q) decreased with decreasing pH in a manner consistent with protonation of the fatty acid carboxyl group. As with I-FABP and CRBP-II(Q109R), the fatty acid remained bound to the protein over the entire pH range examined. The change in chemical shift with ionization for fatty acids bound to I-FABP(R106Q) was similar to that observed for monomeric fatty acid in water in the absence of protein (Figure 2, open inverted triangles) and for fatty acids bound to the homologous proteins liver fatty acid-binding protein and ileal lipid-binding protein (Cistola et al., 1988b, 1989; Sacchettini et al., 1990). However, the apparent pK_a for fatty acids bound to I-FABP(R106Q) was ~6.5, compared to ~4.5–5 for liver fatty acid-binding protein and 4.8 for monomeric octanoate in water in the absence of protein (Cistola et al., 1987). The elevated pK_a value relative to a carboxylic acid in aqueous buffer suggested that the bound fatty acid was in an environment with a dielectric constant lower than that of bulk water. A low dielectric environment is expected for fatty acid bound in the same general location as found for wild-type I-FABP (Sacchettini et al., 1989).

Taken together, these ionization results suggested that fatty acids bound to CRBP-II(Q109R) and I-FABP, but not I-FABP(R106Q), were in a solvent-inaccessible environment, perhaps due to an ion-pair interaction between the bound fatty acid and an Arg residue.

^{13}C NMR Experiments To Monitor Fatty Acid–Arginine Interactions. To further assess the possibility of a specific fatty acid–arginine interaction for CRBP-II(Q109R), carbon-13 NMR spectra of [30%-U-^{13}C]CRBP-II(Q109R) and –I-FABP were analyzed and compared. These uniformly ^{13}C-enriched proteins, which were originally biosynthesized for the purpose of establishing sequence-specific resonance assignments,[3] provided a direct means for monitoring arginine side-chain guanidino carbon resonances and distinguishing them from other types of resonances. Guanidino carbons resonate in an uncrowded region of the carbon spectrum, between 154 and 160 ppm; only tyrosine hydroxyl carbons also resonate in this region (Allerhand, 1979). Because of the uniform and fractional enrichment with ^{13}C, the tyrosine hydroxyl resonances appeared as pseudotriplets, i.e., doublets superimposed on singlets. The doublets arose from one-bond carbon–carbon coupling in molecules where there were ^{13}C nuclei at the hydroxyl position and at least one of the two adjacent ring carbons. The singlets arose from molecules which had ^{13}C enrichment at the hydroxyl position, but not at either of the two adjacent ring carbons. This pattern is best illustrated in Figure 3C, where the four Tyr residues of I-FABP could be distiguished as four partially overlapping pseudotriplets centered between 153.9 and 155.9 ppm. In constrast, the arginine guanidino carbons, which were surrounded by nitrogens, did not exhibit one-bond carbon–carbon coupling and appeared as singlets between 156 and 158 ppm.[3]

The spectra for [30%-U-^{13}C]I-FABP, in apo and holo form, are shown in Figure 3A–D. I-FABP has six arginine residues. For the apoprotein, the arginine guanidino carbon resonances were closely overlapped and were arbitrarily labeled R_1–R_5 and R_6 (Figure 3A). Processing of this spectrum with heavier resolution enhancement revealed that one component, R_6 was partially resolved (Figure 3B). Addition of 1 mol of palmitate/mol of protein resulted in a 1 ppm increase in the chemical shift of peak R_6 to 157.9 ppm (Figure 1C,D). In contrast, R_1–R_5 were not affected by the addition of fatty acid.

