Protein-Membrane Interaction and Fatty Acid Transfer from Intestinal Fatty Acid-binding Protein to Membranes

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Fatty acid transfer from intestinal fatty acid-binding protein (IFABP) to phospholipid membranes occurs during protein-membrane collisions. Electrostatic interactions involving the α-helical “portal” region of the protein have been shown to be of great importance. In the present study, the role of specific lysine residues in the α-helical region of IFABP was directly examined. A series of point mutants in rat IFABP was engineered in which the lysine positive charges in this domain were eliminated or reversed. Using a fluorescence resonance energy transfer assay, we analyzed the rates and mechanism of fatty acid transfer from wild type and mutant proteins to acceptor membranes. Most of the α-helical domain mutants showed slower absolute fatty acid transfer rates to zwitterionic membranes, with substitution of one of the lysines of the α2 helix, Lys27, resulting in a particularly dramatic decrease in the fatty acid transfer rate. Sensitivity to negatively charged phospholipid membranes was also reduced, with charge reversal mutants in the α2 helix the most affected. The results support the hypothesis that the portal region undergoes a conformational change during protein-membrane interaction, which leads to release of the bound fatty acid to the membrane and that the α2 segment is of particular importance in the establishment of charge-charge interactions between IFABP and membranes. Cross-linking experiments with a phospholipid-photoactivatable reagent underscored the importance of charge-charge interactions, showing that the physical interaction between wild-type intestinal fatty acid-binding protein and phospholipid membranes is enhanced by electrostatic interactions. Protein-membrane interactions were also found to be enhanced by the presence of ligand, suggesting different collisional complex structures for holo- and apo-IFABP.

Intestinal fatty acid-binding protein (IFABP) belongs to a family of intracellular lipid binding proteins of low molecular mass (14–15 kDa)

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¶The abbreviations used are: IFABP, intestinal fatty acid-binding protein; FA, fatty acids; FABP, fatty acid-binding protein; FABP, liver fatty acid binding protein; wtIFABP, wild-type intestinal fatty acid binding protein; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; A0FA, anthroyloxy-labeled fatty acid; 12A0, 12-(9-anthroyloxy)oleic acid; EPC, egg phosphatidylcholine; PS, brain phosphatidylserine, CL, bovine heart cardiolipin; NBD-PC, N-[7-nitro-2,1,3-benzoxadiazol-4-yl] egg phosphatidylcholine; 125I-TID-PC, 1-25iodo-o-hexadecanoyl-2-O-[9-125Iiodo-4-(trifluoro-
Lys92 have been highlighted in a stick representation. The protein molecule is oriented to show the positions of mutated residues.

The results demonstrate that no single lysine was solely responsible for FA transfer properties of IFABP. Rather, all of the Lys residues of the α-helical domain contribute to the collisional mechanism of FA transfer from IFABP to model membranes. Lys residues of the α2 segment are of particular importance in the FA transfer process.

**EXPERIMENTAL PROCEDURES**

**Materials**

The mutagenic primers were obtained from Invitrogen (Carlsbad, CA). Pfu DNA polymerase, pGEM-T easy vector, and restriction enzymes XbaI and BamHI were purchased from Promega (Madison, WI). T4 DNA ligase, pET-11a expression vector, and BL21 (DE3) cells were obtained from Novagen (Milwaukeee, WI). Sodium oleate was obtained from Nu-Chek Prep (Elysian, MN). Fluorescently labeled AOFA, 12-(9-anthroyloxy)oleic acid (12AO), and acrylated IFABP (ADIFAB) were purchased from Molecular Probes, Inc. (Eugene, OR). Egg phosphatidylcholine (EPC), N-(7-nitro-2,1,3-benozoxadiazol-4-yl) egg phosphatidylecholine (NBD-PC), brain phosphatidylserine (PS), and bovine heart cardiolipin (CL) were obtained from Avanti Polar Lipids (Alabaster, AL). Lipidex-1000 was purchased from Sigma. Isopropyl-β-D-thiogalacto side was obtained from Fisher. [125I]NaI was from PerkinElmer Life Sciences. All other chemicals were reagent grade or better.

**Construction of Point Mutant IFABPs**

IFABP has four lysine residues in the helix-turn-helix domain, two in each α-helical segment. A series of point mutants were constructed substituting lysine for isoleucine to eliminate the charge and lysine for glutamic acid to reverse the charge. Isoleucine and glutamic acid were chosen to replace lysine due to their respective neutral and negative charges and the relatively similar bulkiness to lysine, so as to maintain the approximate side chain size along the backbone of the protein. Thus, eight mutations in the α-helical region were constructed, and an additional pair of mutants, K92I and K92E, was also generated to assess the effects of a nonhelix domain basic residue; Lys92 is located in β strand G. Recombinant rat pET11d-IFABP plasmid was generously provided by Drs. Alan Kleinfeld and Ron Ogata (Medical Biology Institute, La Jolla, CA). A single or double mutation was introduced in the IFABP sequence employing overlapping PCR methodology (20). The same external primers were used for all constructs: 5′-CGGATAACAT-TCCCTCTGAT-3′ and 5′-TCCCTTTTCGGGGTTTGTAG-3′. Each mutation was verified by sequence analysis. Mutant DNA sequences were subcloned into the pET-11a expression vector by using XbaI and BamHI restriction sites.

