Role of Portal Region Lysine Residues in Electrostatic Interactions between Heart Fatty Acid Binding Protein and Phospholipid Membranes†

Fiona M. Herr, Jed Aronson, and Judith Storch*

Department of Nutritional Sciences, Cook College, Rutgers University, New Brunswick, New Jersey 08903

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ABSTRACT: The structure of heart fatty acid binding protein (HFABP) is a flattened β-barrel comprising 10 antiparallel β-sheets capped by two α-helical segments. The helical cap region is hypothesized to behave as a portal "lid" for the entry and release of ligand from the binding pocket. The transfer of fatty acid from HFABP is thought to occur via effective collisional interactions with membranes, and these interactions are enhanced when transfer is to membranes of net negative charge, thus implying that specific basic residues on the surface of HFABP may govern the transfer process [Wootan, M. G., & Storch, J. (1994) *J. Biol. Chem. 269*, 10517−10523]. To directly examine the role of charged lysine residues on the HFABP surface in specific interactions with membranes, chemical modification and selective mutagenesis of HFABP were used. All surface lysine residues were neutralized by acetylation of recombinant HFABP with acetic anhydride. In addition, seven mutant HFABPs were generated that resulted in charge alterations in five distinct sites of HFABP. Modification of the protein did not significantly alter the structural or ligand binding properties of HFABP, as assessed by circular dichroism, fluorescence quantum yield, and ligand binding analyses. By using a resonance energy transfer assay, transfer of 2-(9-anthroyloxy)palmitate (2AP) from acetylated HFABP to membranes was significantly slower than transfer from native HFABP. In addition, in distinct contrast to transfer from native protein, the 2AP transfer rate from acetylated HFABP was not increased to acceptor membranes of increased negative charge. Transfer of 2AP from HFABP mutants involving K22, located on α-helix I (α-I) of the helical cap region, was 3-fold slower than transfer from wild-type protein, whereas rates from a mutant involving the K59 residue, located on the β2-turn of the barrel near the helical cap, were 2-fold faster than those of wild type. A double mutant involving K22 and K59 resulted in transfer rates identical to those of wild type, indicating that at least two domains are involved in determining the overall rate of ligand transfer. In addition, 2AP transfer rates from HFABP mutated at position 22 were totally unaffected by the charge characteristics of acceptor membranes, in marked contrast to wild type and other members of the mutant series. Further, by introducing a positive charge to α-helix II (α-II) of the helical cap region, 2AP transfer rates increased by 4-fold and properties of HFABP transfer began to approach those seen for AFABP, another member of the FABP family thought to transfer ligand via collisional interactions with membranes, which has a lysine residue in the α-II helix. These studies demonstrate that the helical cap region of HFABP may play an important role in governing ionic interactions between binding protein and membranes.

Fatty acid binding proteins (FABPs) are a family of 14−15 kDa cytosolic lipid binding proteins that includes the cellular retinoid binding proteins (CRBPs). FABPs have been isolated from a variety of tissues, including liver (LFABP), intestine (IFABP), adipose (AFABP), and heart and skeletal muscle (HFABP). Despite disparate primary sequences, those members of the family that have been crystallized exhibit remarkably similar tertiary structures [for recent reviews, see Banaszak et al. (1994) and LaLonde et al. (1994)]. This "β-barrel" motif is a tightly folded structure consisting of 10 strands of antiparallel β-strand twisted such that two, orthogonal, five-stranded antiparallel sheets form the back and sides of a flattened barrel. The front half of the barrel is capped by two short α-helices in a helix−turn−helix motif (Figure 1). The amphiphilic ligands are bound in the interior of the protein in a central cavity. Proteolytic studies of HFABP and the closely related CRBPs indicate differential susceptibility between the apo and holo forms of the binding proteins. This susceptibility was localized to the helical region of the proteins and implied that this region may undergo conformational change upon ligand binding and release (Jamison et al., 1993). In addition, X-ray crystallographic analysis of AFABP and solution NMR studies of bovine HFABP have indicated ligand-induced flexibility in the helical cap region of these proteins (Lucke et al., 1992; Xu et al., 1993).

Despite an abundance of information on their structure, tissue distribution, developmental expression, and equilibrium binding properties, the in vivo functions of FABPs remain unclear (Bass, 1993; Banaszak et al., 1994). They have generally been postulated to play a role in the transport, binding, and/or intracellular metabolism of fatty acids. In particular, it has been proposed that HFABP may be involved in the directed targeting of fatty acids for mitochondrial β-oxidation, a major source of energy for the cardiomyocte (Veerkamp et al., 1991). To address the putative in vivo functions of the FABPs, our laboratory has developed an in

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* Established Investigator of the American Heart Association and author to whom correspondence should be addressed.

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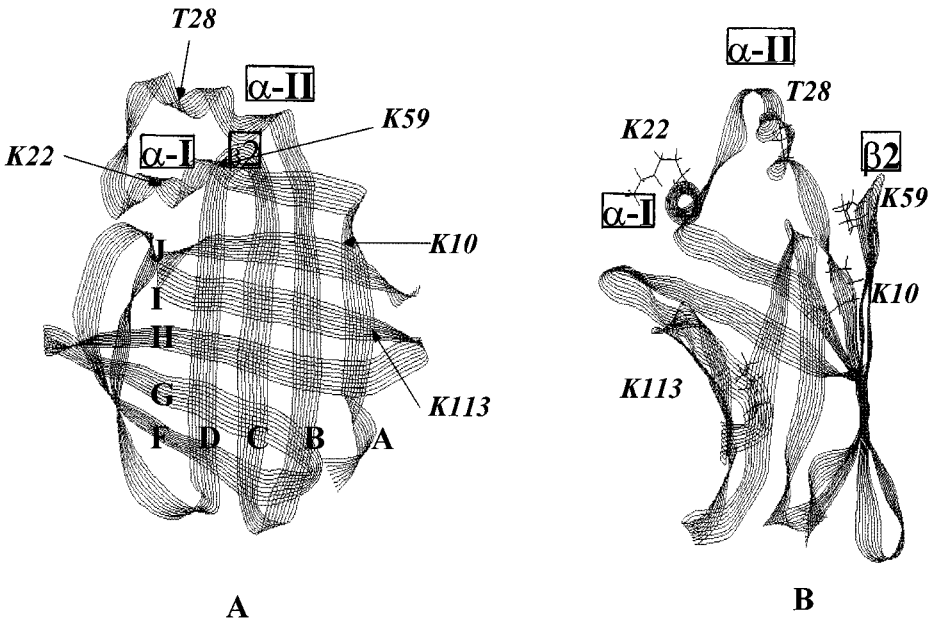

