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Articles

Fatty Acid Binding Sites of Rodent Adipocyte and Heart Fatty Acid Binding Proteins: Characterization Using Fluorescent Fatty Acids†

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ABSTRACT: Murine adipocyte and rat heart fatty acid binding proteins (FABP) are closely related members of a family of cytosolic proteins which bind long-chain free fatty acids (ffa). The physical and chemical characteristics of the fatty acid binding sites of these proteins were studied using a series of fluorescent analogues of stearic acid (18:0) with an anthracene moiety covalently attached at seven different positions along the length of the hydrocarbon chain (AOffa). Previously, we used these probes to investigate the binding site of rat liver FABP (L-FABP) [Storch et al. (1989) *J. Biol. Chem. 264*, 8708–8713]. Here we extend those studies to adipocyte and heart FABP, two members of the FABP family which share a high degree of sequence homology with each other (62% identity) but which are less homologous with L-FABP (approximately 30%). The results show that the fluorescence emission spectra of AOffa bound to adipocyte FABP (A-FABP) are blue-shifted relative to heart FABP (H-FABP), indicating that AOffa bound to A-FABP are held in a more constrained configuration. For both proteins, constraint on the bound ffa probe is highest at the midportion of the acyl chain. Ffa are bound in a hydrophobic environment in both proteins. Excited-state lifetimes and fluorescence quantum yields suggest that the binding site of H-FABP is more hydrophobic than that of A-FABP. Nevertheless, acrylamide quenching experiments indicate that ffa bound to H-FABP are more accessible to the aqueous environment than are A-FABP-bound ffa. The apparent difference between results obtained from spectral properties and those from aqueous phase quenching may reflect differential polarity of adjacent residues in the A- and H-FABP binding sites. All spectral properties varied along the length of the hydrocarbon chain, particularly at the midportion, indicating a structured binding site in both proteins. Although adipocyte and heart FABP appear to bind ffa in a similar manner, the fine structure of their binding sites differs. The differences between A-FABP and H-FABP are less striking than are differences between either protein and the more distantly related L-FABP.

Adipocyte and heart fatty acid binding proteins (FABP)[1] are 15-kDa members of a family of cytosolic proteins which bind hydrophobic ligands. Murine adipocyte FABP (A-FABP) and rat heart FABP (H-FABP) share a remarkable degree of sequence homology. Comparison of their amino acid sequences indicates 62% identity (Bernlohr et al., 1984; Cook et al., 1985; Hunt et al., 1986; Sacchettini et al., 1986; Matarese & Bernlohr, 1988). A-FABP and H-FABP are also highly homologous with bovine myelin P2 protein (P2) (64 and 59%, respectively). Although clearly related to liver FABP

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[1] Abbreviations: FABP, fatty acid binding protein(s); A-FABP, adipocyte FABP; H-FABP, heart FABP; I-FABP, intestinal FABP; L-FABP, liver FABP; P2, myelin P2 protein; ffa, free fatty acid(s); AO, 9-anthroyloxy; AOffa, *n*-(9-anthroyloxy) free fatty acid(s); 2-, 3-, 6-, 7-, 9-, 10-, and 12AS, 2-, 3-, 6-, 7-, 9-, 10-, and 12-(9-anthroyloxy)stearic acid; EPC, egg phosphatidylcholine; τ_F, fluorescence lifetime; Q, fluorescence quantum yield; r, steady-state fluorescence anisotropy; τ_c, rotational correlation time; K_{sv}, Stern–Volmer quenching constant.

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(L-FABP), the sequence homology is less striking (20% and 36%).

An adipocyte FABP was first purified from rat and human adipose tissue (Haq et al., 1982). Also known as AP2, ALBP, and p422, A-FABP was identified in cultured murine adipocytes as one of a group of proteins which increases markedly during differentiation (Spiegelman & Green, 1980; Spiegelman et al., 1983; Bernlohr et al., 1984; Matarese & Bernlohr, 1988). It has been estimated that A-FABP comprises 6% of the soluble protein of mature adipocytes (Spiegelman & Green, 1980). Northern and Western blots of mouse tissue indicate that A-FABP is primarily located in adipose tissue (Bernlohr et al., 1985; Zezulak & Green, 1985). Heart FABP, first purified by Fournier et al. (1978), appears to have a wider tissue distribution. It is abundant in rat heart, skeletal muscle, and mammary gland, with smaller amounts found in stomach, kidney, testis, ovary, and brain (Bass & Manning, 1986; Crisman et al., 1987; Bass, 1988); 4–8% of the cytosolic protein of rat heart appears to be H-FABP (Glatz et al., 1984).

