Role of the Helical Domain in Fatty Acid Transfer from Adipocyte and Heart Fatty Acid-binding Proteins to Membranes

ANALYSIS OF CHIMERIC PROTEINS*

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The adipocyte and heart fatty acid-binding proteins (A- and HFABP) are members of a lipid-binding protein family with a β-barrel body capped by a small helix-turn-helix motif. Both proteins are hypothesized to transport fatty acid (FA) to phospholipid membranes through a collisional process. Previously, we suggested that the helical domain is particularly important for the electrostatic interactions involved in this transfer mechanism (Herr, F. M., Aron, J., and Storch, J. (1996) Biochemistry 35, 1296–1303; and Liou, H.-L., and Storch, J. (2001) Biochemistry 40, 6475–6485). Despite their using qualitatively similar FA transfer mechanisms, differences in absolute transfer rates as well as regulation of transfer from AFABP versus HFABP, prompted us to consider the structural determinants that underlie these functional disparities. To determine the specific elements underlying the functional differences between AFABP and HFABP in FA transfer, two pairs of chimeric proteins were generated. The first and second pairs had the entire helical domain and the first α-helix exchanged between A- and HFABP, respectively. The transfer rates of anthroyloxy-labeled fatty acid from proteins to small unilamellar vesicles were compared with the wild type AFABP and HFABP. The results suggest that the αII-helix is important in determining the absolute FA transfer rates. Furthermore, the αI-helix appears to be particularly important in regulating protein sensitivity to the negative charge of membranes. The αI-helix of HFABP and the αII-helix of AFABP increased the sensitivity to anionic vesicles; the αI-helix of AFABP and αII-helix of HFABP decreased the sensitivity. The differential sensitivities to negative charge, as well as differential absolute rates of FA transfer, may help these two proteins to function uniquely in their respective cell types.

Fatty acids (FA)1 are major substrates for the synthesis of complex lipids and for energy production. Due to their limited solubility, specific carriers, known as fatty acid-binding proteins (FABP), are expressed in various tissues that use FA. Structural analyses of several FABPs have revealed markedly similar three-dimensional folds consisting of ten antiparallel β-strands that form a β-barrel, which is capped by two short α-helices arranged as a helix-turn-helix segment (1–4). It is believed this α-helical domain, along with the β C-D and D-E turns, functions as a “dynamic portal” that regulates FA entry and exit from the internal lipid binding cavity (5–7).

Heart FABP (HFABP) and adipocyte FABP (AFABP) are homologous proteins sharing >60% amino acid sequence identity (8). Both proteins bind one FA in the binding pocket (1, 2), although x-ray crystallographic studies show that the FA adopts an entirely different conformation in their respective binding sites (9, 10). AFABP has generally lower binding affinities for FA than HFABP (11), and it appears that HFABP shows greater affinity for saturated versus unsaturated fatty acids, whereas AFABP does not show such a preference (9). A- and HFABP also exhibit unique patterns of tissue distribution. AFABP is found in adipose tissue and monocytes/macrophages (12, 13), whereas HFABP has a wider distribution, being found in muscle and numerous other tissues (14, 15). Furthermore, HFABP has been suggested to be associated with FA β-oxidation in heart and skeletal muscle (16–18), whereas AFABP may be involved in triacylglycerol storage and lipolysis (19). Thus, these two proteins are hypothesized to have distinct physiological functions. A- and HFABP were found in cardiac muscle cell in Antarctic Teleost fish and in different cell types of bovine mammary gland, suggesting further that the two proteins have specialized functions in the metabolism of fatty acids (20, 21).

The potential role of FABPs in FA trafficking has been investigated in vitro using fluorescent anthroyloxy-labeled fatty acid (AOFA) and a resonance energy transfer assay (22–25). AOFA transfer from both A- and HFABP to membranes appears to occur through a collisional process. The rates of FA transfer were increased by the incorporation of anionic phospholipids, decreased by chemical neutralization of lysines of fatty acid-binding protein; AOFA, anthroyloxy-labeled fatty acid; 2-AP, 2-(9-anthroyloxy)palmitic acid; EPC, egg phosphatidylcholine; PS, phosphatidylserine; CL, cardioplin; NBDPC, 1-palmitoyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine; ADIPAB, acylated-intestinal fatty acid-binding protein; WT, wild type; αA/βH-FABP, chimeric protein containing αI+II+III helices from AFABP and β-barrel from HFABP; αH/αA-FABP, chimeric containing αI+II+III helices from HFABP and β-barrel from AFABP; αI/αH-FABP, chimeric containing αI+II+III helices from AFABP and β-barrel from HFABP; αH/αA-FABP, chimeric containing αI+II+III helices from AFABP and β-barrel from HFABP; I-FABP, intestinal FABP; SUV, small unilamellar vesicle; r.m.s.d., root mean square difference.

