Flip-Flop Is Slow and Rate Limiting for the Movement of Long Chain Anthroyloxy Fatty Acids across Lipid Vesicles†

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ABSTRACT: An issue that is central to understanding cellular fatty acid (FA) metabolism is whether physiologic transport of FA across cell membranes requires protein mediation or can be satisfied by the rate of spontaneous movement through the lipid phase. For this reason, considerable effort has been devoted to determining the rate-limiting steps for transport of FA across pure lipid bilayer membranes. Previously, we found that transbilayer flip-flop was the rate-limiting step for transport of long chain anthroyloxy FA (AOFA) across lipid bilayers and that the times for long chain AOFA flip-flop were ≥100 s, yielding rate constants for flip-flop (k_{ff}) that were ≤0.01 s^{-1} [Storch, J., & Kleinfeld, A. M. (1986) *Biochemistry 25*, 1717−1726; Kleinfeld, A. M., & Storch, J. (1993) *Biochemistry 32*, 2053−2061]. In those studies, k_{ff} values were inferred from the time course of AOFA transfer between lipid vesicles. Recently, Kamp et al. [Kamp, F., Zakim, D., Zhang, F., Noy, N., & Hamilton, J. A. (1995) *Biochemistry 34*, 11928−11937], using pyranine trapped within lipid vesicles to detect flip-flop more directly, have reported that flip-flop rates of long chain AOFA are extremely rapid (k_{ff} > 10 s^{-1}) and are not rate limiting for transbilayer transport. Because no defect was apparent in our previous measurements, we have extended, for AOFA, the pyranine method of Kamp et al. (1995) by using stopped-flow fluorometry to resolve flip-flop rates of both short and long chain AOFA in vesicles. In addition, we have monitored the time course of transbilayer AOFA flip-flop using carboxyfluorescein (CF) trapped within the lipid vesicles as a resonance energy transfer (RET) acceptor of AO fluorescence. The differential quenching of AOFA fluorescence in the outer and inner leaflets of the bilayer allows flip-flop to be separated from the time course of AOFA binding to the vesicles. Results obtained from both the pyranine and CF methods indicate, in agreement with our previous results, that flip-flop of the long chain AOFA is slow relative to either the binding or the rate of dissociation from the vesicle. In particular, we find that the time constant (τ) for pyranine quenching by 2-AO-palmitate (2-AOPA) was >40 s and that k_{ff} obtained from RET in CF vesicles was about 0.003 s^{-1}. Also, in contrast to Kamp et al. (1995) who reported that k_{ff} values were independent of FA chain length or structure for the C-12 to C-18 native and the C-18 AOFA within a factor of 2, we find that the rate of pyranine quenching for the shorter chain 11-AO-undecanoic acid is more than 50-fold faster than for the longer chain AOFA. We conclude, therefore, that transbilayer transport of the AOFA is limited by the rate of flip-flop and that this rate is a sensitive function of the AOFA structure.

Considerable disagreement surrounds the issue of whether fatty acid (FA)[1] transport across cell membranes occurs by diffusion through the lipid phase and/or is mediated by a membrane protein [reviewed in Storch (1990) and Kleinfeld (1995)]. For this reason, understanding fatty acid (FA) transport in model membranes is important because rapid transport across pure lipid bilayers might obviate the need for membrane protein involvement in cellular transport. Thus, considerable effort has been devoted to resolving the rates and mechanism of transbilayer transfer (flip-flop) of FA across lipid bilayers (Doody et al., 1980; Walter & Gutknecht, 1984; Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993; Kamp et al., 1995; Kleinfeld, 1995).

The central question for model membranes is whether transbilayer flip-flop is rate limiting for the net transport of FA between the aqueous compartments on either side of the bilayer. Investigations of this issue have been done with both native and fluorescently modified FA. In particular, we reported previously that the rates of flip-flop of the long chain anthroyloxy FA (AOFA) across lipid vesicles were considerably slower than the rate at which these FA either dissociate from or bind to the bilayer surfaces of these vesicles (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993).

Recently, these results have been called into question by Kamp et al. (1995) who studied AOFA flip-flop by monitoring the pH change in the inner aqueous compartment of lipid bilayer vesicles upon addition of AOFA to the outer aqueous phase. The internal pH was monitored in these studies by

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[1] Abbreviations: AO, anthroyloxy; AOFA, anthroyloxy fatty acid; CF, carboxyfluorescein; EPC, egg phosphatidylcholine; FA, fatty acid; 2-AOPA, 2-AO-palmitic acid; 11-AOUD, 11-AO-undecanoic acid; LUV, large unilamellar vesicles; RET, resonance energy transfer; SUV, small unilamellar vesicles.

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the fluorescent pH indicator pyranine which was trapped within the vesicles. AOFA flip-flop was monitored by the time course of quenching of the pyranine fluorescence as neutral AOFA released an H^+ upon moving from the outer to the inner bilayer leaflet (Kamp et al., 1995). The results of these studies showed that, upon addition of AOFA to these vesicles, pyranine fluorescence was quenched faster than the resolution of the mixing system. Kamp et al. (1995) concluded that long chain AOFA flip-flop times are shorter about 20 ms, at considerable variance with the values of about 100−2000 s found in our previous studies (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). Also in contrast to Kamp et al. (1995), we found in these previous studies that the rate of flip-flop was sensitive to vesicle and FA type, being faster for shorter and/or more unsaturated AOFA.

