

## Fluorescent Fatty Acid Transfer from Bovine Serum Albumin to Phospholipid Vesicles: Collision or Diffusion Mediated Uptake

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**ABSTRACT - Purpose:** The extent of palmitate uptake by hepatocytes is dependent upon the surface charge of the extracellular binding protein. Specifically, hepatocyte uptake is greater when palmitate is bound to cationic binding proteins than when it is bound to anionic proteins. To further understand the role of protein surface charge on the uptake process of protein-bound ligands, we examined the rate of transfer of fluorescent anthroxyloxy palmitic acid (AOPA) in the presence of anionic and cationic extracellular proteins to model membranes containing different surface charged groups. **Method:** AOPA transfer rate in the presence of bovine serum albumin (ALB; isoelectric point pI = 4.8-5.0) or modified ALB (ALB<sub>c</sub>; pI = 7.0-7.5) to negative, positive and neutral lipid vesicles was investigated using a fluorescence resonance energy transfer assay. **Results:** The rate of AOPA transfer from both proteins was decreased when ionic strength was increased; directly dependent on the concentration of acceptor lipid vesicles; and was affected by both the lipid membrane surface charge and protein-bound concentration. **Conclusion:** The data support the notion that AOPA transfer from binding proteins to lipid membranes occurred through two concomitant processes, aqueous diffusion of the unbound ligand (diffusion-mediated process) and a collisional interaction between the protein-ligand complex and acceptor membrane. The contribution of diffusional mediated transfer to the overall uptake process was determined to be 3 to 4 times less than the contribution of a collisional interaction. This study strengthened the hypothesis that charged amino acid residues on proteins are important for effective collisional interaction between protein-ligand complexes and cell membranes through which more free ligand could be supplied for the uptake process.

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### INTRODUCTION

Nonesterified long-chain fatty acids (LCFA) are a major energy source for most mammalian cells. In plasma these substrates are highly protein bound owing to their high lipophilicity. Extracellular protein binding makes understanding their uptake process very complicated. As such uptake of highly lipophilic ligands has been a controversial area of investigation.

Prior to entering the cell, these substrates must traverse several sequential barriers. One of the steps involved in the uptake process is dissociation from the extracellular protein-binding site. Whether the dissociation step is mediated by interactions with the surface charged groups on the outer membrane leaflet of the cell is not clear (1). Although it is generally accepted that uptake does

not involve the protein-ligand complex, several reports provide strong evidence that ligand uptake may occur directly from the protein-ligand fraction (2-6).

Evidence supporting the hypothesis that an ionic interaction between the protein-ligand complex and the cell surface is likely mediating the supply of ligand from the protein-bound fraction to the cell has been reported (2). Hepatocyte uptake of [<sup>3</sup>H]-palmitate was shown to be greater when the binding protein contained a net positive charge at physiological pH compared with a net negative charge. The present studies were undertaken to further explore the uptake process.

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The objective was to determine if an ionic interaction between extracellular binding proteins and acceptor membranes was associated with an increase in uptake rate. The transfer rate of LCFAs from albumin to model membranes was investigated using a fluorescence resonance energy transfer assay. We explored the transfer rate of anthroyloxy labeled palmitic acid (AOPA) bound to ALB (isoelectric point,  $pI=4.8$ ) or modified ALB ( $pI=7.5$ ) to negative, positive, and neutral lipid vesicles. This technique has been successfully used to study the rate and mechanism of transfer of many important physiological substrates such as cholesterol (7); phospholipids (8); sphingomyeline (9); short-chain fatty acids (10); and long-chain fatty acids (11,12) between various binding proteins and phospholipid vesicles.

## MATERIALS AND METHODS

Fluorescently labeled 16-(9-anthroyloxy) palmitic acid (AOPA) was purchased from Molecular Probes, Inc. (Eugene, OR). Synthetic 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), synthetic 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS), synthetic 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-(7-nitro-2,1,3-benzoxadiazole-4-yl) (NBD-DOPE) were obtained from Avanti Polar Lipids (Birmingham, AL). Bovine serum albumin (ALB, essentially fatty acid-free) and stearylamine (SA) were purchased from Sigma (St. Louis, MO.). The aqueous buffer used throughout all experiments was phosphate buffered saline (PBS) having a composition of (in mM: 137 NaCl, 2.68 KCl, 1.65  $KH_2PO_4$ , 8.92  $Na_2HPO_4$  and 3  $NaN_3$ ) with pH adjusted to 7.4 using 0.1 M NaOH.

### *Chemical modification of albumin*

Albumin was modified by activating the carboxyl groups with carbodiimide treatment and then aminating them with ethylenediamine as previously described (13). Briefly, anhydrous ethylenediamine (66.6 ml) was added to 500 ml distilled water. The pH of the resulting solution was adjusted to 4.75 with approximately 350 ml 6 N HCl. ALB (2 g) and 1-ethyl-3-(3-dimethyl-amino propyl)-carbodiimide (0.725 g) was added to this solution. The mixture was allowed to react for approximately

10 minutes with gentle stirring. The reaction was terminated by the addition of 30 ml 4 M acetate buffer ( $pH=4.75$ ). The modified albumin ( $ALB_e$ ) solution was dialyzed against distilled water at  $4^\circ C$ , concentrated, lyophilized, and stored at  $-20^\circ C$  until use.

### *Determination of molecular weight*

Mass measurements of ALB and  $ALB_e$ , dissolved in 5% acetic acid in methanol/water (1:1 v/v), were conducted on an orthogonal injection electrospray ionization time-of-flight (ESI/TOF III) mass spectrometer (14). Prior to analysis, residual sodium, potassium, and phosphate were removed from proteins by ultrafiltration using 25 mM ammonium bicarbonate buffer. Analysis was performed at a declustering voltage of 190 eV. Molecular masses were obtained by deconvolution using a computer program as previously described (14).

