Fatty Acid Flip-Flop in a Model Membrane Is Faster Than Desorption into the Aqueous Phase

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ABSTRACT: Fatty acids (FA) are known to diffuse (flip-flop) rapidly across protein-free phospholipid bilayers in their un-ionized form. However, whether flip-flop through the hydrophobic core of the bilayer or desorption from the membrane into the aqueous phase is the rate-limiting step in FA transport through membranes is still debated. The issue has remained unresolved in part by disagreements over whether some methods of adding FA create artifacts that lead to erroneous conclusions and in part by the lack of fluorescence methods to monitor each individual step. Here we study the kinetics of FA transfer from donors to phospholipid vesicles (small and large unilamellar vesicles) by a dual fluorescence approach that utilizes the probes fluorescein phosphatidylethanolamine (FPE) and pyranine. FPE detects the concentration of FA anions in the outer membrane leaflet, allowing a precise measurement of kinetics of FA adsorption or desorption. Our results showed that as soon as FPE detects adsorption of FA into the outer leaflet, pyranine detects its movement to the inner leaflet. We further demonstrated that (i) flip-flop for FA with 14−22 carbons is much faster than the rates of desorption and therefore cannot be the rate-limiting step of FA translocation across membranes; (ii) fluorescence changes detected by probes located on or in acceptor vesicles are dependent upon the method used to deliver the FA (i.e., uncomplexed, or complexed to albumin or phospholipid bilayers); however, (iii) transfer kinetics observed in the presence of different donors is rate-limited by the desorption of FA from the donor into the aqueous phase rather than by flip-flop.

FA[1] are amphipathic molecules that have alternatively been described as (i) being able to adsorb to and diffuse through lipid bilayers at rates fast enough to support the metabolic needs of cells (*1*−*8*) or (ii) requiring protein transporters to permeate the plasma membrane (*9*−*15*). Proteins such as FAT/CD36 were originally described as catalysts for the movement of the FA anion through the hydrocarbon core of the lipid bilayer (*16*). However, later measurements determined that the pK_a of the FA carboxyl shifts from ∼4 (in solution) to ∼7.5 upon binding to the membrane (*17*), which leads to the protonation of ∼50% of bound FA at physiological pH (*5*). The energy barrier is high for the diffusion of FA anions across the bilayer (*18, 19*), but uncharged FA have a low energy barrier for passage through the hydrophobic membrane core. Transmembrane movement has been shown to occur spontaneously by the "flip-flop" of the uncharged form of the FA (*5*).

Building on the knowledge of physical properties of FA, our research aims to provide a detailed mechanistic description of FA transport across the plasma membrane of cells. A variety of biophysical approaches, including several dual fluorescence assays, have been developed and optimized to study each step of FA diffusion through model membranes (*20, 21*). Fluorescent probes placed in the lipid membrane as well as in the aqueous phase both inside and outside of model membranes and cells have discriminated FA binding, transmembrane movement and intracellular metabolism (*2*−*5, 22*−*26*). We have used these methods to show that FA binding and flip-flop are extremely fast in protein-free phospholipid vesicles ($t_{1/2} \leq 10$ ms) (*2*−*5, 21, 23*−*25*).

Application of some of these assays to biological membranes (i.e., plasma membrane vesicles (*6*), various types of cultured cells (*1, 27, 28*) and isolated rat adipocytes *6, 22*) has led to the working hypothesis that protein transporters are not required to catalyze transport of FA across the membranes and that FA diffusion is sufficient for supplying the demands of intracellular FA metabolism *in vivo* (*6*).

However, biophysical methods to measure FA transport in membrane have provoked controversies, even in simple protein-free phospholipid vesicles. Evidence from several laboratories has led to the conclusion that adsorption and transmembrane movement of FA is fast and that desorption is the rate-limiting step of FA diffusion through membranes (*4, 18, 29*−*34*). In contrast, transmembrane movement has been argued to be the rate-limiting step of FA diffusion by one group (*35*−*38*).

Here we address this controversy by using a fluorescence probe, fluorescein-phosphatidylethanolamine (FPE), that we recently introduced to monitor the arrival of the FA carboxyl

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[1] Abbreviations: FPE, fluorescein-phosphatidylethanolamine; PC, phosphatidylcholine; FA, long chain fatty acids; VLCFA, very long chain fatty acids; OA, oleic acid; BSA, bovine serum albumin; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

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group at the membrane surface of vesicles (*21*). We study vesicles with the membrane-bound FPE alone and in combination with the entrapped water-soluble pH dye pyranine, which monitors the arrival of the headgroup on the inner leaflet of the bilayer (*5*). We incorporated FPE into vesicles that served as either donors or acceptors of oleic acid (OA), a common dietary long-chain FA (LCFA), and other FA of varying chain length. The observed rates of FA transfer between vesicles showed that adsorption to the acceptor is rate-limited by FA desorption from the donor. The kinetics of dissociation from the membrane, referred to here as desorption, of FA with varying chain lengths were similar to those previously reported with our protocol using pyranine (*34*). The method by which FA are delivered to membranes (i.e., complexed with BSA or vesicles) decreases the overall rate at which FA molecules move from the external aqueous phase, traverse the lipid bilayer (flip-flop), and arrive at the internal membrane leaflet, an effect which can be misidentified as slow flip-flop. By using fluorophores that discriminate between the individual steps of FA movement through membranes, we find that flip-flop is always faster than desorption in our model systems.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Alabaster, AL). Fluorescein-phosphatidylethanolamine (FPE or DHPE), 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) and hydroxypyrene trisulfonic acid (pyranine) were obtained from Molecular Probes (Eugene, OR) and Eastman Kodak Co. (Rochester, NY), respectively. BSA (99% fatty acid-free), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) oleic acid (C18:1*cis*), elaidic acid (C18:1*trans*), arachidic acid (C20:0), behenic acid (C22:0) and all buffer materials were purchased from Sigma (St. Louis, MO).