The relative intensity of peak R_6 was 14% of the total arginine guanidino carbon intensity as measured by integration of the proton-decoupled spectrum in Figure 3C. The spin-lattice relaxation time constant for peak R_6, 1.8 s, was identical to that for R_1–R_5. The {^1H}–^{13}C nuclear Overhauser enhancement value for peak R_1–R_5 was 1.2, slightly higher than that of peak R_6, 1.0. Taking this small difference into account, the corrected intensity of peak R_6 was 16.8%, or one-sixth of

[3] For holo-I-FABP, the distinction between Arg guanidino and Tyr hydroxyl carbon resonances has also been established by assigning complete carbon spin systems using 2-D carbon double-quantum NMR (M. E. Hodsdon, J. J. Toner, and D. P. Cistola, manuscript in preparation).

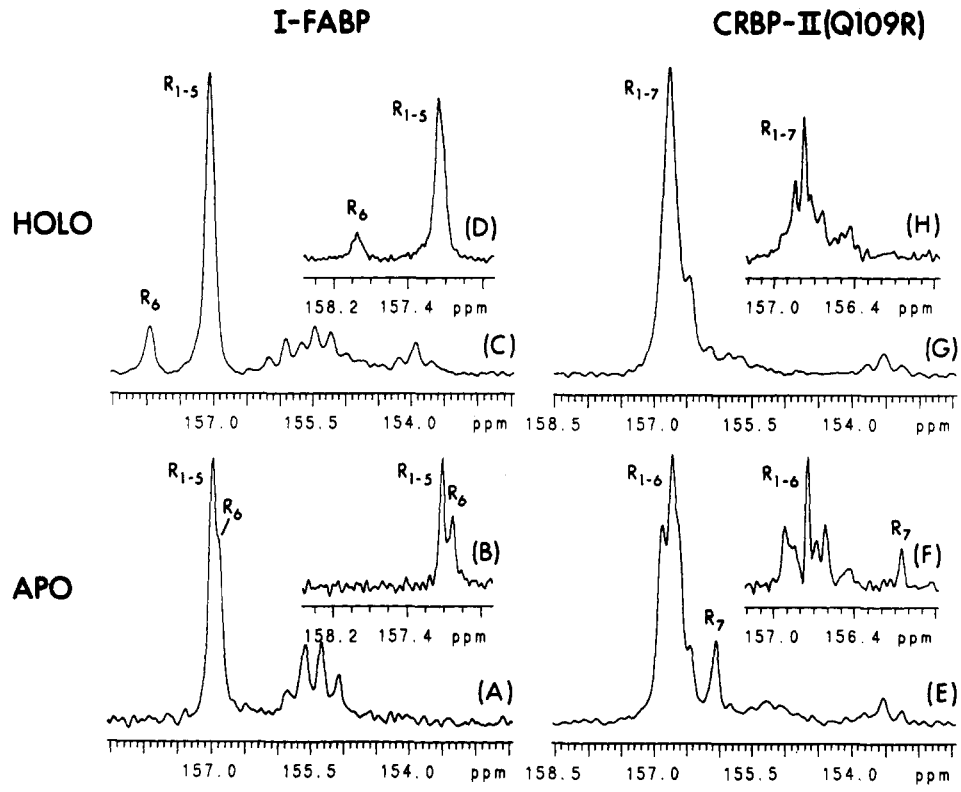

FIGURE 3: Arg guanidino and Tyr hydroxyl region of ^{1}H-decoupled ^{13}C NMR spectra for uniformly ^{13}C-enriched I-FABP and CRBP-II-(Q109R), with the without bound palmitate, all at 25 °C. (A and B) apo-I-FABP; (C and D) holo-I-FABP; (E and F) apo-CRBP-II(Q109R); (G and H) holo-CRBP-II(Q109R). All spectra were processed with Lorentzian–Gaussian apodization for resolution enhancement. A line-broadening factor of –5 Hz was used for spectra A, C, E, and G, and –8 Hz for spectra B, D, F, and H. A Gaussian time constant of 0.05 s was used for spectra A, C, E, and G, and 0.08 was used for spectra for B, D, F, and H. Other conditions for spectra A and B: pulse interval, 2.5 s; spectral width, 25 400 Hz; time domain points, 29 696; protein concentration, 0.5 mM, number of transients, 15 386, pH 7.4. For spectra C and D: interval, 3.0 s; width, 25 100 Hz; points, 26 368; concentration, 4 mM; transients, 512; pH 7.4. For spectra E and F: interval, 2.5 s; width, 27 000 Hz; points, 32 768; transients, 19 648; concentration, 0.9 mM; pH, 7.2. For spectra G and H: interval, 2.5 s; width, 27 000 Hz; points, 32 768; concentration, 0.9 mM; transients, 33 984; pH 7.2.