FIGURE 1: Ribbon representation of heart fatty acid binding protein. This figure is a representation of the crystal structure of HFABP as determined by Zanotti et al. (1992). Panel A: assignment of the primary structural features of HFABP including the upper helix−turn−helix region and β2-turn, which connects β-strands C and D. Panel B: view from the "side" of the β-barrel. Both panels indicate mutated residues in italics. This figure was prepared using the software package Hyperchem (Autodesk).

vitro fluorescence resonance energy transfer assay to examine the rate and mechanism of fatty acid transfer from FABP to membranes. By using fluorescently labeled fatty acid analogs, we have demonstrated that members of the FABP family transfer fatty acids to phospholipid bilayers via experimentally distinct mechanisms (Wootan et al., 1993; Kim & Storch, 1992a,b; Wootan & Storch, 1994). Specifically, LFABP transfers fatty acid to membranes in a diffusional manner involving an initial and obligatory release of ligand to the aqueous milieu (Kim & Storch, 1992b). In contrast, the adipocyte and heart FABPs transfer fatty acid during effective collisional interactions with membranes and not via aqueous diffusion (Kim & Storch, 1992a; Wootan et al., 1993; Wootan & Storch, 1994). The structural elements underlying the collisional transfer of ligand from AFABP and HFABP to membranes, such as specific binding protein−membrane interactions, could have important physiological consequences as they may dictate the directed in vivo intracellular trafficking of fatty acids.

Earlier work has demonstrated that the collisional transfer rate of fluorescently labeled fatty acids from HFABP and AFABP to phospholipid bilayers increased markedly as a function of membrane negative charge and decreased to membranes of positive charge (Wootan & Storch, 1994). These results suggested that cationic surface residues of the protein are involved in ionic interactions with membrane phospholipids. It was further demonstrated that the neutralization of surface lysine residues of AFABP by treatment with acetic anhydride significantly decreased the overall rate of transfer to membranes and completely altered the fatty acid transfer mechanism. Transfer from the acetylated protein no longer occurred via collisional interactions with membranes, but rather proceeded entirely by aqueous diffusion (Herr et al., 1995). The dramatic effect of AFABP acetylation supports the hypothesis that electrostatic interactions play a key role in the formation of effective collisional complexes between fatty acid binding proteins and membranes.

The purpose of the present investigation was to identify specific regions and/or residues of HFABP that are responsible for electrostatic interactions between the protein and phospholipid membranes. Initially, we examined the effect of gross acetylation of surface lysine residues on the fatty acid transfer properties of HFABP. Further, we constructed site-directed mutants of HFABP and, following assessment of their structural integrity, characterized their functional properties by using the fluorescence resonance energy transfer assay. The contributions of five distinct regions of the protein were examined by substitution of a neutral amino acid for a lysine residue, reversal of a lysine charge, or introduction of a net negative charge. We concentrated on the portal, helical lid region of HFABP as this is thought to be involved in fatty acid entry into the ligand binding site and therefore might be involved in ligand transfer to membranes (Zanotti et al., 1992). Seven mutant HFABPs were generated, purified, and characterized, and the effects of altering the electrostatic surface characteristics of particular regions of the protein on fatty acid transfer to phospholipid membranes were examined. The results demonstrate the importance of the helix−turn−helix "portal" domain in ionic interactions of HFABP with membranes.

EXPERIMENTAL PROCEDURES

Construction of Mutant HFABPs. The site-directed mutagenesis of rat heart fatty acid binding protein (HFABP) was performed on pBluescript-rHFABP using a phosphorothioate screening protocol (Amersham, Inc.). The oligonucleotides used for the construction of mutant HFABP were as follows (mutant sequences are underlined): K22I, 5′-GACTACATGATATCACTCGGT-3′; K10I, 5′-GGTACCTGGATCCTAGTCGAC-3′; K59I, 5′-AGTACCTTCATCAACACAGAG-3′; K113I, 5′-AGTGATGGGATCCTCATCCTG-3′; K22E, 5′-GACTACATGGAGTCACTCGGT-3′. All mutated HFABP constructs were verified by sequence analysis (Sanger et al., 1980). The mutant cDNAs were subcloned into pET-11d (Novagen) by using the *Nco*I and

*Eco*RI restriction sites to construct the expression vectors, pEX-HFABP. The pEX-T28K-HFABP mutant construct was kindly provided by Drs. Alan Kleinfeld and Ron Ogata (Medical Biology Institute, La Jolla, CA).

Protein Expression and Purification. Wild-type HFABP, AFABP, and mutant HFABP were expressed and purified as described previously (Herr et al., 1995). Briefly, pEX constructs were transformed into the *Escherichia coli* host strain BL21(DE3)pLysS, and protein expression was induced by the addition of 0.4 mM IPTG to the growing culture. Cells were harvested, resuspended in lysis buffer, and sonicated by using a Branson Model 250 sonifier set to 60 W. Cellular debris was removed by centrifugation at 50000*g* for 30 min, and the supernatant was chromatographed by two sequential Sephadex-G-50 (Pharmacia) columns and anion exchange chromatography (DE-52, Whatman) followed by delipidation (Lipidex-1000, Sigma). FABP purity was assessed by polyacrylamide gel electrophoresis. Protein concentrations were determined by the method of Lowry et al. (1951) and corrected as described previously (Glatz et al., 1985). Yields of purified wild-type and mutant proteins did not differ significantly and ranged from 15 to 35 mg of protein/L of *E. coli* culture.