In our previous studies we had inferred the AOFA flip-flop rates from the time course of transfer of AOFA between vesicles (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993) rather than the more direct measurement of the transport of FA from the outer to the inner aqueous compartments. The extremely rapid rates obtained by Kamp et al. (1995), therefore, raised the possibility that the intervesicle transfer measurements might suffer from an artifact, resulting in the slow rates of flip-flop observed in such measurements. Although no such artifact was identified by either Kamp et al. (1995) or ourselves, we have, in the present study, extended the measurements of Kamp et al. (1995) by using stopped-flow fluorometry to observe the movement of AOFA across the bilayer of vesicles containing pyranine. In addition, we have utilized a second method in which resonance energy transfer (RET) between the AOFA and carboxyfluorescein trapped inside the vesicles allows the direct observation of the flip-flop process. In contrast to Kamp et al. (1995), both methods employed in the present study confirm our previous results and demonstrate clearly the slow flip-flop times for the long chain AOFA. We also provide evidence that the apparent fast rates observed by Kamp et al. (1995) may have resulted from the addition of the AOFA, in an ethanol vehicle, directly to the pyranine-containing vesicles.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (EPC) was purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL). [^{3}H]DPPC (phosphatidylcholine, L-α−dipalmitoyl-, [*choline-methyl*-^{3}H]-) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Anthroyloxy fatty acids (AOFA), (5 and 6)-carboxyfluorescein (CF), and pyranine (8-hydroxyprene-1,3,6-trisulfonic acid) were purchased from Molecular Probes (Eugene, OR). The potassium salt of 2-AO-palmitate was made by dissolving 2-AO-palmitic acid in KOH at 45 °C to a final concentration of 5 mM 2-AO-palmitate and 20 mM KOH. A chloroform extraction of this material was chromatographed by thin layer chromatography using a solvent system of 25:15:4:2 chloroform:methanol:acetic acid:water. The 2-AO-palmitate−K^+ migrated as a single spot with virtually the same mobility as 2-AO- or 16-AO-palmitic acid (in ethanol). Stock solutions of the salt in 20 mM KOH as well as the acid in ethanol were maintained under argon at −20 °C. Standard buffers for CF and pyranine-containing vesicles were 40 mM Tris and 150 mM NaCl at pH 7.6 and 100 mM HEPES and 50 mM NaCl at pH 7.5, respectively.

Vesicle Preparation. Small unilamellar vesicles (SUV) of approximately 200 Å diameter and large unilamellar vesicles (LUV) of approximately 1000 Å diameter were prepared essentially as described previously (Huang & Thompson, 1974; Hope et al., 1985; Storch & Kleinfeld, 1986; Anel et al., 1993). Briefly, EPC lipid in chloroform was first dried by rotary evaporation and was lyophilized overnight. The dried film was then hydrated either with the standard buffers alone, or for CF-containing vesicles, 20 mM CF added to its standard buffer. For pyranine-containing vesicles, 500 μM pyranine was added to its standard buffer. For SUV, the hydrated dispersion was sonicated at 70 W for 30 min at 4 °C and centrifuged at 120000*g* for 45 min to remove titanium and lipid debris. For the LUV preparation the hydrated dispersion was subjected to five cycles of freeze−thawing and was then extruded with a device from Lipex Biomembranes (Vancouver, British Columbia, Canada) five times through a stack of two polycarbonate filters (Nucleopore) with 0.2 μm pore size, followed by 10 extrusions through two polycarbonate filters with 0.1 μm pore size. Both SUV and LUV were chromatographed through sephacryl 1000 to remove free CF or pyranine; trace ^{3}H-DPPC and fluorescence were used to determine the elution profile. Lipid concentration was determined as inorganic phosphate, as described (Gomori, 1942). In most measurements, the vesicle concentration was approximately 100 μM EPC.

Fluorescence Measurements. Fluorescence measurements were done using either an SLM 4800 or an SLM 8100 fluorometer. For both instruments, AOFA fluorescence was measured using excitation and emission wavelengths of 383 and 450 nm, respectively, and pyranine fluorescence was monitored at 455 and 509 nm, respectively. Two types of measurements were done. In the first, a small volume (typically <0.2%) of AOFA solution was added directly to a vesicle dispersion (1.5 mL) in the observation cuvette of the fluorometer. Mixing was accomplished either by drawing the sample in and out of a Pasteur pipette or with a magnetic mixer; equivalent results were obtained by both methods. The time required for adding AOFA to vesicles in this first type of measurement was typically about 15 s (see Figure 5, for example). In the second type of measurement, mixing of vesicles and the AOFA was done using a stopped-flow device with a dead time of <5 ms (Milliflow Stopped Flow Reactor, SLM-AMINCO) coupled to the SLM 8100 fluorometer. In all of the measurements reported in this study, the fluorescence intensity of the aqueous dispersions of the AOFA, whether as the potassium salt or as the acid, was negligible in the absence of vesicles. All measurements were done at 24 or 30 °C (as indicated), and qualitatively similar results were obtained at both temperatures.

Monitoring Transbilayer Transfer of AOFA Using Pyranine-Containing Vesicles. As shown by Kamp et al. (1993), addition of FA to lipid vesicles decreases the internal pH and this change can be monitored by the quenching of the fluorescence of trapped pyranine. Because the neutral form of the FA undergoes faster flip-flop than the ionized form and because the membrane bound pK_a of the FA is approximately 7, about half of the neutral FA will release an H^+ upon transfer to the inner leaflet, assuming the intracellular pH is also about 7 (Kamp et al., 1993). Consequently, if the aqueous medium inside the vesicle is not too highly buffered, the flip-flop of FA will generate a measur-

able change in pyranine fluorescence. We have followed this approach in the present study to monitor the transfer of AOFA across both SUV and LUV, using conditions similar to Kamp et al. (1995), and we have extended the temporal resolution of this approach by using stopped-flow fluorometry for the AOFA. Time constants (τ) were determined from single-exponential fits to the time course of the fluorescence quenching of pyranine. As described in Kamp et al. (1993, 1995), calculating k_{ff} from the observed time constants requires precise information about the intravesicular volume, buffering capacity, amount of FA, and the initial and final intravesicle pH values. Our results are reported in terms of τ rather than k_{ff} because the various parameters required for converting τ to k_{ff} were not specifically determined in this study. However, the preparation of the pyranine-containing vesicles and experimental conditions in the present study were similar to that of Kamp et al. (1995), and therefore, the observed quantities from both studies can be compared. The primary difference in the experimental conditions was, for the reasons explained below, the use of 2-AO-palmitate in this study rather than 6-, 9-, and 12-AO-stearate as in Kamp et al. (1995).

Monitoring Transbilayer Transfer of AOFA Using CF-Containing Vesicles. The rate of transbilayer transfer of AOFA across the lipid bilayer was determined by comparing the time course of AOFA fluorescence after mixing with vesicles that did or did not (blank) contain CF. For blank vesicles, the change in AOFA fluorescence reflects only the time course of AOFA binding to the outer bilayer leaflet because the fluorescence quantum yield of the fluorophore is the same in the outer and inner leaflets. For vesicles containing CF, the quantum yield can be substantially smaller for AOFA in the inner as compared to the outer bilayer leaflet, as a result of resonance energy transfer (RET) quenching of the AOFA donor by the CF acceptor. Thus, if AOFA flip-flop is slow relative to the time course for binding, we would expect the AO fluorescence of the CF-containing vesicles first to increase, with approximately the same time constant as observed for the blank vesicles, and then to decrease, relative to the time course for the blank vesicles, as the AOFA moves from the outer to the inner bilayer leaflet. If flip-flop is fast, the time course should be similar to that of the blank vesicles.