### *Determination of extent of modification*

Amino acid modifications of  $ALB_e$  were identified by peptide mapping as follows. Tryptic digestions of  $ALB_e$  and ALB (as a control) were conducted in 25 mM ammonium bicarbonate solution (1% trypsin, w/w) at  $37^\circ C$  for 24 hours and the digest analyzed by mass spectrometry on a SCIEX prototype tandem quadrupole/TOF mass spectrometer (QqTOF) coupled to a matrix assisted laser desorption ionization (MALDI) ion source. A 2,5 dihydrobenzoic acid (DHB) solution (100 mg/ml in acetone) was used as the matrix. Peptide fragments for sequence identification were formed by collision-induced dissociation (CID) with Argon as the collision gas and collision energies of 50-180 eV.

### *Determination of isoelectric point (pI)*

The isoelectric points (pI) of ALB and  $ALB_e$  were determined on IEF gels using a model 111 Mini IEF-cell (BIO-RAD) and BioLyte 3/10 obtained from Bio-Rad Laboratories (California, USA). The gels were stained with Coomassie blue R-250.

### *Preparation of large unilamellar vesicles (LUVs)*

Lipid vesicles were prepared by the mechanical dispersion method as described by Swairjo et al. (15). Briefly, 65 mol % synthetic 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 10 mol % 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine

-N-(7-nitro-2,1,3-benzoxadiazole-4-yl) (NBD-DOPE), and 25 mol % 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (DOPS, in the case of negatively charged lipid vesicles, -veLUVs), or stearylamine (SA, in the case of positively charged lipid vesicles, +veLUVs), or 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE, in the case of neutral lipid vesicles, neuLUVs) in chloroform were dried in a rotary evaporator under nitrogen gas to form a thin lipid film on the inner wall of a rounded-bottom flask. PBS containing 0.02% sodium azide (NaN<sub>3</sub>), 0.1 mM dithiothreitol (DTT), and 0.1 mM ethylenediamine-tetraacetic acid (EDTA) was added to a final concentration of 3 mM total lipids. The liposome dispersion was extruded 11 times through a polycarbonate membrane (Nuclepore, 200-nm pore size) using a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA) to obtain a homogenous population of large unilamellar vesicles. After extrusion, the vesicles were concentrated in an Amicon centrifugal concentrator (molecular weight cutoff 100 kDa) to total lipid concentration of 10-13 mM. Lipid vesicle preparations were stored at room temperature and used within four weeks. Phospholipid and NBD-DOPE concentrations were assessed by determination of total inorganic phosphate (16) and UV-spectrophotometry using an extinction coefficient 21,700 cm<sup>-1</sup> M<sup>-1</sup>, respectively.

#### **LUV Quality Assessment**

Determination of zeta potential, mean vesicle size and, size distribution were performed using Nicomp 380 ZLS Submicron Particle Sizer / Zeta Potential Analyzer (Particle Sizing Systems, Langhorne, PA, USA). A single zeta potential measurement was conducted, which reflected the value from combined liposome samples. Electron microscopy was used to determine unilamellarity of lipid vesicles.

#### **Binding of AOPA to ALB and ALB<sub>e</sub>**

Binding of AOPA to ALB or ALB<sub>e</sub> was analyzed by fluorimetric titration according to Cogan et al. (17). Briefly, fluorescently labeled AOPA was added to PBS containing 1 μM ALB or ALB<sub>e</sub> from a concentrated ethanolic stock solution. The molar ratio of AOPA to protein in PBS varied between 0.1 and 3.0. The total ethanol concentration was less than 1.5% at the end of the titration. An increase in the fluorescence intensity was observed with

increasing AOPA concentration at 37°C. No correction for background levels of AOPA in PBS was necessary since the contribution of AOPA blank to the observed fluorescence intensity in the presence of protein was less than 5%.

#### **Equilibrium Partitioning of AOPA Between Binding Proteins and Acceptor Vesicles.**

ALB or ALB<sub>e</sub> (1 mM) in PBS, pH 7.4, was incubated with 0.1 mM AOPA at 37°C for 10 minutes and the fluorescence intensity monitored. The protein-AOPA complex was then mixed with negative LUVs, positive LUVs, or neutral LUVs and the mixture was incubated for 20 minutes. Preliminary studies showed that this time period was sufficient to achieve equilibrium partitioning of AOPA. The remaining measured fluorescence reflected only the AOPA bound to protein. The relative partition ratio of AOPA between protein and each lipid vesicle preparation (PR<sub>AOPA</sub>, mol / mol) was calculated as follows:

(1)

$$PR_{AOPA} = \frac{(\% \text{ protein - bound AOPA}) / \text{mM protein}}{(100 - \% \text{ protein - bound AOPA}) / \text{mM phospholipid}}$$

where the numerator is the fluorescence intensity measured after addition of lipid vesicles divided by the fluorescence intensity measured before the addition of lipid vesicles multiplied by 100.

#### **Transfer Assay**

Transfer kinetics of AOPA from ALB or ALB<sub>e</sub> to different acceptor vesicles were measured using a resonance energy transfer assay as described by Wootan et al. (18). Briefly, 1 μM ALB or ALB<sub>e</sub> was incubated with 0.1 μM AOPA at 37°C until binding equilibrium was reached (as measured by maximum fluorescence intensity). AOPA was stored as a concentrated solution containing ethanol. The ethanol concentration in the incubation mixture was < 0.1 % (v/v). Lipid vesicles were added to the protein-palmitate complex so that the final molar ratio of lipid vesicles to protein was 100. Upon mixing for less than 5 seconds, the decrease in AOPA fluorescence with time was monitored with RF-5000 Recording Spectrofluorometer P/N 206-12400 (Shimadzu Scientific Instrument Inc. Columbia, Maryland). Excitation was at 361 nm and emission was monitored at 470 nm. The remaining unchanged

fluorescence signal reflected the protein-bound AOPA. There was no detectable intensity for the binding protein alone, vesicle alone, or unbound AOPA in PBS at the concentrations used.

