

# Fatty acid transfer from intestinal fatty acid binding protein to membranes: electrostatic and hydrophobic interactions

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**Abstract** Intestinal fatty acid binding protein (IFABP) is thought to participate in the intracellular transport of fatty acids (FAs). Fatty acid transfer from IFABP to phospholipid membranes is proposed to occur during protein-membrane collisional interactions. In this study, we analyzed the participation of electrostatic and hydrophobic interactions in the collisional mechanism of FA transfer from IFABP to membranes. Using a fluorescence resonance energy transfer assay, we examined the rate and mechanism of transfer of anthroyloxy-fatty acid analogs *a*) from IFABP to phospholipid membranes of different composition; *b*) from chemically modified IFABPs, in which the acetylation of surface lysine residues eliminated positive surface charges; and *c*) as a function of ionic strength. The results show clearly that negative charges on the membrane surface and positive charges on the protein surface are important for establishing the “collisional complex,” during which fatty acid transfer occurs. In addition, changes in the hydrophobicity of the protein surface, as well as the hydrophobic volume of the acceptor vesicles, also influenced the rate of fatty acid transfer. Thus, ionic interactions between IFABP and membranes appear to play a primary role in the process of fatty acid transfer to membranes, and hydrophobic interactions can also modulate the rates of ligand transfer.—Córscico, B., G. R. Franchini, K.-T. Hsu, and J. Storch. Fatty acid transfer from intestinal fatty acid binding protein to membranes: electrostatic and hydrophobic interactions. *J. Lipid Res.* 2005. 46: 1765–1772.

**Supplementary key words** fatty acid transfer mechanism • chemical modification of proteins • protein acetylation • structure-function analysis • intracellular lipid-binding proteins

Intestinal fatty acid binding protein (IFABP) is an intracellular protein of 15.1 kDa that binds long-chain fatty acids (1). IFABP is abundantly expressed in small intestine enterocyte cytosol and together with liver fatty acid binding protein (LFABP) represents a high proportion of the total cellular protein. It is hypothesized that these fatty acid binding proteins (FABPs) are important in the intracellu-

lar transport of FAs, and accumulating indirect and direct evidence supports this idea (2). Several differences found between these two proteins, including tissue distribution, ligand specificity, and binding stoichiometry, have suggested that they are functionally different. Furthermore, we have demonstrated that they transfer FA to and from phospholipid membranes through markedly different mechanisms: whereas LFABP uses a diffusion-mediated mechanism, IFABP transfers fatty acids through direct collisional interactions (3, 4). We have shown that the  $\alpha$ -helical domain of IFABP is responsible for the collisional mechanism of transfer (5, 6) and have demonstrated that the  $\alpha$ -helical domain of LFABP is responsible for this protein's diffusion-mediated transfer mechanism (7).

The collisional FA transfer mechanism described for IFABP has also been found for several other FABPs examined to date, including heart, adipocyte, keratinocyte, and brain (8–11). As most of the enzymes involved in FA metabolism are membrane-bound, it is possible that IFABP could modulate lipid metabolic enzyme activity via direct interactions with membrane lipids and/or proteins. To gain insight into IFABP function, it is important to determine those characteristics of the protein and the acceptor membrane that regulate the fatty acid transfer process.

In the present study, we analyzed the influence of electrostatic interactions on the collisional mechanism of FA transfer from IFABP to phospholipid membranes. Using a fluorescence resonance transfer assay, the transfer of anthroyloxy-labeled fatty acids (AOFAs) from IFABP to model membranes with different phospholipid head group and acyl chain composition was analyzed. Furthermore, IFABP

Abbreviations: 12AO, 12-(9-anthroyloxy)oleic acid; 12AS, 12-(9-anthroyloxy)stearic acid; AOFA, anthroyloxy-labeled fatty acid; CL, bovine heart cardiolipin; EPC, egg phosphatidylcholine; FA, fatty acid; FABP, fatty acid binding protein; IFABP, intestinal fatty acid binding protein; LFABP, liver fatty acid binding protein; NBD-PC, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) egg phosphatidylcholine; PS, brain phosphatidylserine; SUV, small unilamellar vesicle.

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was chemically modified by complete and selective acetylation of the surface lysine residues to examine the influence of positive surface charges of the protein. Finally, the influence of the ionic strength of the media on the rate of AOFA transfer from the acetylated protein compared with native IFABP was examined.

The results show that the FA transfer rate from IFABP to membranes is influenced by the presence of membrane negative charge and by the acyl chain composition of the acceptor vesicles. Neutralization of IFABP surface lysine residues reduces the rate of FA transfer to acidic membranes and markedly inhibits the collision-mediated transfer. In contrast, the neutralization of positive charge on the surface of the protein produces an increase in the rate of FA transfer to zwitterionic membranes without modifying the collisional mechanism of FA transfer. Experiments varying the ionic strength of the media confirm the important role of electrostatic interactions in the transfer of FA from IFABP to acceptor membranes. These results indicate that, unlike LFABP (3, 4, 8–11), electrostatic interactions are a major determinant of the collisional mechanism of FA transfer from IFABP to membranes. Further modulation by properties of the membrane acyl chain domain also supports a role for hydrophobic interactions in the FA transfer process.

## EXPERIMENTAL PROCEDURES

### Purification of IFABP

Recombinant rat pET11d-IFABP plasmid was generously provided by Drs. Alan Kleinfeld and Ron Ogata (Torrey Pines Institute for Molecular Studies, San Diego, CA). The protein was expressed in the *Escherichia coli* BL21 (DE3) expression system and purified as described previously (3).