Acetylation of HFABP. Neutralization of lysine residues on HFABP was accomplished by reaction with acetic anhydride as described previously (Herr et al., 1995). The modified protein was dialyzed into 10 mM PO_4 and 150 mM KCl (pH 7.4), filtered, and quantitated by the method of Lowry et al. (1951). The extent of lysine modification was determined by using the fluorescent probe fluorescamine (Herr et al., 1995). The >93% decrease in total fluorescamine emission intensity indicated that each of the 14 lysine residues in HFABP had been modified.

Analysis of Wild-Type and Mutant HFABPs. The conformational and ligand binding site integrity of HFABP following acetylation or mutagenesis was examined by several methods. Fluorescent quantum yields (Q_f) of 2-(anthroyloxy)palmitate (2AP) (Molecular Probes, Eugene, OR) bound to wild-type or mutant HFABP were determined by using quininesulfate in 0.1 N H_2SO_4 as the reference fluorophore, with $Q_{ref} = 0.7$ (Scott et al., 1970). Excitation was at 352 nm for quininesulfate and 383 nm for 2AP. Circular dichroism (CD) spectra were measured at 25 °C on an Aviv Model 60DS spectropolarimeter using a 0.1 cm path length quartz cuvette (Hellma). Each spectrum was obtained from seven scans between 195 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. (1986).

Binding of palmitate (Nu-Chek Prep) to wild-type and mutant HFABPs was analyzed by a recently described method employing the fluorescent probe ADIFAB (Molecular Probes) (Richieri et al., 1994). This method allows for the direct measurement of unbound fatty acid in equilibrium with the HFABP. Palmitate, prepared as a 200 μM stock solution of the sodium salt in H_20 containing 50 μg/mL BHT, was titrated into 2.5 mL of 10 mM HEPES, 150 mM NaCl, 5 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4) containing 0.2 μM ADIFAB and 2 μM HFABP. 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*(em505/em432) values were determined. This average was applied to binding equilibrium analyses using standard values of $R_0 = 0.353$ and $R_{max} = 11.5$ (Richieri et al., 1994). Experimental values were fitted to a single-site Scatchard analysis, and K_d values for palmitate binding were obtained. Binding of 2AP to native, acetylated, and mutant proteins was as described by Xu et al. (1991).

Vesicle Preparation. Small unilamallar vesicles (SUVs) were prepared by the method of Huang and Thompson (1974) as described previously (Storch & Kleinfeld, 1986). Typically, vesicles were composed of 90 mol % of egg phosphatidylcholine (EPC) (Avanti Polar Lipids) and 10 mol % of the negatively charged *N*-(7-nitrobenz-2-oxa-1,3-diazolyl)phosphatidylethanolamine (NBD-PE) (Avanti Polar Lipids) (Chattopadhyay & London, 1988). To increase the negative charge density of the acceptor vesicles, either 25 mol % of brain phosphatidylserine (PS) (Avanti Polar Lipids) or 25 mol % of cardiolipin (CL) (Avanti Polar Lipids) was incorporated into the SUVs in place of an equimolar amount of EPC. For studies utilizing zwitterionic SUVs, vesicles were composed of 90 mol % of EPC and 10 mol % of 2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-HPC) (Avanti Polar Lipids). Phospholipid concentrations were determined by quantification of total inorganic phosphate (Gomori, 1942).

Transfer of 2AP from HFABP to Membranes. The rate of transfer of 2-(anthroyloxy)palmitate (2AP) from wild-type, acetylated, and mutant HFABPs to SUV membranes was determined by using a resonance energy transfer assay as detailed previously (Wootan et al., 1993; Storch & Bass, 1990). Transfer was monitored at 25 °C in 10 mM Tris and 150 mM NaCl (pH 7.4). For studies using CL-containing SUVs, the buffer contained 1 mM EDTA. Final assay concentrations were 10 μM HFABP, 1 μM 2AP, and 100 μM acceptor vesicles unless otherwise specified. For studies requiring increased ionic strength, NaCl was added to the SUV and protein samples prior to mixing. The decrease in fluorescence upon mixing of the 2AP−HFABP complex with acceptor SUVs was monitored with a DX-17MV stopped-flow spectrofluorometer (Applied Photophysics) interfaced with an Acorn A5000 computer. The excitation wavelength was 383 nm, and emission was monitored using a 408 nm cutoff filter (Oriel Corporation). The data were analyzed by fitting the Marquant−Levenberg nonlinear regression algorithm, and all curves were well fit by a single exponential with steady state function.