The difference in the time course between blank and CF-containing vesicles is best resolved for conditions that maximize the difference between the inner and outer leaflet quantum yields. Because RET decreases sharply with increasing donor−acceptor separation, AOFA in which the AO group is attached at the $\Delta 2$ position should yield the maximum quantum yield difference. As discussed previously, the critical transfer distance (R_o) for RET between AO and CF is about 35 Å (Storch & Kleinfeld, 1986). Using this R_o and an average position for the 2-AO position of 1 Å from the bilayer surface (Kleinfeld, 1985), we estimate as in our previous study (Storch & Kleinfeld, 1986) that the quantum yield will be reduced by about 15% in the inner relative to the outer leaflet of the LUV vesicles. Although the exact values for these outer and inner leaflet quantum yield differences are not critical to the conclusions concerning flip-flop rates, the observed differences in quenching of 2-AOPA in the CF and blank vesicles are consistent with the predicted values (see Figure 6 for example).

Determination of k_{ff} from the measurements of the time course of AOFA added to CF-containing vesicles was done in two steps. In the first, the time course for binding to blank vesicles was analyzed using the kinetic model described below to determine the rate constants for dissociation of AOFA aggregates in the bulk aqueous phase and binding of AOFA to the vesicle surface. In the second step, the time course for the CF-containing vesicles was analyzed using the kinetic model with the rate constants for aggregate dissociation and vesicle binding fixed by the blank vesicle results. The model used to analyze these results is represented by the following equations:

$$\frac{d[FA_g]}{dt} = -k_{off}^{g}[FA_g] \quad (1)$$

$$\frac{d[FFA]}{dt} = k_{off}[FA]_1 - k_{on}[L][FFA] + [FA_g]k_{off}^{g} \quad (2)$$

$$\frac{d[FA]_1}{dt} = -k_{off}[FA]_1 + k_{on}[L][FFA] + k_{ff}([FA]_2 - [FA]_1) \quad (3)$$

$$\frac{d[FA]_2}{dt} = k_{ff}([FA]_1 - [FA]_2) \quad (4)$$

where [FFA] is the FA concentration in the outer aqueous phase, $[FA]_1$ and $[FA]_2$ are the concentration of FA in the outer and inner bilayer leaflets, respectively, [L] is the vesicle lipid concentration, $[FA_g]$ is the concentration of FA in aggregate form in the aqueous phase, k_{ff} is the flip-flop rate constant, k_{off} and k_{off}^{g} are the rate constants of FA dissociation from the vesicles and aggregate phases, respectively, and k_{on} is the rate constant for binding of FA to the vesicle. In eq 1, we have assumed a negligible rate of formation of the aggregate phase after mixing with the vesicles because the aggregate phase rapidly dissolves in the presence of vesicles at the concentrations used in these studies. Solutions of eqs 1−4 were obtained with the initial conditions: $[FA]_1 = 0$, $[FA]_2 = 0$, $[FFA] = FA_T(1 - f_g)$, and $[FA_g] = FA_T f_g$, where FA_T is the total FA concentration and f_g is the fraction initially in the aggregate state.

Solutions of eqs 1−4 with kinetic parameters typical of those found in this study are shown in Figure 1. In this example, we have used the parameters found for the measurements in Figure 6B in which about half the added FA was initially aggregated and k_{ff} was 0.004 s^{-1}. These results illustrate how rapid binding of soluble FA and slow dissociation of the aggregate form lead to a biphasic increase in FA bound to the outer hemileaflet of the bilayer [$[FA]_1$-(t)]. They also reveal a slow decrease and increase in FA bound to the outer and inner hemileaflet, respectively, that results from the slow rate of flip-flop. Although its functional form is complex, fits to the $[FA]_2(t)$ with the function $[FA]_2(\infty)(1 - e^{-t/\tau})$ provides a reasonable representation of the actual function and returns a value of $1/\tau$ that is within 10% of k_{ff}. This provides the basis for using a single-exponential decay for the analysis of the pyranine quenching results.

The time course of the fluorescence intensity [$F(t)$] can be expressed in terms of the solutions to eqs 1−4 as,

$$F(t) = Q_1[FA]_1 + Q_2[FA]_2 \quad (5)$$

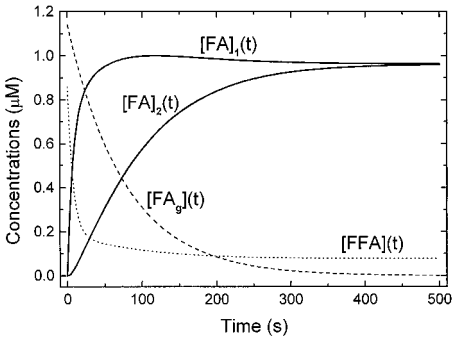

FIGURE 1: Simulations of the time courses of 2-AOPA exchange among the four kinetic FA pools of the AOFA-vesicle mixing experiment. These results indicate how the concentrations of FA in the aggregate state ($[FA_g]$), in the aqueous phase ([FFA]), in the outer hemileaflet of the bilayer ($[FA]_1$), and in the inner hemileaflet ($[FA]_2$) are predicted to change with time after mixing AOFA and vesicles. The plotted values are the solutions of eqs 1−4 with the following parameter set: $f_g = 0.57$, $k_{off}^g = 0.013$ s^{-1}, $k_{off} = 0.01$ s^{-1}, $k_{on} = 5 \times 10^3$ M^{-1} s^{-1}, $k_{ff} = 0.004$ s^{-1}. These values were obtained from fitting to the data of Figure 6B.

where Q_1 and Q_2 are the fluorescence intensities in the outer and inner leaflet, respectively. For the blank vesicles, the measured fluorescence time courses were fitted with eq 5 to obtain values for Q_1, Q_2, k_{on}, k_{off}^g, and f_g, where k_{off} was obtained from k_{on} using a value for the partition coefficient of 5×10^5 (Anel et al., 1993). Using these parameters from the blank vesicle time course, values for k_{ff} were obtained by fitting the time course of the corresponding CF-containing vesicles with eq 5 in which only Q_1, Q_2, and k_{ff} were allowed to vary. These procedures were done using the program MLAB (Civilized Software) as described previously (Richieri et al., 1996) and yielded unique values for k_{ff}.