### Modification of IFABP

The 17 lysine residues of IFABP were selectively neutralized by reaction with acetic anhydride as described previously (12). Ten milliliters of 1 mg/ml IFABP was dialyzed into distilled deionized water and filtered through a 0.22  $\mu\text{m}$  pore filter. Protein concentration was estimated by absorbance at 280 nm using  $\epsilon = 16,900 \text{ M}^{-1} \text{ cm}^{-1}$  (13). The protein solution was titrated to pH 9.0 with NaOH. Acetic anhydride was added to the stirred protein solution in 10 aliquots of 2  $\mu\text{l}$  over 15–30 min to a 50-fold molar excess over lysines. After each addition, the pH was maintained near 9 with the addition of 1 N NaOH. Solid  $\text{NH}_2\text{OH}\cdot\text{Cl}$  was added to the stirred solution to a final concentration of 1 N, and the pH was increased to 10 with 5 N NaOH added in 10  $\mu\text{l}$  aliquots. The solution was incubated at room temperature for 1 h. The acetylated IFABP was dialyzed against phosphate buffer, pH 7.5.

The fluorescent probe fluorescamine (Sigma) was used to quantify lysine modification (14). This probe reacts covalently with primary amines, increasing their fluorescent emission. Briefly, 50  $\mu\text{l}$  of protein was mixed with 1 ml of 0.2 M borate buffer, pH 9, and 0.5 ml of 1 mM fluorescamine. Emission spectra of increasing concentrations of protein were measured using an SLM 8000 spectrofluorimeter, with an excitation wavelength of 390 nm and emission monitored at 478 nm. The linear range of the fluorescamine-dependent fluorescence was determined with a standard curve using native IFABP.

### Ligand binding

Fluorescent 12-(9-anthroyloxy)oleic acid (12AO; Molecular Probes) was used as a probe to assess the fatty acid binding affinity of acetylated IFABP relative to that of native protein, as described (15).

### Protein stability analysis

Circular dichroic spectra in the near ultraviolet range were measured on a Jasco 810 spectropolarimeter (Jasco Corp.). An average of five spectra were obtained for the apo-acetylated protein and the native apolipoprotein. Spectra were recorded from 240 to 180 nm. Secondary structures were predicted from the circular dichroic data using three algorithms, SELCON3, CONTINLL, and CDSSTR, at the online server of the Department of Crystallography, Birkbeck College, University of London (<http://www.cryst.bbk.ac.uk/cdweb>). In agreement with results from X-ray studies (16), native IFABP was predicted to have  $\sim 75\%$   $\beta$  sheet. The estimation for percentage of  $\alpha$ -helix, found to be 10% in the crystallographic analysis, showed substantial variability (22, 23, and 2% for the three algorithms, respectively), likely because of the typically larger errors in the 180–200 nm range of circular dichroic spectra. Each of the three algorithms predicted an  $\sim 30\%$  decrease in the percentage of  $\alpha$ -helix after acetylation; thus, alterations in kinetic properties could be secondary in part to the change in secondary structure. Nevertheless, because the helical domain was basically preserved, results are reasonably interpretable in terms of protein surface properties rather than folding per se.

### Vesicle preparation and characterization

Small unilamellar vesicles (SUVs) were prepared by sonication and ultracentrifugation as described previously (17, 18). All phospholipids were obtained from Avanti Polar Lipids, Inc. The standard vesicles were prepared to contain 90 mol% egg phosphatidylcholine (EPC) and 10 mol% *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) egg phosphatidylcholine (NBD-PC), which served as the fluorescence quencher. To increase the negative charge density of the acceptor vesicles, 25 mol% brain phosphatidylserine (PS), bovine heart cardiolipin (CL), monolysocardiolipin, or dilylcardiolipin was incorporated into the SUVs in place of an equimolar amount of EPC. Vesicles were prepared in TBS buffer (40 mM Tris, 100 mM NaCl, pH 7.4) except for SUVs containing cardiolipin, which were prepared in TBS with 1 mM EDTA. Vesicle preparations containing EPC plus a CL head group species were characterized at 25°C by dynamic laser light scattering using a BI-200SM spectrometer equipped with a BI-9000 AT digital time correlator (Brookhaven Instrument, Inc.) and a solid-state laser (diode pumped solid state, SUWTECH, 200 mW, 532 nm), as described by Zhou et al. (19). Samples were prepared at 3.8–4.4 mM phospholipid in TBS-EDTA and filtered through a 45  $\mu\text{m}$  Millipore Millex-HN filter to remove dust before the measurements. Vesicle surface charge density was estimated according to Pinheiro, Duralski, and Watts (20).