Like other members of the FABP family, adipocyte and heart FABP bind long-chain free fatty acids (ffa) with reported dissociation constants in the range of 0.4–3 μM. Although both proteins are thought to have a single ffa binding site (Glatz et al., 1985a; Paulussen et al., 1986; Veerkamp & Paulussen, 1987; Bass, 1988; Matarese & Bernlohr, 1988), more than one site has been reported for H-FABP (Offner et al., 1986).

Despite the sequence similarities between adipocyte and heart FABP, it is not known whether these proteins perform parallel functions in their tissues of origin. In fact, the precise physiologic function(s) of FABP remain(s) unknown. It is believed they play a role in either transport, metabolism, or storage of ffa [reviewed in Sweetser et al. (1987) and Bass (1988)]. FABP are abundant in tissues where ffa are central to cellular function. These tissues have the common need of handling high concentrations of ffa, but their use of ffa may differ. This functional diversity between tissues, coupled with differences in FABP primary structure and tissue-specific expression, suggests that members of the FABP family may have different functions.

Although the physical nature of the FABP–ffa interaction is beginning to be defined (Keuper et al., 1985; Jones et al., 1988; Sacchettini et al., 1988; Cistola et al., 1989; Storch et al., 1989), little information is available regarding the physical and chemical characteristics of the ffa binding sites of adipocyte and heart FABP. In this report, fluorescent anthroyloxy analogues of stearic acid (18:0) (designated as AOffa) were used to probe the ffa binding site of these FABP at sequential positions along the length of the ffa hydrocarbon chain. This series of AOffa has been used to study the structure of micelles and phospholipid vesicles (Podo & Blasie, 1977; Thulborn & Sawyer, 1978; Chaplin & Kleinfeld, 1983), as well as the binding site of liver FABP (Storch et al., 1989). Anthroyloxy (AO) probes are useful indicators of binding site characteristics because their spectral properties are sensitive to their immediate environment and are independent of the covalent attachment site (Thulborn & Sawyer, 1978; Tilley et al., 1979; Matayoshi & Kleinfeld, 1981; Blatt & Sawyer, 1985). The results provide a comparison of the closely related adipocyte and heart FABP and allow for comparison to the less homologous rat liver FABP, previously studied in our laboratory (Storch et al., 1989).

Experimental Procedures

Materials. The fluorescent probes 2-, 3-, 6-, 7-, 9-, 10-, and 12-(9-anthroyloxy)stearic acid (2AS, 3AS, etc.) were obtained from Molecular Probes, Inc. (Eugene, OR).

FABP Preparation. Delipidated murine A-FABP was purified from mature 3T3-L1 adipocytes as previously described (Matarese & Bernlohr, 1988). The protein concentration was determined by using the molar extinction coefficient at 280 nm, 1.55×10^4 M^{-1} cm^{-1} (Matarese & Bernlohr, 1988). Rat H-FABP was purified by the method of Said and Shultz (1984). H-FABP concentration was determined by colorimetric assay and corrected as previously described (Ockner et al., 1982; Glatz et al., 1985a). Both proteins were stored at −20 °C and diluted just prior to use.

Binding of AOffa to FABP. Binding of AOffa to both A- and H-FABP was analyzed by fluorometric titration. Increasing concentrations of AOffa from ethanolic stock were added to 4 μM delipidated FABP in 5 mM Tris/140 mM NaCl, pH 7.4, in a fluorometric cuvette, with total ethanol always <1% by volume. FABP and AOffa were allowed to reach binding equilibrium (<2 min), and fluorescence intensity was monitored. Binding affinities were calculated by the method of Cogan et al. (1976).

Fluorescence Measurements. All measurements were made at ambient temperature in 3 × 3 mm quartz microcuvettes containing 120 μL of sample. Samples contained 12 μM FABP and 1 μM AOffa in either 5 or 10 mM Tris/140 or 100 mM NaCl, pH 7.4. A blank containing all components except AOffa was measured and subtracted from fluorescence intensity measurements. (The blank represented 2–15% of the total signal.) Fluorescence spectra, steady-state fluorescence anisotropy, and fluorescence quenching were measured using an SLM 8000C fluorescence spectrophotometer. Excitation wavelengths were 383 nm for AOffa, 283 nm for tryptophan, and 352 nm for quinine sulfate. Excited-state lifetimes (τ_F) were determined at 18 mHz by the phase modulation technique (Spencer & Weber, 1969) using an SLM 4800 spectrophotometer, as previously described (Storch et al., 1989). An excitation polarizer set at 35° from the vertical was used to eliminate the effects of Brownian rotation (Spencer & Weber, 1969) and a 390-nm cutoff filter was used in the emission path. A suspension of glycogen in water was used as the light-scattering reference.

