

The α -Helical Domain of Liver Fatty Acid Binding Protein Is Responsible for the Diffusion-Mediated Transfer of Fatty Acids to Phospholipid Membranes[†]

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ABSTRACT: Intestinal fatty acid binding protein (IFABP) and liver FABP (LFABP), homologous proteins expressed at high levels in intestinal absorptive cells, employ markedly different mechanisms for the transfer of fatty acids (FAs) to acceptor membranes. Transfer from IFABP occurs during protein–membrane collisional interactions, while for LFABP, transfer occurs by diffusion through the aqueous phase. Earlier, we had shown that the helical domain of IFABP is critical in determining its collisional FA transfer mechanism. In the study presented here, we have engineered a pair of chimeric proteins, one with the “body” (ligand binding domain) of IFABP and the α -helical region of LFABP (α L β IFABP) and the other with the ligand binding pocket of LFABP and the helical domain of IFABP (α I β LFABP). The objective of this work was to determine whether the change in the α -helical domain of each FABP would alter the rate and mechanism of transfer of FA from the chimeric proteins in comparison with those of the wild-type proteins. The fatty acid transfer properties of the FABP chimeras were examined using a fluorescence resonance transfer assay. The results showed a significant modification of the absolute rate of FA transfer from the chimeric proteins compared to that of the wild type, indicating that the slower rate of FA transfer observed for wild-type LFABP relative to that of wild-type IFABP is, in part, determined by the helical domain of the proteins. In addition to these quantitative changes, it was of great interest to observe that the apparent mechanism of FA transfer also changed when the α -helical domain was exchanged, with transfer from α L β IFABP occurring by aqueous diffusion and transfer from α I β LFABP occurring via protein–membrane collisional interactions. These results demonstrate that the α -helical region of LFABP is responsible for its diffusional mechanism of fatty acid transfer to membranes.

Intestinal enterocytes express high levels of two homologous fatty acid binding proteins (FABPs),¹ liver FABP (LFABP) and intestinal FABP (IFABP). Although their level of primary sequence homology is relatively low, their tertiary structures are very similar (1, 2), consisting of 10 antiparallel β -strands that form a β -barrel, which is capped by two short α -helices arranged as a helix–turn–helix segment. It is believed that this helical domain is part of a “dynamic portal” that regulates the entry of fatty acids (FAs) into and exit of FAs from the internal binding cavity (3, 4). It is hypothesized

that these FABPs are important in intracellular transport of FA; however, neither their precise function nor the reason a single cell type contains more than one distinct FABP is known. Both I- and LFABP bind long chain fatty acids with high affinity; nevertheless, it has been suggested that they are functionally distinct. LFABP is expressed in both small intestine and liver, whereas IFABP is found exclusively in the small intestine (5). IFABP has a single binding site for long chain FA (1), while LFABP contains at least two FA binding sites (2). LFABP binds a number of other endogenous hydrophobic ligands (6–10), whereas IFABP appears to bind exclusively long chain FA (11). L- and IFABP have similar affinities for saturated FAs but LFABP has a greater affinity than IFABP for unsaturated FAs (12). Furthermore, we have shown that these proteins employ markedly different mechanisms to transfer FAs to acceptor membranes (13). Whereas FA transfer from LFABP occurs via aqueous diffusion, FA transfer from IFABP occurs during direct collisional interactions of the protein with acceptor membranes. We have demonstrated, using a helix-less variant of IFABP, that the α -helical region of IFABP is involved in membrane interactions, and appears to play a primary role in the collisional mechanism of the transfer of fatty acids from IFABP to membranes (4, 14). What remains unknown is whether the α -helical domain is also the primary determinant of the diffusional transfer mechanism of LFABP.

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¹ Abbreviations: FABP, fatty acid binding protein; IFABP, intestinal fatty acid binding protein; LFABP, liver fatty acid binding protein; wtIFABP, wild-type intestinal fatty acid binding protein; wtLFABP, wild-type liver fatty acid binding protein; FAs, fatty acids; SUVs, small unilamellar vesicles; AOFA, anthroxyloxy-labeled fatty acid; 12AO, 12-(9-anthroxyloxy)oleic acid; EPC, egg phosphatidylcholine; NBD-PC, egg *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylcholine; PS, brain phosphatidylserine; CL, bovine heart cardiolipin.

In this study, we have engineered a pair of chimeric proteins, one of which consists of the “body” (ligand binding domain) of IFABP and the α -helical region of LFABP (α L β IFABP) and the other of which contains the opposing segments (α I β LFABP). The primary objective of this work was to determine whether the presence of the α -helical domain of LFABP would alter the rate and, in particular, the mechanism of transfer of FAs from the chimeric protein to membranes compared to those of the wild-type proteins. We further hypothesized, on the basis of the aforementioned results with the helix-less IFABP, that the α I β LFABP chimera would assume the kinetic mechanism of the wild-type IFABP.