Stock Solutions. Pyranine stock solution (10 mM) was prepared by dissolving pyranine in deionized water. BCECF acid stock solution (1 mM) was prepared by dissolving BCECF in DMSO. The FPE stock (2.15 mg/mL) was prepared by dissolving lyophilized FPE in a 5:1 (v/v) mixture of $CHCl_3$:methanol as described previously (*39*). FA (C14:0, C16:0, C18:0, C18:1*cis* and C18:1*trans*) were dissolved in enough 0.1 mM KOH to produce 10 or 25 mM stock solutions. The pH of each stock was adjusted to >10 to ensure complete dissolution of the FA into the micellar phase (*40*). The external pH (pH_{out}) was measured with a pH minielectrode purchased from Microelectrodes (Londonderry, NH). Stocks of C18:1*cis* (25 and 80 mM) were also made by dissolving the FA in either DMSO or ethanol.

Preparation of OA/BSA Complexes. BSA (lyophilized powder) was weighed and dissolved in deionized water. The concentration of BSA was determined by measuring optical density (OD) at 279 nm and with an extinction coefficient (ε_{279}) = 0.51 OD/mg/mL. OA was added to a known volume and concentration of BSA (i.e., OA/BSA mole ratios of 2:1, 4:1 and 8:1) in 20 mM Hepes buffer (pH 7.4), vortexed and allowed to equilibrate ~1 h at 20 °C. An 8:1 OA/BSA ratio was used in all stopped-flow measurements to ensure that enough FA was transferred to vesicles, improve signal-to-noise and permit accurate curve fitting of each fluorescence trace.

Preparation of Lipid Vesicles and FA/Vesicle Complexes. Small unilamellar vesicles (SUV) containing 0.1 mM pyranine or 0.5 mM BCECF were prepared from egg phosphatidylcholine (PC) by sonication in 20 or 50 mM Hepes/KOH buffer (pH 7.4) as before (*5*). Large unilamellar vesicles (LUV) with a diameter of 0.1 μm were prepared by extrusion as described (*4*) with similar concentrations of probe and buffer as used for SUV. The external leaflets of SUV and LUV were labeled with FPE (1 mol % relative to PC) as described previously (*21*). Note that in all studies reported here, the FPE was added to preformed vesicles, which restricts its location to the outer leaflet of the bilayer. We followed previous procedures to prepare vesicles containing only pyranine, only FPE, both pyranine and FPE, or no fluorophore. Untrapped pH probe and FPE were removed by washing vesicles through a gel filtration column (G-25 Sephadex) with Hepes/KOH buffer (*5, 25, 41*). A small aliquot of vesicles was then used to measure the exact concentration of PC in the vesicle suspension following collection from the column (*42*).

FA (C14:0, C16:0, C18:0, C18:1*cis* and C18:1*trans*) was added from a stock solution to a known volume and concentration of SUV and LUV (6, 12 or 18 mol % FA with respect to PC), vortexed and allowed to equilibrate overnight at 4 °C, which allows dissipation of the pH gradient that results from addition of FA to preformed vesicles (*5*). If FA are added when $pH_{in} \ll pH_{out}$, inward FA diffusion would become less favorable (*23, 25*). Equilibration of pH across the membrane was confirmed by the lack of pyranine fluorescence change upon addition of nigericin, a technique that has been described previously (*5*).

Cosonication of very long chain fatty acids (VLCFA) with PC was performed as previously described (*34*). Briefly, lyophilized VLCFA (C20:0 or C22:0) was added directly to PC (dissolved in chloroform). The PC−VLCFA mixture was then dried, and SUV or LUV were prepared as described. Since these FA are added prior to sonication, it was not necessary to incubate the vesicles overnight to dissipate the pH gradient.

Fluorescence Instrumentation. Fluorescence measurements were made with a Spex Fluoromax-2 from Jobin Yvon (Edison, NJ) equipped with a stopped-flow apparatus from Hi-Tech Scientific (Salisbury, U.K.). The stopped-flow apparatus consists of a pressure-driven pneumatic drive unit and two glass syringes with a 400 μL total sample mixing volume. The sample compartment of the fluorimeter was temperature-regulated by an external water bath (25 °C), which delivered a continuous flow of water to a small metal plate located beneath the cuvette.

Fluorescence Measurements. Online measurements with a suspension of vesicles (700 μM PC) in 3.0 mL of 50 mM Hepes/KOH buffer (pH 7.4) were used in experiments with uncomplexed FA, to monitor the transfer of C20:0 and C22:0 from FA/vesicle complexes, and to monitor the transfer of OA from BSA at different FA/BSA ratios. FA (complexed and uncomplexed) was delivered into the vesicle suspension through the injection port above the cuvette with continuous stirring using a mini stir bar. In vesicle-to-vesicle transfer studies of C20:0 and C22:0, donor and acceptor vesicles were present in a 1:1 ratio. In BSA-to-vesicle transfer studies, BSA was added to a final concentration of 50 μM to the SUV suspension. The fluorescence of pyranine and FPE was

measured with time using a bandwidth of 3 nm and 3 nm for excitation and emission, respectively. Pyranine was excited at 455 nm, and emission was measured at 509 nm. FPE was excited at 490 nm, and emission was measured at 520 nm. BCECF fluorescence was measured as a ratio of two excitation wavelengths (R = 505 nm/439 nm). When monitoring the fluorescence of a single probe, changes in fluorescence can be resolved to within 2 s.

In stopped-flow studies, solutions containing acceptor and donor were loaded into separate syringes and rapidly mixed within an enclosed cuvette placed inside the temperature-regulated fluorimeter sample compartment. More specifically, suspensions of FA complexed to either SUV (100 μM PC), LUV (100 μM PC), or BSA (50 μM) were mixed with acceptor vesicles (100 μM PC in vesicle-to-vesicle studies and 600 μM PC in BSA-to-vesicle studies). After mixing, the final concentration of SUV in vesicle-to-vesicle and BSA-to-vesicle transfer assays was 100 μM and 300 μM, respectively, and the final concentration of BSA was 25 μM. Similarly, the concentration of LUV in vesicle-to-vesicle transfer assays was 100 μM. This stopped-flow method was used to study the vesicle-to-vesicle transfer of FA (14−18 carbons). Vesicle-to vesicle transfer assays were performed using similar sized vesicles to measure FA transfer (i.e., SUV-to-SUV and LUV-to-LUV). Because small sample volumes were used with this approach, all solutions were prepared with 20 mM Hepes/KOH buffer (pH 7.4). This lower buffer concentration resulted in larger changes in intravesicular pH (pH_{in}) and facilitated kinetic analysis of the data.