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FABP, and decreased by site-directed mutagenesis of specific lysines (23, 26–28). Thus, electrostatic interactions between surface lysine residues and negatively charged membrane lipids were suggested to be involved in the collisional FA transfer process (26–28). Recently, Fourier transform infrared spectroscopy, equilibrium binding studies, and competitive binding assays have also demonstrated direct interactions of AFABP with anionic phospholipid membranes (29, 30). Despite their sharing the same transfer mechanism, however, AFABP and HFABP have several distinct transfer properties. For example, AFABP consistently has approximately 10-fold faster AOFA transfer from HFABP to vesicles, whereas no change in rate from AFABP is observed (31).

Lysine (K) to isoleucine (I) mutagenesis of specific lysines in the portal region have shown distinct effects for each protein on FA transfer (27, 28). A unique lysine (Lys-32) found on the α-II-helix of AFABP but not present in HFABP was shown to be an important determinant of the rate of FA transfer. Furthermore, a K591 mutation on the β-C-D turn of each protein resulted in opposite effects: A decrease in rates from AFABP to neutral phospholipid vesicles whereas a 2-fold increase was found in AOFA transfer from HFABP to vesicles, whereas no change in rate from AFABP is observed (31).

The aim of this study was to examine and compare the structural components or regions that are responsible for the differences in FA transfer properties between α- and HFABP. We generated two pairs of chimeric proteins. In one pair, the complete α-helical region, including the α-II strand, α-II helix, and α-II helix (residues 1–36) of the proteins were switched. In the other pair, the β-A strand plus the first α-helix (amino acids 1–25) were switched between α- and HFABP. Due to the somewhat different sensitivity to membrane-negative charge of the α-helice lysines of α- and HFABP (27, 28), and the different amino acid sequence of the α-helices in the A- and HFABP, we also constructed point mutant E23S-AFABP. This particular mutation makes the hydrophilic side of the amphipathic α-helix of AFABP resemble that of HFABP. The results demonstrate that the entire helix-turn-helix motif is important in determining the absolute rate of FA transfer from protein to membranes. The α-II-helix, in particular, may be the major structural element involved in the rate of FABP interactions with acceptor membranes. Furthermore, the α-I-helices of the two proteins were shown to have different sensitivities to membrane-negative charge, which may help these proteins function uniquely in FA transfer.

**EXPERIMENTAL PROCEDURES**

**Materials—**Vector pBluescript II was from Stratagene (La Jolla, CA). Vectors pET-11a and -11d and competent Escherichia coli BL21(DE3) cells were from Novagen (Milwaukee, WI). Restriction enzymes BstI, BamHI, DraIII, NcoI, XbaI, XhoI, and dNTP were from Promega (Madison, WI). VTF polymerase was from New England BioLabs, Inc. (Beverly, MA). Primers carrying mutations were from Midland Certified Reagents (Midland, TX). Isopropyl-1-thio-β-D-galactoside was from Fisher (Fairlawn, NJ). The fluorescent fatty acid 2-(9-anthroyloxy)palmitate (2-AP) and ADIFAB were from Molecular Probes, Inc. (Eugene, OR). Egg PC (EPC), 1-palmitoyl-2-12-(7-nitro-2,1,3-benzoxadiazol-4-y)aminododecanoyl-sn-glycero-3-phosphocholine, brain phosphatidylserine (PS), and bovine heart cardiolipin (CL) were from Avanti Polar Lipids (Alabaster, AL). All other reagents were of the highest grade available. Vectors pET-AFABP and pET-HFABP for the respective wild type A- and HFABP expressions were provided by Dr. Alan Kleinfeld (Medical Biology Institute, La Jolla, CA).

**Construction of Mutant Genes—**Site-directed and chimeric mutant cDNAs were constructed. The numbering of amino acid residues was with the first methionine as Met-1. Oligonucleotide primers bearing mismatches, indicated by underlining, are listed in Table I. The resultant mutants with various structural elements are listed in Table II. The incorporation of the mutation was confirmed by sequence analysis (32). The AFABP point mutant having a glutamic acid 23 to serine mutation (E23S-AFABP) was generated using specific and outermost primers by overlapping PCR (38). A unique BstI site exists in the cDNA of AFABP (residue 37 next to the α-II-helix end). By ligating different pieces, the α-II-helical region chimeric mutants αII/βH-FABP and αII/αA-FABP were constructed to contain residues 1–37 of AFABP plus 38–133 of HFABP, and 1–36 of HFABP plus 37–132 of AFABP, respectively. To examine the effect of each helix on the transfer of FA to acceptor membranes, we generated a second pair of chimeric constructs. In this pair, the first 25 amino acids were exchanged between A- and HFABP. The helix-turn-helix motif and fatty acid transfer
did not change the overall protein conformation and/or its fatty acid binding properties. Circular dichroism spectra were measured to verify that there were no overall folding modifications in mutant FABP structures as previously described (28). To check the hydrophobic properties of the internal ligand binding pocket, fluorescence quantum yields (Q) of the fluorescent fatty acid-bound to wild type or mutant FABP were determined with an SLM 8000C fluorescence spectrophotometer as previously described (28). Binding of oleate to wild type A- and HFABP and mutant FABPs was analyzed using the fluorescent probe ADIFAB to obtain the FA binding affinity and stoichiometry (11).