**Protein Expression and Purification**

All proteins were overexpressed in Escherichia coli BL21 DE3 harboring pET constructs for wild-type and point mutant proteins, as detailed elsewhere (3, 8); all of the proteins were found in the soluble fraction of the bacterial lysates and were purified from E. coli as described previously (3, 8).

**Analysis of Wild-type and Mutant FABPs**

None of the lysine residues mutated in this study are found within the ligand binding site (21); thus, maintenance of binding site integrity was expected. To assess this directly, the overall conformation and ligand binding site properties of the mutant IFABPs were examined by several methods.

**Molecular Modeling—Crystallographic structures of rat apo-IFABP (Protein Data Bank code 1IFC) (21) and rat holo-IFABP with palmitate bound (Protein Data Bank code 2IFB) (22), solved at 2.0 and 1.2 Å, respectively, were used to model the mutant proteins. Silicon Graphics workstations running the InsightII program (Accelrys, San Diego, CA) were used to replace amino acids to generate initial models, one single...
site replacement for each mutant protein. We then performed conformational energy minimization, first adding hydrogens to the structures, since the Protein Data Bank files contained heavy atoms only. The modeling pH was set to 7.4. The ‘soak’ facility of Insight II was used to create a layer of water molecules around the protein 5.0 Å thick. Water molecules present in the crystallographic files were retained prior to ‘soaking’ as well as the bound palmitate in the holo structure. The energy was then minimized to convergence using default settings. The wild type molecules in the PDB files were also minimized in order that comparisons between wild type and mutants would compare structures that had been treated identically. Bond lengths, bond angles, and Ramachandran plots were normal (data not shown). Analysis of solvent-accessible surface area was by the method of Lee and Richards (23) using the program ACCESS (24). The ACCESS output was sorted and compiled by BINS (25), which classifies each atom as to whether it is aliphatic, aromatic, polar uncharged, or polar charged and aggregates the results for each amino acid and for the protein as a whole. Volumes were computed by the Voronoi method with the program VOLUME, the output being compiled by VOLFMT (26, 27).

**CD Spectra**—CD spectra were obtained at 25 °C on an Aviv model 60DS spectropolarimeter using a 0.1-cm path length quartz cuvette (Hellma). Spectra of the wild-type and mutant proteins were obtained using a standard value of dS.D of 10 pairs of 8000C spectrofluorometer, with excitation at 386 nm. The average and emission intensities at 505 and 432 nm were measured using an SLM-60DS spectropolarimeter using a 0.1-cm path length quartz cuvette (Hellma). Spectra of the wild-type and mutant proteins were obtained using quinine sulfate and 383 nm for 12AO.

**Fluorescent Quantum Yields**—Fluorescent quantum yields (Qo) of 12-(9-anthroyloxy)oleic acid (12AO) bound to wild-type and mutant IFABPs were determined using quinine sulfate in 0.1 N H2SO4, as the reference fluorophore, with Q ref = 0.78 (27). Excitation was at 352 nm for quinine sulfate and 383 nm for 12AO.

**Binding of Oleate**—Binding of oleate to wild-type and mutant IFABPs was analyzed by the method employing the fluorescent probe ADIFAB (29), 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 μM 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 μM ADIFAB and 4 μM wild type or mutant IFABP. Following equilibration at 37 °C for 5 min, fluorescence emission intensities at 505 and 432 nm were measured using an SLM-8000C spectrophotometer, with excitation at 386 nm. The average and S.D. of 10 pairs of R (emission 505 nm/emission 432 nm) values were determined. This average was applied to binding equilibrium analysis using a standard value of R max = 11.5 (29). Experimental values were fitted to a single-site Scatchard analysis, and K' f values for oleate binding were obtained.

**Relative Partition Coefficient**—The relative partition coefficient (Kp) for AOFA partitioning between wild type or mutant IFABPs and small unilamellar vesicles (SUVs) was determined by measuring AOFA fluorescence in a given molar ratio of protein/SUV after titration of SUV into a solution containing 5 μM protein and 0.5 μM 12AO in 40 mM Tris, 100 mM NaCl, pH 7.4 (TBS) at 25 °C (30, 31).

\[
K_p = \frac{([\text{FABP-bound AOFA}]/[\text{FABP}])}{([\text{SUV-bound AOFA}]/[\text{SUV}])} \quad (\text{Eq. 1})
\]

The decrease in AOFA fluorescence upon titration of AOFA-containing FABP with SUVs was related to K p by the following equation,

\[
1/\Delta F = 1/K_p (1/\Delta F_{\text{max}} ([\text{FABP}]/[\text{SUV}]) + 1/\Delta F_{\text{max}} \quad (\text{Eq. 2})
\]

where ΔF is the difference between the initial fluorescence of AOFA in the FABP and the AOFA fluorescence at a given protein/SUV ratio, and ΔF max is the maximum difference in AOFA fluorescence. A plot of 1/ΔF versus (1/ΔF max)([SUV]/[FABP]) gives a slope of 1/K p. The partition coefficient was used to establish AOFA transfer assay conditions so as to ensure essentially unidirectional transfer, as detailed below (32).