Employing a fluorescence resonance energy transfer assay, we analyzed and compared the transfer of anthroyloxy-labeled fatty acids (AOFAs) from the chimeric protein to model membranes to that of wild-type I- and LFABP (wtIFABP and wtLFABP, respectively). The results demonstrate that the apparent mechanism of transfer of FAs from the IFABP binding pocket is changed when the α -helical domain is replaced with that of LFABP, indicating that the helical region was able to impart a completely different FA transfer mechanism to the ligand binding body of the FABP. Furthermore, the results showed a significant modification of the absolute rates of FA transfer from the chimeric protein compared to the wild-type FABPs. This suggests that the slower rate of FA transfer observed for wtLFABP relative to wtIFABP is modulated by the helical domain of the proteins, in part as a consequence of its assumption of a dissociation-limited process. In addition, the replacement of the LFABP helical region with the IFABP helix resulted in not only an increase in the absolute rates of transfer of FAs to membranes but also a clear switch to a collision-mediated transfer mechanism.

These results indicate that the α -helical region of the FABPs plays an important role in determining the rate and, importantly, the mechanism of transfer of fatty acids from these proteins to membranes, and that the helix–turn–helix motif of LFABP imparts the diffusional FA transport mechanism to this protein.

MATERIALS AND METHODS

Materials. The mutagenic primers were obtained from Midland Certified Reagent Co. (Midland, TX). Vent polymerase was from New England Biolabs, Inc. (Beverly, MA). Restriction enzymes *Xba*I, *Bam*HI, and *Eco*RV were purchased from Promega (Madison, WI). T4 DNA Ligase, the pET-11a expression vector, and BL21(DE3) cells were obtained from Novagen (Milwaukee, WI). Vector pBluescript II was purchased from Stratagene (La Jolla, CA). Sodium oleate was obtained from Nu-Chek Prep (Elysan, MN). Fluorescently labeled AOAFA, 12-(9-anthroyloxy)oleic acid (12AO), and ADIFAB (acylodated IFABP) were purchased from Molecular Probes, Inc. (Eugene, OR). Egg phosphatidylcholine (EPC), egg *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylcholine (NBD-PC), brain phosphatidylserine (PS), and bovine heart cardiolipin (CL) were obtained from Avanti Polar Lipids (Alabaster, AL). Lipidex-1000 was purchased from Sigma (St. Louis, MO). Isopropyl β -D-thiogalactoside was obtained from Fisher (Fairlawn, NJ). All other chemicals were reagent grade or better.

Construction of Chimeric FABPs. Recombinant rat pET11d-IFABP and pET11a-LFABP plasmids were generously provided by A. Kleinfeld and R. Ogata (Medical Biology Institute, La Jolla, CA). A unique restriction site (*Eco*RV) was generated in the region between α II and β B in both of the plasmids to allow subsequent separation and exchange of segments. Employing the overlapping PCR methodology (15), we introduced a single silent mutation in position 141 of the LFABP cDNA sequence; in the IFABP sequence, it was necessary to introduce two point mutations, one silent (position 117 of the cDNA sequence) and the other one resulting in a substitution of Ile for Asn35 (position 119 of the cDNA sequence). Energy minimization analysis to assess the effect of this mutation was performed using HyperChem software (Hypercube, Inc., Gainesville, FL), and virtually no changes were predicted for this conservative substitution. The sequences of the primers employed to construct the restriction site mutations were as follows (point mutations underlined): 5'-CGGATAACAATTCCTCTA-3' and 5'-TTCCTTCGGGCTTTGTAG-3' as external primers (the same external primers were used for both constructs), 5'-GTTTCAAGATATCATGAGCT-3' and 5'-CTCATGATATCTTGAAACTG-3' as internal primers for IFABP cDNA, and 5'-CCCCCTTGATATCCTTCCCT-3' and 5'-GAAGGATATCAAGGGGGTGT-3' as internal primers for LFABP cDNA. The mutated FABP constructs were verified by sequence analysis. Prior to the treatment with *Eco*RV, the mutant cDNAs were subcloned into pBS vectors by using the *Xba*I and *Bam*HI restriction sites (pET vectors have two restriction sites for *Eco*RV). The mutant cDNAs were treated with restriction enzymes *Eco*RV and *Bam*HI to separate the β -barrel region from the rest of the construct. The β -barrel of IFABP was ligated to the rest of the construct belonging to LFABP employing T4 DNA ligase, generating in this way a chimeric cDNA with β A, α I, and α II from LFABP and β B– β J from IFABP. Similarly, ligation of the β -barrel of LFABP to the rest of the construct belonging to IFABP generated a chimeric cDNA with β A, α I, and α II from IFABP and β B– β J from LFABP. The chimeric cDNAs were subcloned into the pET11a vector by using the *Xba*I and *Bam*HI restriction sites to construct the expression vectors. The chimeric FABP constructs were verified by sequence analysis.

Protein Expression and Purification. The wild-type and chimeric proteins were overexpressed in *Escherichia coli* harboring pET11d-IFABP, pET11a-LFABP, pet11a- α L β IFABP, and pet11a- α I β LFABP, respectively, as described in detail elsewhere (13). The wild-type proteins and the α L β IFABP chimeric protein were purified from *E. coli* as described previously (13). The α I β LFABP chimeric protein was not expressed as a soluble protein, and several attempts to avoid the formation of inclusion bodies were unsuccessful. The pellet was therefore dissolved in 6 M guanidinium hydrochloride Tris-HCl (pH 7.4), chromatographed on a Sephadex-G-50 column under denaturing conditions, and finally renatured by dilution and slow dialysis against Tris-HCl (pH 7.4) with 10% glycerol (16). The amount of protein obtained by this procedure was small; thus, only a few studies were able to be accomplished on properly folded α I β LFABP.