Quantum yields (Q) were determined relative to quinine sulfate in 0.1 N sulfuric acid for AOffa (Q_{ref} = 0.7) and to L-tryptophan in buffer for the tryptophan residues in FABP (Q_{ref} = 0.13) (Scott et al., 1970; Chen, 1967a). Spectra were corrected for lamp and photomultiplier variation with wavelength (Parker & Reese, 1960).

The Perrin equation was used to calculate the rotational correlation time (τ_c) of AOffa bound to FABP using experimental values for fluorescence lifetime (τ_F) and anisotropy (r): $\tau_c = \tau_F[r/(r_0 - r)]$. The limiting anisotropy (r_0) in the absence of rotational diffusion is 0.29 (Matayoshi & Kleinfeld, 1981; Vincent et al., 1982).

Fluorescence Quenching of AOffa Bound to FABP. Quenching of AOffa fluorescence was performed by sequential addition of 5-μL aliquots of concentrated acrylamide to 120 μL of FABP-bound AOffa in a fluorometric cuvette. Fluorescence intensity was measured at 450 nm before and after acrylamide additions. Inner filter effects due to acrylamide were negligible at probe excitation and emission wavelengths. Intensities were corrected for dilution of the fluorophore and for an acrylamide blank, and the data were analyzed by the Stern–Volmer equation:

$$I_0/I = 1 + K_{sv}[Q] \quad (1)$$

where I_0 and I are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the molar

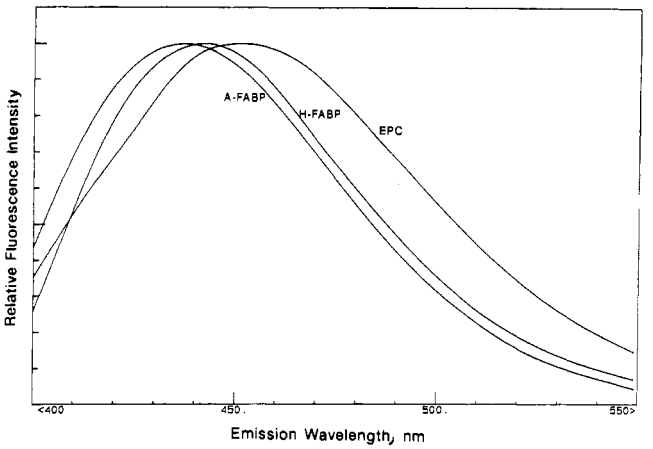

FIGURE 1: Emission spectra of 7AS bound to A-FABP, H-FABP, and EPC. 12 μM FABP or 0.2 mM EPC vesicles were incubated with 1 μM 7AS. Excitation wavelength was 383 nm. Spectra were normalized to 1.

concentration of quencher, and K_{sv} is the Stern–Volmer quenching constant in M^{-1} (Lakowicz, 1983; Campbell & Dwek, 1984).

RESULTS

Spectroscopic Characteristics of Adipocyte and Heart FABP. Adipocyte and heart FABP share a common emission maximum at 331 nm (data not shown). The spectra reflect the two tryptophan residues found in each protein (Bernlohr et al., 1984; Hunt et al., 1986; Sacchettini et al., 1986; Matarese & Bernlohr, 1988). The tryptophan quantum yield (Q) for A-FABP was 0.35 ± 0.01 and for H-FABP was 0.32 ± 0.01 (mean ± standard deviation, $n = 4$). The blue shifts of the emission spectra compared to tryptophan in water suggest that at least one of the tryptophan residues in both A-FABP and H-FABP is buried in a hydrophobic region of the protein (Konev, 1967).

Binding of AOffa to FABP. The binding of 9AS and 12AS to A- and H-FABP was analyzed by fluorometric titration (Cogan et al., 1976). A linear increase in fluorescence intensity was observed with the addition of increasing concentrations of AOffa to both adipocyte and heart FABP (data not shown). Apparent saturation of binding occurred between a 0.6 and a 1.3 molar ratio of ligand to protein. No consistent differences between AOffa or between FABP were found, and the average number of binding sites was 0.9 ± 0.3. In addition, only small differences were observed between the binding affinities of the AOffa for each FABP, and all values were within experimental error (0.6 ± 0.3 μM). The affinities and stoichiometries for AOffa binding to FABP are within the reported range obtained with native ffa (Bass, 1988; Glatz et al., 1985a,b; Veerkamp & Paulussen, 1987). [The technical aspects of FABP binding assays have recently been addressed by Vork et al. (1990).] Our AOffa binding experiments suggest that differential binding characteristics do not explain the differences in the spectral characteristics between AOffa bound to A- and H-FABP, or between AOffa as a function of AO covalent attachment site.