Characteristics of AOFA Binding to Lipid Vesicles. It is critical in these studies that one know accurately the time course of AOFA binding to the vesicles. Previous studies (Storch & Kleinfeld, 1986) have demonstrated that the membrane-bound AOFA concentration is directly proportional to the AOFA fluorescence intensity at the low mole fractions (<0.01) of FA to lipid vesicle concentration that were used in the present study. Furthermore for the AOFA and vesicle concentrations employed in this study, the contribution of aqueous phase AO fluorescence is negligible. As shown in the Results, the time course for binding is generally biphasic, especially for the longer chain AOFA. This behavior arises because, in the aqueous phase, the AOFA exist as monomers in equilibrium with aggregates. Binding to the vesicle surface presumably involves the AOFA monomer, and therefore the aggregate form must first dissociate before these AOFA can bind to the vesicle. As discussed in the Results, this dissociation occurs relatively slowly ($\tau \approx 50$s) and might obscure faster binding and flip-flop rates. To minimize the effects of AOFA aggregates in the stopped-flow studies, conditions for the aqueous dispersion of AOFA in the reservoir syringes were chosen so as to maximize the monomer fraction (solubility). First, most of the studies with long chain AOFA were done using 2-AO-palmitate (2-AOPA), whose solubility is significantly greater than the 2-AO-stearate analogue. Second, measurements were done using the lowest AOFA concentrations compatible with adequate signal to noise values, either for changes in pyranine fluorescence or for AO fluorescence in studies with CF-containing vesicles. Third, the AOFA dispersion was prepared in weakly buffering media at pH values considerably higher (up to pH 11 as indicated in the text) than in the more strongly buffered vesicle medium. The pH after mixing the higher pH AOFA dispersion with the vesicles was virtually identical with the vesicle pH of 7.5.

RESULTS

Stopped-Flow Pyranine-Fluorescence Yields Slow Rates of Long Chain AOFA Flip-Flop. To determine the rate of transbilayer flip-flop of the long chain AOFA, we have extended the method of Kamp et al. (1995) by using stopped-flow fluorometry to monitor the time course of pyranine fluorescence as AOFA flips from the outer to inner bilayer leaflet. Accurate resolution of the flip-flop rate requires two measurements: the time course of AOFA binding to the vesicles and the time course of pyranine fluorescence. We therefore measured, following stopped-flow mixing of AOFA and the pyranine containing-vesicles, the binding of AOFA by the increase in AOFA fluorescence and the rate of AOFA movement from outer to inner leaflet, by the decrease in pyranine fluorescence. Results typical of such measurements for SUV are shown in Figure 2A where it is seen that AOFA fluorescence exhibits a biphasic increase upon mixing the potassium salt of 2-AOPA with SUV. Approximately 40% of the FA binds to the vesicles with a time constant (τ) of about 1 s while the remaining 60% binds with a 40-fold slower τ of about 40 s. The insert in Figure 2A shows the time courses for both 2-AOPA and pyranine fluorescence in the first second after stopped-flow mixing and indicates clearly the rapid phase of the 2-AOPA time course. Similar studies using LUV (Figures 2B−D) also reveal a biphasic increase in 2-AOPA fluorescence. These measurements demonstrate that the fraction of 2-AOPA that binds within about 10 s ranges from about 15% at 1 μM 2-AOPA to more than 60% at 0.1 μM 2-AOPA and the remaining fraction binds with a τ of about 70 s. As discussed below, the fast (1−10 s) time constants likely reflect the rate at which 2-AOPA monomers bind to these vesicles, while the slower rates (40−70 s) reflect the time course for dissociation of 2-AOPA aggregates, which for all measurements yielded an average of about 50 s.

In contrast to the biphasic binding of 2-AOPA, which has a fast component <10 s, the pyranine fluorescence for the SUV vesicles (Figure 2A) is quenched with a time course well described by a single τ, with an average value for all SUV measurements of about 60 s. This contrast is especially clear in the insert to Figure 2A where it is seen that within 1 s after mixing, the 2-AOPA fluorescence exhibits a substantial increase while the pyranine fluorescence is almost unchanged. Quenching of the pyranine fluorescence in the LUV (Figure 2B−D) at all three of the 2-AOPA concentrations is also well described by a single exponential, with an average τ for all LUV measurements of about 100 s. Thus, for both types of vesicles, stopped-flow mixing of AOFA and vesicles reveals a time course for transbilayer flip-flop that is considerably slower than for AOFA binding.

Origin of the Biphasic Time Course for AOFA Binding to Lipid Vesicles. Understanding the origin of the biphasic nature of the time course for AOFA fluorescence is important because we have assumed that the increase in AOFA fluorescence accurately reflects the increase in the concentration of AOFA bound to the vesicle. Given the limited