**Veisicle Preparation**—Small unilamellar vesicles (SUV) were prepared by sonication and ultra-centrifugation, according to the method of Huang and Thompson (39). The standard vesicles were prepared to contain 90 mol % of EPC and 10 mol % of NBDFC. Two anionic vesicles were also used; 25 mol % of phosphatidylserine (PS) or cardiolipin (CL) was incorporated into the neutral SUVs in place of an equimolar amount of EPC. The phospholipid concentrations were determined as previously described (30).

**Fluorescent FA Transfer Assay**—The rate of 2-AP transfer from wild type and mutant FABPs to acceptor vesicles was determined using a fluorescence resonance energy transfer assay as detailed previously (28). AOF transfer was monitored at 25 °C. The conditions were developed prior to each experiment such that no photobleaching of FA was observed. Final concentrations in a typical transfer assay were 1 μM AOF in 200 mM acetate at pH 5.0. AOF transfer from protein to membranes was monitored and analyzed as a time-dependent decrease in fluorescence, as described (28). The rates of AOF transfer are represented as means ± S.E. from at least three or more separate sets of experiments.

**Statistical Analysis**—Two-tailed paired t tests were used to analyze the differences among rates of FA transfer from mutant proteins versus their respective wild type FABPs, to various concentrations and compositions of acceptor phospholipid membranes. Results were considered significant at p < 0.05.

## RESULTS

### Homology Modeling of Chimeric Proteins

The solvent-accessible surface areas and volumes are shown in Fig. 1. It is seen that the differences between the chimeras and the wild type proteins are small and comparable to those between the two proteins for which crystallographic structures are known. All the values are well within the range seen for high resolution globular proteins of this size (37).

By way of further illustration, we superimposed the backbone atoms of the human muscle protein (PDB file 1HMS) after removing its oleate and minimizing its energy, onto those of the chimera whose β-body was derived from residue 38–133 of the rat protein (αβ/βH). The superimposition is shown in Fig. 2. The two molecules tracked one another quite closely. The backbone atoms used for the superimposition were the peptide N, Ca, peptide C, and peptide O for all residues. The root mean square difference (r.m.s.d.) for these 1048 atoms is 1.42 Å. Table III shows the r.m.s.d. values for all four chimeras after superimposition onto the protein from which the chimera’s β-body was taken. They are all reasonably small given the number of atoms involved and given the close tracking shown in Fig. 2. For all chimeras, subtle differences from wild type FABP were found in many parts of the tertiary structure, rather than a single domain exhibiting large structural alterations.

### Controls for Structural Integrity

It is critical to ensure that any significant experimental results were due solely to the specific alterations introduced. Several methods were used to examine the physical-chemical properties of mutant FABPs, circular dichroic spectroscopy, AOF transfer, and equilibrium binding of native FA to FABP.

Circular dichroic spectra were measured to check the mean residue ellipticity for α-helical content at 222 nm (θ222) for wild type A- and HFABP and mutant FABPs were approximately −8127 and −8623 deg cm2/ dmol, respectively, representing 19 and 21% of the amino acids in α helices. Furthermore, these two proteins displayed 71 and 75% β-sheet secondary structure, in close agreement with their known tertiary structures (1, 2). Each mutant essentially

| Protein         | Mutation | Domain                      |
|-----------------|----------|-----------------------------|
| Point mutant    | E23S-AFABP | α1-helix                    |
| Chimeric        | α/β-H-FABP (1–37/28–133) | α-β A, α1, II/H-β B-J       |
|                 | α/β-A-FABP (1–36/37/123) | α-H A, α1, IIA-β B-J        |
|                 | α/β-A/FABP (1–25/26/133) | α-H A, α1H-αIIJ-β B-J       |
|                 | α/H-β/A-FABP (1–25/26–132) | H-B A, α1/A-IIJ-β B-J       |

**TABLE II**

### FABP Helix-Turn-Helix Motif and Fatty Acid Transfer

**Homology Modeling of Chimeric Proteins**—The solvent-accessible surface areas and volumes are shown in Fig. 1. It is seen that the differences between the chimeras and the wild type proteins are small and comparable to those between the two proteins for which crystallographic structures are known. All the values are well within the range seen for high resolution globular proteins of this size (37).