### Transfer of AOFA from FABP to SUVs

A fluorescence resonance transfer assay was used to monitor the transfer of AOFA from native and acetylated IFABPs to acceptor model membranes as described in detail elsewhere (3, 8, 9). Briefly, FABP with bound AOFA was mixed with SUV using a Stopped-Flow Spectrofluorometer DX-17MV (Applied Photophysics Ltd.). The NBD moiety is an energy transfer acceptor of the anthroyloxy group (AO) donor; therefore, the fluorescence of the AOFA is quenched when the ligand is bound to SUV that contains NBD-PC. Upon mixing, transfer of AOFA from protein to membrane is monitored directly by the time-dependent decrease in AO fluorescence. Final transfer assay conditions were

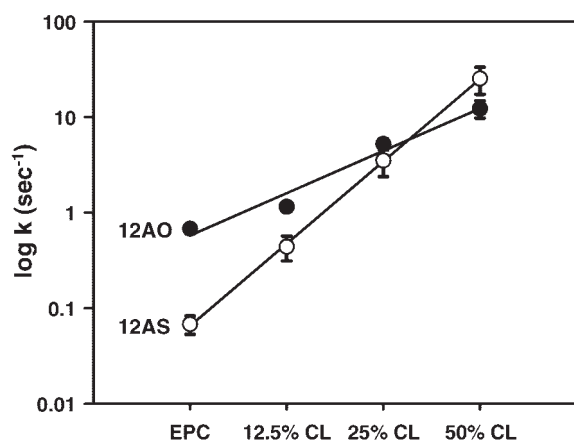
15  $\mu\text{M}$  IFABP with 1.5  $\mu\text{M}$  12AO or 12-(9-anthroyloxy)stearic acid (12AS) and 150–600  $\mu\text{M}$  SUV. Transfer was monitored at 25°C. Controls to ensure that photobleaching was eliminated were performed before each experiment. Data were analyzed using software provided with the instrument, and all curves were well described by a single exponential function. For each experimental condition, at least five replicates were done during each experiment. Average values  $\pm$  SD for three or more separate experiments are reported.

## RESULTS

### Effect of acceptor vesicle composition

The surface charge density of acceptor vesicles can modulate ligand transfer rates if electrostatic interactions between donor protein and acceptor membranes are involved. In previous studies, we observed a substantial increase in AOFA transfer rates from IFABP by incorporation of 25% CL into phospholipid vesicles compared with zwitterionic vesicles (3, 5, 7). Here, two AOFA analogs, 12AS (18:0) and 12AO (18:1), were used to evaluate the effect of the CL content of the acceptor vesicles. In agreement with previous results (3), the monounsaturated probe, 12AO, transferred 10-fold more rapidly than the saturated probe, 12AS, to zwitterionic vesicles. As increasing amounts of CL were incorporated in the vesicles, transfer rates of both 12AO and 12AS from IFABP showed logarithmic increases, although with a greater slope for 12AS (Fig. 1). Thus, as membrane CL content increases, the difference in transfer rate between 12AO and 12AS decreases. Both ligands show approximately the same transfer rate for vesicles with 25% CL, and at 50% CL the rate of 12AS transfer slightly exceeded that of 12AO (Fig. 1).

We also examined the effects of CL species on the AOFA transfer rate from IFABP. Monolysocardiolipin and



**Fig. 1.** Effect of vesicle bovine heart cardiolipin (CL) content on anthroxyloxy-labeled fatty acid (AOFA) transfer from intestinal fatty acid binding protein (IFABP) to small unilamellar vesicles (SUVs). Transfer of 1.5  $\mu\text{M}$  12-(9-anthroyloxy)oleic acid (12AO; closed circles) and 12-(9-anthroyloxy)stearic acid (12AS; open circles) from 15  $\mu\text{M}$  IFABP to 150  $\mu\text{M}$  egg phosphatidylcholine (EPC) SUV containing 10% *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) egg phosphatidylcholine (NBD-PC) and increasing concentrations of CL. Averages from three different experiments  $\pm$  SD are shown.

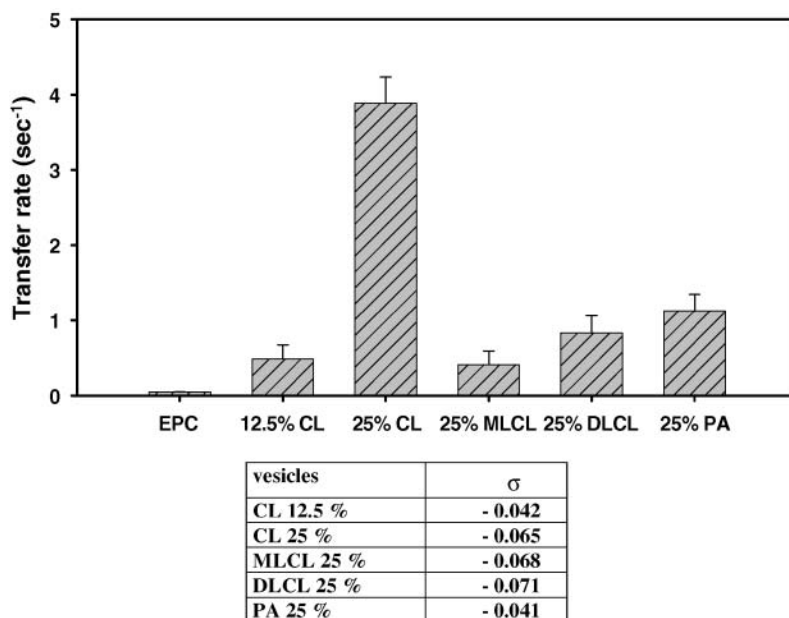
dilysocardiolipin are CL analogs formed by the hydrolysis of one and two *sn*-2 acyl chains, respectively, present on the glycerol backbone of heart CL. Dynamic light-scattering measurements on 100% PC vesicles, 25% monolysocardiolipin/75% PC vesicles, and 25% CL/75% PC vesicles indicated that the preparations were monodisperse, with mean diameters of  $23.9 \pm 4.3$ ,  $28.4 \pm 5.2$ , and  $36.9 \pm 6.6$  nm, respectively. Estimation of the surface charge (20) showed that all vesicles containing 25% of a CL species had approximately the same charge, as did 12.5% CL vesicles and 25% phosphatidic acid vesicles (Fig. 2, table).