Spectroscopic Properties of FABP-Bound AOffa. The emission spectra of 7AS bound to adipocyte and heart FABP and, for comparison, to egg phosphatidylcholine (EPC) vesicles are shown in Figure 1. The emission maxima of A-FABP- and H-FABP-bound 7AS are blue-shifted relative to 7AS in EPC vesicles. Figure 2 shows the fluorescence emission maxima as a function of the covalent attachment site of the anthracene moiety. Emission is blue-shifted for A-FABP-bound AOffa relative to AOffa bound to H-FABP and to EPC

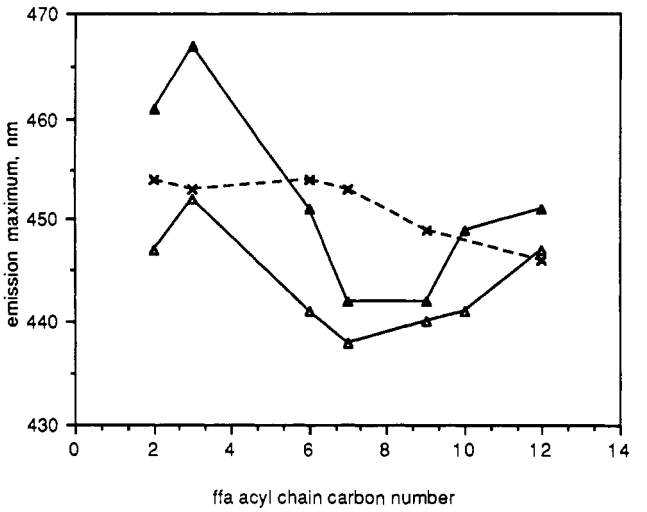

FIGURE 2: Emission maxima of AOffa bound to A-FABP (▲), H-FABP (▲), and EPC (×). 12 μM FABP or 0.2 mM EPC vesicles were incubated with 1 μM AOffa. Excitation wavelength was 383 nm.

vesicles at all positions along the ffa acyl chain, with the exception of 12AS. Emission of AOffa bound to H-FABP is blue-shifted relative to EPC vesicles only when AO was attached to the middle region of the hydrocarbon chain. In addition, the emission maximum of both A-FABP- and H-FABP-bound AOffa varied with the position of the AO moiety, with observed blue shifts most pronounced with attachment to the midportion of the acyl chain. Little variation was observed for EPC-bound AOffa (Figure 2) or for AOffa in isotropic solvents (Thulborn et al., 1979). In contrast to L-FABP-bound AOffa (Storch et al., 1989), no vibrational structure was apparent in the emission spectra of AOffa bound to either adipocyte or heart FABP (Figure 1).

Variations in quantum yield and excited-state lifetime with AO attachment site were observed for AOffa bound to A-FABP and H-FABP (Figure 3). The lifetimes for AOffa bound to both proteins were long (Figure 3A). A comparison to values in nonpolar solvents (Waggoner & Stryer, 1970; Thulborn & Sawyer, 1978; Matayoshi & Kleinfeld, 1981) suggests that AOffa are bound to A-FABP and H-FABP in a hydrophobic environment. The lifetimes also suggest that the binding site of H-FABP is more hydrophobic than that of A-FABP. The observed quantum yields provide additional evidence that the binding site of H-FABP is more hydrophobic than that of A-FABP (Figure 3B). For each protein, the Q and τ_F curves had similar shapes.

AOffa Fluorescence Quenching. Quenching of FABP-bound AOffa fluorescence was measured by adding increasing amounts of acrylamide and monitoring the decrease in fluorescence intensity. The results are presented as Stern–Volmer plots (Figure 4, Table I). With the exception of 2AS and 12AS for a A-FABP and possibly 2AS for H-FABP, all curves were linear, indicating a single quenching mechanism (Lakowicz, 1983). AO fluorescence was least quenched in the middle region of the acyl chain for AOffa bound to either A-FABP or H-FABP, as compared with probe attached to the carboxy and methyl termini. In addition, quenching of AOffa bound to H-FABP was greater than when bound to A-FABP. The Stern–Volmer quenching constants of H-FABP-bound AOffa were greater than 20-fold those of A-FABP-bound AOffa at all positions. (Note that in Figure 4 the concentration of acrylamide was 30-fold higher for A-FABP.)