Data were collected in photon counting mode at 10-s intervals for 600 to 1000 s and analyzed by plotting the variation of fluorescence intensity versus time, fitting to a single exponential equation (18):

$$F(t) = C_0 \exp^{-Kt} + C \quad (2)$$

Where,  $C_0$  is the initial (maximum) fluorescence intensity at zero time,  $K$  is the transfer rate of AOPA from binding protein to acceptor vesicles (units of  $s^{-1}$ ),  $t$  is the time interval (s), and  $C$  is the fluorescence intensity of AOPA which remained bound to the protein at equilibrium. Goodness of fit was assessed by the correlation coefficient which was  $> 0.9$ , absolute sum of squares which was  $< 0.1$ .

#### Data Analysis

Data are represented as mean  $\pm$  SEM unless otherwise stated.  $N$  is the number of replicates performed for each experiment. Whenever appropriate, data were subjected to non-linear least square regression analysis or linear regression analysis. For statistical comparison between groups student t-test or one-way analysis of variance (ANOVA) was used taking  $p < 0.05$  for the level of significance.

## RESULTS

#### Molecular Weight (MW) and protein isoelectric point (pI)

The molecular weight of ALB and ALB<sub>e</sub> was

determined to be 66,400 and 67,300, respectively. The range in isoelectric point (pI) for ALB and ALB<sub>e</sub> was 4.8-5.0 and 7.0-7.5, respectively. Thus, ALB contained a net negative charge while ALB<sub>e</sub> was neutral at physiological pH. Peptide mapping of ALB<sub>e</sub> showed that approximately 37% of the glutamic acid residues were modified by the addition of the ethylenediamine groups.

#### LUVs Physical Properties

Vesicle diameter and zeta potential of the lipid vesicles are shown in Table 1. There was no statistical difference in the mean vesicle size of the three lipid vesicle preparations. Freeze-fracture electron microscopy confirmed unilamellarity of vesicles (data not shown).