It was possible to incorporate the two probes FPE and pyranine into the same vesicles. However, the instrument cannot switch between wavelengths in the fast time resolution (1 ms) of stopped-flow experiments. Thus, our protocol was to divide the sample preparation into two equal portions and measure the fluorescence of each probe separately upon rapid mixing of the FA and vesicle solutions (*21*). All stopped-flow measurements are limited by a mixing time of 10 ms.

Data Analysis. Analysis of fluorescence data was performed to determine the rate constant and the $t_{1/2}$ for FA transfer. All fluorescence traces were well fit by a single exponential function. The observed rate constant (k_{obs}) of the fluorescence change was obtained by fitting the fluorescence trace to a first order decay function:

$$F(t) = F(\infty) + F(0)\exp(-tk_{obs}) \quad (1)$$

where t is time, $F(0)$ is the initial fluorescence intensity and $F(\infty)$ is the fluorescence at $t = \infty$. The rate constant is related to the half-time of fluorescence change ($t_{1/2}$) by the following equation:

$$t_{1/2} = \ln 2/k_{obs}) \quad (2)$$

RESULTS

The primary goals of this study were (i) to determine how measurements of the adsorption and transbilayer movement of LCFA are influenced by the donor composition and (ii) to utilize a new approach with two fluorescence probes to test our hypothesis that FA desorption is much slower than flip-flop of FA between membrane leaflets.

Measuring the Binding and Transmembrane Diffusion in Acceptor Vesicles with Pyranine. We first compared the kinetics of adsorption (arrival of OA) to vesicles when OA was presented as a complex with BSA or as the uncomplexed FA presented below its solubility limit in the buffer at pH 7.4 (~6 μM for OA (*26*)). Using online fluorescence to monitor the fluorescence of the pH probe pyranine entrapped in the vesicles, we observed a pH drop that was complete within the time limit imposed by the online fluorimeter, 2 s (Figure 1A). This result indicated that OA partitioned into SUV from BSA and translocated to the inner membrane leaflet within 2 s. Similar results were obtained when uncomplexed FA were added to vesicles. Figure 1A shows that the pH drop produced by uncomplexed OA (1 μM) has a magnitude intermediate to that produced by OA/BSA complexes at a 2:1 and 4:1 OA/BSA ratio. Comparing the pH drops shows that only ~1% of the OA bound to BSA equilibrated to the vesicles, in excellent agreement with thermodynamic predictions (*2*).

When complexes with low (<2:1) OA/BSA ratios were used as donors, the pH drops were very small (data not

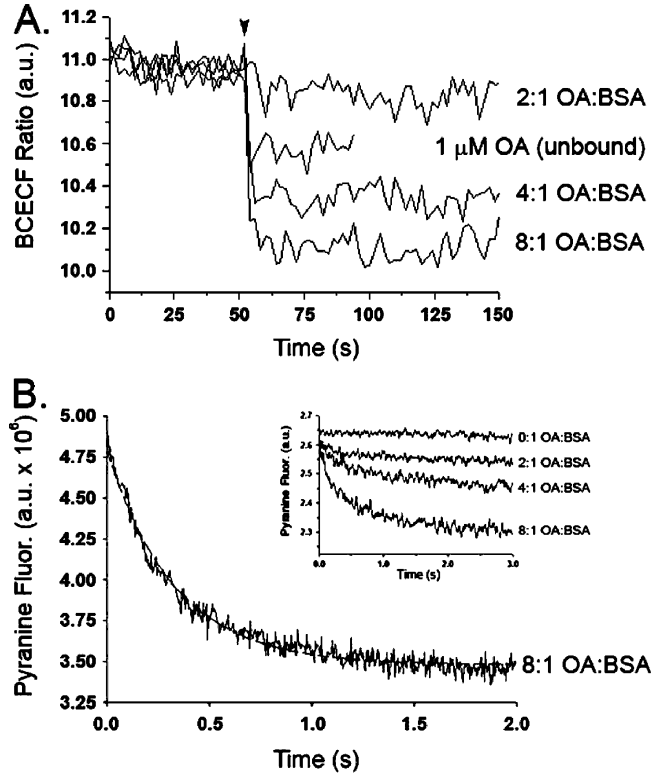

FIGURE 1: Transfer of OA from BSA to SUV. Pyranine or BCECF entrapped in the SUV measures the release of H^+ as fatty acids reach ionization equilibrium at the inner leaflet. The kinetics of fluorescence of an entrapped pH probe reflect the sum of the following steps: (1) desorption from BSA, (2) binding to the outer leaflet of SUV and (3) transmembrane movement to the inner leaflet. (A) In online fluorescence measurements, the addition of OA:BSA complexes (50 μM BSA and the indicated ratio of OA to BSA) to a suspension of vesicles (700 μM) produced rapid pH drops (<2 s). The addition of uncomplexed OA (1 μM) produced an equally rapid response intermediate in magnitude to that produced by OA/BSA complexes at ratios of 2:1 and 4:1, in which the total OA concentration is 100 μM and 200 μM, respectively. (B) In stopped-flow measurements, the transfer of OA from BSA (8:1 OA:BSA ratio) was measurably slower (k_{obs} = 3.4 s^{-1}; $t_{1/2}$ = 204 ms) than previously published data for uncomplexed FA using similar conditions (Table 1). OA:BSA < 4:1 did not produce fluorescence changes that were large enough to perform accurate curve fitting (inset). All fluorescence traces are the average of 4−8 measurements following a mixing dead time of 10 ms. Stopped-flow data were fit to a first-order decay function (dashed line) with R^2 > 0.975 and a SE of 1.1%.