RESULTS

Acetylation of HFABP. We have shown that transfer of fluorescently labeled fatty acid from AFABP and HFABP to membranes occurs during a collisional interaction between the protein and the membrane (Wootan et al., 1993; Kim & Storch 1992a). Recently, we demonstrated that acetylation of lysine residues on AFABP, which in effect masks the surface cationic charges, dramatically alters the rate and mechanism of transfer of anthroyloxy fatty acids to phospholipid membranes. Transfer from acetylated AFABP was significantly slower than transfer from unmodified protein and occurred by a diffusional mechanism involving release of the ligand to the aqueous milieu (Herr et al., 1995). As AFABP and HFABP are hypothesized to transfer fatty acid

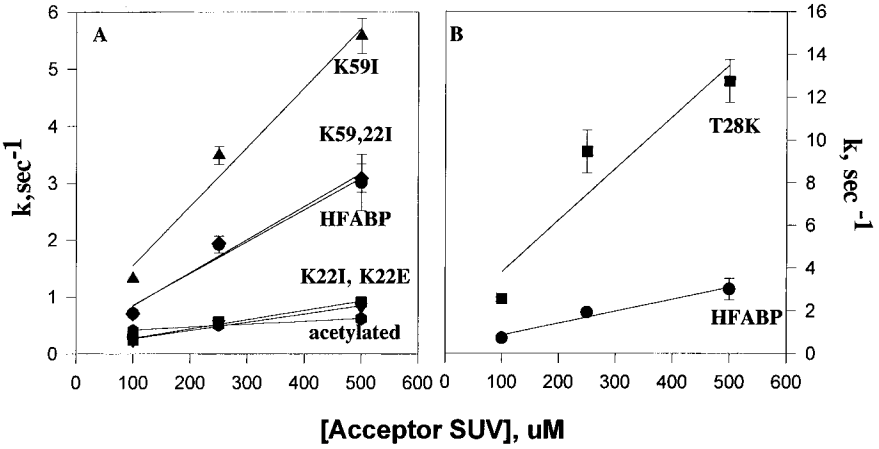

FIGURE 2: Effect of acceptor membrane concentration on the rate of transfer from native and mutant HFABPs. The transfer of 1 μM 2AP from 10 μM protein was monitored as a function of the concentration of acceptor EPC/NBDPE SUV phospholipid. The transfer was measured at 25 °C in 10 mM Tris and 150 mM NaCl (pH 7.4) using the resonance energy transfer assay described under Experimental Procedures. The proteins examined were (A) native HFABP (●) (and B), acetylated HFABP (⬢), pEX-K22I-HFABP (■), pEX-K59I-HFABP (▲), pEX-K22E-HFABP (▼), pEX-K59,22I-HFABP (◆); (B) pEX-T28K-HFABP (■). Averages from at least four separate determinations ± SEM are shown.

via a similar mechanism (Wootan & Storch, 1994), we directly examined the effect of neutralization of lysine residues on the transfer of fatty acid from HFABP to membranes. Circular dichroism spectroscopy indicated that acetylation of HFABP did not alter the β-barrel structure of the binding protein (θ_{222} native = −7620 deg M^{-1} cm^{-1}; θ_{222} acetylated = −7900 deg M^{-1} cm^{-1}), maintaining 67−70% β-sheet and approximately 20% α-helix, in agreement with the structure of human HFABP determined by X-ray crystallography (Zanotti et al., 1992). In addition, the acetylation procedure did not affect the binding affinity of the protein for fluorescently labeled 2-(9-anthroyloxy)palmitic acid (K_d native = 0.32 ± 0.09 μM; K_d acetylated = 0.33 ± 0.08 μM).

Effect of Acetylation on 2AP Transfer to Membranes. Transfer of 2AP from HFABP to phospholipid vesicles is thought to occur via direct collisions of protein with acceptor phospholipid membranes. This mechanism can be demonstrated experimentally by increasing the concentration of acceptor vesicles, effectively increasing the number of theoretical collisions between protein and membrane and resulting in an increased rate of transfer. Indeed, as shown previously (Kim & Storch, 1992a), the rate of 2AP transfer from native HFABP increased proportionally with an increase in the concentration of acceptor vesicles (Figure 2A). Acetylation of HFABP resulted in a 2−5-fold decrease in the rate of transfer, depending on the protein:membrane ratio examined (Figure 2A). Transfer from acetylated HFABP increased only minimally as a function of acceptor concentration and was no longer directly proportional to the membrane:protein ratio. We have previously shown that 2AP transfer from HFABP to membranes increases when the overall negative charge density of the membranes is increased by the incorporation of anionic phospholipids such as phosphatidylserine (PS) into acceptor SUVs (Wootan & Storch, 1994). As expected, in the present studies the rate of 2AP transfer from native protein increased from 0.61 ± 0.06 s^{-1} to EPC/NBDPE vesicles to 1.23 ± 0.10 s^{-1} to EPC vesicles containing 25 mol % of phosphatidylserine. In contrast, no increase in transfer rate was found for acetylated HFABP (0.37 ± 0.006 s^{-1} to EPC/NBDPE versus 0.34 ± 0.11 s^{-1} to EPC/PS/NBDPE vesicles), consistent with what was observed for acetylated AFABP (Herr et al., 1995).

Table 1: Physical and Binding Parameters of Wild-Type and Mutant HFABP[a]

	Q_f	θ_{222} (deg M^{-1} cm^{-1})	app K_d[b] (nM)	app K_d[c] (μM)
pEX-HFABP	0.27 ± 0.03	−9569	10 ± 2	0.32 ± 0.09
pEX-K22I	0.29 ± 0.04	−8405	10 ± 4	0.18 ± 0.03
pEX-K59I	0.30 ± 0.04	−9420	9 ± 1	0.28 ± 0.03
pEX-K59,22I	0.29 ± 0.02	−10040	12 ± 3	ND
pEX-K22E	0.32 ± 0.03	−8730	8 ± 2	ND
pEX-K10I	0.30 ± 0.03	−8728	ND	ND
pEX-K113I	0.32 ± 0.07	−9300	ND	ND
pEX-T28K	0.34 ± 0.04	−10265	42 ± 10	ND

[a] Expressed wild type and mutant HFABP were analysed for fluorescent quantum yield (Q_f)(n=3), circular dichroism spectroscopic properties (θ_{222}) and apparent ligand dissociation constants (app K_d) as outlined under "Experimental Procedures". Ligand binding was assessed using the following two methods. [b] ADIFAB binding analysis (n=3). [c] 2AP binding determinations (n=2), as described. N. D. = not determined.