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|                 | α/β-A-FABP (1–36/37/123) | α-H A, α1, IIA-β B-J        |
|                 | α/β-A/FABP (1–25/26/133) | α-H A, α1H-αIIJ-β B-J       |
|                 | α/H-β/A-FABP (1–25/26–132) | H-B A, α1/A-IIJ-β B-J       |
preserved the β-barrel and α-helical content of their respective wild type proteins, when spectra were analyzed using the reference spectra of Chang et al. (40) and Yang et al. (41) (Table IV). Overall, the two pairs of chimeric proteins as well as point mutant E23S-AFABP were found to maintain similar secondary structures in comparison with native A- and HFABP (Table IV).

The quantum yield ($Q_\lambda$) of the fluorescent fatty acid analog 2-AP was determined to assess the hydrophobicity of the ligand binding cavity in wild type and mutant proteins, and the values are listed in Table IV. The observed quantum yields provide evidence that the binding site of HFABP is more hydrophobic than that of AFABP, in agreement with previous findings (42). $Q_\lambda$ for native AFABP was 0.28 ± 0.01, and mutant protein E23S-AFABP had a similar $Q_\lambda$ value, 0.29 ± 0.01. The $Q_\lambda$ value of chimeric αH/βA-FABP was 0.32 ± 0.03; although the value was somewhat greater, the difference was not statistically significant relative to wild type. Chimeric αH/βA-FABP, which also has the β-body of AFABP, had a slightly greater $Q_\lambda$ value of 0.37 ± 0.02 ($p < 0.05$), suggesting a somewhat more hydrophobic FA binding environment. Mutants that have the β-body of HFABP and the helical region from AFABP preserved the properties of HFABP, with similar $Q_\lambda$ values of 0.41 ± 0.03, 0.49 ± 0.03, and 0.44 ± 0.02, for wild type HFABP, αA/βH-FABP, and αL/βH-FABP, respectively.

Using the ADIFAB assay developed by Richieri et al. (11), the affinity of oleate binding to mutant proteins and their

![Fig. 2. Ribbon diagrams of proteins containing HFABP β-body. Ribbon diagram of human HFABP superimposed on chimeras of residues 1–57 of AFABP and 38–133 of HFABP of the muscle protein. The file used for the heart protein is PDB file 1HMS after oleate removal and conformational energy minimization. The chimera was constructed as described in the text and was also minimized. The two helices of the cap domain are at the top. The N termini are at the bottom in the rear and lead into the first β-strand before the polypeptide enters the first helix at the upper right. The carboxyl termini are at the lower right.](http://www.jbc.org/Downloadedfrom)
respective native FABPs was examined, and results are shown in Table IV. The $K_v$ values for oleate binding to the E238FABP mutant and to chimeric proteins oH/$\beta$-A and oL-H/$\beta$-FABP were 85 ± 19, 81 ± 11, and 86 ± 9 nM, respectively, indicating that the FA binding affinity of these three proteins was slightly higher but not statistically different from wild type AFABP (110 ± 11 nM). The calculated number of binding sites (n) was consistent with a 1:1 stoichiometry, in agreement with the x-ray crystal structure (1) and other ADIFAB studies (11). However, mutant oL-H/$\beta$A-FABP, which has the first 25 amino acids from HFABP ligated to the $\beta$-body of AFABP, seemed to have “n” close to two (1.8 ± 0.1). The oleate binding stoichiometries for native rat HFABP, the $\alpha$A/$\beta$H-, and the oL-A/$\beta$H-FABP were also consistent with one binding site. In addition, the $K_v$ values obtained for mutants possessing the $\beta$-body of HFABP reflected a similar oleate binding affinity (31 ± 6 and 14 ± 4 nM) relative to the native HFABP (17 ± 1). These results are consistent with the HFABP tertiary structure determined by Zanotti et al. (2) and with earlier reports of FA equilibrium binding affinities (11). Overall, the results indicate that mutants largely retain the properties of their “parent” FABP, i.e. their $\beta$-barrel domain.

Effect of the Helix-Turn-Helix Motif on FA Transfer—Studies of lysine to isoleucine point mutants of AFABP (28) and HFABP (27) strongly suggested that the helical domain is the primary region involved in the effective collisional mechanism of FA transfer from FABPs to model membranes. Therefore, the present studies, in which we exchanged the entire helical cap between the two proteins, were designed to examine the structure-function relationships in this domain. The rates of 2-AP transfer from the A- and HFABP and the first pair of chimeric proteins ($\alpha$A/$\beta$H- and oL-H/$\beta$A-FABP) to SUVs are shown in Fig. 3. As previously found, A- and HFABP transferred FA via a collisional mechanism, as seen by the increase in the absolute rates of 2-AP transfer as a function of acceptor vesicle concentration, as well as the modulation of 2-AP transfer to SUVs containing negatively charged phospholipid relative to neutral SUVs. Based on their qualitatively similar regulation of AOFA transfer rates, the results indicate that the two chimeric proteins retain the collisional FA transfer mechanism. Notably, as previously observed, 2-AP transfer from AFABP is markedly faster than from HFABP (23).