It was found that the rate of transfer of 12AS from IFABP to membranes composed of 25 mol% monolysocardiolipin and dilysocardiolipin decreased by 90% and 80%, respectively, compared with that of 25% CL vesicles (Fig. 2). Although all of the CL species carry the same net charge, they are reported to differ in acyl chain packing (21). In addition, transfer to vesicles composed of 25 mol% CL was  $\sim$ 8-fold faster than transfer to 12.5 mol% vesicles, further indicating that the AOFA transfer rate is not strictly a linear function of membrane charge. Finally, the rate of transfer of 12AS to membranes composed of 25% phosphatidic acid was approximately double the rate to 12.5% CL vesicles, despite equivalent surface head group charge. Thus, alterations in phospholipid acyl chain properties of the bilayer appear to significantly influence the ability of IFABP to associate with membranes, in addition to the very major effects of phospholipid head group charge. Because the average vesicle size was found to increase with increasing numbers of CL acyl chains, it is possible that changes in fatty acid transfer rates could arise secondary to changes in vesicle curvature. However, we previously examined the effect of vesicle size on fatty acid transfer from FABPs and showed that for a given acceptor lipid concentration, transfer rates are slower with increasing size (22). In the results obtained in the present work, fatty acid transfer rates from the CL vesicles were dramatically higher than those for transfer from PC vesicles, indicating that the effect of phospholipid properties rather than vesicle size per se is predominant.

### Acetylation of IFABP

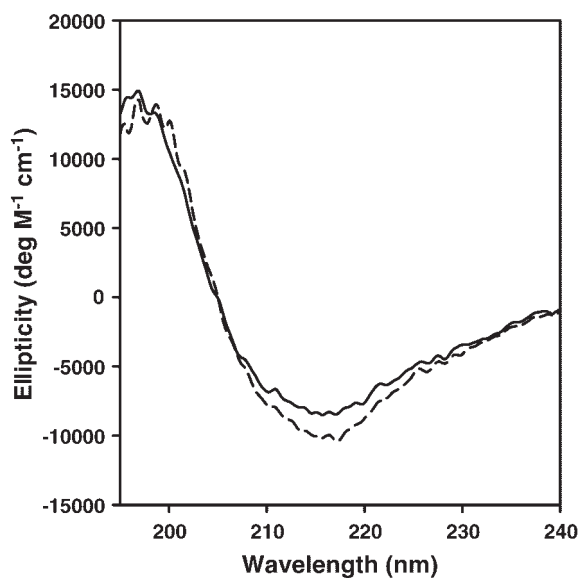
The importance of IFABP cationic surface residues is suggested by the increase in fatty acid transfer rate from IFABP to negatively charged acceptor vesicles relative to zwitterionic vesicles, as observed previously (3, 5, 7). Therefore, we directly examined the effect of neutralization of lysine residues on the transfer of fatty acids from IFABP to membranes. To control for potential alterations in the structure of acetylated IFABP, binding affinities for the fluorescent fatty acid 12AO were assessed, and circular dichroism spectra of acetylated IFABP were obtained and compared with those of the native protein. 12AO bound to both native and acetylated proteins in a saturable manner, and Scatchard analysis indicated apparent binding constants of  $0.16 \pm 0.04$   $\mu\text{M}$  and  $0.15 \pm 0.05$   $\mu\text{M}$  for native and modified IFABP, respectively. Circular dichroic spectroscopy of the wild-type protein agrees in shape and intensity with previously published results (7, 23) (Fig. 3).





**Fig. 2.** 12AS transfer from IFABP to membranes of different phospholipid composition. 12AS (1.5  $\mu\text{M}$ ) and 15  $\mu\text{M}$  IFABP were mixed with 150  $\mu\text{M}$  SUV composed of EPC/NBD-PC (90:10), 12.5% CL (EPC/CL/NBD-PC, 77.5:12.5:10), 25% CL (EPC/CL/NBD-PC, 65:25:10), 25% monolysocardiolipin (MLCL; EPC/MLCL/NBD-PC, 65:25:10), 25% dilyocardiolipin (DLCL; EPC/DLCL/NBD-PC, 65:25:10), or 25% phosphatidic acid (PA; EPC/PA/NBD-PC, 65:25:10). Averages from three different experiments  $\pm$  SD are shown. In the table at bottom, surface charge density ( $\sigma$ ) was estimated as  $(e_0 z_n X_n)/A(1 + PX_n + X_n)$ , where  $X_n$  is the mole fraction of negatively charged lipid,  $z_n$  is the charge of the anionic phospholipids,  $e_0$  is the elementary charge,  $A$  is the cross-sectional area for the phospholipid molecule, and  $P$  is the ratio of the surface areas of negative to neutral lipids (20).