Analysis of Wild-Type and Chimeric FABPs. The conformation and ligand binding site integrity of the chimeric FABP

were examined by several methods. Fluorescent quantum yields (Q_f) of 12-(9-anthroyloxy)oleic acid (12AO) bound to wild-type IFABP, LFABP, and the chimeric FABP were determined using quinine sulfate in 0.1 N H₂SO₄ as the reference fluorophore, with a Q_{ref} of 0.7 (17). Excitation was at 352 nm for quinine sulfate and 383 nm for 12AO. Circular dichroism (CD) spectra were recorded at 25 °C on an Aviv model 60DS spectropolarimeter using a 0.1 cm path length quartz cuvette (Hellma). Each spectrum was obtained from five scans between 200 and 260 nm. Protein structural analysis was performed with a least-squares fitting program utilizing the protein secondary structural analysis reported by Yang et al. (18).

Binding of oleate to wild-type and chimeric FABPs was analyzed by the method employing the fluorescent probe ADIFAB (12), which allows for the direct measurement of unbound fatty acid in equilibrium with FABP. Oleate prepared as a 25 mM stock solution of the sodium salt in water at pH 9.7 containing 25 μ M BHT was titrated into 2.5 mL of 10 mM HEPES, 150 mM NaCl, 5 mM KCl, and 1 mM Na₂HPO₄ (pH 7.4) containing 0.2 μ M ADIFAB and either 4 μ M IFABP, 4 μ M α L β IFABP, or 2 μ M LFABP. Following equilibration at 37 °C for 5 min, fluorescence emission intensities at 505 and 432 nm were measured using an SLM-8000C spectrofluorometer, with excitation at 386 nm. The average and standard deviation of 10 pairs of R (emission at 505 and 432 nm) values were determined. This average was applied to binding equilibrium analysis using a standard R_{max} value of 11.5 (12). Experimental values were fitted to a single site (IFABP and α L β IFABP) or double component (LFABP); Scatchard analysis and K_d values for oleate binding were obtained. Binding of 12AO to wild-type and chimeric FABPs was assessed using a fluorescent titration assay (19). Emission spectra of 12AO bound to the FABPs were obtained at an excitation wavelength of 383 nm.

Vesicle Preparation. Small unilamellar vesicles (SUVs) were prepared by sonication and ultracentrifugation as described previously (20, 21). The standard vesicles were prepared so they would contain 90 mol % EPC and 10 mol % NBD-PC, which served as the fluorescence quencher. To increase the negative charge density of the acceptor vesicles, either 25 mol % PS or CL was incorporated into the SUVs in place of an equimolar amount of EPC. Vesicles were prepared in TBS buffer [40 mM Tris and 100 mM NaCl (pH 7.4)] except for SUVs containing cardiolipin which were prepared in TBS with 1 mM EDTA.

Transfer of AOFA from FABP to SUVs. A fluorescence resonance transfer assay was used to monitor the transfer of AOFA from the wild-type and chimeric FABPs to acceptor model membranes as described in detail elsewhere (13, 22, 23). Briefly, FABP with bound AOFA was mixed with SUVs using a DX-17MV stopped-flow spectrofluorometer (Applied Photophysics Limited, Staines, U.K.). The NBD moiety is an energy transfer acceptor of the anthroyloxy group (AO) donor; therefore, the fluorescence of the AOFA is quenched when the ligand is bound to SUVs which contain NBD-PC. When they are mixed, transfer of AOFA from the protein to the membrane is directly monitored by the time-dependent decrease in AO fluorescence. Final transfer assay conditions were 15 μ M wtIFABP with 1.5 μ M 12AO and 150–600 μ M SUVs, 5 μ M wtLFABP with 0.5 μ M AOFA and 300–

Table 1: Physical and Binding Parameters of Wild-Type and Chimeric FABP^a

	K_d (μ M) ^b	K_d (nM) ^c	quantum yield	Θ_{222} (deg M ⁻¹ cm ⁻¹)
wtIFABP	0.16 \pm 0.04	31 \pm 8	0.11 \pm 0.03	–7472
α L β IFABP	0.08 \pm 0.04	61 \pm 6	0.40 \pm 0.15	–8253
wtLFABP	0.02 \pm 0.01	8 \pm 1 (K_{d1}) 65 \pm 2 (K_{d2})	0.53 \pm 0.13	–15812

^a Data are the mean \pm standard deviation of values obtained in three separate experiments. ^b Fluorescence titration assay. ^c ADIFAB binding assay.