The rates of FA transfer from oH/$\beta$A-FABP were compared with those from wild type AFABP, to both neutral and acidic vesicles (Fig. 3). For all vesicle compositions, 3- to 4-fold reductions in rates were found from the chimeric protein relative to AFABP. Likewise, replacement of the $\alpha$H → $\alpha$A helical domain on HFABP with $\alpha$A (resultant chimeric oA/$\beta$H-FABP) led to 2- to 4-fold increases in 2-AP transfer rates relative to those of wild type HFABP. These results indicate that the helical segment plus the $\beta$-A strand (first 36 amino acids) of AFABP is an important determinant of the more rapid rate of FA transfer from AFABP compared with HFABP.

The fact that the helix-domain chimera did not entirely convert the rate of 2-AP transfer to that of its respective FABP indicated that the $\beta$-body of AFABP also aided in determining the absolute rates of ligand transfer to membranes. When comparing chimeric oA/$\beta$H-FABP with native AFABP, i.e. proteins sharing the same cap region, a 2- to 3-fold reduction was found in the rate of FA transfer to both neutral and acidic vesicles. Similarly, a comparison of oH/$\beta$A-FABP with wild type HFABP reveals a 3- to 4-fold increase in AOFA transfer rates from the chimeric relative to wild type HFABP. This agreed with our earlier Lys → Ile point mutagenesis studies, which suggested that several $\beta$-strands of AFABP were involved to a modest extent in the collisional transfer process (28). Although the changes in absolute rates of FA transfer were relatively small (20–40%) from individual site-directed mutant proteins (28), the severalfold changes observed in the present chimeric studies likely reflect the collective effects of several residues in the $\beta$-body.

Effect of Single Helix Domains on FA Transfer—The helical lid consists of two short $\alpha$ helices, from residues 17–24 and 27–35, and a small turn (Gly-Val-Gly) that links them together. The first helix of A- and HFABP is an amphipathic helix whereas the second one of both proteins is not. Two differences

![Graph](http://www.jbc.org/Downloaded_from/http://www.jbc.org/by_guest_on_July_25,2018.png)
in amino acid residues are found between A- and HFABP in each helix. In the αI-helix, glutamic acid 23 and valine 24 are found in AFABP, whereas serine 23 and leucine 24 are present in HFABP. In the second helix, lysine 32 and glycine 35 exist in AFABP, but HFABP contains the corresponding glutamine 32 and serine 35. Notably, lysine (Lys-32), which displayed a unique effect on FA transfer, exists in the αII-helix of AFABP, whereas no lysine is found in the same region of HFABP. Neutralization of Lys-32 resulted in increased rates to neutral vesicles but decreased rates to acidic membranes relative to the rates of 2-AP transfer from wild type AFABP (28). Hence, we sought to distinguish the different contributions of each helix to the FA transfer process. Thus, two additional chimeric mutants were constructed in which the first 25 amino acids were exchanged (β-A strand and αI-helix) between the two proteins.

The rates of 2-AP transfer from this pair of mutants relative to the two native proteins are shown in Fig. 4. As seen by the increases in transfer rates with acceptor membrane concentration, a collisional process was maintained for these two chimeric proteins (results for αI-A/βH-FABP shown in the inset). Qualitatively, the pattern of FA transfer from these two mutants to neutral vesicles was similar to the αII/βH- and αH/βA-FABP chimeras, but the effects were of lesser magnitude. Adding the αI-helix of HFABP to the remaining structure of AFABP (αI-helix and β B-J strands), to obtain mutant αI-H/βA-FABP, resulted in a 2-fold reduction in rates relative to wild type AFABP. Changing the αI-helix from HFABP to AFABP, to obtain mutant αI-A/βH-FABP, showed small but statistically significant increases in the transfer rates (13–25%) to 250 and 500 μM neutral vesicles relative to native HFABP (p < 0.05 and 0.01, respectively) (Fig. 4, inset).