Mutagenesis and Comparison of Physical Properties with Native HFABP. Results with acetylated HFABP implied that surface lysine residues on HFABP were involved in electrostatic interactions between protein and membranes. Mutagenesis studies were then undertaken to locate specific regions of the protein that may be responsible for this interaction. Regions of the protein of interest included those near the hypothesized portal ligand entry/exit region and the amino acid residues in the helix−turn−helix and β2-turn (Figure 1). A series of lysine mutants in these areas was prepared by the method of Eckstein (Sayers et al., 1992) (Table 1) and subcloned into the pET expression system to obtain high yields of protein for analysis. For all mutants generated we examined whether alterations in certain physical characteristics of the protein had occurred. The following methods were utilized to examine HFABP structure and binding: (a) fluorescence quantum yield measurements of bound anthroyloxy fatty acid; (b) circular dichroic spectroscopy; and (c) ligand binding studies.

Fluorescence quantum yields (Q_f) are often indicative of the hydrophobicity of the environment surrounding a fluorophore. Quantum yields of bound anthroyloxy fatty acids differ between members of the FABP family, indicating

relative differences in the physicochemical properties of their binding sites (Storch et al., 1989; Storch 1990, 1993; Wootan et al., 1990). Here we measured 2AP Q_f values for native and mutant HFABPs to determine whether mutagenesis of HFABP had altered the dielectric environment of its binding pocket. Virtually no differences were found in the Q_f values for native and mutant proteins (Table 1), demonstrating that the hydrophobic environment of the bound fatty acid remained unchanged with mutagenesis. The integrity of the binding environment with mutagenesis was not surprising, as X-ray crystallographic data indicate that the lysine residues of HFABP are not in the vicinity of the binding cavity (Zanotti et al., 1992).

For each mutant, the circular dichroic spectrum in buffer was similar to that of the native protein. Values of the molar ellipticity at 222 nm, θ_{222}, for native HFABP and mutant proteins did not vary significantly (Table 1). When the experimental spectra were analyzed by using the reference spectra of Yang et al. (1986), all of the proteins displayed 64−70% β-conformation, in agreement with the known tertiary structure of the protein (Zanotti et al., 1992).

The binding affinity of native and mutant HFABPs for fatty acids was assessed by two separate methods. The first involved the fluorescent probe ADIFAB (acrylodated intestinal fatty acid binding protein). This probe allows for the examination of binding characteristics of native fatty acids rather than fluorescently labeled compounds. The emission spectrum of the acrylodan fluorophore in ADIFAB undergoes a red shift when ADIFAB is bound to fatty acid, and this probe therefore can be used to determine the concentration of unbound fatty acid (FFA) in a solution. In addition, the sensitivity of the probe is such that it allows for the determination of FFA levels without perturbing the binding equilibrium between bound and free ligand (Richieri et al., 1992). Given the known K_d of ADIFAB for a particular ligand (Richieri et al., 1994), equilibrium binding properties can be determined for an unknown HFABP. The K_d of 10 ± 2 nM obtained here for wild-type rat HFABP is in agreement with previously determined values (Richieri et al., 1994). The series of proteins demonstrated apparent dissociation constants for palmitate of 8−12 nM (Table 1). The only mutant protein with a dissociation constant significantly different from that of the native was pEX-T28K-HFABP (42 ± 10 versus 10 ± 2 nM, $0.02 < p < 0.01$).

A second method of binding analysis was employed that used the fluorescently labeled fatty acid 2AP. The fluorescence of the anthroyloxy moiety increases markedly when bound in the hydrophobic environment of the ligand binding domain (Storch et al., 1989; Wootan et al., 1990). 2AP bound to native and mutant HFABPs in a saturable manner, and Scatchard analysis indicated no significant differences in apparent binding affinity for the proteins examined (Table 1). Comparison of the absolute K_d values obtained from the two binding analyses reflects the disparity that exists in the literature regarding fatty acid equilibrium binding constants of FABPs (Richieri et al., 1994; Maatman et al., 1994). However, for the purpose here, both methods clearly demonstrate little to no difference in fatty acid binding affinity between native and all but one of the mutant proteins examined.

Effect of Lysine Mutations on the 2AP Transfer Rate to Phospholipid Membranes. Having established that mutagenesis did not markedly alter conformational or binding characteristics of the mutant proteins, we next examined their relative AOFA transfer properties. Transfer of 2AP from HFABP is hypothesized to occur via a direct collisional mechanism, wherein effective interactions between protein and membranes determine the rate of ligand transfer (Kim & Storch, 1992a; Wootan & Storch, 1994). As mentioned, a distinction of such collisional mechanisms is the increase in transfer rate with an increase in the number of acceptor phospholipid vesicles. Here, transfer of 2AP from native and mutant HFABPs increased to varying degrees with an increase in acceptor membrane concentration (Figure 2). Transfer from native, pEX-K59I-HFABP, and pEX-K59,22I-HFABP increased linearly as a function of vesicle concentration, while the increase in transfer rate from pEX-K22I-HFABP and pEX-K22E-HFABP was not directly proportional to the membrane level. In addition, the absolute rates of transfer differed among the proteins. Transfer of 2AP from 10 μM native HFABP to 100 μM acceptor SUVs was 0.71 ± 0.05 s^{-1}, which is consistent with published data for HFABP isolated from rat heart (Kim & Storch, 1992a). Neutralization of the lysine positioned at residue 113 on β-strand I, pEX-K113I-HFABP, and residue 10 on β-strand A, pEX-K10I-HFABP, resulted in 2AP transfer rates identical to that of native (data not shown), and these mutations were therefore considered null.