1200 μ M SUVs, 15 μ M α L β IFABP with 1.5 μ M 12AO and 150–600 μ M SUVs, or 5 μ M α L β LFABP with 0.5 μ M AOFA and 300–1200 μ M SUVs. Transfer was monitored at 25 °C. Controls to ensure that photobleaching was eliminated were performed prior to each experiment, as previously described (13). Data were analyzed using software provided with the instrument, and all curves were described well by a single-exponential function. For each experimental condition, at least five replicates were carried out. Average values \pm the standard deviation for three or more separate experiments are reported, unless otherwise indicated.

RESULTS

Construction of Chimeric Proteins and Comparison of Their Physical Properties with Those of Native FABPs. To examine whether the primary determinant of the FABP fatty acid transfer mechanism resides in the helix–turn–helix domain, we undertook the exchange of the α -helical regions between I- and LFABP. To control for potential alterations in the overall folding of the chimeric α L β IFABP protein, the following methods were employed in analyzing structure and binding properties: (a) circular dichroism spectroscopy, (b) fluorescence quantum yield measurements of bound anthroyloxy fatty acid, and (c) native and fluorescent ligand binding studies. For the α L β LFABP chimeric, control (c) was conducted. A summary of the results for these experiments is presented in Table 1.

The CD spectra of the wild-type proteins agree in shape and intensity with previously published results (24, 25) (Figure 1). Values of the molar ellipticity at 222 nm (θ_{222}) for wtIFABP and the α L β IFABP chimeric protein are quite close, but different from those of wtLFABP (Table 1). While wtLFABP exhibits a minimum at 220 nm, IFABP and the α L β IFABP chimeric protein minima are shifted to 215 nm. The maximum in the spectra for the three proteins is between 200 and 205 nm, but the chimeric maximum is closer to that of wtLFABP.

Fluorescence quantum yields (Q_f) for the AOFA are used to assess the relative hydrophobicity of the environment surrounding the fluorophore (17, 26–28). The comparison of 12AO Q_f values for the chimeric and native proteins could, therefore, indicate whether the modifications introduced into the native protein may have altered the dielectric environment of its binding pocket. We found that the Q_f value for the α L β IFABP chimeric protein was between those obtained for the wild-type proteins (Table 1), suggesting that the introduction of the wtLFABP α -helical domain increased to some extent the hydrophobicity of the IFABP ligand binding site.

Two methods were employed to analyze proteins' fatty acid binding affinities. The first involved the fluorescent

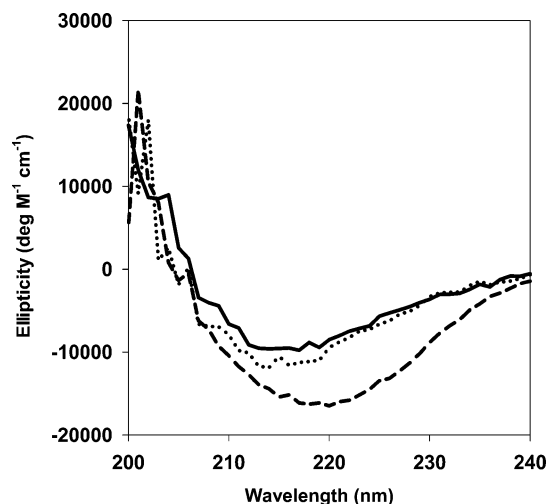


FIGURE 1: CD spectra of wtIFABP (—), α L β IFABP (···), and wtLFABP (---). Spectra were obtained at 25 °C, using each protein at 0.15 mg/mL.

probe ADIFAB, an IFABP covalently modified with an acrylodan fluorophore. Binding of FA to ADIFAB induces a red shift in the acrylodan emission spectrum, and this is used to provide a measure of the amount of unbound FA in solution (12). Using the known K_d of ADIFAB for a particular ligand (12), equilibrium binding properties can be determined for an unknown FABP. The K_d values for oleate binding to wtIFABP (31 ± 8 nM) and wtLFABP (8 ± 1 and 65 ± 2 nM for the high- and low-affinity sites, respectively) are in agreement with those previously determined (12). Data for the chimeric α L β IFABP demonstrated a single binding site with a K_d value of 61 ± 6 (Table 1).

The second method used to analyze ligand binding employs the fluorescently labeled fatty acid 12AO (19). The fluorescence of the anthroyloxy moiety increases markedly when bound in a hydrophobic environment such as an FABP ligand binding domain (17, 26). 12AO bound to wild-type and chimeric proteins in a saturable manner, and the Scatchard analysis showed that the K_d values for the chimeric proteins were between those determined for the wild-type proteins. K_d values obtained for wild-type I- and LFABP were 0.16 ± 0.04 and 0.02 ± 0.01 μ M, respectively. The K_d for binding of 12AO to α L β IFABP was 0.08 ± 0.04 and to α I β LFABP 0.04 ± 0.01 μ M.

Taken together, the controls suggested no major alterations in the conformation and binding site properties of the chimeric α L β IFABP relative to its parent wild-type proteins. The α L β I chimeric FABP folds properly and binds a single FA molecule in a relatively hydrophobic binding site. In addition, the binding analysis indicates that α I β LFABP has also maintained the integrity of its ligand binding site. It must be noted that preparations of α I β LFABP that did not demonstrate intact ligand binding were not used for further studies and that fatty acid transfer studies were performed immediately upon completion of binding analysis for those samples that were further examined.