In contrast to the pattern observed for the α-helical (αI + αII) domain chimera, a completely opposite trend was found for 2-AP transfer from the αI chimera to anionic vesicles (Fig. 4). Transfer rates from these mutant proteins were not between the values for wild type A- and HFABP. Instead, the rates were either 2-fold faster for the αI-H/βA-FABP, or 2-fold slower for the αI-A/βH-FABP, compared with their respective wild type proteins sharing the same αI-helix plus β-barrel structure (p < 0.05). For instance, mutant αI-H/βA-FABP transferred 2-AP to 500 μM PS-vesicles at a rate of 94 ± 3.8 s⁻¹, whereas the rate of 2-AP transfer from native AFABP (“αI-A/βA-FABP”) was 53.4 ± 3.9 s⁻¹. Similarly, when compared with native HFABP, αI-A/βH-FABP transferred FA at 2-fold slower rates to PS-incorporated membranes. For example, the rates of FA transfer to 100 μM PS vesicles were 0.45 ± 0.02 s⁻¹ and 0.90 ± 0.04 s⁻¹ from αI-A/βH-FABP and HFABP, respectively (inset of Fig. 4). The same pattern was found in transfer to CL-containing membranes, although, as for all proteins examined, faster absolute rates were observed (Fig. 4). The transfer rates from mutant αI-H/βA-FABP were faster when compared with those from wild type AFABP, however, the differences were statistically significant only when FA transferred to 100 μM CL-SUVs. This may be due to the very rapid rates of FA transfer to acceptor CL membranes, which approach the limit of detection range by the stopped flow mixing chamber and therefore may lead to large variability. Collectively, these results suggest that the αI- and αII-helices play distinct roles in the regulation of collision-mediated fatty acid transfer from A- and HFABP and further suggest that helix αI of HFABP has a greater sensitivity than that of αII-helix to the negative charge of acceptor phospholipid membranes.

Comparison of the rates of 2-AP transfer from native AFABP with those from chimeric αI-A/βH-FABP, as well as native HFABP with mutant αI-H/βA-FABP, allows us to examine the impact of the αII-helix plus the β-body of the two proteins in the transfer process. Rate 3- to 6-fold faster were found for proteins that have the αII/β-body from AFABP, implying that α-II helix plus β B-J strands are the primary determinants of the absolute rates of FA transfer. Moreover, these results suggest that αI-helix is important in determining the sensitivity to

![Fig. 4. 2-AP Transfer from A- and HFABP and αI-chimeric FABPs to neutral and acidic membranes.](http://www.jbc.org/)

The transfer of 1 μM 2-AP from 10 μM proteins to 100–500 μM SUV was measured at 25 °C, pH 7.4, as described under “Experimental Procedures.” 2-AP transfer to (A) neutral EPC vesicles, (B) PS vesicles, and (C) CL vesicles from wild type AFABP (○), HFABP (●), chimeric αI-A/βH-FABP (○), and αI-H/βA-FABP (●) are shown. *, p < 0.05; #, p < 0.01; and +, p < 0.005 for chimeric proteins versus their respective native proteins sharing the same β-barrel structure. The inset enlarges the data for HWT and αI-A/βH-FABP.
negative charge for ligand transfer from A- and HFABP to membranes.

Effect of Glutamic Acid 23 to Serine Mutation on FA Transport—A higher sequence identity (77.5%) between A- and HFABP is found in the first 40 amino acid residues relative to the overall protein (62%). Among those residues, the first helix from both proteins is amphipathic, and such helices are thought to be involved in protein-membrane interactions (43–45). Six out of eight residues in the α-helix of HFABP are identical between adipocyte and heart FABP, with one different residue on the hydrophobic side (valine and leucine 24 on A- and HFABP, respectively) and one on the hydrophilic side (glutamic acid and serine 23 on A- and HFABP) of the helix. To mimic the hydrophilic side of the α-helix of HFABP, an E → S mutation was introduced into AFABP at residue 23, changing the negatively charged glutamic acid to the neutral polar serine. Having verified that this mutation did not alter the conformational integrity and binding properties of the protein relative to native AFABP (Table IV), 2-AP transfer to acceptor membranes was examined.

Like other mutants studied so far, FA transfer from E23S-AFABP to model membranes occurs via a collisional process. Unlike the dramatic effects of lysine to isoleucine mutations in AFABP to those of HFABP. This suggests that the charge of the glutamic acid may not be its significant property, but, rather, the polarity of the residue may be necessary for maintaining the amphipathic character of the helix for protein-membrane interactions.

Sensitivity of Chimeric Mutants to Negative Charge—As expected, 2-AP transfer rates were faster from all proteins examined to acceptor membranes containing 25 mol % of the negatively charged phospholipids phosphatidylserine and cardiolipin, relative to neutral vesicles. Transfer of 1 µm 2-AP from 10 µm protein to 100 µm SUV is shown as a representative for the relative effect of membrane charge on FA transfer rate (Fig. 5). AFABP showed a large response to negative charge, with rates increasing 5– and 40-fold to PS and CL vesicles, respectively, relative to neutral membranes. HFABP demonstrated increases of about 2- and 20-fold to PS- and CL-SUV, respectively. Those proteins with the faster absolute transfer rates also showed more sensitivity to membrane-negative charge. For example, the greatest sensitivity to negative charge was found in mutant of α-I-H/βA-FABP, whereas α-I-βH-FABP was the least sensitive protein (Fig. 5). Mutant α-I-H/βA-FABP showed increases about 22.5- and 137-fold to PS- and CL-SUV, respectively, and displayed the highest 2-AP transfer rates observed (Fig. 4). These increases are ~3.5- and 7-fold greater in sensitivity to anionic vesicles compared with those of wild type A- and HFABP. In contrast, mutant α-I/β-H-FABP displayed only 1.5- and 8.5-fold increase in rates of FA transfer to PS and CL vesicles, respectively, and showed the slowest 2-AP transfer rates (Fig. 4). This protein had only 20 and 40% of the sensitivity to anionic phospholipids compared with native AFABP and HFABP, respectively.