**Vesicle Preparation for AOF A Transfer Experiment**

SUVs were prepared by sonication and ultracentrifugation as described previously (33, 34). The standard vesicles were prepared to contain 90 mol % of EPC and 10 mol % of NBD-PC, which served as the fluorescent quencher. To increase the negative charge density of the acceptor vesicles, either 25 mol % of PS or CL were incorporated into the SUVs in place of an equimolar amount of EPC. Vesicles were prepared in TBS buffer except for SUVs containing cardiolipin, which were prepared in TBS with 1 mM EDTA.

**Transfer of AOFA from FABP to SUV**

A fluorescence resonance energy transfer assay was used to monitor the transfer of AOFA from the wild type and mutant IFABPs to acceptor model membranes as described in detail elsewhere (3, 4, 8). Briefly, FABP with bound AOFA was mixed at 25 °C with SUV using a stopped-flow spectrophotometer DX-17MV (Applied Photophys-ics Ltd.). The NBD moiety is an energy transfer acceptor of the anthroyloxy group donor; therefore, the fluorescence of the AOFA is quenched when the ligand is bound to SUVs that contain NBD-PC. Upon mixing, transfer of AOFA from protein to membrane is directly monitored by the time-dependent decrease in anthroyloxy group fluorescence. Final transfer assay conditions were 15 μM wild-type or mutant IFABP with 1.5 μM 12AO and a range of 150–600 μM SUV. Controls to ensure that photobleaching was eliminated were performed prior to each experiment, as previously described (8). Data were analyzed using software provided with the instrument, and all curves were well described by a single exponential function. For each experimental condition within a single experiment, at least five replicates were done. Average values ± S.D. for three or more separate experiments are reported, unless otherwise indicated.

**Preparation of Photoactivable Reagents**

125I-TID-PC was prepared by radioiodination of its nonradioactive tin-containing precursor 1-O-hexadecanoyl-2-O-[9-[[2-(tributylstannyl)-4-(trifluoromethyl)-(3H)-diazirin-3-yl]benzyl]oxy]carbonyl]-nonanoyl]-sn-glycerol-3-phospho-choline according to Weber and Brunner (35) and our previous work (36). The precursor was generously donated by Prof. J. Brunner from the Swiss Federal Institute of Technology (Zurich, Switzerland). The dried tin-containing precursor (~20 nmol) was dissolved in 10 μl of acetic acid in a 1:1 Reacti-Vial (Pierce). [125I]NaI (1 mCi) was added, and the iodination was started by the addition of peracetic acid (2 μl of a 32% solution in acetic acid). After 2 min at room temperature, the reaction was quenched with 50 μl of 10% Na2S2O3. Then 40 μl of chloroform/methanol (2:1) were added and vortexed. The organic phase was collected and concentrated using a charcoal filter to adsorb volatile radioactivity. The residue was dissolved in 20 μl of methanol/chloroform/H2O (9:1.1) and subjected to reverse-phase high pressure liquid chromatography using the same solvent and a 208HSS4 C8 column (Vydac) in a Merck-Hitachi apparatus with UV detection at 254 nm. The flow rate was 1 ml/min, and fractions of 0.5 ml were collected. [125I]-TID-PC eluted at ~20 min, whereas the excess of tin-containing precursor eluted at ~40 min. An aliquot (5 μl) of each fraction in the elution region of [125I]-TID-PC was analyzed by TLC on silica gel plates (LK6D, 60 Å; Whatman, Clifton, NJ) and subjected to autoradiography.
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Fractons containing radioactivity were pooled and concentrated by co-evaporation with toluene/ethanol (1:1). 125I-TID-PC was dissolved in ethanol/toluene (1:1) at ~1 mCi/ml and stored at −20 °C.

Preparation of Lipid Vesicles Containing 125I-TID-PC

Large unilamellar vesicles (LUVs) of EPC, EPC/PS (3:1, mol/mol), or EPC/CL (3:1, mol/mol) were prepared (0.5 mM in phospholipids) by extrusion through polycarbonate membranes of 100-nm pore diameter (Avestin Inc., Ottawa, Canada). To prepare the LUVs containing 125I-TID-PC (200 μCi/μmol of phospholipid), the photoreagent was mixed with the lipids in chloroform. Lipids in chloroform were mixed, dried under a stream of N2, and resuspended in 40 mM Tris, 100 mM NaCl, 50 mM glutathione (buffer A) by vortexing. Cardiolipin-containing vesicles also had 1 mM EDTA included in the buffer A. Then lipid suspensions were incubated at 37 °C for 30 min and passed 11 times through the polycarbonate filters using a Liposofast extruder system (Avestin).