Table 1: Calculated Rate Constants for OA Transfer between Albumin and Lipid Membranes[a]

donor system	rate constant	value
unbound FA	k_{ff}	$\geq 69\ s^{-1}$
FA/SUV Complex	$k_{off(SUV)}$	$7.2 \pm 0.8\ s^{-1}$
	calcd $k_{on(SUV)}$	$3.60 \times 10^6\ M^{-1}\ s^{-1}$
FA/BSA complex	$k_{off(BSA)}$	$3.4 \pm 0.3\ s^{-1}$
	calcd $k_{on(SUV)}$	$1.7 \times 10^6\ M^{-1}\ s^{-1}$

[a] Donor SUV were labeled with FPE or contained entrapped pyranine. Data for unbound OA were determined previously (*21*). Fluorescence traces were fit to a single exponential equation: $F(t) = F(\infty) + F(0)\ \exp(-tk_{obs})$, and $t_{1/2}$ was calculated with the following equation: $t_{1/2} = \ln 2/k_{obs}$. For further details on these calculations, refer to the Materials and Methods. When unbound OA below its solubility limit is used, the rate constant for flip-flop (k_{ff}) is the rate-limiting step. Since we have shown that $k_{off} \ll k_{ff}$, we can safely assume that $k_{obs} \approx k_{off}$ for each donor used. Adsorption rates (k_{on}) to EggPC vesicles were calculated using the known Kp for OA in model membranes ($0.5 \times 10^6\ M^{-1}$) (*2, 6, 25*) and the experimentally determined k_{off} from SUV or BSA using the following equation: $Kp = k_{on}/k_{off}$. The estimated SD values were determined from at least three independent experiments with different vesicle preparations. Calculated values of k_{on} are similar to those previously reported (*8*)

shown). This is expected because of the high affinity binding of LCFA to BSA at these ratios. At LCFA:BSA ratios >3: 1, the 3 highest affinity binding sites in albumin become saturated, the concentration of unbound FA begins to increase nonlinearly (*25, 43, 44*), and the binding sites in the PC membrane become more competitive with the lower affinities of the remaining albumin binding sites (*2*).

The preceding results show that delivery of LCFA from albumin might not be a rate-limiting step for LCFA uptake into cells because the rates are fast compared to metabolism (*6*). However, the precise kinetics were not observed by online measurements, because kinetic events that occur in <2 s are not detected. We therefore measured the transfer of OA from BSA to SUV using stopped-flow fluorescence with a mixing dead time of 10 ms. SUV with entrapped pyranine were rapidly mixed with an equal volume of a suspension containing OA complexed to BSA at various mole ratios. Figure 1B shows the mixing of 8:1 OA/BSA complex and SUV. Lower ratios gave similar kinetic values but poorer signal-to-noise for the same reasons described above in the online experiments (Figure 1B inset). Upon mixing, a rapid decrease in pyranine fluorescence was observed, but with kinetics that were more than an order of magnitude slower ($t_{1/2} = 204$ ms; $k_{obs} = 3.4\ s^{-1}$) than those reported for addition of uncomplexed OA under otherwise similar experimental conditions (*21*). Increasing the amount of the 8:1 OA/BSA complex to give a 6-fold increase in the albumin:SUV ratio resulted in a further decrease in the $t_{1/2}$ of FA transfer (data not shown). This comparison suggests that FA desorption from BSA significantly slows the transfer of FA to acceptor SUV, even under conditions where acceptor membrane binding sites are in excess of albumin FA binding sites (as in Figure 1B). This result is expected since the transfer of FA from albumin occurs by a unimolecular mechanism (*30, 45*); i.e., OA desorbs from BSA into the aqueous buffer, an energetically unfavorable step, prior to fast adsorption to acceptor vesicles.

Parallel experiments using vesicles as FA donors have been previously carried out with stopped-flow fluorescence for several FA, but without studying dose-dependency (*34*). Here we show the dose-dependency for transfer of OA from

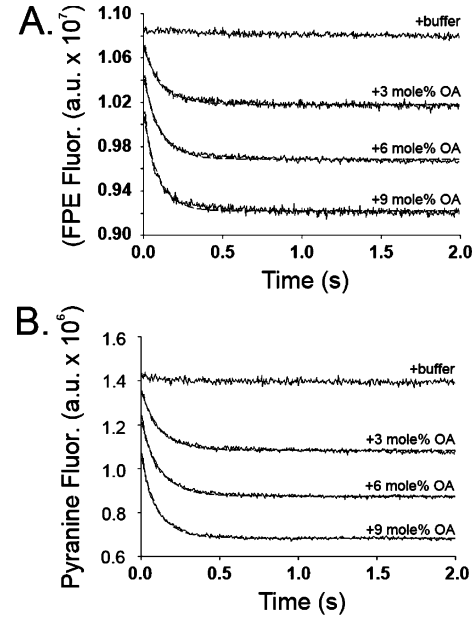

FIGURE 2: Transfer of OA from donor to acceptor SUV monitored by stopped-flow fluorescence spectroscopy. The vesicle-to-vesicle FA transfer assay measures the following kinetic steps: (1) desorption from donor SUV, (2) diffusion through the aqueous medium, (3) binding to the outer leaflet of acceptor SUV and (4) transmembrane movement of FA to the inner leaflet. The probes FPE and pyranine were present in the same vesicles. (A) FPE in the outer leaflet of the acceptor SUV measures steps 1−3. (B) Entrapped pyranine also measures step 4. Both probes produced dose-dependent changes in fluorescence with the same kinetics ($k_{obs} = 7.2\ s^{-1}$; $t_{1/2} = 96$ ms). All fluorescence traces are the average of 4−8 measurements following a mixing dead time of 10 ms. Data were fit to a first-order decay function (dashed line) with $R^2 > 0.97$ and SE ranging from 1.0 −to 4.3% for the different OA doses.