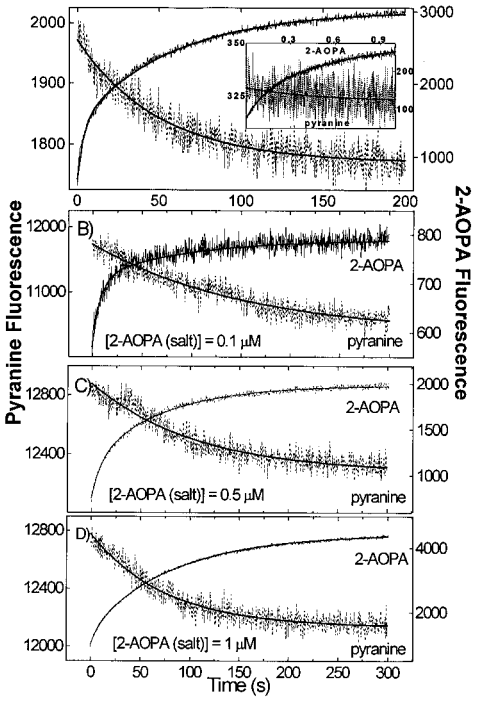

FIGURE 2: Time course of pyranine and 2-AOPA fluorescence following stopped-flow mixing. In these scans, the temperature was 24 °C and EPC concentrations were 100 μM. Solid lines through the data represent respectively a single-exponential decay [$f(t) = \alpha\ e^{-t/\tau} + c$] fitted to the pyranine fluorescence and a double-exponential fit ([$f(t) = f(0) + \alpha_1(1 - e^{-t/\tau_1}) + \alpha_2(1 - e^{-t/\tau_2})$] for the AO fluorescence. (A) A dispersion of 1 μM 2-AOPA (salt) mixed with SUV. The fits for these data yield a decay with $\tau = 50$ s for pyranine and 43% of a $\tau < 1.5$ s and 57% of a $\tau = 37$ s increase for AO fluorescence. We also found that fitting the pyranine fluorescence with a two-exponential decay does not yield a unique solution nor are the χ^2 values for these fits significantly smaller than those obtained with a single exponential. In contrast, the χ^2 value for a single-component fit to the time course for 2-AOPA fluorescence was 10-fold greater than for the two-component fit. Moreover, because for the pyranine decay the two exponential solutions had fractional intensities for the short component that were <15% and a time constant >10 s, we concluded that the pyranine decay was well described by a single exponential with $\tau \approx 50$ s. These same considerations apply to all the pyranine time courses measured in this study. Further evidence that the pyranine decay is homogeneously slow in comparison to 2-AOPA binding, which contains a large fraction of a very rapid component, is provided by the insert to Figure 2A. This shows the time courses for pyranine and 2-AOPA in the first second after stopped-flow mixing and reveals virtually no change for pyranine (<2%) but a large (>300%) increase in 2-AOPA fluorescence. (B) A dispersion of 0.1 μM 2-AOPA (salt) mixed with LUV. The fit parameters are $\tau = 152$ s for pyranine and 64% of a $\tau = 8$ s and 36% of a $\tau = 93$ s increase for AO fluorescence. (C) A dispersion of 0.5 μM 2-AOPA (salt) mixed with LUV. The fit parameters are $\tau = 104$ s for pyranine and 35% of a $\tau = 10$ s and 65% of a $\tau = 74$ s increase for AO fluorescence. (D) A dispersion of 1.0 μM 2-AOPA (salt) mixed with LUV. The fit parameters are $\tau = 74$ s for pyranine and 15% of a $\tau = 11$ s and 85% of a $\tau = 85$ s increase for AO fluorescence. Although we observed some variability in the 2-AOPA time courses because of variable loss of AOFA in the stopped-flow device, and therefore the actual and nominal 2-AOPA concentrations in the mixing chamber may differ, this had no effect on the determination of the pyranine decay rate.

solubility of the long chain AOFA, an aqueous dispersion composed of aggregates and monomers of the fatty acids might reveal rapid binding to the vesicle for monomeric AOFA while binding from the aggregates would be limited by the rate of dissociation from the aggregates, k_{off}^{g}. To test this, we measured the time course of AOFA fluorescence as

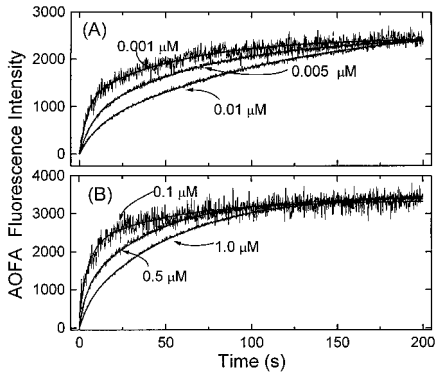

FIGURE 3: Time course of 2-AOPA acid and salt binding to LUV. In these stopped-flow measurements done at 24 °C, 200 μM LUV were mixed with 2-AOPA dispersed in the weak buffer at pH 11 so that the pH after mixing was 7.5 and the final LUV concentration was 100 μM. The acid form was dispersed by adding an ethanolic solution of 2-AOPA so that the final ethanol concentration in the syringe was <1% by volume. Solid lines through the data are fits using the-two exponential function described in the caption for Figure 2. (A) The acid form of 2-AOPA was added so that the indicated concentrations are those in the observation chamber. For all concentrations, τ for the fast component was 7 ± 2 s and its relative fraction decreased with increasing 2-AOPA concentration as 49, 31, and 18% for the 0.001, 0.005, and 0.01 μM amounts, respectively. (B) The potassium salt from of 2-AOPA at the indicated concentrations. The fast component was 5 ± 1 s, and its relative fraction decreased with increasing 2-AOPA concentration as 64, 29, and 16% for the 0.1, 0.5, and 1.0 μM amounts, respectively. The average τ value for the slow component for all conditions was about 50 s.

a function of the total AOFA concentration and ionization state. The results in Figure 3A reveal that increasing the total concentration of the acid form of 2-AOPA from 0.001 to 0.01 μM results in a monotonic decrease in the fraction of the fast component from 49 to 18%. Furthermore, the same type of measurements done with the potassium salt of 2-AOPA show that increasing the total concentration from 0.1 to 1 μM results in a monotonic decrease in the fraction of the fast component from 64 to 16% (Figure 3B). Both these sets of measurements were done by adding AOFA in a weakly buffered suspension at pH 11 to vesicles suspended in a highly buffered medium at pH 7.5 and, as mentioned in the Materials and Methods, the pH of the mixture is virtually unchanged from 7.5 after mixing. In addition, measurements were also done using weakly buffered AOFA suspensions at pH 7.4 and 9, and for both SUV and LUV, increasing the pH of the AOFA suspension increases the fraction of the fast component (data not shown). Thus, we conclude that the fraction of the fast component increases in proportion to the solubility of the AOFA and, therefore, the fast component of the increase in AOFA fluorescence is likely due to monomeric AOFA binding to the vesicle, while the slower component is related to the fraction of AOFA in aggregate form. Although the proportion of fast and slow binding components varies with AOFA concentration and method of dispersion, quenching of the pyranine fluorescence is in all cases consistent with a single exponential having a $\tau >$ 50 s, indicating that the rate-limiting step for pyranine quenching is not binding.