Effect of Vesicle Concentration on the Transfer of AOFA from FABPs to Membranes. The effect of the acceptor membrane phospholipid concentration on the rates of ligand transfer can be used to distinguish between an aqueous diffusion mechanism, where no effect is observed, and a collision-mediated mechanism, where the ligand transfer rate

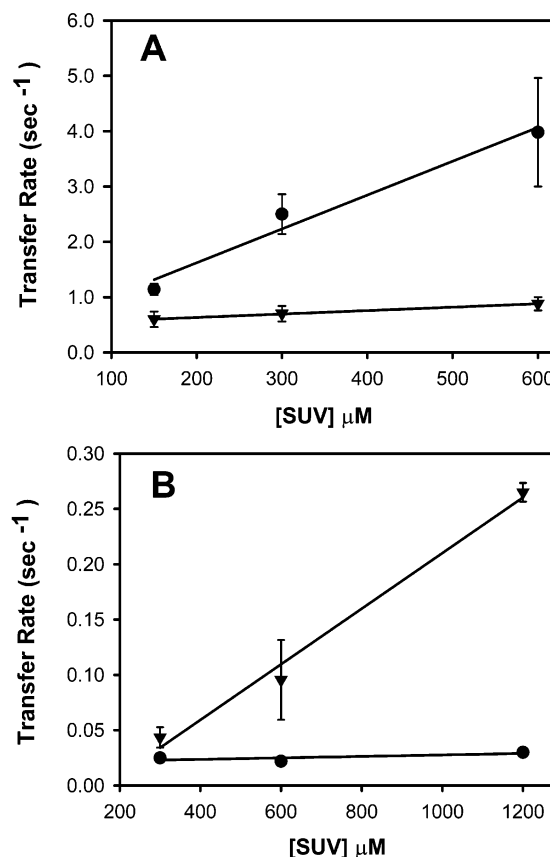


FIGURE 2: Effect of acceptor membrane concentration on the transfer of AOFA from FABP. (A) Transfer of (●) 1.5 μ M 12AO from 15 μ M wtIFABP to EPC/NBD-PC SUVs and (▼) 1.5 μ M 12AO from 15 μ M α L β IFABP to EPC/NBD-PC SUVs. Average transfer rates from three different experiments \pm the standard deviation are shown. (B) Transfer of (●) 0.5 μ M 12AO from 5 μ M wtLFABP to EPC/NBD-PC SUVs and (▼) 0.5 μ M 12AO from 5 μ M α I β LFABP to EPC/NBD-PC SUVs. Average transfer rates from three and two different experiments \pm the standard deviation are shown for wtLFABP and α I β LFABP, respectively.

is directly related to the donor–acceptor collisional frequency and, hence, the vesicle concentration. To distinguish between these transfer mechanisms, transfer of AOFA from the chimeric proteins to model membranes was examined as a function of increasing membrane concentration, and results were compared to those for wild-type LFABP and IFABP. Figure 2 shows the results obtained when constant concentrations of these FABP–AOFA donor complexes were mixed with increasing concentrations of EPC SUVs. Increasing the concentration of the acceptor lipid over a 4-fold range did not affect substantially the rate of transfer of 12AO from the chimeric α L β IFABP, whereas wild-type IFABP showed an almost proportional increase in the transfer rate with SUV concentration (Figure 2A), in agreement with previous results (4, 13). The very minimal increases in the AOFA transfer rate as a function of SUV phospholipid concentration for α L β IFABP are quite similar to the effect observed for wtLFABP, suggesting that the FA transfer mechanisms for these two proteins are similar and likely to be aqueous diffusion-mediated. In addition, at a 10:1 EPC SUV:FABP molar ratio, the absolute rate of 12AO transfer from the chimeric FABP was approximately 2-fold lower than the rate from wtIFABP. The slower transfer rate may reflect the higher ligand binding affinity of the α L β IFABP chimeric

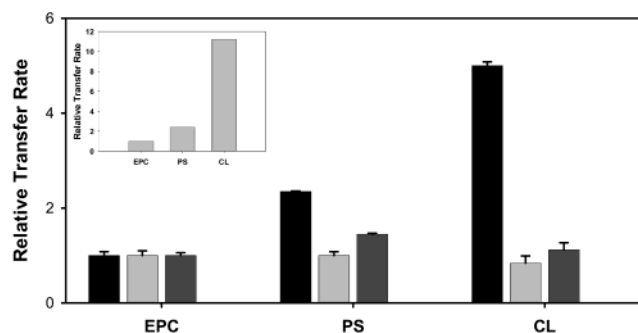


FIGURE 3: Effect of vesicle charge on transfer of AOFA from FABP. Transfer of 1.5 μ M 12AO from 15 μ M wtIFABP to 150 μ M EPC/NBD-PC SUVs containing 25 mol % PS or CL (black bars), 1.5 μ M 12AO from 15 μ M α L β IFABP to 150 μ M EPC/NBD-PC SUVs containing 25 mol % PS or CL (light gray bars), or 0.5 μ M 12AO from 5 μ M wtLFABP to 300 μ M EPC/NBD-PC SUVs containing 25 mol % PS or CL (dark gray bars). Results are expressed relative to the rate of transfer of 12AO to EPC/NBD-PC membranes. Averages from three different experiments \pm the standard deviation are shown. The inset is a single transfer experiment for transfer of 0.5 μ M 12AO from 5 μ M α L β LFABP to 300 μ M EPC/NBD-PC SUVs containing 25 mol % PS or CL relative to the rate of transfer of 12AO to EPC/NBD-PC membranes.