Photolabling Analysis of FABP-Membrane Interactions

Experiments were conducted in a 37°C incubator as previously described (36). Briefly, 120 μL of 0.5 μM photoreagent-containing LUVs (30 μCi/ml) were incubated with the 60 μg of FABP in 200 μl of buffer A at room temperature for 30 s. In these experiments where oleate was included, we employed a 10:1 protein/ligand (mol/mol) ratio, and the protein-ligand complex was prepared prior to the incubation with LUVs. After the indicated incubation time, mixtures (0.3 ml) in glass cuvettes were irradiated for 30 s. In those experiments where oleate was included, we employed a 10:1 protein/ligand (mol/mol) ratio, and the protein-ligand complex was prepared prior to the incubation with LUVs. After the indicated incubation time, mixtures (0.3 ml) in glass cuvettes were irradiated for 30 s with a xenon lamp (450 watts) at a distance of 25 cm. As a control, the photoreagent-containing LUVs were irradiated prior to their mixture with IFABP. After irradiation, 3 volumes of CHCl3/methanol (2:1) were added and vortexed, and the organic phase was discarded. FABPs were precipitated with 5 volumes of acetone and redissolved in 25 μl of sample buffer for direct analysis by SDS-PAGE (37). Following Coomassie Blue staining, gels were dried and exposed to X-Omat film (Eastman Kodak Co.) at −80 °C for different times, depending on the amount of radioactivity.

RESULTS

Construction of Mutant Proteins and Comparison of Structural and Ligand-binding Properties with Native IFABP—To examine whether the primary determinant of the IFABP fatty acid transfer mechanism resides in the lysine residues of the helix-turn-helix domain, we undertook the construction of point mutants that neutralized or reversed the charges of the four lysines of the α-helical domain and one of the lysine residues of the β-barrel. To control for potential alterations in the overall folding of the point mutants, molecular modeling, circular dichroism spectroscopy, fluorescence quantum yield measurements of bound anthroyloxy fatty acid, equilibrium binding affinity of oleate using the ADIFAB method, and determination of the relative partition coefficient of 12AO between the IFABPs and SUVs were used. A summary of the results for these experiments is presented in Table 1.

Conformational energy minimization calculations of the mutant IFABPs were performed for both apo and holo forms of the mutant proteins and compared with the wild-type protein, which was also subjected to energy minimization. As shown in Table 1, the differences in the overall conformational energy of the mutant proteins compared with the native IFABP were small, ranging from 0.01 to 1.87%. The solvent-accessible surface areas were found to be similar to that calculated for the native protein, and the mutant apo-IFABP forms also showed protein volume values similar to that of the wild-type apo-IFABP (Table 2). For the holoproteins, K27I was found to have a 0.42% increase in protein volume, whereas all other mutations resulted in somewhat lower volumes than the wild type. When the 95% confidence limit for the mean values and volumes is calculated, it is seen that none of the individual mutant or wild type values fall outside the limits. This is consistent with the absence of significant conformational change due to the mutations.

To examine the likely conformation of the portal domain (residues 24–33, 54–55, and 73–74), the backbone of the rest of the protein (residues 2–22, 35–52, 57–71, and 76–130) was superimposed, and the result for the minimized holo structures showed that little change was introduced by the point mutations to the conformation of the protein. Table 3 shows the root mean square displacement for the protein’s heavy atoms, grouped by structural regions. Not surprisingly, relatively larger root mean square displacement than any of the point mutants. Together, these estimations suggest that, as anticipated, the point mutations did not change the overall protein structure to a great extent.

A one-tailed t test comparing the root mean square displacements of the portal and nonportal regions of the protein was performed. Because the variance for the different groups of regions is not the same (F-test, p < 0.01), the t test for unequal variance was used. It shows that the portal region displacements exceed the nonportal displacements (p < 0.02).

The CD spectrum of the wild-type protein was found to agree in shape and intensity with previously published results (4, 38). The
mutant IFABP spectra were similar to the wild type protein spectrum, with all showing a minimum at 215 nm (not shown). Values of the molar ellipticity at 222 nm, \( \theta_{222} \), for wtIFABP and the mutant IFABPs are shown in Table 1. It is likely that the variability in these data, as described under “Experimental Procedures,” showed a preferential partitioning of 12AO to the SUVs (Table 1), in agreement with previous results (40). The partition coefficients for 12AO distribution between the mutant proteins and EPC SUs are very similar to that for the wild-type protein. These results indicate that, as for the native ligand, oleate, the relative affinity of the mutant proteins for 12AO is essentially unchanged compared with the wild-type IFABP. The similar \( K_p \) values obtained indicated that in the AOFAB transfer assays, the same protein/SUV ratios as those employed for the wild-type protein, could be employed.

Overall, the controls suggest no major alterations in the conformation and binding site properties of the mutant IFABPs relative to their parent wild-type protein. All mutants fold properly and bind a single FA molecule in a relatively hydrophobic binding site.

The fluorescent probe ADIFAB, an IFABP covalently modified with an acrylodan fluorophore, was used to assess the equilibrium binding affinity of the fatty acid oleate. Binding of FA to ADIFAB induces a red shift in the acrylodan emission spectrum, and this is used to provide a measure of unbound FA in solution (29). Using the known \( K_d \) of ADIFAB for a particular ligand (29), equilibrium binding affinities can be determined for another protein. The \( K_d \) obtained for oleate binding to wtIFABP (37 ± 1 nM) is in agreement with those previously determined (4, 29). Data for the mutants demonstrated a single binding site with \( K_d \) values similar to that obtained for the wild type protein (Table 1).