Estimation of the Rotational Correlation Time (τ_c) of FABP. τ_c was calculated using the Perrin equation and the

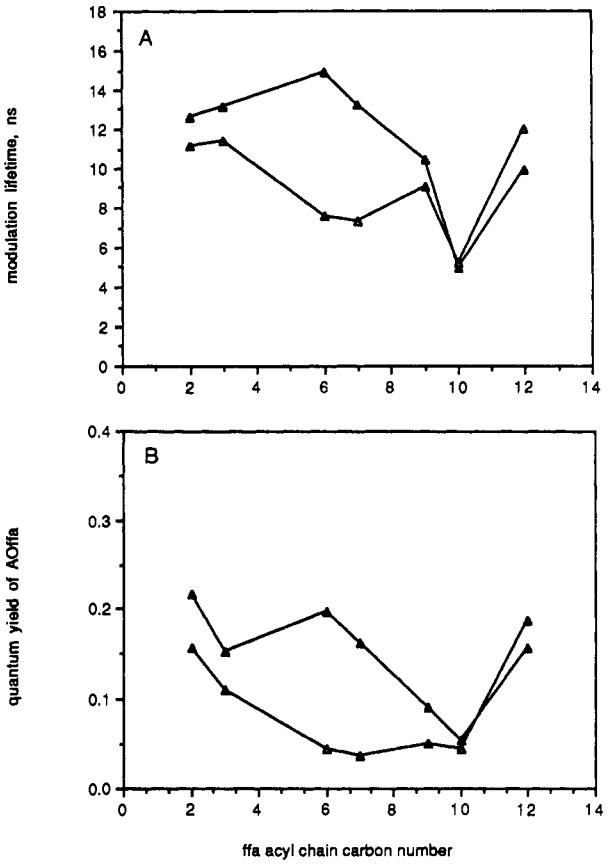

FIGURE 3: Spectroscopic properties of 1 μM AOffa bound to 12 μM A-FABP (△) and H-FABP (▲). (A) Excited-state lifetimes (τ_F) were determined by the phase modulation technique at 18 mHz (Spencer & Weber, 1969). (B) Fluorescence quantum yields (Q) were determined relative to quinine sulfate in sulfuric acid (Parker & Rees, 1960), with Q_{ref} = 0.7 (Scott et al., 1970).

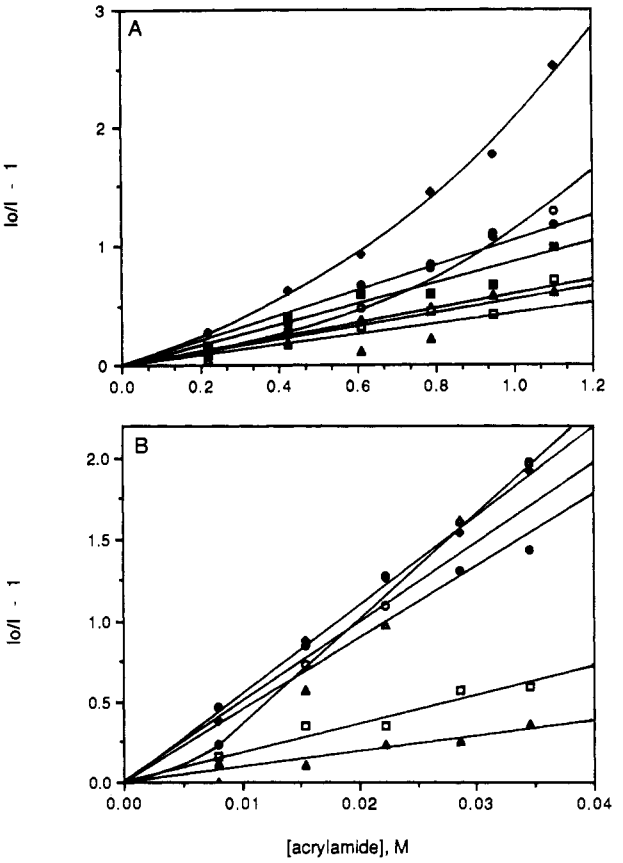

FIGURE 4: Stern–Volmer plots of fluorescence quenching. (A) Quenching of AOffa bound to A-FABP. (B) Quenching of AOffa bound to H-FABP. 5.5 M (A-FABP) or 0.2 M (H-FABP) acrylamide was added in 5-μL increments to FABP bound to 2AS (○), 3AS (●), 6AS (△), 7AS (△), 9AS (□), 10AS (■), and 12AS (♦). Values from one representative experiment are shown.

Table I: Stern–Volmer Quenching Constants[a]

AOffa	K_{SV} A-FABP	K_{SV} H-FABP
2AS	*c*	*c*
3AS	1.1	47.2
6AS	0.5	52.1
7AS	0.6	9.5
9AS	0.6	18.4
10AS	0.8	ND[b]
12AS	*c*	55.5

[a] 5.5 M (A-FABP) or 0.2 M (H-FABP) acrylamide was added in 5-μL increments. Stern–Volmer quenching constants (K_{SV}) were calculated using eq 1. Data from one representative experiment are shown. Correlation coefficients of linear regression ranged from 0.95 to 1.0. [b] ND, not determined. [c] Nonlinear plot.