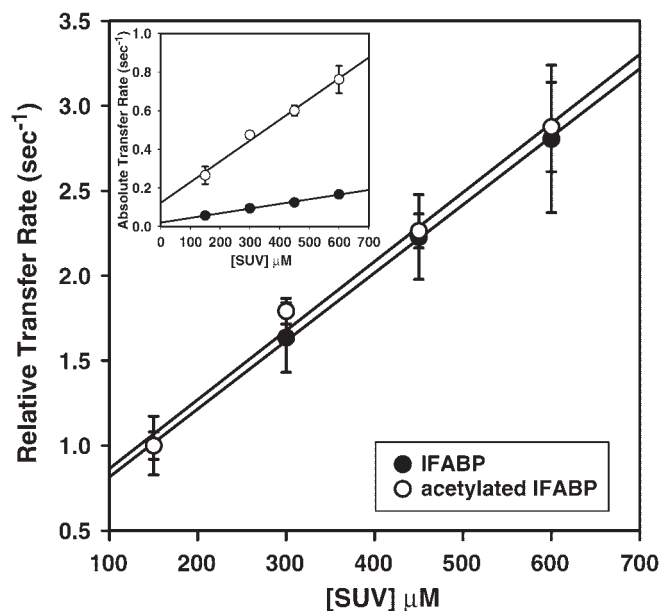
Values of the molar ellipticity show a minimum at 215 nm for both the native and acetylated proteins. These results confirm that acetylation of surface lysine residues did not alter IFABP folding or ligand binding properties.



**Fig. 3.** Circular dichroic spectra of IFABP (solid line) and acetylated IFABP (broken line). Spectra of 0.15 mg/ml IFABP and acetylated IFABP in 10 mM potassium phosphate buffer, pH 7.4, were obtained at 25°C by repetitive scans between 180 and 250 nm.

#### Effect of vesicle concentration on AOFA transfer from acetylated IFABP to zwitterionic membranes

Acetylation of IFABP induced a 5-fold increase in the transfer rate of 12AS from the protein to zwitterionic EPC phospholipid vesicles. Transfer of 1.5  $\mu\text{M}$  12AS from 15  $\mu\text{M}$  native or acetylated IFABP to 150  $\mu\text{M}$  EPC/NBD-PC SUVs were  $0.057 \pm 0.001$  or  $0.265 \pm 0.046$ , respectively. The effect of acceptor membrane phospholipid concentration on rates of ligand transfer can be used to distinguish between an aqueous diffusion mechanism, in which no effect is observed, and a collision-mediated mechanism, in which the ligand transfer rate is directly related to the donor-acceptor collisional frequency and, hence, the vesicle concentration. To distinguish between these transfer mechanisms, AOFA transfer from the acetylated protein to model membranes was examined as a function of increasing membrane concentration, and the results were compared with those for the native IFABP. **Figure 4** shows the results obtained when constant concentrations of these FABP-AOFA donor complexes were mixed with increasing concentrations of EPC SUVs. Increasing the concentration of acceptor lipid over a 4-fold range showed an almost proportional increase in transfer rate with SUV concentration for native IFABP (Fig. 4), in agreement with previous results (3, 5, 7). The result obtained for the acetylated protein are completely equivalent to those of the native IFABP, indicating that, in spite of the modification in the absolute transfer rate [which is maintained for the whole range of SUV concentrations tested (Fig. 4, in-

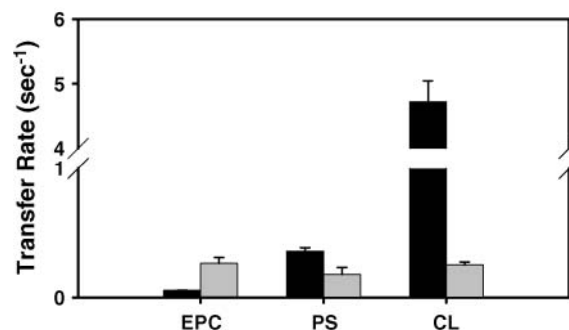


**Fig. 4.** Effect of acceptor membrane concentration on AOFA transfer from IFABP and acetylated IFABP. Transfer of 1.5  $\mu\text{M}$  12AS from 15  $\mu\text{M}$  IFABP (closed circles) and 15  $\mu\text{M}$  acetylated IFABP (open circles) to EPC/NBD-PC SUV. Results are expressed relative to the 12AS transfer rate from IFABP or acetylated IFABP to 150  $\mu\text{M}$  EPC/NBD-PC SUV. Absolute transfer rates are shown in the inset. Averages from three different experiments  $\pm$  SD are shown.

set)], the mechanism of FA transfer from acetylated IFABP to zwitterionic vesicles appears unchanged compared with that of the native protein.

#### Effect of vesicle charge on AOFA transfer from acetylated IFABP to membranes

As mentioned above, changes in the surface charge density of the acceptor vesicles can influence ligand transfer rates if electrostatic interactions between donor protein and acceptor membranes are involved. **Figure 5** shows that, as expected, the 12AS transfer rate from native IFABP was increased significantly by the incorporation of PS or CL into EPC/NBD-PC acceptor membranes (3, 5, 7). In sharp contrast, 12AS transfer from acetylated IFABP was not increased by the presence of negatively charged phospholipids, which suggests a repulsive effect between the acetylated protein and the acidic acceptor vesicles. To further investigate the effect of negative charge of the acceptor vesicles on the FA transfer mechanism from acetylated IFABP, we analyzed the modification of transfer rates with increasing concentrations of negatively charged acceptor vesicles. The rate of FA transfer from acetylated IFABP showed no proportional increase in transfer rate with acceptor membrane concentration when PS, with a single negative charge, or CL, which is doubly charged, were incorporated in the vesicles, in marked contrast to native IFABP, which always, and independently of the net charge of the vesicles, showed the classical proportional increase in transfer rate with acceptor concentration (**Fig. 6**). It is interesting that the lack of response to acceptor vesicle concentration observed for acetylated IFABP increased with