Flip-Flop of Short/Medium Chain Is Faster Than Long Chain AOFA. Kamp et al. (1995) have suggested that flip-flop rates are independent of FA structure and chain length. In contrast, our previous study of intervesicle transfer of AOFA suggested that flip-flop rates of the short chain AOFA

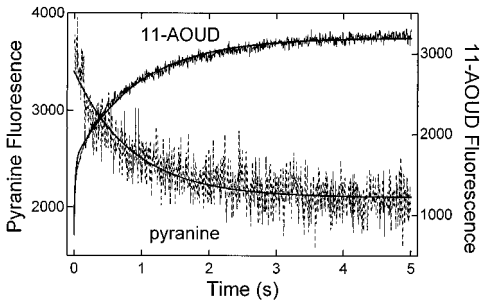

FIGURE 4: Stopped-flow time course of pyranine and AO fluorescence for short chain FA interacting with SUV. These measurements were done by mixing the acid form of 11-AOUD and EPC-SUV at 24 °C so that the concentrations in the observation chamber were 10 and 100 μM, respectively. Higher concentrations of the shorter chain AOFA were used because of its greater solubility and correspondingly lower level of membrane partition. Fits to these data yielded $\tau = 0.9$ s for pyranine and 40% of a 0.02 s component and 60% of a 1.0 s component for the AO time course. Although a two-exponential fit to the pyranine decay yielded 47% of a 0.17 s and 56% of a 1.4 s decay, the χ^2 for this fit was only 6% smaller than for the single-exponential fit. Given that even this negligible improvement in fit quality is obtained with a fast component that is 8-fold slower than for the 11-AOUD time course, we conclude that the single-exponential representation of the pyranine decay is appropriate.

were significantly greater than for the longer chain AOFA (Storch & Kleinfeld, 1986). The observed transfer times for the short chain AOFA were too fast to resolve the flip-flop step in this previous study, which was done without the aid of stopped-flow mixing. We have therefore in the present study used stopped-flow mixing of pyranine-containing SUV to assess the flip-flop rate of the 11 carbon AOFA, 11-AO-undecanoic acid (11-AOUD). As Figure 4 demonstrates, 11-AOUD binding is composed of a fast component ($\tau = 0.06$ s) that comprises about 56% of the intensity and a slower one with $\tau = 1.1$ s, while the time course of pyranine quenching by 11-AOUD is well described by a single component with a time constant of about 1 s. These results indicate that the time constant for 11-AOUD flip-flop is about 1−2 orders of magnitude faster than for the longer chain AOFA.

Ethanol May Promote the Rapid Quenching of Pyranine Fluorescence When AOFA Are Added Directly to Vesicles in an Ethanol Vehicle. The stopped-flow results presented here are consistent with our previous results from measurements of AOFA transfer between vesicles (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993) but differ by 3−4 orders of magnitude with the values reported by Kamp et al. (1995). Both studies were done with the same type of pyranine-containing vesicles, but in Kamp et al. (1995), an ethanol solution of the acid form of the AOFA was added directly to the pyranine-containing vesicles while, in the present study, either AOFA were added as the potassium salt or the ethanol solution of the AOFA (acid) was first added to an aqueous buffer before mixing with vesicles. To help resolve the discrepancy in these two methods of mixing AOFA and vesicles, we also carried out studies similar to those of Kamp et al. (1995). As seen in Figure 5, we reproduced the results of Kamp et al. (1995), revealing virtually completed time courses for pyranine (Figure 5A) and 2-AOPA fluorescence (Figure 5B) within the time required to add an ethanol solution of 2-AOPA to a cuvette containing pyranine vesicles. Clearly, adding AOFA acid in its ethanol vehicle directly to a vesicle dispersion involves

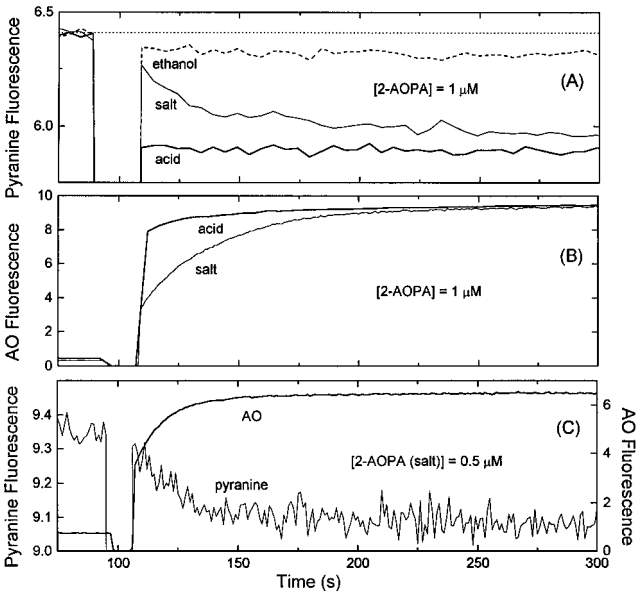

FIGURE 5: Time course of pyranine and 2-AOPA fluorescence following direct addition of AOFA to LUV. In these measurements, 1.5 mL of a 100 μM pyranine−LUV was placed in a cuvette and either pyranine or AO fluorescence was monitored at 24 °C for about 300 s. At about 100 s, a small volume (0.003 mL) of either ethanol (0.2% by volume), 2-AOPA (acid) as an ethanolic solution, or 2-AOPA salt was added to the sample volume and mixing was accomplished by drawing the sample volume in and out of a Pasteur pipette. (A) This shows that within the time required to close the fluorometer shutters, mix, and open the shutters (<20 s), equilibrium levels of quenching of pyranine fluorescence are reached following ethanol or 2-AOPA acid, but not when adding the same concentration of the 2-AOPA salt. (B) The time course of AO fluorescence for the same additions as in panel A. This shows that binding of the acid 2-AOPA in ethanol is almost complete within the mixing time when added directly, in contrast to the slow time course observed when the acid 2-AOPA in ethanol is first dispersed in buffer (Figure 2A). Furthermore, direct addition of 1 μM 2-AOPA salt displays a biphasic response, about 40% of the response occurs within the mixing time while less than 30% of the corresponding pyranine quenching (A) occurs within this time. (C) A measurement similar to that of panel A but using a lower concentration of the 2-AOPA salt to demonstrate that slow pyranine quenching occurs even when a greater percent (50%) of the 2-AOPA salt binds rapidly to the vesicles.

a distinctly different mechanism of AOFA−vesicle interaction than does mixing an aqueous dispersion of AOFA/ethanol and a vesicle dispersion. The time courses for pyranine and AOFA fluorescence are complete within the time of the measurement by the direct addition method (Figure 5) but require >200 s for equilibration, even when much lower concentrations of 2-AOPA are used, in measurements done by stopped-flow mixing (Figure 3A, for example).