An apparent partition coefficient value was also obtained for each protein, describing the relative distribution of 12AO between an FABP and EPC-SUVs. This value was determined by adding SUVs containing the energy transfer quencher NBD-PC to a solution containing a preformed 12AO-FABP complex. With the successive addition of increasing amounts of the SUVs, a decrease in fluorescence emission was observed upon net displacement of fatty acid to the SUVs. Analysis of these data, as described under “Experimental Procedures,” showed a preferential partitioning of 12AO to the SUVs (Table 1), in agreement with previous results (40). The partition coefficients for 12AO distribution between the mutant proteins and EPC SUVs are very similar to that for the wild-type protein. These results indicate that, as for the native ligand, oleate, the relative affinity of the mutant proteins for 12AO is essentially unchanged compared with the wild-type IFABP. The similar \( K_p \) values obtained indicated that in the AOFAB transfer assays, the same protein/SUV ratios as those employed for the wild-type protein, could be employed.

Overall, the controls suggest no major alterations in the conformation and binding site properties of the mutant IFABPs relative to their parent wild-type protein. All mutants fold properly and bind a single FA molecule in a relatively hydrophobic binding site.

**Effect of Vesicle Concentration on AOFAB Transfer from FABPs to Membranes**—The effect of acceptor membrane phospholipid concentration on rates of ligand transfer has been used to distinguish between an aqueous diffusion mechanism, where no effect is observed, and a collision-mediated mechanism, where the ligand transfer rate is directly related to the donor-acceptor collisional frequency and, hence, the vesicle concentration (3–5, 8). To distinguish between these transfer mechanisms, AOFAB transfer from the mutant proteins to model zwitterionic...
FIGURE 2. Effect of acceptor membrane concentration on 12AO transfer from FABPs to zwitterionic SUVs. Final concentrations were 15 μM FABP with 1.5 μM 12AO and 150–600 μM EPC-NBD acceptor vesicles. Each panel represents the results of the neutralization (∗) and reversion (■) mutants for a specific position, compared with the wild-type IFABP (wI). A, K16I and K16E; B, K20I and K20E; C, K27I and K27E; D, K29I and K29E, E, K92I and K92E. The values shown are the mean ± S.D. from three sets of experiments. Two-tailed paired t-tests were used to determine the significant differences for each mutant versus wild-type IFABP for each concentration. p < 0.05 (*) and p < 0.01 (#) are presented.
membranes was examined as a function of increasing SUV concentration, and results were compared with those for the wild-type IFABP, a well-characterized example of an FABP with a collisional ligand transfer mechanism. Fig. 2 shows the results obtained when constant concentrations of these FABP-AOFA donor complexes were mixed with increasing concentrations of EPC SUVs. Wild-type IFABP showed an almost proportional increase in transfer rates, ranging from 0.56 ± 0.10 to 1.69 ± 0.17 s⁻¹, to 150–600 μM SUV, similar to previous results (3, 8). All point mutants examined exhibit a nearly proportional increase in 12AO transfer rate as a function of vesicle concentration. This suggests that they all maintain the collisional mechanism of FA transfer characteristic of the wtIFABP. Nevertheless, the position of the lysine modification was important in determining the absolute AOFA transfer rates. Transfer rates from the α₁ helical mutants were lower than rates from the wild-type IFABP. For both Lys₁⁶ and Lys₂⁰, a larger impact was observed when lysine was substituted with isoleucine (∼36%) than the substitution for a glutamic acid (∼25% decrease). Notably, the neutralizations (Lys → Ile mutants) in positions 16 and 20 produce exactly the same effects, and the same happens with the charge reversals (Lys → Glu mutants) in the same residues.

In contrast to the α₁ helical lysine mutants, the behavior of the two lysine residues of the α₂ helix was divergent. The largest impact of any of the point mutations was found for K27I, exhibiting up to a 70% decrease in transfer rate to EPC SUV relative to wild type IFABP. In contrast, AOFA transfer rates from K29I and K29E mutants were little different from those of IFABP.

Nonportal neutralization mutant at position 92 did not show any difference compared with the wtIFABP. Surprisingly, reversion of the positive charge at the same position (K92E) resulted in a significant increase in AOFA transfer rates compared with those of wtIFABP (Fig. 2E).

Effect of Vesicle Charge on AOFA Transfer from FABPs to Membranes—Changes in the surface charge properties of the acceptor vesicle can also influence ligand transfer rates if electrostatic interactions between donor protein and acceptor membranes are involved, whereas in the case of aqueous diffusion, characteristics of the acceptor membrane would not be expected to modulate the transfer rate. Fig. 3 shows that, as expected from previous studies, the 12AO transfer rate from wtIFABP is substantially increased by incorporation of 25 mol% PS or CL into EPC/NBD-PC acceptor membranes (4, 8). A 2-fold increase for wtIFABP, on average, was observed in the absolute rate of 12AO transfer to PS-containing vesicles, from 0.56 ± 0.10 to 0.93 ± 0.05 s⁻¹ for transfer to 150 μM EPC and PS acceptor SUVs, respectively. Incorporation of negatively charged CL in acceptor phospholipid vesicles resulted in a dramatic 8-fold increase in AOFA transfer rate from IFABP relative to transfer to zwitterionic vesicles.