FABP. In contrast to results for α L β IFABP, chimeric α L β LFABP exhibited a proportional increase in the AOFA transfer rate with SUV concentration (Figure 2B), very similar to the behavior of wtIFABP, indicating that the FA transfer mechanism is likely to be collisional. The absolute transfer rate of 12AO was also modified compared to that of wtLFABP, showing in this case an almost 2-fold increase at a 10:1 EPC SUV:FABP molar ratio, which may reflect a lower ligand binding affinity of the chimeric α L β LFABP.

Effect of Vesicle Charge on the Transfer of AOFA from FABPs to Membranes. Changes in the surface charge density of the acceptor vesicles can also influence ligand transfer rates if electrostatic interactions between the donor protein and acceptor membranes are involved. By contrast, in the case of aqueous diffusion, characteristics of the acceptor membrane would not be expected to modulate the transfer rate under the donor:acceptor ratios used in these experiments. Figure 3 shows that, as expected from previous studies, the rate of transfer of 12AO from wtIFABP is substantially increased by incorporation of 25 mol % PS or CL into EPC/NBD-PC acceptor membranes, whereas transfer of 12AO from LFABP was essentially unaffected by the presence of negatively charged phospholipids (13). The results for the α L β I chimeric FABP resembled completely the results for LFABP, further implying that the mechanism of the transfer of fatty acid from the chimeric FABP is likely aqueous diffusion. Results from a single experiment with α L β LFABP showed that, in direct contrast to the case with wtLFABP, the rate of transfer of AOFA to anionic SUVs was markedly increased [2.4 times relative to EPC for transfer to PS vesicles and 11.4 times for transfer to CL vesicles (Figure 3 inset)].

Effect of Ionic Strength on the Transfer of AOFA from FABPs to Membranes. The aqueous solubility of fatty acids is inversely related to the ionic strength of the solvent (29, 30). Thus, the rate of transfer of AOFA from a binding protein through an aqueous space to an acceptor membrane is expected to decrease with the salt concentration of the media. Wild-type LFABP shows a logarithmic decrease in

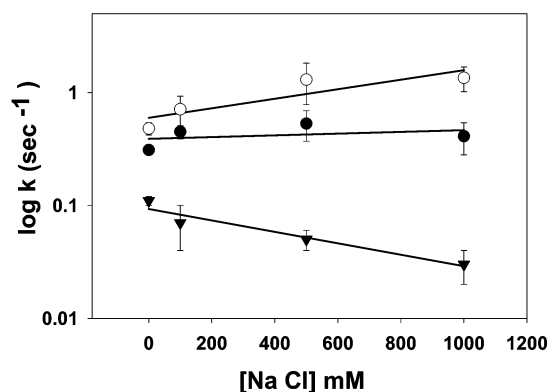


FIGURE 4: Effect of ionic strength on transfer of AOFA from FABP. Transfer of (○) 1.5 μ M 12AO from 15 μ M wtIFABP to 150 μ M EPC/NBD-PC SUVs, (●) 1.5 μ M 12AO from 15 μ M α L β IFABP to 150 μ M EPC/NBD-PC SUVs, and (▼) 0.5 μ M 12AO from 5 μ M wtLFABP to EPC/NBD-PC SUVs. The NaCl concentrations of the acceptor and donor were adjusted before mixing. Average transfer rates from three different experiments \pm the standard deviation are shown.

the rate of transfer of 12AO to SUVs (Figure 4), in agreement with previous findings (13, 22). Such behavior was also observed for the transfer of fatty acids from donor SUVs to LFABP (31), and is supportive of the hypothesis that transfer takes place through an aqueous phase intermediate. As previously observed, a small increase in the rate of transfer of 12AO from wtIFABP to SUVs is detected with increasing NaCl concentrations (Figure 4), supporting the absence of an aqueous intermediate and thus indicative of collisional interaction between FABP and SUVs during AOFA transfer (22, 27). The effect of ionic strength on the transfer of 12AO from the α L β IFABP chimeric protein shows virtually no alteration of the rate of transfer from the protein to zwitterionic SUVs, over the same range of NaCl concentrations (Figure 4), presumably reflecting the combined influence of factors contributed from each of the wild-type proteins.

DISCUSSION

Intestinal and liver FABPs are homologous intracellular proteins expressed in small intestinal enterocytes. Several lines of evidence indicate relevant roles in cellular fatty acid transport and metabolism, but their precise functions remain unclear. Differences with regard to their structure, binding specificities, and ligand transfer properties suggest they may each play a unique role in the enterocyte.