This fast response (Figure 5A and B) observed when AOFA/ethanol is added directly to the vesicles might simply reflect the ability of the ethanol vehicle to maintain AOFA in soluble form so that the rate of flip-flop is not obscured by aggregate formation. However, other means of increasing the fraction of soluble 2-AOPA by, for example, using the AOFA salt at low concentrations and high pH all yield slow rates of pyranine quenching. An alternative possibility is that the fast responses observed in Figure 5 might be related to perturbation by the ethanol vehicle itself. Evidence for a perturbation of the pyranine/vesicle system is shown in Figure 5A where it is seen that direct addition of 0.2% ethanol alone results in a virtually instantaneous reduction

in pyranine fluorescence, although ethanol should not be able to quench pyranine fluorescence by the mechanism described by Kamp et al. (1995). This quenching of trapped pyranine fluorescence by ethanol, at about 15% of the level of quenching observed with AOFA, is reproducible and was observed in both SUV (data not shown) and LUV (Figure 5A). Addition of 0.2% ethanol directly to pyranine reduces the fluorescence <3% (data not shown). Further evidence for a perturbing role of the ethanol vehicle is provided by the contrasting results obtained when adding the 2-AOPA *salt* and the 2-AOPA in *ethanol*, directly to the vesicle dispersion. Figure, panels 4 A and B, shows that the same concentration of the AOFA added as the salt results in a slow monoexponential ($\tau \approx 70$ s) decrease in pyranine fluorescence (Figure 5A) but a biphasic increase in AO fluorescence (Figure 5B) in which about 30% of the increase occurs within the 15 s resolution of the addition procedure and the remaining 70% increase occurs with a τ of 70 s. The decoupling of the rate of AOFA binding and the rate of change of pyranine fluorescence is even more apparent when the concentration of the 2-AOPA salt is reduced to 0.5 μM (Figure 5C). In this case, although more than half the binding occurs within 15 s, the time course of pyranine fluorescence is unchanged ($\tau \approx 70$ s). Thus, at the levels used here and in Kamp et al. (1995), direct addition of ethanol to lipid vesicles may transiently alter bilayer structure, and in so doing facilitate rapid transbilayer flip-flop of AOFA and any EPC derived FA, present in the vesicles.

The Time Course of AOFA Interacting with Carboxyfluorescein-Containing Vesicles also Indicates Slow Flip-Flop. Because pyranine fluorescence monitors the time course of pH change rather than AOFA flip-flop per se, we used carboxyfluorescein-containing vesicles to monitor more directly flip-flop of the AOFA. Moreover, because AO fluorescence is monitored in this type of measurement, rather than the effect of AOFA on pyranine fluorescence, much lower 2-AOPA concentrations can be used and therefore the effects of AOFA aggregate formation can be reduced. Thus, as described in the Materials and Methods, we compared the time course of AOFA fluorescence in vesicles containing trapped CF with vesicles without CF (blank). Measurements were done by stopped-flow mixing of vesicles with aqueous suspensions of both the acid and potassium salts of 2-AOPA at concentrations between 0.001 and 0.03 μM. Typical results of these measurements (Figure 6) reveal that the time course of 2-AOPA fluorescence from blank vesicles increases monotonically and at the lowest FA concentrations (0.001 μM), approaches a constant fluorescence within the time of the measurement. In contrast, the time course for 2-AOPA in CF-containing vesicles, while showing the same rapid ($\tau \approx 5$s) initial increase as the blank vesicles, the slow portion of the time course *decreases* following the initial rapid increase.

This behavior, as discussed in the Materials and Methods, is expected for binding followed by slow AOFA flip-flop in CF-containing vesicles. We have, therefore, analyzed these time courses using the model described by eqs 1−5, and fits obtained from this analysis are shown as the solid curves in Figure 6. As described in the Materials and Methods, we used this model first to obtain the parameters that describe the AOFA aggregate state [aggregate fraction (f_g) and

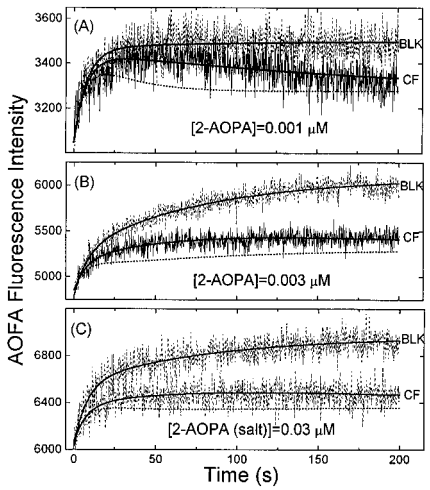

FIGURE 6: Determination of flip-flop by RET between 2-AOPA and carboxyfluorescein. In these studies, 2-AOPA as either the acid or the salt at the indicated concentrations was mixed by stopped-flow with 100 μM LUV that either did not contain (BLK) or contained approximately 20 mM of trapped carboxyfluorescein (CF). The time course of the AO fluorescence intensities (λ_{ex} = 386 nm, λ_{em} = 450 nm) was monitored at 30 °C. The solid lines through the blank (dashed) and CF (solid) vesicle data are least-squares fits of the solutions to eq 5. The dotted lines are time courses calculated using the same parameters obtained from the least-squares fits, except that the k_{ff} value was set equal to 5 times the fit value. To better illustrate the differences between the blank and CF scans, only the upper 15−20% of the intensities are shown; for all measurements the initial intensity (without 2-AOPA) was approximately zero. Representative scans from the more than four collected for blank and CF vesicles at each 2-AOPA concentration are shown. (A) The acid form of 2-AOPA added at 0.001 μM. Average values of f_g and k_{ff} from fits to all scans were 0.19 and 0.003 s^{-1}, respectively. (B) The acid form of 2-AOPA added at 0.003 μM. Average parameters from these fits were $f_g = 0.57$ and $k_{ff} = 0.004$ s^{-1}. (C) The 2-AOPA salt added at 0.03 μM. Average parameters from these fits were $f_g = 0.37$ and $k_{ff} = 0.003$ s^{-1}. Measurements were also done using the acid form of 2-AOPA at 0.005 and 0.015 μM and both gave results consistent with those shown here.