Fig. 3A shows the relative transfer rates from wtIFABP and the α₁ helix mutants to EPC-containing, PS-containing, and CL-containing vesicles, normalized to the EPC acceptor for each protein. Overall, point mutants of lysine residues in the α₁ helix resulted in modest decreases in sensitivity to vesicle negative charge. Incorporation of CL into the acceptor vesicles resulted in approximately 3–4-fold increases in AOFA transfer rate relative to rates to EPC, as compared with the 8-fold stimulation observed for wtIFABP. K16I showed the greatest effect, withceptor membrane CL causing only a 2-fold increase in AOFA transfer rate. For transfer to PS-containing membranes, only K16I showed a decrease in relative transfer rate.

Point mutations in the α₂ region showed a consistent pattern for neutralization versus charge reversal for both of the lysines. Neutralization did not induce significant decreases in AOFA transfer rate to either PS or CL vesicles, compared with wtIFABP (Fig. 3B). Moreover as for wtIFABP, both K27I and K29I showed a significant increase in 12AO transfer rate to CL vesicles relative to EPC vesicles. On the other hand, reversion mutants showed substantial decreases in AOFA transfer rates to PS vesicles and the most dramatic decreases in transfer rates to CL vesicles (78% (p < 0.01) for K27E, and 86% for K29E (p < 0.01)) compared with the wild-type protein (Fig. 3B). Indeed, charge reversal mutants K27E and K29E showed no significant modification of AOFA transfer rates to vesicles of different composition, as confirmed by analysis of variance (p > 0.05).

Neutralization of Lys²⁰ to isoleucine or conversion to a negatively charged glutamate had little effect on the absolute rates of AOFA transfer to membranes or on the sensitivity to negative charge (Fig. 3C).
IFABP Helix Lysines and Fatty Acid Transfer

FIGURE 4. Photolabeling of apo- and holo-IFABP by incubation with membranes of different composition containing 125I-TID-PC. Each panel corresponds to vesicles of different composition: EPC-125I-TID-PC (I), PS-125I-TID-PC (II), and CL-125I-TID-PC (III). Experiments were conducted as indicated under “Experimental Procedures.” Results of one representative experiment of three are presented. SDS-PAGE (A) and autoradiography (B) are shown in each panel. Quantification of the radioative labeling in the autoradiography was performed using the ImageJ program (developed by the National Institutes of Health) as described under “Experimental Procedures.” Values are expressed relative to the mass of the protein in the SDS-polyacrylamide gel, quantified with the same program, and normalized (1.0) to apo-wtIFABP incubated with EPC LUVs. Results of the fatty acid transfer assay and examined their interaction with acidic membranes. The mutant proteins generated showed no major structural or ligand-binding differences compared with the wild-type IFABP (Tables 1 and 2). The structural stability of the mutant proteins was not unexpected, since point mutations of other members of the FABP family have also been shown to be remarkably stable (12, 14), and the covalent incorporation of several fluorescent moieties into the IFABP structure did not alter its folding and ligand binding properties (41). The absence of effects on the ligand binding site was also expected, since IFABP lysines are all oriented toward the aqueous milieu (22) and do not interact with the ligand located in the binding pocket. Instead, they are accessible for interaction with the polar head groups of acceptor membrane phospholipids.

Charge neutralization of three of the four Lys residues of the α-helical domain (K16I, K20I, and K27I) decreases the FA transfer rate to zwitterionic vesicles, compared with the wild-type protein. K29I, in contrast, shows a behavior indistinguishable from the wild type protein. Neutralization of lysine 27 (K27I) results in the most dramatic effect, decreasing the absolute transfer rate and markedly diminishing the sensitivity to SUV concentration. Modifications in the two Lys residues of the α helix induce very similar changes in the FA transfer kinetics. Neutralization of residues 16 and 20 decreases the ligand transfer rate to EPC-SUVs; however, the charge reversal mutants were less affected. It is possible that maintaining the polar face of the α amphipathic helix results in a kinetic behavior similar to the wild type, whereas a disruption of the amphipathicity caused by the incorporation of Ile, disrupts the putative protein-membrane interactions.

The dramatic increase in AOFA transfer rate from wild-type IFABP to negatively charged CL vesicles was blunted 2–4-fold for the α helix mutants, indicating that loss of each of the positively charged residues diminished somewhat the protein’s sensitivity to acceptor charge. Thus, unlike FA transfer to zwitterionic acceptor membranes, maintaining the α helix amphipathicity by retaining a charged residue of either sign has the same effect on transfer to acidic vesicles as did the disruption of the amphipathicity by replacement of a charged residue with an uncharged residue. This suggests the importance of the interactions of cationic residues with anionic phospholipids. For the α2-helix, such charge-
charge interactions appear even more essential for FA transfer properties. For both K27E and K29E, sensitivity to acceptor vesicle charge is almost completely abolished relative to wild-type IFABP, probably demonstrating electrostatic repulsion between the Glu residues and the acidic groups in the membrane. In contrast, replacement of the basic residues Lys27 and Lys29 with isoleucine resulted in less than a 2-fold decrement in the proteins’ sensitivity to acceptor negative charge. This systematic and significant difference observed for charge reversal compared with neutralization highlights the importance of charge-charge interaction of the α2 helix with membranes and suggests that the charged face of the α2 helix is critical for membrane interactions that lead to the dramatic increase in AOFAR transfer rates from IFABP to anionic membranes.