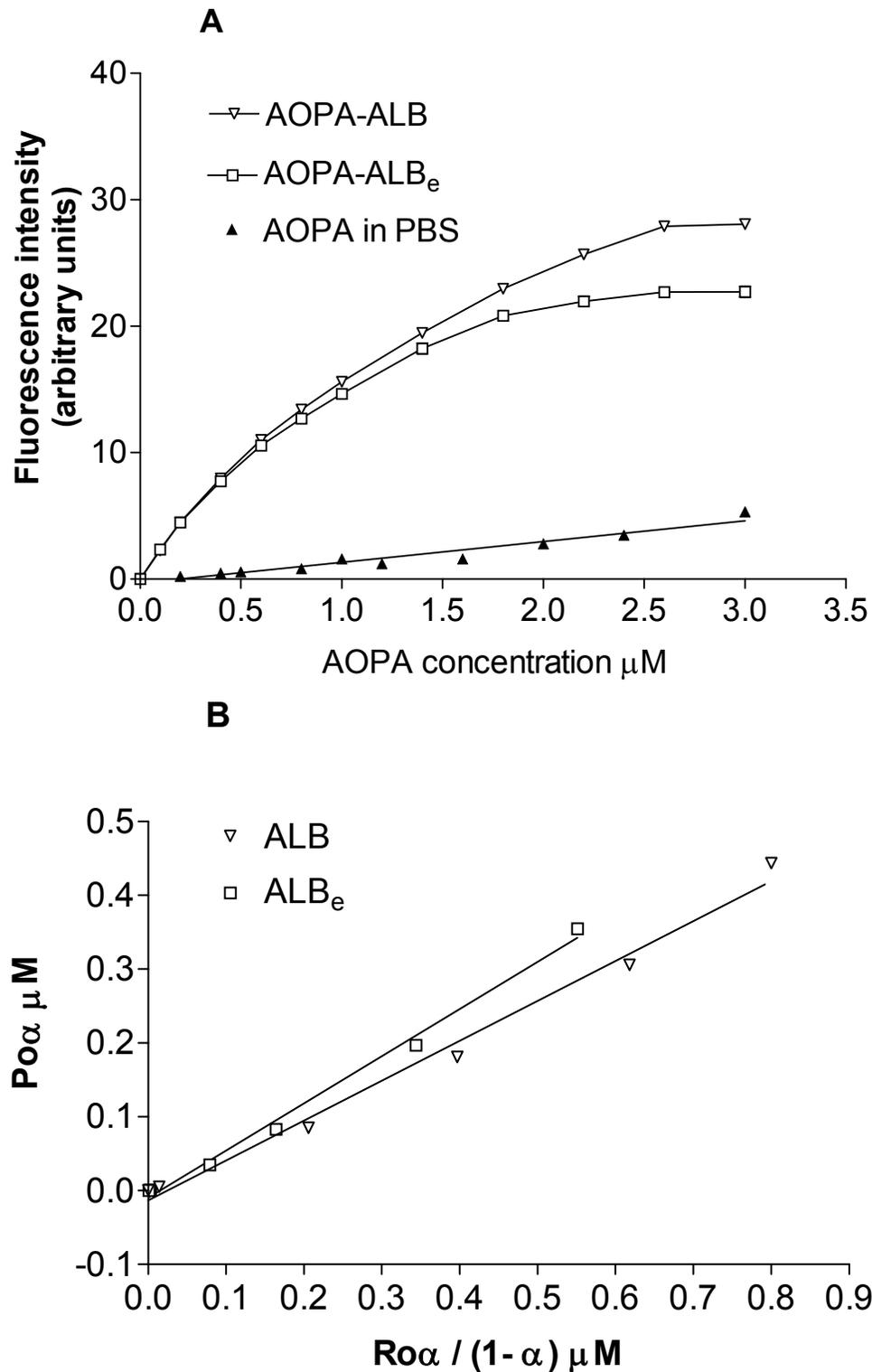
#### AOPA Binding

Figure 1A shows the fluorescence intensity of AOPA in the presence of ALB, ALB<sub>e</sub>, and PBS. Binding of AOPA to ALB and ALB<sub>e</sub> is shown in Figure 1B and was analyzed according to the method of Cogan et al. (17). The calculated apparent dissociation constant ( $K_d$ ) for ALB and ALB<sub>e</sub> was  $0.024 \pm 0.003$  and  $0.016 \pm 0.001$  mM ( $n=4$ ), respectively. The apparent number of ligand binding sites for ALB and ALB<sub>e</sub> was calculated to be  $n=2$ . The data show that ALB and ALB<sub>e</sub> bind AOPA with comparable affinities. Based on these  $K_d$  values, the protein:ligand molar ratio used in all transfer assays was 10:1, a ratio at which more than 99% of the AOPA was bound to ALB or ALB<sub>e</sub>.

**Table 1.** Physical properties of lipid vesicle preparations.

Physical properties	Negatively charged vesicles (negative LUVs)	Neutral vesicles (neutral LUVs)	Positively charged vesicles (positive LUVs)
Average zeta potential (mV)	-18	-5	+10
Mean diameter (nm $\pm$ SD)	199 $\pm$ 37*	200 $\pm$ 39* 232 $\pm$ 66**	222 $\pm$ 35*

\* = LUVs in PBS and \*\* = neutral LUVs in PBS containing 1 M NaCl.



**Figure 1.** Binding of AOPA to proteins. A: Increase in fluorescence intensity of 1  $\mu\text{M}$  ALB (▽) or ALB<sub>e</sub> (□) when titrated with increasing concentrations of AOPA. Fluorescence intensity of AOPA alone in PBS is also shown (▲). B: binding data of AOPA to ALB or ALB<sub>e</sub> were analyzed as described in Materials and Methods.  $Po$  is the total protein concentration,  $Ro$  is the total AOPA concentration, and  $\alpha$  is the fraction of free binding sites on protein molecule. The apparent  $K_d$  for ALB and ALB<sub>e</sub> were 0.024 and 0.016  $\mu\text{M}$ , respectively.

### **Partitioning of AOPA Between Binding Proteins and Lipid Vesicles**

The relative distribution of AOPA between ALB or ALB<sub>e</sub> and lipid vesicles was calculated by measuring the equilibrium fluorescence intensity before the addition of vesicles and the remaining fluorescence intensity after the addition of vesicles according to equation 1. Data in Table 2 are presented as the relative partitioning of AOPA between binding proteins and lipid vesicles. While partitioning of AOPA in the presence of ALB and ALB<sub>e</sub> between the negative and positive lipid vesicles was highly significant ( $p < 0.0001$ ), there was no statistical difference in the partitioning of AOPA between ALB or ALB<sub>e</sub> and the neutral lipid vesicles. AOPA had the greatest preference for positively charged lipid vesicles followed by neutral and lastly negatively LUV. In all cases, partitioning favored binding proteins rather than vesicles.

### **Effect of Lipid Vesicle Concentration on AOPA Transfer to neutral LUV**

To discriminate between AOPA transfer occurring by aqueous diffusion and that occurring through a direct interaction of protein and acceptor membrane, we examined AOPA transfer from binding protein as a function of increasing acceptor membrane concentration. Figure 2 shows the transfer of AOPA from ALB or ALB<sub>e</sub> to neutral LUV. Prior to the addition of vesicles, there was no statistical difference in the maximum fluorescence intensity between the two proteins. Over a range of phospholipid:protein (mol/mol) of 20:1 to 100:1, the rate of transfer from both proteins increased linearly as the lipid vesicle concentration increased, suggesting that one possibility for the mechanism of fatty acid transfer from proteins is through a collisional interaction of the protein-fatty acid

complex with the phospholipid membranes. If the transfer mechanism was solely through aqueous diffusion, we should not expect to see a change in the transfer rate as the number of acceptor vesicles was increased (11). Interestingly the Y-intercepts for the regression lines in Figure 2 were  $0.013 \pm 0.001 \text{ s}^{-1}$  and  $0.009 \pm 0.0003 \text{ s}^{-1}$  for ALB and ALB<sub>e</sub>, respectively ( $p < 0.05$ ). Transfer rates at zero lipid vesicles concentration were estimated to be equivalent to the dissociation of AOPA into an aqueous phase ( $K_{\text{off}}$ ) (12). The  $K_{\text{off}}$  of AOPA-ALB<sub>e</sub> was slower than the  $K_{\text{off}}$  of AOPA-ALB supporting the above results showing that  $K_{\text{d}}$  of AOPA-ALB<sub>e</sub> is lower than the  $K_{\text{d}}$  of AOPA-ALB.

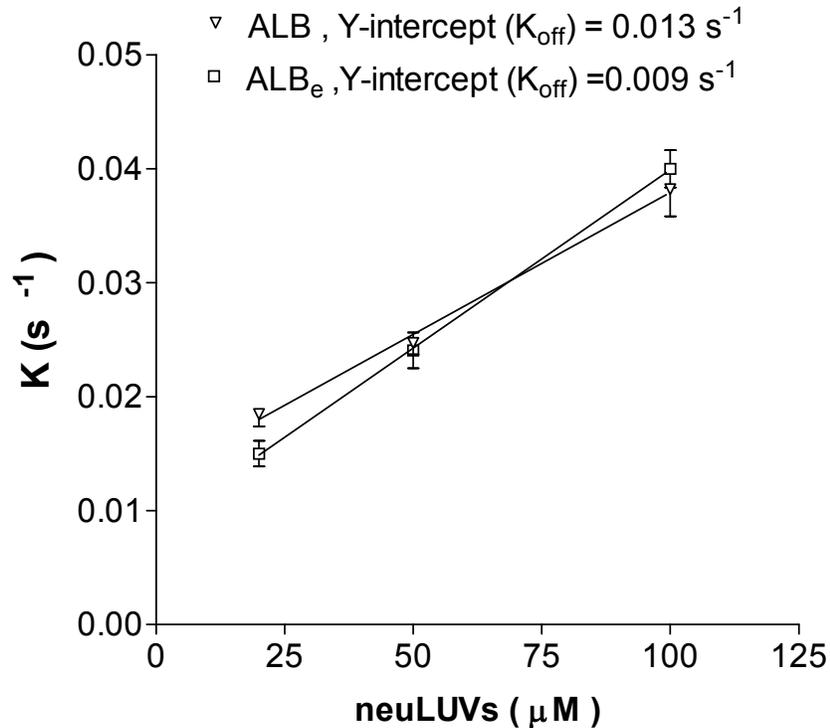
### **Effect of Ionic Strength on AOPA Transfer to neutral LUV.**