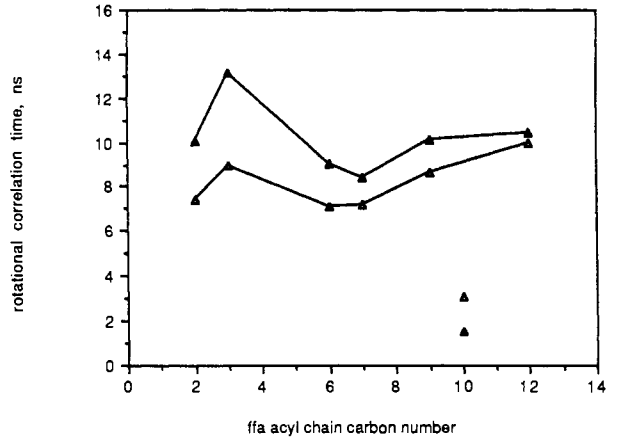

FIGURE 5: Rotational correlation time (τ_c) of AOffa bound to A-FABP (△) and H-FABP (▲). τ_c values were calculated from the measured values of fluorescence anisotropy (r) and modulation lifetime (τ_F) using the Perrin equation, as described under Experimental Procedures.

experimental values of steady-state fluorescence anisotropy (r) and excited-state lifetimes (τ_F). Values for A-FABP- and H-FABP-bound AOffa varied in a small but similar manner with the position of the AO moiety along the bound ffa chain (Figure 5). The τ_c values for AOffa bound to H-FABP were longer than when bound to A-FABP at all positions. The average values for AOffa bound to H-FABP and A-FABP were 10.2 ± 1.6 and 8.2 ± 1.1 ns, respectively (mean ± standard deviation of all measured positions, excluding 10AS).

Discussion

The physical and chemical characteristics of the fatty acid binding site of adipocyte and heart FABP were analyzed by using a series of fluorescently labeled long-chain free fatty acids. These closely related members of the FABP family were compared in order to determine if the ligand binding domain differs between highly homologous FABP. These studies also allowed for comparison to the more distantly related liver FABP, previously studied in our laboratory (Storch et al., 1989).

AOffa are useful indicators of the physical nature of the fatty acid binding site of FABP because their spectral properties are sensitive to the immediate environment of the AO moiety. The intrinsic properties of these probes are independent of the AO covalent attachment site (Thulborn & Sawyer, 1978; Tilley et al., 1979; Matayoshi & Kleinfeld,

1981; Blatt & Sawyer, 1985), and thus a series of AOffa, with AO attached at various positions along the acyl chain, can be used to probe the environment of the binding site at different positions. The use of this series of AOffa to study the structural properties of micelles, vesicles, and biological membranes at a graded series of depths is well documented (Podo & Blasie, 1977; Thulborn & Sawyer, 1978; Chaplin & Kleinfeld, 1983; Storch & Schachter, 1985; Collins et al., 1990). In addition, we recently used AOffa to obtain information about the ffa binding site of liver FABP (Storch et al., 1989). It is noteworthy that the binding stoichiometry and affinity of AOffa for the two FABP reported here, as well as for liver FABP (Storch et al., 1989), are within the range of values reported for native ffa (Bass, 1988; Glatz et al., 1985a,b; Veerkamp & Paulussen, 1987). In all these biological systems, the presence of the anthroyloxy moiety may influence the behavior of the ffa. Thus, these probes are not used for obtaining absolute dimensions of the FABP binding sites or of acyl chain distances in bilayers, but instead are used for comparing relative environmental characteristics.

AOffa emission maxima are minimally affected by solvent polarity but are quite sensitive to the degree of rotational constraint on the AO moiety (Werner, 1976; Werner et al., 1976; Thulborn et al., 1979; Matayoshi & Kleinfeld, 1981). The blue shift observed for AOffa bound to adipocyte FABP suggests that AOffa are bound in a configuration that is more constrained than are AOffa in membranes (Figure 2). A comparison of emission maxima indicates that the binding site of H-FABP is less constrained than that of A-FABP. In contrast to emission maxima of AOffa in EPC vesicles and to the observation that covalent attachment site has no effect on the emission maximum of AOffa in a single solvent (Thulborn et al., 1979), the emission maxima of AOffa bound to both A-FABP and H-FABP varied with position. The spectral shifts were most pronounced with the AO moiety attached to the midportion of the acyl chain, indicating that probe in this region of the bound fatty acid is most constrained. A similar variation in AOffa emission was seen for L-FABP, with a blue shift approximately 6 nm greater than observed for A-FABP (Storch et al., 1989).