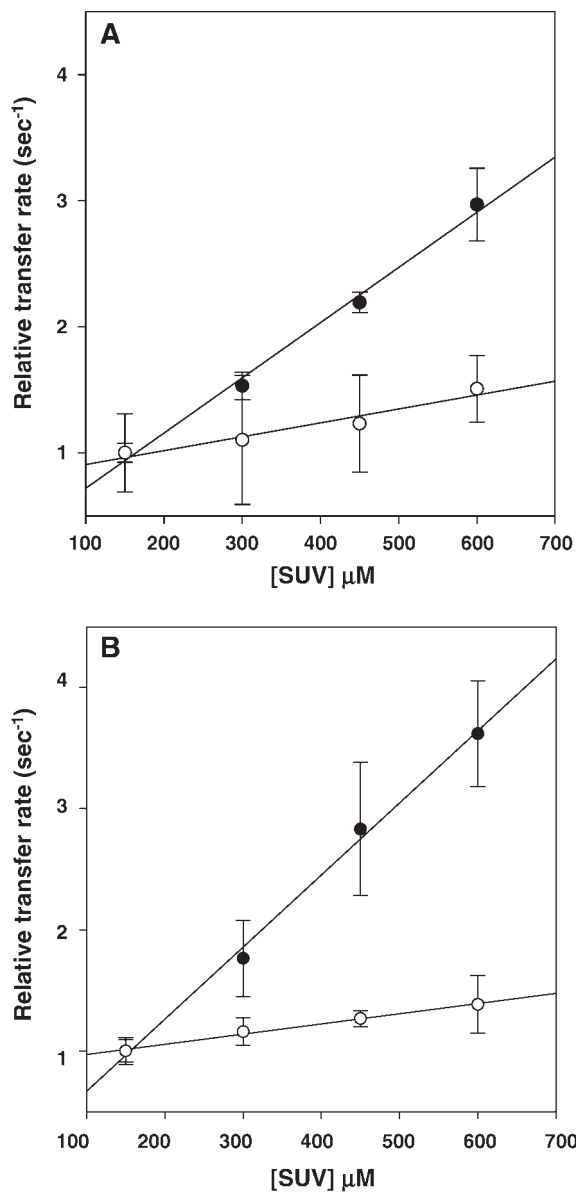
**Fig. 5.** Effect of vesicle charge on AOFA transfer from IFABP and acetylated IFABP. Transfer of 1.5  $\mu\text{M}$  12AS from 15  $\mu\text{M}$  IFABP (black bars) or 15  $\mu\text{M}$  acetylated IFABP (gray bars) to 150  $\mu\text{M}$  EPC/NBD-PC SUV containing 25 mol% brain phosphatidylserine (PS) or CL. Averages from three different experiments  $\pm$  SD are shown.

the net negative charge of the vesicles, emphasizing the possibility of a repulsive effect between the acetylated protein and the acidic acceptor membranes.

#### Effect of ionic strength on AOFA transfer from acetylated IFABP to membranes

Transfer of AOFA from wild-type and acetylated IFABP to membranes was examined as a function of increasing concentrations of NaCl. Circular dichroic spectra for native and acetylated protein at 100 and 1,000 mM NaCl showed essentially equivalent spectra (data not shown). The molar ellipticities at 215 nm were  $-7,846$  and  $-8,096$   $\text{M}^{-1} \text{cm}^{-1}$  for native and acetylated IFABP at 100 mM NaCl and  $-10,064$  and  $-9,303$   $\text{M}^{-1} \text{cm}^{-1}$  for native and acetylated IFABP at 1,000 mM NaCl. Thus, any small changes in helical content secondary to ionic strength were the same for both proteins, indicating that differences in fatty acid transfer were likely attributable to the change in surface properties rather than to differential protein folding.

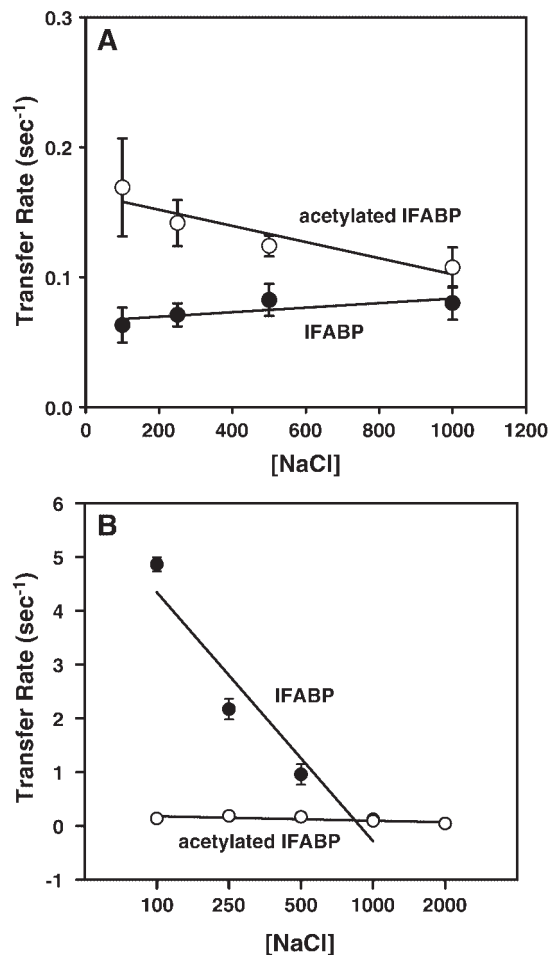
In agreement with previous results (7), a small increase in 12AS transfer rate from native IFABP to neutral SUVs was detected with increasing NaCl concentrations. Increasing ionic strength caused a statistically insignificant decrease in AOFA transfer rate from acetylated IFABP to zwitterionic vesicles (**Fig. 7A**). When negative charge was added to the acceptor vesicles, a drastic modification was observed for the native protein at high salt concentrations. As shown in Figs. 5, 6, native IFABP exhibited an 80-fold increase in AOFA transfer rate to CL vesicles compared with EPC vesicles at low ionic strength. Upon increasing the ionic strength, a marked decrease from the very high values observed at low ionic strength was found (**Fig. 7B**). This suggests a masking of electrostatic interactions, which play a very important role at low ionic strength, caused by the high salt content of the media. On the other hand, the fatty acid transfer rate from acetylated IFABP did not change with increasing ionic strength (**Fig. 7B**), and rates were not different from transfer to zwitterionic vesicles at any value of ionic strength.