If transfer of AOPA from extracellular binding proteins to acceptor vesicles occurred through aqueous diffusion, then the rate of transfer may be affected by changing the aqueous solubility of the ligand (12). Conversely, if transfer occurred solely by a collisional interaction, then the transfer rate ought to be unaffected by changes in ligand solubility. Figure 3 shows that an increase in sodium chloride concentration resulted in a nonlinear decrease in the rate of AOPA transfer from ALB or ALB<sub>e</sub>. The decrease in transfer rates was linear up to 540 mM sodium chloride. At higher concentrations ( $>540 \text{ mM}$ ), the decrease in transfer rate appeared to reach a nadir. Although the results indicate that AOPA transfer must be occurring through diffusion, the observed decrease in the transfer rates due to possible changes in protein binding and/or lipid vesicles integrity by increasing ionic strength cannot be ruled out (see discussion).

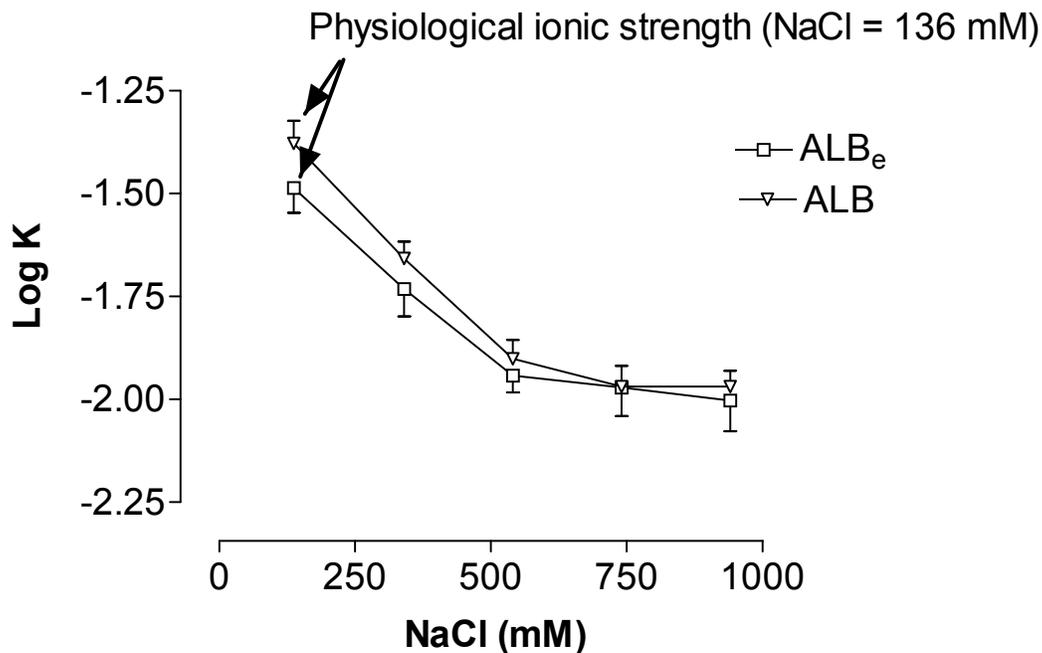
**Table 2.** Relative partitioning of AOPA between protein and lipid vesicles.

Protein	Negatively charged vesicles (negative LUVs)	Neutral vesicles (neutral LUVs)	Positively charged vesicles (positive LUVs)
ALB	*270 ± 9	229 ± 9	*43 ± 9
ALB <sub>e</sub>	*360 ± 37	219 ± 3	*66 ± 1.3

Proteins (1 μM) were incubated with 0.1 μM AOPA at pH 7.4 and 37° C, as described in Materials and Methods. The fluorescence intensity was measured and the protein-AOPA complex was mixed with neutral, negatively, or positively charged lipid vesicles. At equilibrium, the remaining fluorescence reflected only the AOPA, which remained bound to the protein. The relative distribution of AOPA between protein and vesicles was then calculated using equation 1. Values are mean ± SEM, n = 4 to 8. \*  $p < 0.0001$ ,



**Figure 2.** Effect of acceptor membrane concentration on AOPA transfer from binding proteins. The transfer of 0.1 μ M AOPA from 1.0 μ M ALB (▽) or ALB<sub>e</sub> (□) to neutral acceptor vesicles. Transfer was monitored at 37° C. The estimated K<sub>off</sub> (Y-intercept at zero lipid vesicles concentration) for ALB and ALB<sub>e</sub> were 0.013 ± 0.001 and 0.009 ± 0.0003 s<sup>-1</sup>, respectively. Data are mean ± SEM, n = 4.



**Figure 3.** Effect of ionic strength on AOPA transfer from binding proteins. Transfer of 0.1 μ M AOPA from 1.0 μ M ALB (▽) or ALB<sub>e</sub> (□) to 100 μ M neutral acceptor vesicles. Sodium chloride concentration of lipid vesicles and ligand-protein solutions were adjusted before mixing. Data are mean ± SEM, n = 4.