Changing the residue at position 22 from the positively charged lysine to the neutral isoleucine, pEX-K22I-HFABP, resulted in a rate of fatty acid transfer approximately 3-fold slower (0.25 ± 0.06 s^{-1}) than that of wild type, while neutralization of the lysine residue at position 59, pEX-K59I-HFABP, resulted in a rate of transfer 2-fold greater (1.32 ± 0.02 s^{-1}). When a double mutant incorporating both changes was examined, pEX-K59,22I-HFABP, the transfer rate returned to that obtained for the native protein, 0.71 ± 0.04 s^{-1}. Results with this double mutant suggest that at least two distinct regions of HFABP, helix α-I, which contains K22, and β2-turn, which contains K59, are involved in determining the absolute rate of transfer of ligand, and differences in rate between the two regions appear additive. Changing the net charge at the 22 position of α-I from positive to negative via substitution of glutamic acid for lysine, pEX-K22E-HFABP, did not further alter the transfer rate from that seen for the K22I neutral mutant, 0.24 ± 0.01 s^{-1} (Figure 2A). Finally, the addition of a positive charge to the α-II strand of HFABP by substitution of threonine at position 28 with lysine, pEX-T28K-HFABP, resulted in transfer rates a minimum of 4-fold greater than those seen for the native protein (2.6 ± 0.1 s^{-1} at the lowest protein: membrane ratio examined) (Figure 2B). This T28K mutation results in an α-II domain of HFABP that more closely resembles the AFABP α-II region.

Effect of Lysine Mutations on Transfer to Membranes of Negative Charge. We have previously found that 2AP transfer from HFABP is faster to acceptor membranes containing 25 mol % of the negatively charged phospholipids phosphatidylserine and cardiolipin (EPC/PS or CL/NBDPE, 65:25:10) relative to EPC/NBDPE (90:10) membranes (Wootan & Storch, 1994). As expected, the transfer rate from native HFABP increased with the incorporation of increasing membrane negative charge (Figure 3). Transfer from pEX-K59I-HFABP and pEX-T28K-HFABP was affected similarly. Transfer from the pEX-T28K-HFABP mutant protein appeared more sensitive to the increased

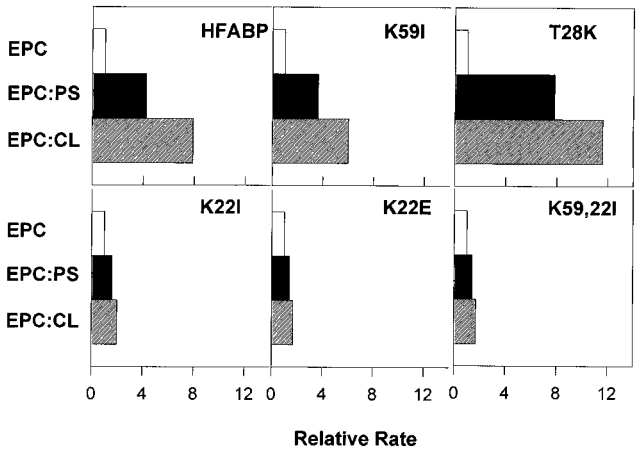

FIGURE 3: 2AP transfer from native and mutant HFABPs to negatively charged SUVs. Transfer of 1 μM 2AP from 10 μM protein to 100 μM acceptor EPC:NBDPE SUV (EPC) or EPC:NBDPE SUV containing 25 mol % of phosphatidylserine (EPC:PS) or cardiolipin (EPC:CL) was monitored as described for Figure 2. Results are the average of at least three separate experiments, and the rates are expressed relative to the transfer rate of 2AP from each individual HFABP to EPC:NBDPE vesicles. Variability was <7% for all conditions.

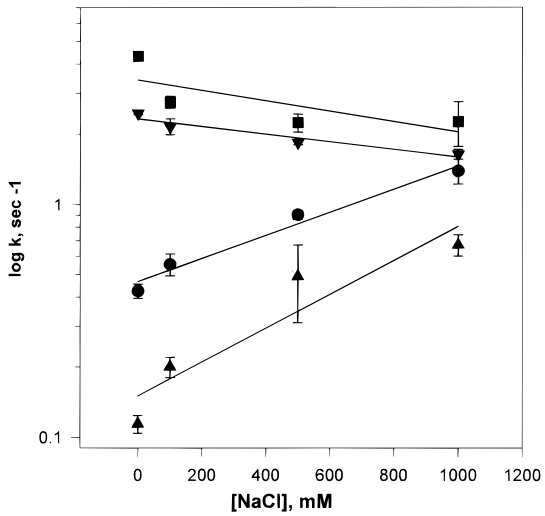

FIGURE 4: Effect of ionic strength on 2AP transfer rate. Transfer of 1 μM 2AP from 10 μM AFABP (■), HFABP (●), pEX-T28K-HFABP (▼) or pEX-K22E-HFABP (▲) to 100 μM EPC/NBDPE SUV was measured as a function of increasing NaCl concentration. All conditions are as described for Figure 2. Averages from three separate determinations ± SEM are shown.

negative charges in comparison to native HFABP. In distinct contrast, despite the above-mentioned difference in absolute rates of 2AP transfer, mutations that involved neutralization or alteration of lysine 22 of HFABP yielded proteins that did not respond to the charge characteristics of the membranes (Figure 3, lower panels).

Separate experiments compared the negatively charged EPC/NBDPE vesicles (effective charge of 10 mol %) with zwitterionic SUVs (EPC/NBD-HPC), and the results were quite similar. 2AP transfer from wild-type HFABP to the negatively charged EPC/NBDPE vesicles was 2-fold faster than transfer to the zwitterionic SUVs. Moreover, in agreement with the results in Figure 3, the transfer rate of 2AP from pEX-K59I-HFABP and pEX-T28K-HFABP increased by 2-fold in the presence of 10 mol % of negative phospholipid, whereas transfer from pEX-K22I-HFABP was not affected (data not shown).