Using an *in vitro* fluorescence resonance energy transfer assay, we have previously shown that the movement of fluorescent AOFA from LFABP to acceptor membranes was regulated entirely by the aqueous solubility of the FA (13, 32). On the other hand, we have found that transfer of FAs from IFABP to a phospholipid membrane appears to involve an intermediate in which IFABP and the membrane are in physical contact. The collisional FA transfer mechanism observed for IFABP, although markedly different from that for LFABP, has also been found for several other FABPs examined to date, including HFABP, AFABP, and KFABP (22, 33, 34). The only member of the FABP superfamily that exhibits an aqueous diffusion mechanism similar to LFABP is CRBP II (35).

Earlier work demonstrated the importance of cationic surface residues in the ionic interactions with membrane

phospholipids, for those FABPs that transfer a ligand via a collisional mechanism. The FA transfer rate increases as a function of membrane negative charge (13, 23) and decreases for membranes with a positive charge (23). Neutralization of surface lysine residues decreases the rate of transfer to membranes and completely eliminates the collisional FA transfer mechanism (27, 36). The large effects of membrane and protein surface charge on AOFA transfer rate suggest that positively charged residues on the surface of these FABPs are important for the formation of a collisional complex with the membrane. An analysis of surface electrostatic potential topologies for several "collisional transfer" FABPs demonstrated a net positive potential across the helix–turn–helix portal region of the proteins which supports the suggestion that this region is important for the interactions with the membranes. The presence of both negative and positive electrostatic potential in the helix–turn–helix cap of LFABP (37) may be preventing such an interaction. Moreover, it has been recently shown that the paralogous protein from toad, Lb-FABP, which also exhibits negative and positive electrostatic surface potential, shares with LFABP the diffusional mechanism of transfer of AOFA to phospholipid membranes (38). It is noteworthy that the α -I helices of I-, H-, and AFABP are amphipathic, while α -I helices of LFABP and CRBP II are not. The significance of amphipathic helices in the targeting of proteins to membranes is well appreciated, and charge characteristics of a helix are believed to modulate interactions with membranes (39). Moreover, NMR studies have demonstrated that the ligand portal region, which includes the helix–turn–helix domain, shows the greatest degree of conformational difference between holo- and apo-IFABP (3, 40), and we have found similar differences in root-mean-square deviations between holo- and apo-LFABP (Y. He, H. Wang, J. Storch, and R. E. Stark, unpublished observations). Taken together, these observations have focused our attention on the helical "portal" domain of the two proximal intestine FABPs.

Employing a helix-less mutant of IFABP, we showed the crucial importance of the IFABP helical domain for its collisional transfer of FA to phospholipid membranes (4, 14). The absence of the helical domain almost completely abolished the collisional mechanism of transfer and markedly diminished the capacity to interact with membranes. Site-directed mutagenesis of specific lysine residues in HFABP (27), AFABP (28), and IFABP (B. Córscico and J. Storch, unpublished observations) also showed the importance of the positively charged residues of the portal domain of these FABPs in the collisional transfer of FA to membranes. Here we assessed whether the unique LFABP mechanism (only found in CRBP II and Lb-FABP) also resides in properties of the helix–turn–helix region, by engineering a pair of chimeric proteins with the body of IFABP and the helical domain of LFABP (α L β IFABP), and the body of LFABP and the helical domain of IFABP (α I β LFABP).

Intestinal and liver FABPs have very similar overall conformations, as shown by the comparison of rms distance differences, the superposition of crystallographic structural models (2), and the secondary structure assignments from their solution structures (3). Their similar secondary structures allowed us to proceed with the domain exchange approach despite their very low level of sequence homology (25% for the overall structure and 33% in the α -helical

domain). The α L β IFABP chimeric protein showed no major structural differences compared to the wild-type proteins. The molar ellipticity and CD spectral minimum for the chimeric form are very close to those of wtIFABP, reflecting the large contribution of the β -barrel binding pocket of the parent protein. The increase in the hydrophobicity of the binding pocket relative to wtIFABP, as shown by the Q_f (Table 1), reflects the participation of helix–turn–helix domain residues in determining binding site properties, as the LFABP binding site hydrophobicity is considerably greater than that of IFABP (Table 1 and ref 32). The structural stability of a chimeric protein possessing the IFABP β -barrel structure was not unexpected, since the deletion of the helical domain of IFABP results in an all- β -sheet variant which folds properly and is remarkably stable (42–44). Furthermore, it was found that the covalent incorporation of several fluorescein moieties into the IFABP did not alter its folding and ligand binding properties (45).