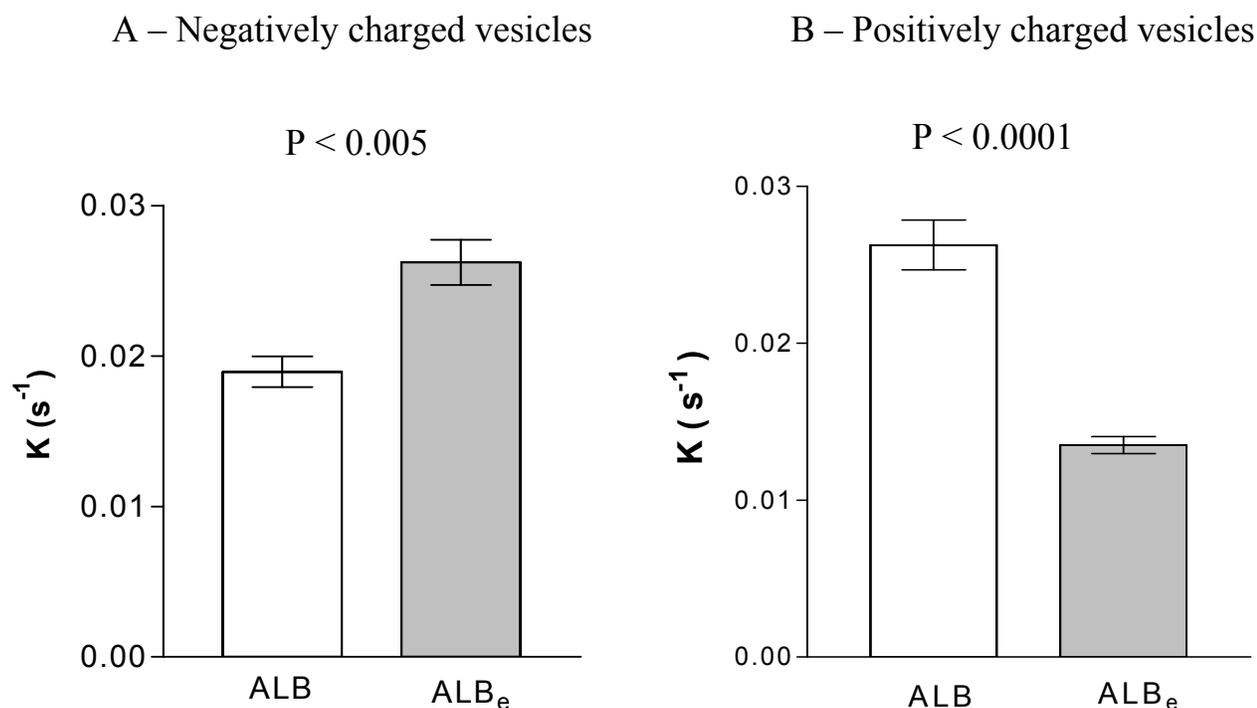
### **Effect of Phospholipid Surface Charge on AOPA Transfer.**

To further explore the effect of membrane properties on the transfer rate of AOPA from ALB or ALB<sub>e</sub>, we measured the AOPA transfer rates to acceptor membranes of different surface charge. If transfer occurred solely through diffusion then the rate should not be significantly affected by acceptor membrane characteristics. Figure 4A and 4B show a statistical difference in the transfer rate of AOPA from ALB and ALB<sub>e</sub> to negatively and positively charged vesicles. Also, the AOPA transfer rate to negatively charged vesicles in the presence of ALB was significantly lower ( $0.019 \pm 0.001 \text{ s}^{-1}$ , n=12) than that to positively charged vesicles ( $0.026 \pm 0.0016 \text{ s}^{-1}$ , n=8,  $p < 0.0007$ ). In contrast, the AOPA transfer rate in the presence of ALB<sub>e</sub> was significantly higher ( $0.026 \pm 0.0015 \text{ s}^{-1}$ , n=4) when negatively charged vesicles were used as acceptor

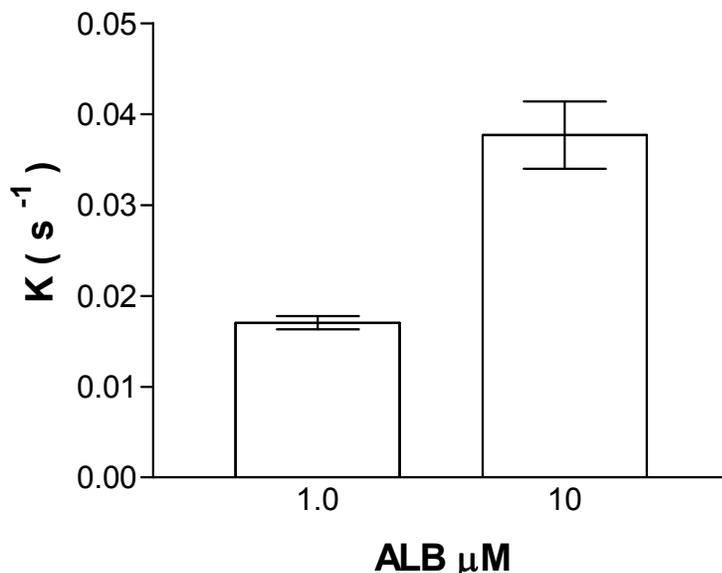
membranes as compared to positively charged vesicles ( $0.0135 \pm 0.00054 \text{ s}^{-1}$ , n=10,  $p < 0.0001$ ).