the total guanidino intensity. A similar percentage was obtained by integrating a fully-relaxed spectrum (pulse interval, 26 s) obtained with inverse-gated proton-decoupling. Therefore, R_6 represented one Arg residue of I-FABP, and one of the six Arg guanidino resonances was selectively perturbed by the binding of fatty acid. On the basis of these results and the crystal structure of I-FABP complexed with palmitate, we assigned peak R_6 to the guanidino carbon of Arg^{106}.

A similar, selective increase of 1 ppm was observed for a resonance corresponding to a single arginine residue of CRBP-II(Q109R), as shown in Figure 3E–H. This protein has 7 arginine residues. In the apoprotein, the resonance labeled R_7 was well resolved from the other six partially resolved arginine guanidino resonances (R_1–R_6, Figure 3E,F). Upon binding of palmitate, the chemical shift of peak R_7 increased by 1 ppm to overlap with peaks R_1–R_6 (Figure 3G,H). The chemical shifts of peaks R_1–R_6 did not change with added fatty acid. On the basis of the analogy with I-FABP, peak R_7 was provisionally assigned to arginine-109 of CRBP-II-(Q109R).

Fluorescence Experiments To Assess Retinoid Binding. Because of the very low solubility of retinoids in aqueous buffer, titration calorimetry could not be used to determine retinoid binding parameters for these proteins. Therefore, a fluorescence spectroscopy method was employed, where the quenching of protein tryptophan fluorescence upon ligand binding was monitored. As shown in Figure 4, distinctly different results were obtained for I-FABP and I-FABP(R106Q). For the latter, a linear decrease in fluorescence intensity was observed upon addition of retinol, retinal, or retinoic acid until saturation

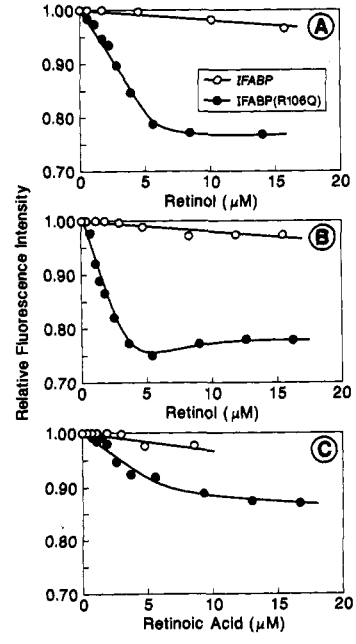

FIGURE 4: Fluorometric titration of I-FABP and I-FABP(R106Q) with retinoids. The protein concentrations were 6.4 and 6.3 μM for I-FABP and I-FABP(R106Q), respectively. The protein solutions were titrated with *all-trans*-retinol (A), *all-trans*-retinal (B), or *all-trans*-retinoic acid (C). Fluorescence emission was measured at 330 nm after excitation at 290 nm. See Materials and Methods section for further details.

was reached (Figure 4, filled symbols). The degree of quenching, which is related to the relative location and

orientation of the ligand and tryptophan residues, was similar to that observed for retinoid binding to plasma retinol-binding protein but considerably less than that for CRBP-II (Cogan et al., 1976; Li et al., 1987). These fluorescence results indicated that retinol, retinal, and retinoic acid bound to I-FABP(R106Q) in a saturable manner with a 1:1 ligand: protein mole ratio. Although the binding affinities could not be accurately quantitated, the dissociation constants for retinol and retinal binding to I-FABP(R106Q) were estimated at $\leq 10^{-6}$ M. This estimate is not dissimilar to that reported for retinol binding to wild-type CRBP-II (10^{-7}–10^{-8}; Li et al., 1987).