SUV (Figure 2). The rate for OA transfer from vesicles is not dose dependent and is faster than when BSA is used as a donor ($k_{obs} = 7.2\ s^{-1}$; $t_{1/2} = 96$ ms), as expected (*34*). Below, we extend our approaches to measure the desorption rates for several other FA (Figure 3).

Measuring the Adsorption of LCFA using FPE in Acceptor Vesicles. The pyranine assay measures both adsorption and transmembrane movement of the LCFA. To focus solely on the adsorption step, we added FPE to preformed vesicles to monitor the binding of OA. FPE localized to the outer leaflet detects the arrival of charged FA carboxyl group at the external membrane-water interface; moreover, its fluorescence is a quantitative measure of the negative charges (*39, 46−48*). Adsorption, as detected by FPE, occurs very rapidly ($t_{1/2} < 10$ ms) when unbound OA is added to acceptor vesicles (*21*).

For our experiments, designed to demonstrate the rate-limiting effect of desorption from a donor when adsorption to the acceptor vesicles is measured, we did not use BSA. Addition of FA-free BSA to a suspension of FPE-labeled vesicles causes the fluorescence of FPE to decrease in a dose-dependent manner (*39*), a consequence of the high surface charge of albumin. Rather, we used phospholipid vesicles as a delivery vehicle, which has the added advantages that donor and acceptor particles can have the same composition and can be present at the same concentration. Analysis of FA partitioning between like particles is more straightforward than between vesicles and albumin, which contains more than eight binding sites of varying affinities for long-chain

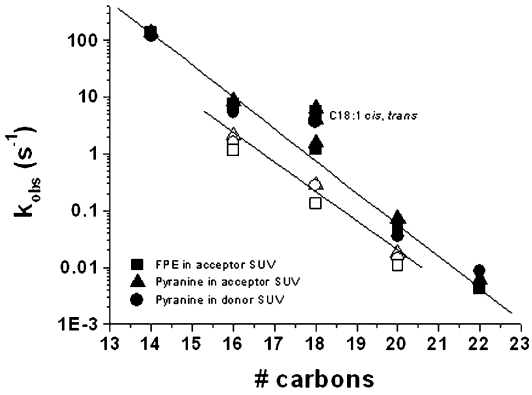

FIGURE 3: Desorption of FA from vesicles as a function of FA chain length. Desorption rate constants were derived from fluorescence measurements using FPE in acceptor vesicles and pyranine entrapped in either donor or acceptor vesicles. After incorporation of FA (12 mol % relative to PC) into donor SUV (closed symbols) or LUV (open symbols), these vesicles were mixed with an equal concentration of acceptor vesicles containing no added FA. The FA molecules transfer between donor and acceptor vesicles until an equilibrium distribution is reached. This transfer of FA produced fluorescence changes that were fit using a first order decay function. The measured rate constants were identical within experimental error in all assays for a FA of particular chain length and saturation. The desorption rate constants for saturated FA decreased linearly with acyl chain length with a slope (ΔG) of -775 kcal/CH_2 for both SUV and LUV. The absolute rates of FA transfer between LUV were approximately 5-fold slower for FA with 16−20 carbons. The presence of one double bond was equivalent to a decrease of one CH_2 group from the acyl chain. The estimated SD were determined from at least three independent vesicle preparations and ranged from 12 to 29% and from 4 to 15% for all FA in the SUV and LUV transfer assays, respectively.

FA (*49*−*54*). This is highlighted by the fact that the vesicle-to-vesicle transfer rate for OA does not change significantly with the ratio of donor:acceptor vesicles (data not shown).

Upon mixing SUV containing various mounts of added OA (e.g., 6, 12 or 18 mol %) with an equal concentration and volume of a suspension of SUV containing 1 mol % FPE and pyranine (acceptor vesicles), the fluorescence of FPE (Figure 2A) rapidly decreased in a dose-dependent manner as half of the FA desorbed from the donor SUV, diffused through the aqueous media, and adsorbed to the acceptor SUV ($t_{1/2} = 96$ ms; $k_{obs} = 7.2$ s^{-1}). As shown in Figure 2B, the kinetics were the same when pyranine response was measured in the same FPE-labeled vesicles (Materials and Methods) but are also similar to those reported for experiments with pyranine alone in the acceptor vesicles (*34*). Thus, our new results suggest that the measured rate must reflect desorption of OA from the donor vesicles, and that flip-flop of FA between leaflets in the acceptor vesicles must occur on the same time scale as binding (adsorption). By comparison, this rate for OA desorption is ∼2 times faster than from BSA (Table 1).

Measuring the Desorption of LCFA by FPE in Donor Vesicles. To complement the previous strategy, we used stopped-flow fluorescence to measure the desorption of OA from donor SUV labeled with FPE without prior assumptions about flip-flop kinetics. Upon mixing the donor vesicles with acceptor vesicles containing no fluorophore, FPE fluorescence increased rapidly, with a calculated $t_{1/2} \sim 100$ ms ($k_{obs} = 6.9$ s^{-1}). An increase in fluorescence is expected as FA anions leave the outer leaflet of the SUV. The same kinetic result was observed in a parallel experiment in which OA was transferred from unlabeled donor vesicles to acceptor vesicles containing 1 mol % FPE in the outer leaflet to detect adsorption, further supporting our hypothesis that adsorption, and the subsequent flip-flop step, are rate-limited by FA desorption from the donor (See Discussion, Figure 5).