The higher AOFAR transfer rates obtained for the neutralization mutants compared with the reversion mutants could also be a consequence of a contribution of Ile in positions 27 and 29 to the hydrophobic patch observed in the IFABP tertiary structure; the α2 hydrophobic residues Val25, Val26, and Leu30 point away from the interior of the protein, forming the only hydrophobic patch on the protein surface (21). We have recently shown that both electrostatic and hydrophobic interactions contribute to the collision-mediated FA transfer mechanism for IFABP (16). Thus, an increase in the hydrophobic character of the α2 helix could offset to some extent the loss of the electrostatic interactions of Lys27 and Lys29 with acidic membranes.

The α2 helix is a key structural element of the putative fatty acid portal, and forms long range interactions with the β2 turn between strands C and D. The present studies indicate that it is likely to play a role in the fatty acid transfer process as well. NMR solution structures of the apo and holohelix forms of IFABP have demonstrated considerably greater differences from those initially suggested by crystallographic analysis (7). Notably, the distal half of the α2 helix and the turn between β-strands C and D are the regions of the protein that exhibit the largest structural differences; both of these portal domain elements were found to be more disordered in the absence of bound ligand and to exhibit diminished long range interactions (7). This suggests that during ligand exit/entry, a conformational change may take place in this region of the protein, allowing the fatty acid to pass through the portal. In addition, mutations in the α2 helix and C-D turn of heart FABP and adipocyte FABP were shown to alter the rate of AOFAR transfer to membranes, further indicating the participation of α2 in collisional transfer of fatty acids (12, 13). For IFABP, mutations at lysine 27 show the largest changes in response to both acceptor vesicle concentration and charge. Neutralization of lysine 27 markedly diminishes the sensitivity to SUV concentration but maintains sensitivity to SUV charge, whereas substitution for glutamic acid at position 27 eliminates the sensitivity to charge but maintains some sensitivity to SUV concentration, which is probably secondary to diminished protein-membrane interactions. In the holoprotein, Lys27 is oriented across the portal, forming an interaction with Asn24, which is located at the end of the turn between the two helices. In the apoprotein, by contrast, Lys27 points toward the exterior of the molecule (21, 22). Substitution of this lysine is likely to modify the interaction with Asn24 and/or with the polar head groups of the phospholipids, thereby contributing to the changes in FA transfer rate observed in the present experiments (Fig. 6A).

Lysine 29 is located in one of the most dynamic regions of backbone mobility (7). As for Lys27, neutralization of the residue reduced IFABP sensitivity to acceptor membrane negative charge; charge reversal resulted in a drastic (>85%) loss in sensitivity to CL-containing vesicles. Thus, disruption of the basic character of the α2 helix disrupts the effective interaction of holo-IFABP with membranes. In contrast to the large differences in AOFAR transfer rates from Lys29 IFABP mutants to acidic vesicles, mutations in the Lys29 position did not produce significant changes in transfer rates to zwitterionic vesicles. It is possible that this unique behavior may be explained by the existence of side chain interactions. Unlike the other lysines examined, in the native protein, lysine 29 forms a surface salt bridge with glutamate 15 on the α1 helix, a highly conserved residue in the superfamily of cytosolic lipid-binding proteins (21). The salt bridge may help to hold the two helices together, thereby maintaining the stability of the helical cap. The role of surface salt bridges in local and overall protein stability has been described in other proteins (42, 43). A helical cap that lacks the Lys29-Glu15 salt bridge, caused by neutralization or reversal of the positive charge, results in an altered behavior compared with the rest of the mutants of the α-helical domain, which can be attributed both to the destabilization of the α-helical domain and to the liberation of the Glu15 charge to the aqueous medium. This suggestion is supported by energy minimization of the K29I and K29E mutant structures, as shown in Fig. 6B.

In contrast to the α-helix domain lysine mutants, little effect was found following modifications of Lys92, present in β-strand G, with the K92I showing AOFAR transfer rates identical to those of the wild-type IFABP for all conditions examined and K92E substitution resulting in a stimulation of AOFAR transfer rate identical to that of wild-type IFABP upon CL incorporation into acceptor membranes. K92E substitution did result in an increase in absolute AOFAR transfer rates to zwitterionic membranes; however, suggesting that whereas Lys27 is not an essential element of the collisional transfer process, the introduction of a negative charge at this position generates the possibility of an additional interaction of this group with the positive charge of the phosphatidylcholine head group on the zwitterionic vesicle.

Physical interaction between the IFABP forms and membranes was directly investigated by analyzing the radioactive labeling of the protein after incubation with a photoactivable reagent, followed by cross-linking photolabeling. Membrane insertion of several proteins has been identified using these reagents (36, 44, 48). The results showed that native IFABP interacted with membranes in an acceptor vesicle charged and lipid-dependent manner. The dependence on the membrane charge is coincident with our transfer experiments, where the AOFAR transfer rate is increased to acceptor membranes containing negatively charged phospholipids. Since the net surface charge of all cytosol-facing membranes is believed to be negative, it seems likely that charge-charge interactions are the primary driving force for IFABP-mediated FA transfer within the cell. It is interesting to note that CL is a relevant constituent of the inner and outer mitochondrial membranes (49), and its involvement in protein-membrane interactions is well appreciated (50). It is possible that the apparently strong interaction and rapid FA transfer rates from IFABP to CL-containing membranes could be sug-

Supplementary Material

FIGURE 6. Superimposition of rat intestinal FABP and α2 Lys mutant structures after energy minimization based on the IFABP-palmitate complex x-ray crystal structure (19) (Protein Data Bank code 2IFB). The protein molecule is oriented to show the different orientation of residues in the α2 helix most clearly. A, residues 24 and 27 have been highlighted to show the altered interaction caused by the K27I and K27E mutations observed in the putative ligand portal domain. B, residues 15 and 29 have been highlighted to show the likely disruption of the stabilizing salt bridge between them caused by the K29I and K29E mutations.