Effect of Ionic Strength. Rates of fatty acid transfer from HFABP and AFABP, both thought to transfer ligand via collisional interactions with membranes, are affected differently by increasing the ionic strength of the buffer (Wootan et al., 1993; Kim & Storch, 1992a). Transfer rates from rat HFABP increase logarithmically with increasing ionic strength (Kim & Storch, 1992a), while transfer from rat AFABP is not affected by increases in ionic strength (Wootan et al., 1993). The effect of ionic strength on the fatty acid transfer rates from pEX-HFABP, pEX-AFABP, pEX-K22E-HFABP, and pEX-T28K-HFABP was assessed by measuring the rate of 2AP transfer to EPC/NBDPE membranes in the presence of increasing concentrations of NaCl (Figure 4). As expected (Kim & Storch, 1992a), transfer from pEX-HFABP increased with increasing ionic strength. This was also observed for transfer from the mutant pEX-K22E-HFABP (Figure 4) and other remaining members of the mutant series (data not shown), with the exception of pEX-T28K-HFABP. The transfer rate of 2AP from pEX-AFABP was minimally affected by the ionic strength of the buffer (Figure 4), in agreement with previous results. Interestingly, the pEX-T28K-HFABP mutant behaved identically to pEX-AFABP and markedly differed from pEX-HFABP and the other members of the mutant series, in response to changes in ionic strength.

DISCUSSION

The functions of members of the FABP family in vivo remain unclear. Our laboratory has elucidated distinct mechanisms by which different members of this protein family transfer fatty acid to phospholipid membranes in vitro. We hypothesize that these mechanisms may play relevant physiological roles in the intracellular trafficking and metabolism of fatty acid. In contrast to LFABP, which releases ligand to the aqueous milieu prior to their insertion into membranes, AFABP and HFABP appear to utilize direct effective collisions with phospholipid membranes to transfer fatty acids (Kim & Storch, 1992a,b; Wootan et al., 1993). Previous studies of HFABP and AFABP suggested that ionic interactions between charged residues on the protein surface and membrane phospholipids may govern these collisional interactions (Wootan & Storch, 1994). Here, we examined both chemically modified HFABP and site specific mutants of HFABP to elucidate regions of the protein involved in collisional interaction with membranes. Circular dichroism, fluorescent quantum yield, and ligand binding analyses demonstrated that neither the acetylation procedure nor the targeted mutagenesis imparted significant changes to the gross structure of the protein or to its ligand binding characteristics (Table 1). This structural stability was not unexpected as the FABP β-barrel structure seems to be stable to modification and mutagenesis. Indeed, Frieden et al. (1995) recently reported that incorporation of bulky fluorescein moieties throughout the structure of IFABP did not alter folding of the protein, indicating that the protein can accommodate major perturbations and still fold into its stable conformation.

In the present study, it was found that any mutation or modification that involved lysine 22 resulted in a virtual absence of HFABP sensitivity to membrane surface charge, such that the rate of AOFA transfer from these proteins was not regulated by the charge composition of the acceptor

vesicles. In contrast, wild-type and other mutant HFABPs that contained the K22 residue all demonstrated a concomitant increase in transfer rate with an increase in the negative charge density of acceptor membranes, similar to that described previously for native HFABP (Wootan & Storch, 1994). The K22 residue lies within a short 8-amino acid α-helix, α-I, which helps to cap the ligand binding cavity of HFABP. Importantly, α-I appears as an amphipathic helix (Zanotti et al., 1992), a unique structural motif known to be involved in protein−lipid interaction via its ability to orient proteins at the polar/nonpolar interface of a membrane. In HFABP, K22 is the only positively charged residue on the hydrophilic, exterior face of the α-I helix. The significance of amphipathic helices in protein targeting to membranes has been studied extensively. These helices are involved in membrane binding by cholesteryl ester transfer protein (Wang et al., 1995) and CTP:phosphocholine cytidyltransferase (Craig et al., 1994), lipid associations with apolipoproteins, and the targeting of mitochondrial proteins, among other interactions [for recent reviews, see Anantharamaiah et al. (1993) and Roise (1993)]. In many instances, the charge characteristics and distribution of positively charged lysine residues on the helix influence electrostatic interactions with membranes either by slightly altering the helical content of the region or by altering its relative hydrophilicity. On the basis of the transfer results obtained with K22-HFABP mutants, it appears that K22 on the amphipathic α-I helix of HFABP is necessary for the formation of an effective electrostatic interaction between HFABP and membrane acidic phospholipids. A comparison of the two related proteins, AFABP and HFABP, both of which transfer ligand via collisional interactions with membrane, shows 75% sequence homology in the α-I helical region, which is somewhat higher than that found overall (64% homology). AFABP has a singular lysine residue in the same location on its amphipathic α-I, and studies are presently underway to examine whether this specific lysine directs ionic interactions between AFABP and membranes. The tertiary structure of LFABP, which does not transfer ligand via collisions with membranes, is not yet known; however, it has only 37% similarity with HFABP.

Mutation in the second α-helix of HFABP (Figure 1) also altered the ligand transfer properties of the protein. The substitution of a basic lysine residue for the polar threonine residue at position 28 of HFABP, pEX-T28K-HFABP, resulted in transfer characteristics significantly different from those of the wild type. The faster rate of 2AP transfer (Figure 2B) and its relative insensitivity to the ionic strength of the medium (Figure 4) are results similar to those obtained for AOFA transfer from AFABP (Wootan et al., 1993). In addition, the binding affinity of pEX-T28K-HFABP for palmitate, as determined with the ADIFAB assay, was lower than that of wild-type HFABP (Table 1) and approaches the value reported for AFABP (Richieri et al., 1994). Interestingly, sequence and structural data show that the AFABP α-II domain has a unique lysine residue at position 30 (Xu et al., 1992). This residue is one of only two sequence dissimilarities in α-II between HFABP and AFABP, whereas there is no sequence similarity at all between HFABP and the putative α-II region of LFABP. AOFA transfer data from the T28K-HFABP mutant again demonstrate that charge characteristics of the helix−turn−helix segment of HFABP regulate, in part, the rate of collisional transfer of ligand to phospholipid membranes. In the case of α-II, the presence of specific basic residues may explain unique differences in transfer and binding properties observed between HFABP and AFABP.