Unlike I-FABP(R106Q), wild-type I-FABP exhibited no significant interaction with retinoids (Figure 4, open symbols). Similarly, CRBP-II(Q109R) exhibited a markedly decreased binding of retinoids relative to wild-type CRBP-II (Cheng et al., 1991).

DISCUSSION

The molecular and thermodynamic features of ligand–protein interactions for several mutant and wild-type lipid-binding proteins have been compared using titration calorimetry, carbon-13 NMR, and fluorescence spectroscopy. These comparisons have yielded information regarding the presence or absence of ligand–protein electrostatic interactions and their contribution to binding specificities and affinities.

The mutant protein CRBP-II(Q109R), unlike the wild type, was found to bind long-chain fatty acids. Its binding stoichiometry and affinity were indistinguishable from those for the wild-type fatty acid-binding protein, I-FABP. In addition, the binding of fatty acids to CRBP-II(Q109R), as with I-FABP, was characterized by an ion-pair electrostatic interaction involving the cationic side chain of a single arginine residue and the anionic carboxyl moiety of the fatty acid.

For I-FABP, this fatty acid–arginine interaction has been well established by evidence from X-ray crystallography and NMR (Sacchettini et al., 1989; Cistola et al., 1989). Three lines of evidence from the present study point to the existence of a similar fatty acid–arginine interaction for CRBP-II-(Q109R). First, the NMR ionization curve for fatty acid bound to CRBP-II(Q109R) was nearly identical to that for wild-type I-FABP; both showed that the fatty acid was negatively charged and exhibited only a slight change in ionization state over a wide pH range. These results indicated a solvent-inaccessible environment for the bound fatty acid carboxyl group. Second, the binding of fatty acids selectively perturbed the local environment of a single arginine residue for both CRBP-II(Q109R) and I-FABP, as monitored by the ^{13}C resonance of the arginine guanidino carbons. Finally, the free energies of binding for both CRBP-II(Q109R) and I-FABP were dominated by enthalpic contributions, unlike those observed for related proteins that lack the corresponding arginine (see below).

The reciprocal mutant protein, I-FABP(R106Q), also bound long-chain fatty acids with a stoichiometry of 1. However, the binding affinity was 20-fold lower than those for CRBP-II(Q109R) and wild-type I-FABP. In addition, the binding of fatty acids to I-FABP(R106Q) was characterized by the lack of a detectable fatty acid–protein electrostatic interaction. As illustrated in Figure 2, the NMR ionization curve for fatty acids bound to I-FABP(R106Q) was clearly distinct from that of wild-type I-FABP and CRBP-II(Q109R). The former exhibited a complete ionization shift which indicated that the bound fatty acid carboxyl moiety was solvent-accessible. In addition, the free energy of binding for I-FABP(R106Q) contained a much lower enthalpic contribution than did that for CRBP-II(Q109R) and wild-type I-FABP. This low enthalpic contribution was similar to that observed for fatty acids bound to two other wild-type lipid-binding proteins that lack an arginine in this position: ileal lipid-binding protein and liver fatty acid-binding protein (K. R. Miller and D. P. Cistola, manuscript in preparation). Finally, retinoids bound with high affinity to I-FABP(R106Q), but not to wild-type I-FABP.