Liver and intestinal FABP exhibit somewhat different binding affinities for unsaturated fatty acids. Using the ADIFAB assay, K_d values of IFABP for oleate were found to be more than 4-fold higher than those of LFABP, as expected (Table 1 and ref 12). Moreover, binding characteristics of LFABP are unique among the FABPs, as this protein binds two long chain fatty acids rather than one (12). Employing the fluorescent probe ADIFAB, the chimeric α L β IFABP demonstrated an apparent dissociation constant closer to the value obtained for wtIFABP (Table 1) and the existence of a single ligand binding site, properties which likely reflect the fact that the β -barrel of the chimeric protein corresponds primarily to IFABP structure. The assessment of ligand binding using the fluorescently labeled fatty acid 12AO showed that both chimeric proteins had intermediate K_d values compared to the wild-type proteins. Thus, both binding methods clearly demonstrate that the binding cavities of the chimeric proteins maintain their affinity for the ligands. The binding affinity changes suggest that the α -helical region could contribute to the binding properties of these proteins. Indeed, NMR analysis of the backbone dynamics of apo- and holo-IFABP revealed that the C-terminal half of helix II interacts with the bound fatty acid, resulting in a stabilization of the portal region (3, 40). Moreover, since it appears that the mechanism of ligand transfer for the α L β IFABP is via aqueous diffusion, its lower equilibrium binding affinity for 12AO is consistent with a rate of dissociation that is greater than the rate of dissociation of 12AO from LFABP.

Employing an *in vitro* fluorescence resonance transfer assay, we have previously shown that the FA transfer mechanism of IFABP is completely different from that of LFABP. Here we demonstrate that exchanging the α -helical domain of IFABP for that of LFABP drastically modifies the collisional mechanism characteristic of IFABP. The absence of an acceptor membrane concentration effect suggests that the transfer of AOFA from α L β IFABP to acceptor vesicles is mediated by a diffusional mechanism. Moreover, the decrease in the AOFA transfer rate observed for α L β IFABP compared to that for wtIFABP shows that the α -helical region of the FABPs plays a role not only in establishing the transfer mechanism but also to some extent in determining the absolute rate of FA transfer. Furthermore, the substitution of the α -helical domain of LFABP for that

of IFABP dramatically changes the LFABP response to increasing vesicle concentrations, imparting to it the collisional transfer properties of the IFABP and thereby showing, once again, the critical role of the IFABP α -helical domain in the collisional mechanism of transfer of FA to acceptor membranes.

The lack of sensitivity to the acceptor vesicle charge of α L β IFABP is further evidence of the role of the helical region of IFABP in the collisional mechanism of transfer of fatty acids to phospholipid membranes. The number and distribution of charged residues in the helical domain could account for the difference. In particular, the α -I helix of IFABP is expected to be membrane interactive due to its amphipathicity (46), a characteristic which is also present in other collisionally interactive proteins of this family (47). In IFABP, the α -I helix polar face contains two basic lysine residues at positions 16 and 20 and two negatively charged glutamate residues at positions 15 and 19 (46). On the other hand, the LFABP α -I helix is not amphipathic, containing a Lys residue at position 19 and one Glu at position 15. Thus, the results presented here suggest that the charged face of the α -I helix is critical for membrane interactions, leading to the dramatic increase in the rate of transfer of AOFA to anionic membranes for IFABP but not LFABP.

The rate of diffusion of a hydrophobic molecule through an aqueous milieu will be modulated by factors that alter its aqueous solubility (30). Thus, in agreement with our previous findings (13, 32), an increase in ionic strength results in a logarithmic decrease in the rate of transfer of 12AO from LFABP to acceptor membranes. In marked contrast, we reported that IFABP shows an opposite relationship, which suggests that the aqueous solubility of fatty acids does not govern the rate of transfer from this protein to phospholipid membranes (13). We hypothesize that one possible explanation for the observed increase in the rate of transfer from IFABP might be that the extent of hydrophobic interactions could be increased by a high salt concentration, thereby stabilizing the protein membrane collisional complex (48). While electrostatic interactions are likely of primary importance for collisional transfer of AOFA from IFABP, several observations indicate that hydrophobic interactions may also occur between IFABP and acceptor membranes. The characteristic increase in the transfer rate with a proportional increase in the acceptor membrane concentration is found in the absence or presence of a net charge on the membranes. Moreover, we have previously demonstrated that the phospholipid acyl chain, sterol, and zwitterionic phospholipid composition of acceptor membranes modulated rates of transfer of AOFA from IFABP, and such effects are likely to arise, at least in part, from hydrophobic interactions (13). Further, our experiments with the helix-less IFABP indicated that, in the absence of the helical domain, additional associations of the β -barrel with membranes may exist, which may be hydrophobic in nature (4, 14). In the studies presented here, the chimeric α L β IFABP protein shows no substantial modification of the transfer rate with an increase in ionic strength. It is therefore possible that hydrophobic domains present in the β -barrel of IFABP may be stabilizing the interaction with phospholipid membranes in a high-ionic strength environment. Another possible source of the salt effects observed for the chimeric α L β IFABP is the fact that high-salt conditions are known to influence the compactness

and stability of protein structure (49–51), and this conformation-induced modulation of protein surface properties could result in some degree of protein–membrane interaction.

In summary, these results show that the helical domain of LFABP was able to convert the FA transfer properties of IFABP to those of LFABP, and the helical domain of IFABP was able to confer a collisional mechanism of transfer to the ligand binding domain of LFABP. These short (six to eight residues) helical segments can now be appreciated as novel motifs that are likely to be important in determining the existence and extent of protein–membrane and/or protein–protein interactions, thereby contributing to vectorial trafficking of the ligand. Under conditions of altered ionic strength, in particular, other nonhelix domains may also have a relevant role in the ligand transfer process.

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