The variation of quantum yields and excited-state lifetimes with AO attachment site and the coincident shapes of the Q and τ_F curves for each protein suggest a structured ffa binding site in both adipocyte and heart FABP (Figure 3). In contrast to the emission maxima, Q and τ_F of AOffa are polarity-sensitive parameters (Werner & Hoffman, 1973; Matayoshi & Kleinfeld, 1981), both decreasing with increasing polarity. The long lifetimes observed for AOffa bound to adipocyte and heart FABP were similar to values in nonpolar solvents (Waggoner & Stryer, 1970; Thulborn & Sawyer, 1978; Matayoshi & Kleinfeld, 1981), suggesting a hydrophobic ffa binding site (Figure 3A). Quantum yields of A-FABP and H-FABP (Figure 3B) were low compared to values for AOffa bound to L-FABP (Storch et al., 1989) and to values in nonpolar solvents (data not shown). In view of the long lifetimes, the low Q values indicate a large contribution of nonradiative processes to the decay of the excited state of AOffa bound to A-FABP and H-FABP. The lower quantum yields and lifetimes obtained for AOffa bound to A-FABP as compared to H-FABP may in part reflect the higher degree of constraint on A-FABP-bound AOffa (Werner, 1976). It should be noted, however, that despite the very high degree of motional constraint on L-FABP-bound AOffa, quantum yields for the liver protein were considerably higher than those for both A-FABP- and H-FABP-bound AOffa (Storch et al., 1989). Thus, the lower Q and τ_F obtained for A-FABP-bound AOffa compared to H-FABP are likely a reflection of a less hydrophobic fatty acid binding site. This could be due to either a higher prevalence of polar functional groups in the ligand binding site of A-FABP or to greater contact of bound AOffa with the bulk aqueous environment.

The average rotational correlation time calculated for AOffa bound to H-FABP was 2 ns longer than for A-FABP (Figure 5) and 5 ns longer than for L-FABP (Storch et al., 1989), despite the fact that all three proteins have similar molecular weights (14–15K). It is not likely that FABP aggregation, as reported for high concentrations of H-FABP (in the range of 65–135 μM) (Fournier & Rahim, 1983), is responsible for the longer τ_c values, since FABP concentrations in this study were <15 μM. Rather, these data suggest either that the heart protein occupies a larger volume and therefore rotates more slowly or that in addition to FABP rotation, other motions, such as the rotation of AOffa within the binding site, contribute to the observed τ_c. Since τ_c values of AOffa in hydrophobic solvents are long (Matayoshi & Kleinfeld, 1981; Vincent et al., 1982), greater rotational freedom of the bound fluorophore might be expected to lead to longer τ_c because probe rotation would contribute more to observed values. Either possibility is consistent with the hypothesis that AOffa bind to H-FABP in a more open pocket than in A-FABP and that both have a more open binding pocket than L-FABP. This model is strengthened not only by the lower degree of motional constraint observed for AOffa bound to H-FABP but also by acrylamide quenching experiments.

Acrylamide was used to assess the degree of exposure of FABP-bound AOffa to the aqueous environment. This neutral quencher is very soluble in water but quite insoluble in nonpolar media (Eftink & Ghiron, 1976, 1981) and has been shown to quench AOffa fluorescence in membranes and micelles by a collisional mechanism (Chalpin & Kleinfeld, 1983). Both A-FABP- and H-FABP-bound AOffa were quenched by acrylamide (Figure 4, Table I). In contrast, acrylamide was unable to quench the fluorescence of AOffa bound to L-FABP (Keuper et al., 1985; Storch et al., 1989). The data indicate that AO attached to the middle region of the bound acyl chain has less contact with the aqueous environment than at the carboxy or methyl termini for both A-FABP- and H-FABP-bound AOffa. In addition, the overall quenching of AOffa fluorescence was greater for AOffa bound to H-FABP than bound to A-FABP.

Although τ_F and Q suggest that the fatty acid binding site of H-FABP is more hydrophobic than that of A-FABP, it also appears to be more accessible to solvent. This suggests that the difference in hydrophobicity between the ligand binding sites may be due to differences in the molecular composition of the sites rather than to the degree of contact with the aqueous environment. This difference could be explained by a higher prevalence of polar or charged amino acid residues proximal to A-FABP-bound AOffa, or by the presence of a greater number of ordered solvent molecules in the A-FABP binding site, similar to the ordered water molecules observed in the binding site of intestinal FABP (I-FABP) (Sacchettini et al., 1989).

Up to this point, discussion of the spectroscopic properties of FABP-bound AOffa has excluded 10AS. 10AS bound to both adipocyte and heart FABP exhibited fluorescence behavior deviant from observed trends. For example, the quantum yield, excited-state lifetime, and rotational correlation time for 10AS bound to H-FABP were lower than values for the other probes (Figures 3 and 5), suggesting that the AO

moiety of bound 10AS is in a more polar microenvironment than AO at the other measured positions. The quantum yields for AO attached to C6–10 of A-FABP-bound AOffa indicate that AO attached to the entire midportion of the bound acyl chain are in a more polar environment than are H-FABP-bound AOffa. Nevertheless, the short lifetime of A-FABP-bound 10AS, in addition to the identical values of Q and τ_F observed for 10AS bound to both proteins, suggests that a polar residue and/or ordered solvent molecules may be present in the binding site of both adipocyte and heart FABP near C10 of the bound ffa. No such effects were observed for 10AS bound to L-FABP (Storch et al., 1989).