These results provided evidence that the presence or absence of a single Arg residue, at a position corresponding to residue 106 in I-FABP or 109 in CRBP-II, is an important determinant of ligand-binding specificity and affinity for these retinol- and fatty acid-binding proteins. Its influence appears to be mediated by an ion-pair electrostatic interaction with the anionic carboxyl moiety of the bound fatty acid. This interaction is necessary for the binding of fatty acids to CRBP-II. It is not necessary for fatty acid binding to I-FABP, although its absence results in a 20-fold reduction in affinity.

There are several possible explanations to account for the differences in ligand-binding properties exhibited by different members of the lipid-binding protein family. One explanation is that some members may have different backbone conformations and, therefore, different binding site conformations. A second explanation is that these proteins have similar backbone conformations, but differences in ligand binding are conferred by differences in the amino acid side chains of residues lining the binding cavity. The three-dimensional structures of five homologues reveal Cα tracings are nearly superimposable (Jones et al., 1988; Sacchettini et al., 1989; Müller-Fahrnow et al., 1991; Xu et al., 1992).[2] Therefore, the second explanation seems more likely. The results from the present study extend this explanation further and indicate that just *one* key residue in the binding cavity can govern ligand specificity. With respect to binding properties, the single-site mutant CRBP-II(Q109R) was found to be nearly identical to wild-type I-FABP. Likewise, the reciprocal mutant I-FABP(R106Q) was similar to wild-type CRBP-II. This is in spite of the fact that the two wild-type proteins bind different ligands and share only 31% sequence identity.

Sequence alignments of all members of the cytoplasmic lipid-binding protein family indicate that five contain this important Arg residue (Jones et al., 1988; Böhmer et al., 1987; Gantz et al., 1989). One of these five members, CRABP, binds retinoic acid. Two others bind fatty acids, I-FABP and heart fatty acid-binding protein. The myelin P2 protein and adipocyte lipid-binding protein bind either a fatty acid or retinoic acid. The four members that do not contain this Arg residue are liver fatty acid-binding protein, ileal lipid-binding protein, CRBP-II, and cellular retinol-binding protein (CRBP-I). They contain Thr, Ser, Gln, and Gln, residues, respectively, at the corresponding position.

There is growing evidence to suggest that the latter four proteins function in the binding and transport of ligands other than fatty acids or retinoic acid. Ileal lipid-binding protein binds bile salts and appears to function in their enterohepatic circulation (Sacchettini et al., 1990; Vodenlich et al., 1991). It also binds fatty acids, but with a 100-fold lower affinity than I-FABP (K. R. Miller and D. P. Cistola, manuscript in preparation). Similarly, liver fatty acid-binding protein binds a variety of other ligand such as lysophospholipids, bile salts, heme, and, possibly, cholesterol (Bass, 1985; Scallen et al., 1985; Burrier & Brecher, 1986; Nemecz & Schroeder, 1991). CRBP-I and -II exhibit no detectable binding of fatty acids but bind retinol and retinal. Therefore, the residue at position

106/109 may influence the ligand preferences and individual functions of other members of the lipid-binding protein family besides CRBP-II and I-FABP.

X-ray crystal structures of the myelin P2 protein and heart fatty acid-binding protein indicate that a different buried arginine residue at position 126 is involved in ligand binding (Jones et al., 1988; Zanotti et al., 1992). In these proteins, both Arg^{126} and Arg^{106} participate in a hydrogen-bonding and electrostatic network with the bound fatty acid. The Arg at position 126/129 is conserved in all homologues except CRBP-I and CRBP-II, which contain Gln. However, substitution of Arg for Gln at position 129 in CRBP-II yielded a protein that did not bind fatty acids, perhaps due to a general perturbation in the interior of the β-clam structure (Cheng et al., 1991). Also, Arg^{126} in I-FABP forms an ion-pair interaction with a protein side-chain, Asp^{34}, rather than the ligand. It is possible that CRBP-II and I-FABP have evolved as part of a distinct subgroup within this protein family. In this subgroup, the type of residue at position 106, but not 126, governs ligand-binding properties.

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