The AOFA transfer rate from all mutant proteins increased as a function of acceptor membrane concentration. This suggests that transfer of ligand from mutant HFABP still occurred during collisional interactions of the protein with the acceptor membrane. Nevertheless, for pEX-K22I-HFABP and pEX-K22E-HFABP, the increase in the rate of 2AP transfer was not directly proportional to the increase in the acceptor vesicle concentration. The response of these proteins to SUV concentration was dampened by approximately 25% relative to the response of wild-type HFABP and all other mutants. Moreover, the transfer rates from these two modified proteins were nearly identical. It is possible that these modifications result in the alteration of structural elements, which define the effectiveness of a particular collisional interaction, by altering the orientation of the protein with respect to the membrane bilayer. The decrease in the linearity of ligand transfer rate with acceptor concentration may also indicate some alteration in the lifetime of a particular protein conformation, such that the formation of effective collisional complexes occurs to a somewhat lesser extent. For example, K22 forms a hydrogen bond with glycine 25 in human HFABP (Zanotti et al., 1992), and the loss of this bond through mutagenesis or modification may impart a change in the helical content, which decreases the fraction of collisions during which FA transfer can occur. However, the observation that the 2AP transfer rate from the double mutant, pEX-K59,22I-HFABP, was indeed linear with respect to acceptor concentration and occured at a rate identical to that of wild-type protein indicates that effective collisional interactions are likely to be governed by more than one protein domain. Thus, if more than one region of the HFABP surface can interact with membranes, perhaps resulting in the formation of membrane−protein−membrane complexes, then alteration in one of these surface domains could dampen the response of ligand transfer rate to membrane concentration. Recent observations for AFABP suggest that the wild-type protein forms stable complexes with membranes, and the nature and stoichiometry of this interaction is under investigation (E. R. Smith and J. Storch, unpublished observations). Of interest is the fact that the 2AP transfer rate from acetylated HFABP was almost completely unaffected by acceptor membrane concentration, indicating that this global alteration in protein charge almost completely eliminates the domains required for collisional fatty acid transfer. Further mutant HFABPs are being studied to elucidate the regions and/or structural features of the protein necessary for these effective collisions.

The collisional interaction between binding protein and membrane is not likely to be dependent on only one residue or domain of the protein. The site specific mutations on β2-turn, helix α-I, and helix α-II of HFABP all altered the absolute rate of transfer of ligand, whereas mutations on β-strands A and I did not. A comparison of the transfer rate for the double mutant K59,22I with the rates for the single mutants K59I and K22I illustrates that two distinct regions, in this case β2-turn and helix α-I, can have additive effects on ligand transfer rates. The high sequence homology

between the helical regions of AFABP and HFABP and the poor homology with LFABP suggest that these helices may be key for electrostatic interaction between protein and membranes. At this point, the molecular dynamics of the collisional transfer mechanism is wholly speculative, but may involve a multiphase docking of the protein with the membrane. The first phase may be governed by electrostatic interactions involving the helical lid of the protein with specific membrane components, which may then be followed by a repositioning involving the β2-region and the resultant hinged opening of the portal to release ligand. Such transient conformational flexibility in the helical cap region of the protein is supported by both crystallographic and solution structure analyses (LaLonde et al., 1994; Lucke et al., 1992).

The effects of alterations in ionic strength underscore the multifaceted nature of the putative collisional complex. The previously observed decrease in AOFA transfer rate from LFABP, which transfers FA via an aqueous diffusion mechanism, likely results from decreased AOFA solubility in the bulk aqueous phase as the salt concentration is increased (Kim & Storch, 1992b). It might also be expected that electrostatic interactions would be disrupted by high ionic strength and, hence, that the rate of collisional AOFA transfer from HFABP would decrease as a function of salt concentration as well. We find, however, that the AOFA transfer rate from HFABP and all of the K22 mutants actually increases with salt concentration and that this increase is observed using membranes of net negative charge. In addition, the identical AOFA transfer rates for the K22I and K22E-HFABP proteins indicate that the introduction of a negative charge has no different effect on transfer than does charge neutralization at this residue. Furthermore, collisional transfer from HFABP to zwitterionic membranes occurs, albeit at a slower rate than transfer to acidic membranes. Collectively these results suggest that forces in addition to those arising from electrostatic interactions are likely to play a role in the fatty acid transfer process (Wootan et al., 1994; Herr et al., 1995).

Cellular membranes are remarkably heterogeneous entities. The lipid compositions of intracellular organelles differ from one another with respect to the relative proportion of lipid species, the assymmetry observed between leaflets of the bilayer, and possibly the presence of lipid domains. In some instances, a particular lipid occurs only in a specific biological membrane. For example, the anionic phospholipid cardiolipin is found in high abundance only in mitochondrial membranes. Cardiolipin is thought to be vital for many mitochondrial membrane-bound enzymes, and its composition closely correlates with fatty acid β-oxidation levels (Berger et al., 1993; Daum, 1985). The results with HFABP and its mutants indicate that the presence of cardiolipin on the mitochondrial outer membrane (Hovius et al., 1990) may help facilitate specific and directed transfer of cellular fatty acids from HFABP to mitochondria via directed ionic interactions between protein and membranes. Other membrane lipid or acidic protein components may also be involved in the collisional transfer of fatty acid from FABPs to particular organelles. The observations here regarding the role of the helical domains of the binding protein in the electrostatic interactions between protein and membranes have provided the framework for ongoing analysis aimed at elucidating additional structural features of FABPs involved in directing intracellular fatty acid trafficking.

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