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gesting one aspect of its function in FA trafficking to specific metabolic pathways. Indeed, the cross-linking results indicate that IFABP interacts more strongly with membranes to deliver FA to zwitterionic and PS-containing membranes than to extract FA but interacts strongly with CL-containing membranes to facilitate FA transfer in both directions. As expected (8–10), the helixless IFABP showed a markedly diminished degree of membrane interaction compared with wtIFABP. In contrast, cross-linking analysis of the Lys point mutants showed that the interaction of IFABP with membranes is not affected by individual amino acid substitutions. These results are not unexpected, since a cooperative effect of the lysines appears to be responsible for effective interaction with membranes to take place and since no single point mutation completely abolished the effect of acceptor membrane properties on AOFA transfer. It is also important to note that this method may not be sensitive enough to detect small changes in the degree of IFABP-membrane interaction.

The interaction of IFABP with membranes appears to be greater for the holoprotein than the apoprotein. This suggests the formation of different pathways. It is also important to note that this method may not be sensitive enough to detect small changes in the degree of IFABP-membrane interaction.

Previously, we suggested (8, 12, 14) that the collision–based transfer of fatty acids from the FABP binding site to model membranes may occur in a multistage process, as follows: stage 1, interaction of FABP with the acceptor membrane; stage 2, conformational transition of the dynamic portal region from the ordered closed state to a more disordered open state; stage 3, dissociation of FA from the ligand binding cavity; and stage 4, association of the FA with the acceptor membrane. For wild-type IFABP, stage 1 would be rate-limiting, and the transfer process exhibits collisional kinetics. For the helixless protein (9), we hypothesized that step 3 was rate-limiting due to complete elimination of steps 1 and 2. Based on the present results, we hypothesize that a stable $\alpha_1$ helix is necessary for step 1 to occur and that the $\alpha_2$ helix is of critical importance for steps 1 and 2. Modification of either lysine in $\alpha_2$ resulted in a decreased rate of transport that was probably caused by decreases in helix stability, decreased interaction with the membrane, or both. For mutant K27I in $\alpha_2$, it appears that step 1 is markedly dampened, probably reflecting a weak protein-membrane interaction when the acceptor membranes are net neutral. When membranes are negatively charged, the K27I and K29I mutants in $\alpha_2$ each demonstrated a 50% decrease in sensitivity to this charge, suggesting that the remaining basic residue maintained its charge–charge interactions with the acidic acceptors. In contrast, the $\alpha_3$ charge reversal mutants K27E and K29E showed a drastic decrease in sensitivity to membrane charge, probably reflecting repulsive effects with negative charges on the membrane that diminished the strength of the protein-membrane interaction, stage 1. Given that the side chain of lysine 27 extends over the portal region and that the side chain of lysine 29 stabilizes helix 2-helix 1 interactions, it is likely that step 2 could be modified when either of these Lys are substituted with Ile, resulting in a change in the protein-membrane complex and/or decrease in the rate of the conformational transition of the dynamic portal, thereby causing slower delivery of FA to membranes. The present results also suggest that the collisional complex formed in step 1 and the conformational transition of step 2 may be different, depending on the acceptor vesicle composition. In all experiments to date, however, the transfer data are well fit by a single exponential function, implying that in the case of the Lys point mutants, the rate-limiting step is likely to reflect step 1, a specific protein-membrane interaction. Thus, we hypothesize that the lysine mutations modify the conformation of the IFABP-membrane “collisional complex,” resulting in altered fatty acid transfer rates. Although there are no tertiary structures yet available for an FABP-membrane complex, evidence for conformational changes with membrane binding has been obtained using infrared reflection-absorption spectroscopy, where we showed that the secondary structure of wtIFABP in lipid monolayers differed from its solution structure (10). Changes in IFABP structure upon ligand binding, greatest in the ligand portal region, are also supportive of a conformational change (7, 51). Finally, we have shown that fluorescent fatty acid transfer from membrane donors to IFABP occurs via membrane–protein interaction; however, modulation of the transfer rate by membrane and solution properties was different than for transfer from protein to membrane, implying that the collisional complexes for holo-IFABP and apo-IFABP are different (40).

In summary, the present results provide support for the multistep process of FA transfer from IFABP to membranes and support the hypothesis of Hodsdon and Cistola (7) regarding the existence of a portal domain that undergoes conformational changes during FA release. Our results suggest that IFABP interactions with vesicles induce this conformational change and further indicate the possibility of different conformational changes when the protein is delivering ligand to or extracting ligand from membranes of different composition.

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