### **Effect of ALB Concentration on AOPA Transfer Rate**

To further investigate the role of ALB in AOPA transfer to model membranes, we measured the transfer rate of AOPA to positive LUVs in the presence of 1.0, and 10.0  $\mu\text{M}$  ALB and at low AOPA to ALB molar ratio e.g. 0.1. Using these protein concentrations and low AOPA to ALB molar ratio, the free AOPA concentration is predicted to be unchanged and independent on ALB concentration (19). Figure 5 shows that the transfer rate of AOPA from 10  $\mu\text{M}$  ALB was significantly higher than from 1.0  $\mu\text{M}$  ALB ( $p < 0.0001$ ). These data imply that the AOPA-ALB bound fraction is an important determinant in transfer.



**Figure 4.** Effect of vesicle surface charge on AOPA transfer. Transfer of 0.1  $\mu\text{M}$  AOPA from 1.0  $\mu\text{M}$  ALB or ALB<sub>e</sub> to 100  $\mu\text{M}$  negative LUVs (in A) or to 100  $\mu\text{M}$  positive LUVs (in B). Rates were  $0.019 \pm 0.001 \text{ s}^{-1}$  and  $0.026 \pm 0.0015 \text{ s}^{-1}$  from ALB and ALB<sub>e</sub>, respectively to negative LUVs, (A) or  $0.026 \pm 0.0015 \text{ s}^{-1}$  and  $0.014 \pm 0.0005 \text{ s}^{-1}$  from ALB and ALB<sub>e</sub>, respectively to positive LUVs, (B). Data are mean  $\pm$  SEM, n = 4 to 12.



**Figure 5.** Effect of ALB concentration on AOPA transfer. Transfer of AOPA from 1.0, and 10.0  $\mu\text{M}$  ALB to 63  $\mu\text{M}$  positive LUVs. For the two protein concentrations, AOPA-to-ALB molar ratio was 0.1. Data are mean  $\pm$ SEM,  $n=4$ . \* $p<0.0001$

## DISCUSSION

The objective of the present study was to elucidate whether the initial transfer process for long-chain fatty acids bound to proteins that differ in their surface charge characteristics is affected by acceptor vesicle membrane surface charge, concentration, and properties of the aqueous phase. One of the first barriers that cellular substrates must overcome prior to cellular entry is binding to the outer membrane leaflet followed by transmembrane flux. Transmembrane flux for long-chain fatty acids is known to occur via diffusion and membrane transport proteins and has been well studied, however, the mechanism responsible for substrates to gain access to and interact with the outer plasma membrane leaflet is less clear. Thus, our study examined the initial transfer of fluorescently labeled palmitic acid (AOPA) from ALB and ALB<sub>e</sub> to LUVs. Results show that: (a) the transfer is a first order process and is best described by a monoexponential equation; (b) the mechanism of transfer involves two processes; an aqueous diffusion-mediated process and a collisional-mediated process; (c) the observed kinetic rate is estimated to be the arithmetic sum of the slower dissociation rate (dissociation of AOPA from its protein binding site into solution,  $K_{\text{off}}$ ) and the

faster collisional transfer rate; (d) AOPA transfer that is mediated by a membrane-protein interaction providing the possibility of regulating the movement of these substrates by changing in the membrane composition and structure or the characteristics of the plasma delivery vehicle (e.g., lipid vesicle). These two processes are independent of each other and occur at the same time. Various factors affecting both kinetic processes are discussed below.

We have previously determined the high affinity binding constants ( $K_a$ ) for ALB and ALB<sub>e</sub> using tracer radiolabeled fatty acid, e.g. [<sup>3</sup>H] palmitic acid, and the heptane: buffer partitioning method (19, 20). In this method, the [<sup>3</sup>H] palmitic acid to protein molar ratio was less than 0.1. Thus, the calculated binding constant ( $K_a$ ) represents binding of palmitate to the high affinity-binding site on the protein. In the present study we used anthroyloxy labeled fatty acid and fluorimetric titration (0 to 3 moles of AOPA per 1 mole of protein) to determine the equilibrium binding constants for AOPA binding to ALB or ALB<sub>e</sub>. The binding constants calculated in this study were 3-fold lower than our previously obtained binding constants for the same proteins. Thus, it may be that, in this study, two fatty acid molecules bind to the first two high affinity binding sites or to the

high affinity and low affinity binding sites on the protein molecule; the calculated apparent dissociation constants would be the average value of these binding sites. Our calculated binding constants are, however, comparable to the lower values reported in the literature (21, 22). To estimate valid transfer rates, it was necessary to determine the  $K_a$  values for both AOPA-ALB and AOPA-ALB<sub>e</sub> in order to calculate the protein concentrations that produce the same unbound ligand concentrations. Since the measured  $K_a$  values were similar, the ALB and ALB<sub>e</sub> concentrations were also similar in the transfer experiments.

Measurement of equilibrium partitioning of AOPA between ALB or ALB<sub>e</sub> and each lipid vesicle preparation shows that partitioning of AOPA favors binding protein over membrane phospholipids by an order of magnitude. Therefore, it was necessary to use higher acceptor to donor ratios in the transfer assay experiments to ensure that unidirectional transfer is monitored. Data in Table 2 show that AOPA has the greatest preference for positively charged lipid vesicles. Second preference was for negatively charged lipid vesicles and the least preference was for neutral lipid vesicles. While there is no difference in the partitioning of AOPA between ALB or ALB<sub>e</sub> and neutral LUVs, the observed significant difference in the partitioning of AOPA between both proteins and charged lipid vesicles indicates that charged surface groups might regulate the distribution of AOPA between protein and charged lipid vesicles. Our AOPA partitioning data using neutral lipid vesicles are comparable with the equilibrium distribution of [<sup>3</sup>H] palmitic acid between albumin and lipid vesicles prepared from phosphatidylcholine (palmitate: albumin molar ratio = 2;  $K_{eq} = 310$ ) (23).