**Fig. 6.** 12AS transfer from IFABP and acetylated IFABP to acidic membranes. Transfer of 1.5  $\mu\text{M}$  12AS to PS vesicles (A) and CL vesicles (B) from 15  $\mu\text{M}$  IFABP (closed circles) and acetylated IFABP (open circles). Results are expressed relative to the 12AS transfer rate from FABP or acetylated IFABP to 150  $\mu\text{M}$  SUV. Averages from three different experiments  $\pm$  SD are shown.

## DISCUSSION

It has long been hypothesized that FABPs participate in the intracellular transport of long-chain fatty acids. Using an *in vitro* fluorescence energy transfer assay, we have suggested that FA transfer to and from IFABP occurs via collisional interaction between the FABP and the vesicles (3, 4). Direct interactions of IFABP with membranes were also shown using biochemical and spectroscopic approaches (5, 6), supporting this hypothesis. IFABP-membrane interactions imply that the protein may be involved in the extraction of FA from donor sites, for example, their removal from the plasma membrane after transmembrane transport and the delivery



**Fig. 7.** Effect of ionic strength on AOFA transfer from IFABP and acetylated IFABP. Transfer of 1.5  $\mu\text{M}$  12AS from 15  $\mu\text{M}$  IFABP (closed circles) or acetylated IFABP (open circles) to 150  $\mu\text{M}$  EPC/NBD-PC SUV (A) or 150  $\mu\text{M}$  EPC/NBD-PC SUVs containing 25 mol% CL (B). The NaCl concentrations of acceptor and donor were adjusted before mixing. Average transfer rates from three different experiments  $\pm$  SD are shown.

of ligand to acceptor sites (e.g., mitochondria and/or endoplasmic reticulum) for further metabolism. The rate of FA transfer from IFABP was found to be highly sensitive to acceptor vesicle charge characteristics, indicating that ligand transfer from IFABP may be regulated by ionic interactions between positively charged residues on the surface of the protein and negative charges on membranes.

In the present work, we analyzed the influence of electrostatic and hydrophobic interactions between IFABP and phospholipid membranes by 1) modifying the charge density and composition of acceptor vesicles, and 2) modifying the surface charge of IFABP. Particular attention was focused on cardiolipin because of its dramatic stimulation of FA transfer rates from IFABP (3, 5, 7) as well as its known propensity for protein-lipid interactions (24). Cardiolipin is a singular phospholipid with dimeric structure, carrying four acyl groups and two negative charges. In cells, it is localized exclusively in mitochondria, with approximately one-fourth found in the outer mitochondrial membrane, where it may be presumed accessible to cytoplasmic proteins (25). In these

studies, we found that increasing the CL concentration of acceptor vesicles resulted in a logarithmic increase in the transfer rate of both 12AO and 12AS from IFABP. This could be attributed to the higher proportion of negative charge and, hence, increased electrostatic interactions with positive residues on the surface of the protein, resulting in the faster FA transfer rate. Nevertheless, it is clear that absolute charge density alone cannot fully explain the CL effect, because despite similar surface charge properties, transfer rates to CL were faster than to monolysocardiolipin or dilyso-cardiolipin species. In addition, the difference in transfer rate between 12AO and 12AS, as observed previously (3), indicates that changes in ligand structure, which are unrelated to membrane or protein charge, also affect the apparent transfer rate. It is worth noting that for a collision-mediated transfer process, we would not predict such an effect of acyl chain properties; however, the data indicating direct protein-membrane interactions were otherwise compelling (3–6). It is proposed that this ligand-dependent behavior likely indicates some degree of interaction of the fatty acids with the aqueous milieu, perhaps at the surface of the protein during the formation of the putative protein-membrane collisional complex. Because 12AS is less soluble, it displays a slower transfer rate but nevertheless is transferred via the collisional mechanism.

Interestingly, we found that the difference in transfer rate of 12AO versus 12AS to zwitterionic vesicles disappears as increasing amounts of CL are incorporated in the acceptor vesicles, indicating that as the likelihood of electrostatic interactions increases, the effect of the ligand structure becomes far less pronounced. Although the phase polymorphism of cardiolipin is well appreciated, phase changes are not expected under the conditions of pH, ionic strength, and the absence of divalent cations used in these experiments (21, 26, 27).