Measuring the Transfer of FA as Function of Chain Length by Both FPE and Pyranine Methods. To test both the applicability of our methods and the validity of our hypotheses, we extended our methods to a wide range of FA. Previously, we measured desorption (designated "dissociation") kinetics with our protocol using vesicles with pyranine in either donor or acceptor vesicles. With our novel surface probe, we were presented the opportunity to perform parallel experiments with FPE, for comparison with our previously published results (*34*). Furthermore, in these new experiments we used pyranine together with FPE in the acceptor vesicles, as well as a separate set of experiments with pyranine in the donor vesicles to test the consistency of results. The rates of vesicle-to-vesicle transfer of C14:0, C16:0, C18:0, C18:1*cis*, C18:1*trans*, C20:0, and C22:0 are plotted as a function of FA chain length in Figure 3. For each individual FA molecule examined, the rate constants derived from pyranine and FPE traces were the same, and were independent of whether pyranine was entrapped in donor or acceptor vesicles. Not surprisingly, the kinetics for transfer of OA between SUV in the present experiments at 22 °C was slower than that reported for OA at 37 °C (*30*). A 5-fold decrease in the transfer rate for each FA was observed when using LUV, which may be the result of decreased membrane curvature, but trends for the FA were parallel. The data for all FA fit a log-linear trend ($R^2 > 0.99$), with a slope for both SUV and LUV very similar to that found previously for SUV (*34*). Since desorption is the first step common to all assays, and must occur before fluorescence changes can be observed, these new data with FPE further suggest that FA desorption is rate-limiting. It should also be noted that elaidic acid (C18:1*trans*), which was not previously measured by Zhang et al. (*34*), was incorporated into this study to assess whether acyl chain conformation (linear or bent) of the two 18-carbon monounsaturated FA would affect desorption rate, but no differences were observed.

DISCUSSION

The transport of FA across the plasma membrane and within cells is vital for the functions and metabolism of these key nutrients. A clearer understanding of the molecular details of transport in membranes is emerging, largely from fluorescence approaches that can track these small molecules at different stages of transport. Our previous work established new strategies for monitoring adsorption, transmembrane movement and desorption of FA, and provided thermodynamic and kinetic information for transport in model and biological membranes (*1, 4*−*6, 24, 25, 55*). This topic has also been extensively discussed in numerous reviews (*2, 3, 8, 19, 23, 30, 45*).

We recently applied novel dual fluorescence methods to focus on the adsorption of FA to phospholipid bilayers and established that the FA adsorbs very rapidly when presented in the unbound form (without a carrier) (*21*). Under the conditions of our stopped-flow experiments, the vesicles

showed no leakage or any indication of disruption. We concluded that this is the fastest step of membrane transport in the model membrane (*4*). Delivery of unbound FA to membranes and cells can provide essential information about individual steps of FA transport without the potential complications of an additional constituent that acts as a FA donor. However, such an approach is also limited by the fact that FA seldom reach concentrations near their solubility limit in aqueous media (low μM), except possibly during rapid intra- or extracellular lipolysis of triglyceride.

The Rate of Fatty Acid Transfer to Vesicles Is Dependent on the FA Donor. The present studies examined transport in the presence physiological donors and acceptors of FA (albumin and membranes) that also maintain a low concentration of unbound FA at any instant. We showed that transfer of FA from either albumin (Figure 1) or a phospholipid vesicle to an acceptor phospholipid vesicle (Figure 2) was measurably slower compared to the rate when FA was presented in an uncomplexed form (*21*) (Table 1). Whereas our previous protocols used only a water-soluble pH dye trapped inside vesicles to measure the arrival of FA in the acceptor vesicles (*34*), this study also used an additional surface probe, FPE. This probe can be localized to the outer leaflet of the vesicles and provides unambiguous detection of how fast FA anions intercalate into the outer leaflet (*21*). Comparison of our results with different donors allows the conclusion that none of the methods of donating OA to vesicles (unbound, BSA, vesicles) introduced any artifact in our measurements, and that our method of measuring transfer using the entrapped pH dye is valid, since the flip-flop occurs as rapidly as adsorption (see below). Slower kinetics are expected when BSA or vesicles are used as donors, compared to unbound FA, because of the energy barrier for solvation of the FA, as discussed below in detail.

Our interpretation of the kinetic data when albumin is used as a donor, that desorption from albumin is rate-limiting, is consistent with those of most prior independent investigations using albumin as a donor of FA for transfer to vesicles (*2, 30, 45, 56*). Another group has argued that desorption of FA from albumin is extremely fast, and has postulated that the rates measured by pyranine entrapped in the acceptor vesicles correspond to slow flip-flop (*35*−*37, 57, 58*). However, these conclusions have yet to be supported by any direct measurement of FA desorption from membranes in the absence of other kinetic steps. Under the conditions used in our study (Figure 1A), the rate of desorption of OA from BSA is faster than some rates measured by other methods ($t_{1/2} \sim 5-17.3$ s; $k \sim 0.04-0.14$ s^{-1}), but in accord with our previous online measurements (*25*) and those in this study which showed a $t_{1/2} < \sim 1$ s. When measured on a faster time scale by stopped flow, our rate of transfer of OA from albumin to vesicles ($t_{1/2} \sim 204$ ms; Table 1) is similar to the value recently reported by Kleinfeld et al. (*57*). More important, the new results with FPE eliminate the possibility that the observed transfer kinetics are due to slow flip-flop in the acceptor vesicles, as postulated in (*57*).

Desorption of FA from a Phospholipid Bilayer Is Slower than Flip-Flop across the Bilayer. Our new study also addresses a major question that has been contentious for more than 20 years: whether desorption of LCFA from a phospholipid bilayer is slower or faster than transmembrane movement (flip-flop). The first studies of desorption and flip-flop derived rate constants from the vesicle-to-vesicle transfer of two different fluorescent analogues of FA, pyrene-labeled (*59*) and anthroyloxy-labeled (*60*), and came to opposite conclusions. The anthroyloxy FA was later shown by the same investigators to predict flip-flop rates that were slower than other estimates by 2 orders of magnitude ($k < 0.01$ s^{-1}), calling into question the use of this analogue for estimating the kinetics of natural FA (*61*). Our kinetic and thermodynamic data for a wide range of FA (14−26 carbons) (*34*) showed that desorption is slower than flip-flop in model membranes, a conclusion reached in earlier studies (*59, 62*). Parallel studies with SUV and LUV revealed that although membrane curvature does have a small effect on the absolute rates of FA transfer between vesicles, the ΔG of membrane desorption is unchanged, suggesting that the physical chemical properties which govern FA desorption are dependent on the dissociating molecule rather than on membrane curvature.