The possibility that AOPA transfer from binding protein to acceptor lipid vesicles occurs via a collisional mechanism was examined by increasing the concentration of the acceptor membranes and thereby the number of vesicles available for collisional interaction with the AOPA-protein complex. If this mechanism is important in the transfer process, then we expect an increase in the AOPA transfer rate as the acceptor membrane concentration increases (12). This is clearly the case, as seen in Figure 2. By increasing lipid vesicles concentration, there was an increase in the quenching of AOPA fluorescence, which reflect

more transfer of AOPA to the lipid vesicle membrane. The observed rates are estimated to be equal to the dissociation rates of AOPA from binding proteins into water ( $K_{off}$ , Y-intercepts in Figure 2) plus collision-mediated transfer rates.

Our estimated low  $K_{off}$  values are reasonably comparable to the lower values reported in the literature if one considers the differences in FFA: ALB molar ratios used in this study and other studies (24-26). Using albumin-agarose as an acceptor, we previously determined the albumin-palmitate dissociation rate constant to be approximately  $0.07 \text{ s}^{-1}$  (2, 20). This value is higher than that estimated in this study. The higher  $K_{off}$  value determined previously may be overestimated if part of the FFA transfer occurred through direct collisional interaction between FFA-ALB complex and the agarose beads.

To further examine the mechanism(s) through which AOPA is transferred to lipid vesicles, we altered its aqueous solubility by increasing the solution ionic strength (Figure 3). If the aqueous solubility of AOPA is not an important factor in the transfer process, we do not expect a linear change in the transfer rate with increasing ionic strength. The data in Figure 3 demonstrate that the transfer rate decreases nonlinearly with increasing salt concentration, suggesting that one possible form of the transfer process may occur via aqueous phase diffusion. Another possible explanation for the effect of high ionic concentration on the transfer rate is that the hydrophobic and electrostatic protein-membrane interactions are disrupted by high salt concentrations, which may affect the transfer rate (27). The observation that the decrease in the transfer rate appears to level off with further increase in salt concentration suggests that the diffusion-mediated transfer is not the only mechanism taking place in the transfer process and that some interaction of the ligand-protein complex with lipid vesicles (collision-mediated transfer) may occur. We have determined the possibility that increasing salt concentration might affect the lipid bilayer structure and / or lipid vesicle mean size. Increasing NaCl concentration causes vesicle fusion, as indicated by the increase in vesicle mean size and a broader particle size distribution (Table 1). The changes in physical structure of the lipid vesicles may, in part, explain the decrease in the transfer rate of AOPA from ALB or ALB<sub>e</sub>. It also has been shown that fluorescence polarization of

DPH incorporated into SUVs is increased as ionic strength increases, indicating an increase in the phospholipid acyl chain order (28). The possibility that the binding of AOPA to ALB or ALB<sub>e</sub> is affected by increasing NaCl concentration is unlikely, since the observed relative maximum fluorescence intensity at each NaCl concentration compared to the maximum fluorescence intensity at physiological ionic strength was similar.

The hallmark difference between transfer occurring through collision and that occurring through aqueous diffusion is the effect of acceptor membrane properties and / or the ligand-protein surface charge on the transfer rate (12, 29). In order to examine the effect of lipid vesicle surface charge on the transfer rates, we used three lipid vesicle preparations with similar properties and different net surface charge. Zeta potential measurements (Table 1) confirmed the proper vesicle charge. Data in Figure 4 show that the transfer of AOPA bound to ALB (pI=4.8-5.0) was significantly faster to positive LUVs than to negative LUVs. In contrast, the transfer rate of AOPA bound to ALB<sub>e</sub> (pI=7.0-7.5) was significantly faster to negative LUVs than to positive LUVs. The observed effect of the surface charge may reflect an electrostatic interaction between charged phospholipid head groups in the membrane and opposite charges on the AOPA-protein complex. Another possible explanation is that the presence of charged head groups on the membrane phospholipids might cause secondary changes in the membrane bilayer structure e.g. phospholipid head group packing order in the membrane. These secondary changes could increase or decrease the accessibility of binding protein to the lipophilic site of the membrane.

The observed differences in AOPA transfer rates to charged LUVs are unlikely due to differences in vesicle size and / or lamellarity since the particle size measurement (Table 1) and electron microscopic studies showed that the three vesicle preparations were similar.

In an attempt to elucidate the role of ALB in the transfer process, we measured the AOPA transfer rate in the presence of two low ALB concentrations at a fixed AOPA to ALB molar ratio. Under this condition, the free AOPA concentration is predicted to be unchanged for the two ALB solutions (19). If the transfer process occurs solely from the unbound AOPA fraction and independent of ALB

concentration, we do not expect to see a difference in the measured transfer rates. Data in Figure 5 show that transfer rate increased significantly with increasing ALB concentration. This result suggests that transfer of AOPA occurs from both the free and the protein-bound pools.

In summary, we provide evidence for the direct transfer mechanism of FFA bound to bovine serum albumin to lipid membranes. In contrast to the known aqueous diffusion mechanism observed for transfer of lipophilic compounds such as cholesterol and phospholipids, bovine serum albumin does not only function as an extracellular buffer for FFA levels but also as a delivery vehicle via its direct interaction with membranes. This provides a mechanism through which an efficient targeting of tightly bound substrates to the cell membranes is preserved. Our results support other reports showing the effect of albumin binding on cellular uptake (30, 31) as well as our previous report that electrostatic interactions between the albumin-ligand complex and hepatocyte or myocyte surfaces modulate the kinetics of free fatty acid uptake (2, 20). Furthermore, our work showed that at a constant concentration of unbound palmitate, there is a positive relationship between hepatocyte-<sup>3</sup>H palmitate uptake and total albumin concentration (32). Thus, the unique function of serum albumin to interact with the cell surface is particularly important during cell differentiation when a change in plasma membrane composition occurs which may affect the targeting of extracellular FFA by influencing the rate of FFA transfer from albumin. Overall, the results presented in this study set the framework for future work directed at elucidating the specific structural features of drug delivery vehicles involved in targeting cells.

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