Our previous studies also suggested that hydrophobic interactions might be involved in the formation of the IFABP-membrane complex (3, 5, 22). Here, we analyzed the effect of hydrophobic volume on the FA transfer rate from IFABP to CL-containing vesicles using CL molecules of different acyl chain number. We observed a striking decrease in the rate of 12AS transfer to vesicles containing the same head group composition and charge but diminished hydrophobic volume. Thus, hydrophobic interactions are also likely to be important for the collisional mechanism of FA transfer from IFABP to membranes. A reduction in hydrophobic volume, as in the lysocardiolipin species, can also lead to a micellar phase; thus, it is possible that the observed changes could be secondary to micelle formation. This is unlikely, however, because micelle formation would probably lead to a decrease in particle diameter for the lysocardiolipin-containing vesicles, which was not observed. Moreover, micelle formation has not been found in mixed PL preparations, and in the present studies, all phospholipids used were prepared in the presence of 75 mol% PC. Indeed, it has been observed that in vesicles of mixed composition, CL has a stabilizing effect on PC bilayers (20, 28), by which the incorporation of the CL head group stabilizes the intermolecular hydrogen bond network at the bilayer surface.

When transfer from acetylated IFABP to zwitterionic acceptor membranes was assessed, an increase in AOFA transfer rate relative to wild-type protein was unexpectedly observed. This is not what we had previously found for surface-neutralized adipocyte FABP, in which transfer rates were diminished to zwitterionic EPC membranes. It is possible that elimination of the positively charged lysines disrupts existing charge-charge interactions on the protein surface, resulting in increased electrostatic interactions between acetylated IFABP and zwitterionic vesicles, relative to the native protein. For example, lysine 29 and glutamic acid 15 form a salt bridge in the native IFABP structure; thus, disruption of this interaction may leave residue 15 available to interact with the cationic moiety of the choline head group. Another possibility is that the increase in surface hydrophobic character caused by the additional acetyl moieties promotes increased hydrophobic interactions between the protein and the zwitterionic EPC membranes. The basic collision transfer mechanism was clearly maintained, as seen by the proportional increase in AOFA transfer rate as a function of vesicle concentration. In contrast to the zwitterionic membranes, FA transfer to acidic vesicles was markedly slower from the acetylated IFABP, in keeping with what we had observed for acetylated adipocyte FABP. Indeed, the acetylated IFABP exhibited diminished collisional transfer properties to negatively charged membranes, as seen by the relatively small changes in FA transfer rate that accompany increased acceptor membrane concentrations. It is suggested that these changes arise as a result of repulsion between negative charges on the membrane surface and the net negative surface charge of acetylated IFABP.

It is generally thought that electrostatic interactions at surfaces are diminished and hydrophobic interactions are stimulated as a function of increasing ionic strength. The minimal effect of ionic strength on the rate of AOFA transfer from native and acetylated protein to zwitterionic vesicles suggests that the elimination of electrostatic interactions by salt shielding is compensated by an increase in hydrophobic interactions; thus, there is little net change in transfer rate. In the acetylated protein, by contrast, acetyl groups have eliminated the positive charges on the surface of the protein. Thus, at low ionic strength, negatively charged groups and hydrophobic residues (including acetylated lysine residues) contribute to the protein-membrane interactions. As the ionic strength increases, the contribution of electrostatic interactions is lessened. At very high salt concentrations, the native and acetylated proteins reach closer AOFA transfer rates, perhaps because only hydrophobic interactions remain for both proteins.

In agreement with our previous observations, the native IFABP shows a large increase in FA transfer rate to CL vesicles compared with zwitterionic vesicles at low salt concentration (3, 5, 7). When the ionic strength of the media increases, the electrostatic interactions, which are important in driving the transfer, are masked, thereby diminishing the rate of FA transfer from the native protein to anionic vesicles. On the other hand, the increase in ionic strength did not cause a net modification of AOFA transfer rate from the acetylated protein to acidic vesicles. As shown



above, at low salt concentrations, the acetylated protein did not show an increase in transfer rate to acidic vesicles, which we attribute to a possible repulsive effect. By not having electrostatic interactions at low salt levels, the rate of transfer is maintained by hydrophobic interactions. In this regard, it is noteworthy that for transfer to zwitterionic and acidic vesicles at high salt concentrations, both native and acetylated IFABP proteins tend to reach the same final transfer rate, indicating that when electrostatic interactions are eliminated, the differences in behavior observed at physiological ionic strength are also eliminated.

Collectively, these results indicate that electrostatic interactions play an important role in FA transfer from IFABP to acceptor membranes and that hydrophobic interactions also participate in the process. In the complete absence of surface lysine positive charges, it appears that hydrophobic interactions are sufficient to form a putative protein-membrane collisional complex with net neutral phospholipids. A comparison of the present results with those from other members of the FABP superfamily that also exhibit a "collisional" FA transfer mechanism, such as heart, keratinocyte, and adipocyte FABP, indicates that hydrophobic interactions probably play a more important role in the case of the IFABP interaction with model membranes. Nevertheless, because 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. Moreover, it is also possible that IFABP forms charge-charge interactions with acidic domains on membrane proteins, thereby facilitating the targeted transport of intracellular fatty acids. In the intestinal enterocyte, IFABP may use such a mechanism to specifically target fatty acids to or from particular organelles. ■

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