However, disagreements on this fundamental issue continue (*57, 61, 63, 64*), and it has been suggested that our approach of adding FA without albumin (*5, 6, 25*) to measure fast kinetics by stopped-flow creates an artificial environment that allows the FA to permeate the bilayer more rapidly. We have recently demonstrated this speculation to be erroneous by demonstrating the integrity of vesicles with various probes, including entrapped calcein (*21*).

The present study with physiological donors, together with our recent detailed study with unbound FA (*21*), provides further clarification of the kinetics of flip-flop. Furthermore, all of our results lead to the conclusion that flip-flop is faster than desorption in model membranes. Three comparative results provide conclusive evidence for this conclusion.

First, when donor vesicles with OA were mixed with acceptor vesicles containing the surface probe FPE and entrapped probe pyranine, either singly or in combination, the kinetics fit a single exponential function, and were identical for each probe. This can be gleaned for the dose-dependence results in Figure 2, and graphically by the superimposition of results for the two probes at a single dose of OA (Figure 4). The only conclusion that can be made is that OA flip-flops rapidly, as soon as binding to the outer leaflet is detected.

Second, we compared the transfer kinetics using donor vesicles containing FPE or acceptor vesicles containing FPE. As expected, the FPE fluorescence increased as FA left the FPE-labeled donor vesicles and the number of FA anions decreased; when FPE was present in the acceptor, its fluorescence decreased as the number of FA anions increased in the outer leaflet (Figure 5). To our knowledge, FPE is the only probe that measures desorption of natural FA from the outer leaflet of a bilayer membrane. It yielded the result that adsorption to acceptor vesicles occurs only as fast as the rate at which FA desorb from the donor (i.e., desorption, and not flip-flop, is the rate-limiting step of FA diffusion through membranes). In these experiments, the same mole % OA was transferred from donor or to acceptor vesicles. Since donor and acceptor SUV were mixed at a 1:1 ratio, the OA should redistribute equally among donor and acceptor SUV at equilibrium. In accordance with this expectation, the final FPE fluorescence intensity after transfer was the same

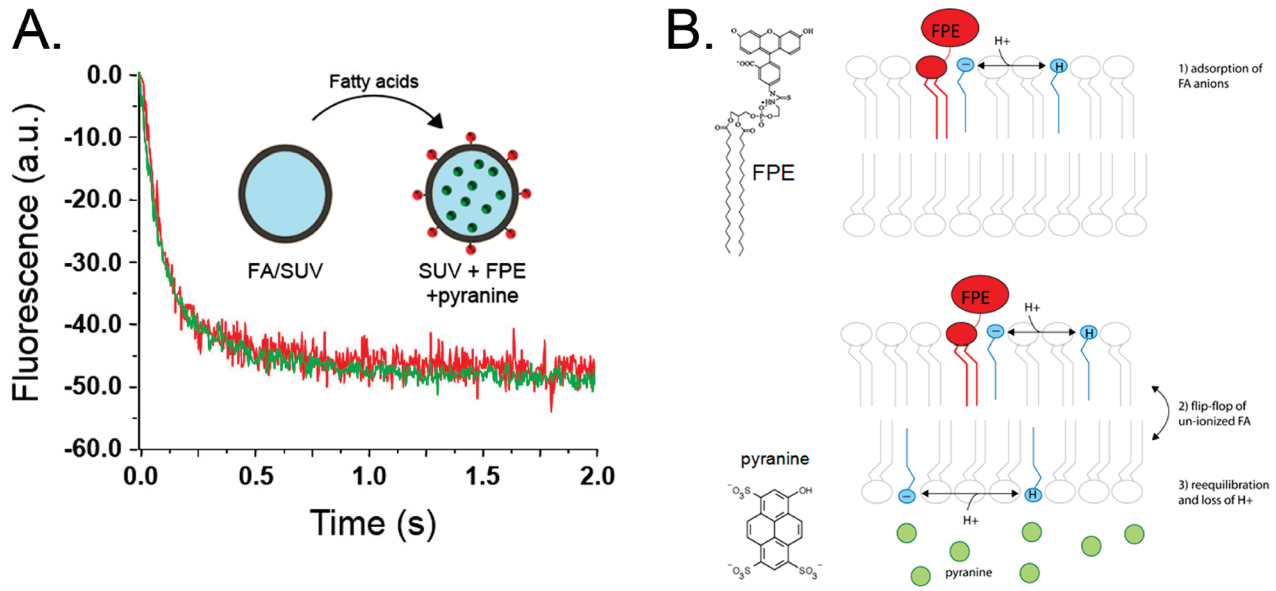

FIGURE 4: Binding and flip-flop of OA in SUV occur on the same time scale as analyzed by stopped-flow fluorescence spectroscopy. SUV with 6 mol% OA were mixed with SUV with FPE incorporated into the outer leaflet and pyranine in the inner aqueous volume (ratio of donor: acceptor SUV = 1:1). (A) The kinetics of fluorescence changes were the same for the surface probe that detects adsorption [FPE (red)] and the pH probe [pyranine (green)] that detects flip-flop. Each fluorescence trace is the average of 4−8 measurements following a mixing dead time 10 ms. (B) Schematic showing the structures of the outer membrane leaflet probe, FPE (top), and the entrapped pH probe, pyranine (bottom) at the opposite side of the bilayer. FPE fluorescence is linearly proportional to the number of FA anions that intercalate into this leaflet (adsorption) and alter the membrane surface potential. There is an extremely fast equilibration of protons between the ionized and un-ionized forms of the FA. Following flip-flop of un-ionized FA, pyranine in the aqueous volume inside the vesicle detects the release of protons as the FA reach ionization equilibrium (*5*). The fluorescence responses of FPE and pyranine in the same vesicles show that both events occur so rapidly that they are not resolved in the time resolution of the stopped-flow measurement (ms).