The tertiary structures of rat intestinal FABP (Sacchettini et al., 1988) and bovine myelin P2 protein (Jones et al., 1988) have been determined by X-ray crystallography and are strikingly similar. Both proteins are composed of 10 antiparallel β-strands. Two nearly orthogonal β-sheets form a barrel which is open on one side. The barrel is lined with primarily nonpolar residues, creating a hydrophobic pocket in which one ffa is bound. Fatty acids bound to both I-FABP and P2 appear to be in a bent configuration within the binding site. The high degree of primary sequence homology between adipocyte and heart FABP and myelin P2, in particular, suggests that these FABP are likely to share a similar tertiary structure. A higher degree of steric constraint in the middle region of AOffa bound to both adipocyte and heart FABP is indicated by the lower emission maxima, and is consistent with the bent configuration of ffa bound to I-FABP and P2. The present results also indicate that A-FABP and H-FABP bind AOffa in a structured hydrophobic binding site, in agreement with the hydrophobic pocket found in I-FABP and myelin P2.

Although the general character of the fatty acid binding sites of FABP appears to be similar, existing evidence and results of the present study suggest differences in fine structure. A comparison of the spectroscopic properties of liver FABP (Storch et al., 1989) with those of adipocyte and heart FABP suggests that while all three proteins bind AOffa in a bent configuration within a hydrophobic pocket, AOffa bound to L-FABP experience a higher degree of motional constraint than either A-FABP- or H-FABP-bound AOffa. In addition to a larger blue shift in AOffa emission maxima, the presence of vibrational structure in the emission spectra of AOffa bound to L-FABP but not A-FABP or H-FABP provides further evidence that ffa in the binding site of L-FABP are more constrained (Werner, 1976; Storch et al., 1989).

Fluorescence quantum yields suggest that the binding site of L-FABP is more hydrophobic than either A-FABP or H-FABP. In addition, acrylamide quenching data indicate that the binding site of L-FABP is not accessible to the aqueous phase, compared with those of A-FABP and H-FABP. Finally, the rotational correlation time for L-FABP-bound AOffa was 4.5 ns, similar to that predicted for a 14-kDa protein (Storch et al., 1989) and shorter than the τ_c of both A-FABP and H-FABP. Thus, it appears that of the three FABP studied, the ffa binding pocket of L-FABP is most hydrophobic and most shielded from the aqueous environment, and its ligand is bound in the most constrained configuration. Although there are differences between A-FABP and H-FABP, in keeping with their high degree of sequence homology, they are more similar to each other than either is to L-FABP. Cistola et al. (1989) have also reported structural differences between the binding sites of two members of the FABP family, L-FABP and I-FABP. For example, the carboxy terminus of ffa bound to L-FABP was found to be accessible to solvent, whereas the carboxy terminus of ffa bound to I-FABP did not appear to be in contact with the aqueous environment.

In addition to the distinct fine structure of the FABP binding sites, we have found differences in the rate of AOffa transfer from FABP to phospholipid membranes (Storch & Bass, 1990). These studies demonstrate that AOffa transfer from H-FABP is an order of magnitude faster than from L-FABP. Not only are these results consistent with the lower degree of motional constraint and greater aqueous phase accessibility of H-FABP-bound ffa discussed above, they suggest that differences in the physical nature of FABP binding sites may be an indication of functional differences between these proteins.

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Multiple Heme Pocket Subconformations of Methemoglobin Associated with Distal Histidine Interactions

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ABSTRACT: Electron paramagnetic resonance spectra of methemoglobin reveal that, in addition to the major tetragonal high-spin aqueous complex and the low-spin hydroxide complex, three other complexes associated with the interaction of the distal histidine are resolved. These are a rhombic high-spin and two classes of low-spin bis-histidine complexes. By freeze-quenching experiments it is shown that the rhombic high-spin and one of the low-spin bis-histidine complexes (B) are at equilibrium with the dominant species. Incubation in the 210–260 K temperature range shifts the total equilibrium toward a low-energy state with the distal histidine coordinated to the iron (complex C).

On the distal side of the heme pointing into the ligand pocket on almost all mammalian hemoglobins and myoglobins is a histidine, the distal histidine. The role of this histidine has been discussed by many investigators (Giacometti et al., 1980; Mims et al., 1983; Tucker et al., 1978; Shaanan, 1983; Rifkind, 1988). It has been suggested that the histidine acts