These results suggested that mutant α-I-H/βA-FABP may contain the structural elements from each FABP protein that provide increased sensitivity to phospholipid membrane-negative charge, i.e. the α-II-helix and β-barrel of AFABP, and the α-helix of HFABP. In contrast, mutant α-I-βH-FABP may consist of structural components that are relatively less sensitive to phospholipid-negative charge. The chimeric proteins having their entire helical domains (residues 1–36) exchanged displayed sensitivities between those of AFABP and HFABP (left panel in Fig. 5), thus they appear to contain a mixture of sensitive and insensitive elements.
DISCUSSION

AFABP and HFABP each display a remarkable N-terminal sequence identity among different species (46, 47). Indeed, AFABP isolated from five different mammals (murine, rat, human, porcine, and bovine) is identical from position 1 to 39 of the N-terminal end (46). Although the exact importance of the sequence identity at the N terminus is not yet understood, it may include the maintenance of specific collagenous fatty acid transfer properties. It is well established that α-helical motifs are often involved in protein-membrane interactions (43). Hence, we focused on the N terminus helix-turn-helix motif, believed to serve as a portal for FA entering and exiting the molecule (across the helix-turn-helix motif), which extends to include the opening to the binding cavity. In addition, upon ligand binding, subtle but consistent changes were found only in the shape of the large positive potential contour across the top of the protein, and no significant changes were observed in the electrostatics outside this positive ridge. It is possible that this positive cap on the holo-protein directs the effective protein-membrane interactions is supported by a recent study by LiCata and Bernlohr (49), who used structure-based mutagenesis studies of AFABP (28), which indicated that the lysine residues located in β-strands A, G, and I (β-body) also participate to some extent in the electrostatic interactions between the protein and phospholipid membranes.

Each helix appears to have unique attributes in transporting FA to membranes. A comparison of the αI chimera and the “αI + αII” chimera suggests that the αI-helices are the primary determinants of absolute rates of AOFA transfer from A- and HFABP to anionic acceptor membranes (Figs. 3 and 4). To further dissect the contribution of each helix, we directly compared the impact of the helical domains on 2-AP transfer rates from the FABPs (Fig. 6). Proteins that share the body of AFABP or HFABP are shown in the left and right panels, respectively. Taking into consideration that (a) the entire helical region of AFABP has a greater sensitivity to negative charge than that of HFABP (αA > αII), and (b) the αI-helix (plus β-A strand) of HFABP was more sensitive to negative charge than that of AFABP (αI-H > αI-A), the αI-helix of AFABP must be very sensitive to anionic vesicles relative to that of HFABP to overcome the contribution by helix I (αII-A > αII-H). Collectively, the αI-helix (plus β-A strand) of HFABP, and the αII-helix of AFABP, displayed the greatest ability in sensing negative charge of acceptor phospholipid membranes. The overall abilities of AFABP and HFABP to respond to acceptor membrane charge, therefore, appears to be an average function of the αI- and αIII-helix sensitivities.

HFABP and AFABP both contain six hydrophilic residues in the helical cap region, however, differences in these residues could result in different properties of FA transfer to neutral versus anionic membranes. In particular, the hydrophilic side of helix I of AFABP has more available negatively charged side chains than that of HFABP, and may therefore interact with positive charges on the zwitterionic phosphatidylcholine trimethylamino groups, resulting in faster AOFA transfer rates to zwitterionic SUVs. In contrast, for transfer to negatively charged vesicles, the greater number of negative charges on the αI-helix of AFABP may result in a more repulsive interaction, resulting in slower FA transfer from AFABP relative to αI/β-FABP. This speculation does not contradict the proposal that the helix-turn-helix cap of AFABP has a higher positive electrostatic potential than that of HFABP (49), because a unique lysine (Lys-32) located on the αII-helix of AFABP, re-

![Fig. 6. Effect of α-helical segments on FA transfer from FABPs sharing the same β-barrel structure to negatively charged PS vesicles.](http://www.jbc.org/)

Results here are in agreement with our previous site-directed mutagenesis studies of AFABP (28), which indicated that the lysine residues located in β-strands A, G, and I (β-body) also participate to some extent in the electrostatic interactions between the protein and phospholipid membranes.