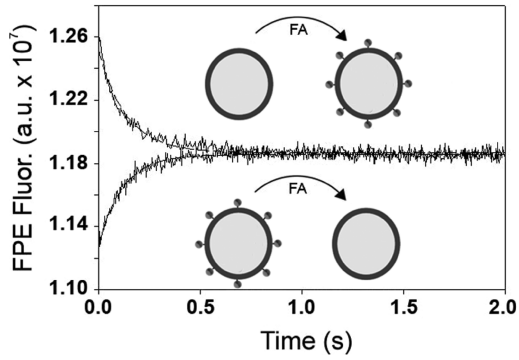

FIGURE 5: Comparison of the transfer of OA from SUV with FPE in acceptor or donor vesicles. A decrease or increase in concentration of FA anions in the membrane results in an increase or decrease of FPE fluorescence, respectively. For FPE-labeled acceptor SUV, the kinetics of FPE fluorescence reflects the sum of the following kinetic steps: (1) desorption from donor SUV, (2) transfer through the aqueous phase, and (3) binding to the outer leaflet of acceptor SUV (top trace). For FPE-labeled donor SUV, the kinetics of the FPE fluorescence change reflect only desorption from donor SUV into the aqueous phase (lower trace). The donor SUV initially contained 18 mol % OA, and the final amount of OA transferred to the acceptor is expected to be 9 mol % in this experiment with mixing equivalent amounts of donor (100 μM) and acceptor (100 μM). In stopped-flow experiments, the transfer of OA between vesicles occurred with a (k_{obs} = 6.9 s^{-1}; $t_{1/2}$ = 100 ms) and the final fluorescence intensity obtained in each experiment was the same, as expected. In experiments with lower initial concentrations of OA relative to phospholipid, the same results were obtained. Fluorescence traces are the average of 4−8 measurements following a mixing dead time of 10 ms. Stopped-flow data were fit to a first-order decay function (dashed line) with $R^2 > 0.98$ and SE ranging from 1.0 to 1.3% for the different transfer experiments.

in both of these experiments (Figure 5). Note that the design of this experiment requires no assumptions about the kinetics of flip-flop.

Third, we used SUV and LUV to study the transfer of FA with different chain lengths with a combination of FPE and pyranine for comparison with our previous study in SUV using only pyranine (*34*). The fluorescence changes for both FPE and pyranine were well fit by a single exponential function and, in the case of SUV, gave identical rate constants (at room temperature) to those previously published for all FA (Figure 3). Rates obtained with LUV were nearly 5-fold slower for all FA tested (C16:0, C18:0 and C20:0), which is expected from the tighter lipid packing as a consequence of reduced membrane curvature (*65*).

As discussed above for OA, the conclusion can be made that desorption is slower than flip-flop for this very wide range of FA. Although small differences were observed in the desorption rates for each FA from LUV when compared to SUV, the data obtained with FPE and pyranine indicated that flip-flop is still too fast to detect in LUV despite reduced membrane curvature. This is consistent with our previous measurements estimating half-times for flip-flop of long chain FA in LUV to be shorter than ~25 ms (*4*).

The thermodynamic analysis of the log-linear dependence in both SUV and LUV further supports this conclusion. The pseudo-unimolecular rate constants (k_{off}) for dissociation of C14:0 to C22:0 showed a 10-fold decrease for each addition of two CH_2 groups to the acyl chain, corresponding to a ΔG of −775 kcal/CH_2. This value is close to the value derived from analysis of our previous data for chain lengths of 12−26 carbons (−740 kcal/CH_2) and to that from independent studies using a different methodology (−640 kcal mol^{-1}) (*30*). The partitioning of saturated FA (7−22 carbons) between heptane and water (*66*) showed a linear relationship between the logarithm of the true distribution constant and the FA chain length. Moreover, the solubility (as the free

energy of transfer from the hydrocarbon solvent to water) is a linear function of the alkyl chain length for alkanes, alkenes, *n*-alkanols, and *n*-aliphatic acids, with a slope of about −850 kcal/CH_2 (*40, 66*). Therefore, as we concluded previously, our thermodynamic result is consistent with the complete transfer of FA from the hydrophobic environment of the bilayer into the water phase.

The large difference in the energy barrier for transfer of FA from the lipid to water phase compared to movement across the lipid bilayer is best exemplified by the FA with chains of >20 carbons. We have recently demonstrated fast flip-flop ($t_{1/2}$ < 1 s) of FA with 20, 22, 24 and 26 carbons using cyclodextrin as a delivery vehicle (Hamilton, Pillai and Brunaldi; unpublished). The 24-carbon FA desorbs with a half-time that is 3 orders of magnitude slower than the upper limit for flip-flop and the 26-carbon FA, 4 orders of magnitude slower (*34*).

CONCLUSIONS

The fluorescence strategies employed and the rate constants (k_{obs}) and $t_{1/2}$ values for uncomplexed OA and OA complexed to albumin or vesicles obtained in this study clearly demonstrate that (i) fluorescence changes detected by probes located on acceptor vesicles are dependent upon the method used to deliver the FA (i.e., uncomplexed or complexed) and (ii) flip-flop is extremely fast and cannot be the rate-limiting step for FA permeability in phospholipid bilayer vesicles (*67*). The results reinforce our previous questions about the roles of membrane proteins that have been postulated to catalyze the transmembrane movement of FA (*5, 23, 68*). However, the lack of fluorescent probes that can measure each individual step of transport of FA has impeded progress in deciphering mechanisms in biological membranes, and our new approaches could provide new insights into the functions of such proteins. The FPE probe can be used as a sensor for FA binding to the extracellular leaflet of the plasma membrane, and an intracellular pH probe as a sensor for the movement of the FA to the cytosolic leaflet. Thus, for example, it could be determined whether FA accumulates on the extracellular leaflet without translocation to the cytosolic leaflet in the absence of adequate levels of CD36 in the plasma membrane (*69*). Similarly, the hypothesis that caveolin-1 expression mediates slow FA accumulation in the cytosolic leaflet of HEK cells (*28*) could be tested by measuring the rate of FA binding to the extracellular leaflet and assessing whether the FPE fluorescence decreases at the same slow rate as the intracellular pH, which has been postulated to reflect FA movement into binding sites on caveolin-1.

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