![DISCUSSION.](http://www.jbc.org/)

Neither of the two helical region mutants was able to convert AFABP or HFABP to anionic acceptor membranes (Figs. 3 and 4). To further dissect the contribution of each helix, we directly compared the impact of the helical domains on 2-AP transfer rates from the FABPs (Fig. 6). Proteins that share the body of AFABP or HFABP are shown in the left and right panels, respectively. Taking into consideration that (a) the entire helical region of AFABP has a greater sensitivity to negative charge than that of HFABP (αA > αII), and (b) the αI-helix (plus β-A strand) of HFABP was more sensitive to negative charge than that of AFABP (αI-H > αI-A), the αI-helix of AFABP must be very sensitive to anionic vesicles relative to that of HFABP to overcome the contribution by helix I (αII-A > αII-H). Collectively, the αI-helix (plus β-A strand) of HFABP, and the αII-helix of AFABP, displayed the greatest ability in sensing negative charge of acceptor phospholipid membranes. The overall abilities of AFABP and HFABP to respond to acceptor membrane charge, therefore, appears to be an average function of the αI- and αIII-helix sensitivities.

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sults in an overall greater positive potential for the entire cap relative to HFABP.

We have hypothesized that the FA collisional transfer mechanism is likely to be a multistep process that includes electrostatic interactions between the entire helix motif with target phospholipid membranes, a resultant conformational change in the putative cap from an ordered closed state to a more disordered open one, long chain fatty acid release from the internal ligand binding cavity, and finally, the association of FA with the acceptor membrane (48). The present investigations suggest a modified model, in which the first step may be directed by the α-II-helix alone (or perhaps with some contributions of the β-body) to affect a close proximity to acceptor phospholipid membranes. Second, the entire helical domain may interact with phospholipid membranes by electrostatic interactions, causing a conformational change in this domain from the ordered closed state to a disordered open state (6, 7, 51). FABP then releases its bound ligand from the binding cavity, and finally the FA associates with the acceptor membrane. Cistola and Hodson (6) have suggested that the binding of fatty acid shifts the order-disorder equilibrium of FABP toward the ordered state by stabilizing a series of cooperative interactions resembling a C-terminal helix capping box (C cap), in the region that links the α-II-helix and the β- strand of intestinal FABP (6). We hypothesize that, upon interacting with membranes at the α-helical region, FABP may shift from an ordered state to a disordered state, which allows the FA to be released from FABP. Unlike the typical C cap, with glycine or proline in the location next to the last residue of the α-helix (52, 53), A- and HFABP do not have such a motif to stabilize their α-helices. Therefore, as proposed for IFABP (6), A- and HFABP may also use side-chain and/or backbone amide hydrogen bonds and hydrophobic interactions to stabilize the helical lid. Our results suggest that the modified helices result in altered interactions between the α-II-helix and the β-C D turn, leading to differences in FA transfer rates. However, these presumably subtle conformational changes cannot yet be defined, due to the absence of actual three-dimensional structures for the chimeric AHFABPs.

Equilibrium binding studies have shown that fatty acid binding affinities are higher for HFABP than for AFABP (11). Thus, the release of FA from the binding cavity may play a role in the absolute rates of FA transfer to membranes. Woolf and Tychko (54) suggested that the extended form of ligand bound in the AFABP cavity, compared with the bent form in the HFABP pocket, contributed to a more rapid release from AFABP. In our assay system, the fluorescence signal is lost when the AOFA leaves the relatively hydrophobic FABP binding pocket (31). Although this is suggested to occur during the final step of the proposed multistep process, the rate at which the fluorescence quenching occurs will reflect the slowest step that occurs prior to including the desorption/membrane association. Because the present mutations resulted in little or no alterations in binding site properties or equilibrium binding affinities, it is likely that the FA release from the binding pocket is not the rate-limiting step in the FA transfer process, although proportional changes in off- and on-rates cannot be ruled out.

In summary, these studies show that the FABP α-II-helix is important in determining the absolute FA transfer rates to membranes; the α-II-helix appears to be central in regulating protein sensitivity to the negative charge of membranes. Within the cell, A- and HFABP may interact with specific domains on phospholipid membranes, and/or specific domains on target proteins. Several studies have, indeed, provided experimental support for this hypothesis (55, 56). Based on our in vitro studies, it is suggested that electrostatic interactions, between cationic FABP surface residues and anionic functional groups on membranes or proteins, are involved in targeted intracellular movement of FA. The differential sensitivities of A- and HFABP to negative charge appear to be largely a combinatorial effect from both their α-I- and α-II-helices. The differential FA transfer properties may result in unique physiological functions of these proteins in their respective cell types, resulting, for example, in FA targeting to sites of FA storage or oxidation.

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Role of the Helical Domain in Fatty Acid Transfer from Adipocyte and Heart Fatty Acid-binding Proteins to Membranes: ANALYSIS OF CHIMERIC PROTEINS
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