dissociation rate constant k_{off}^{g}] and binding to the vesicles (k_{on}). Results of this analysis, as seen for example in Figure 6, indicate that the time courses for the blank vesicles at all 2-AOPA concentrations used, are well described by values for $k_{off}^{g} = 1.3 \times 10^{-2}$ s^{-1} and $k_{on} = 5 \times 10^{3}$ M^{-1} s^{-1}. Using the value of f_g determined for each 2-AOPA concentration and the global kinetic parameters for the blank vesicles, the time courses for the corresponding CF vesicle measurements were used to obtain k_{ff}. A result of this analysis for all 2-AOPA concentrations yields a $k_{ff} = 0.0025 \pm 0.001$ s^{-1}. An indication of the accuracy of this result is provided by the dotted lines shown in Figure 6, which are time courses calculated using k_{ff} values that are 5-fold greater than the values obtained for each of the fits. Even this modest increase in k_{ff} is clearly inconsistent with the values obtained from the fits. Thus, the time course of 2-AOPA interacting with blank and CF-containing vesicles is consistent with the slow rate of transbilayer flip-flop revealed by the pyranine vesicle measurements.

DISCUSSION

In this study the rate of AOFA flip-flop was measured across lipid vesicles composed of egg phosphatidylcholine using two different methods. One method used pyranine trapped within vesicles to detect the pH change caused by

the release of H^+ as the neutral AOFA transfers from the outer to the inner bilayer leaflet of the vesicles, as described by Kamp et al. (1995). In the second method, carboxyfluorescein was trapped within the vesicles and RET between the anthroyloxy donor and CF acceptor was used to monitor directly the transbilayer movement of the AOFA. Both methods yielded results that are consistent with a rate of long chain AOFA flip-flop that is much slower than the rate at which these AOFA bind to the vesicles. In particular, we find that τ for pyranine quenching by 2-AOPA was longer than about 40 s and that k_{ff} obtained from RET in CF vesicles was about 0.003 s^{-1}. The results of this study also show clearly that the rate of flip-flop is very sensitive to AOFA chain length, being greater for shorter chain as compared to longer chain AOFA. These results are in excellent agreement with our previous determinations of the rate of AOFA flip-flop, which were inferred from measurements of the transfer of AOFA between lipid vesicles (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993) and therefore strongly support our assertion that flip-flop is the rate-limiting step in the transbilayer movement of the AOFA across lipid bilayers.

Comparison with Previous Measurements of Long Chain AOFA Flip-Flop across Lipid Bilayer Vesicles. Previously, we reported that the transfer of long chain AOFA between lipid vesicles was biexponential and was well described by a mechanism that involved slow flip-flop followed by a much faster off step (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). In addition to the biexponential nature of the kinetics, evidence for slow flip-flop was obtained by generating donor vesicles with a greater outer than inner leaflet concentration of AOFA, either by quenching the inner leaflet fluorescence (Storch & Kleinfeld, 1986) or by preferentially populating the outer leaflet (Kleinfeld & Storch, 1993). The alteration in the transfer kinetics from these vesicles with asymmetric AOFA distributions relative to vesicles with equal concentrations of AOFA in both leaflets was exactly that predicted for a rate of transfer of the long chain AOFA between lipid vesicles that is limited by slow transbilayer flip-flop.

Previously, we determined a k_{ff} value of 0.002 s^{-1} for 2-AO-palmitate in SUV (Storch & Kleinfeld, 1986). Although this is in excellent agreement with the value 0.003 s^{-1} found for LUV in the present study, we reported previously that k_{ff} for 12-AO-stearate was about 10-fold larger in SUV than in LUV (Kleinfeld & Storch, 1993). This lack of sensitivity to vesicle size and/or structure is probably due, in part, to our use in Kleinfeld and Storch (1993) of LUV that were prepared by detergent dialysis and yield 2000 Å diameter vesicles, while in the present study the LUV were prepared by the extrusion method. Preliminary studies comparing the transport of native FA across these two types of vesicles is consistent with considerably faster (>3-fold) rates of flip-flop in the vesicles prepared by extrusion (Kleinfeld, 1995). Additionally, 2-AO-palmitate may simply be less sensitive to vesicle size and/or structure than is 12-AO-stearate.

Kamp et al. (1995) reinvestigated these intervesicle transfer studies using three different configurations of SUV donor and acceptor systems. In the first, they used donor vesicles containing 12-AO-stearate and acceptor vesicles that were doped with NBD-PE and found biexponential kinetics, as in our previous studies (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). However, in contrast to our previous studies in which the observed fast and slow rate constants were 0.04 and 0.007 s^{-1}, respectively, Kamp et al. (1995) obtained the *smaller* values of 0.0078 and 0.0017 s^{-1}, respectively. Although in our previous studies we ensured that photobleaching was negligible, in Kamp et al. (1995) photobleaching constituted a significant portion of the decay [Figure 4C of Kamp et al. (1995)], leading to the conclusion that the very slow rate (0.0017 s^{-1}) they observed was a result of photobleaching. Consequently, they *assumed* that the faster component (0.007 s^{-1}) reflected the vesicle off rate constant. Implicit in this conclusion is that were photobleaching eliminated, the time course of intervesicle transfer would be monoexponential with a rate constant of 0.007s^{-1}. This assumption was not tested either by experimentally eliminating photobleaching or by deconvoluting the observed time course of photobleaching from the intervesicle transfer time course. We suggest that application of either of these procedures would have resulted, not in a monoexponential time course, but in a biexponential one in which the *slower* rate constant would have been equal to the 0.0078 s^{-1} value observed by Kamp et al. (1995) and equivalent to that found in our previous studies (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). The *faster* rate constant (0.04 s^{-1}) that we observed in every one of the more than 100 such kinetic traces that we have measured previously (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993) was presumably obscured by the photobleaching contribution in the study of Kamp et al. (1995).

In the second and third configurations, Kamp et al. (1995) used pyranine-containing SUV either as the donor or as the acceptor vesicles to monitor transfer of 12-AO-stearate. In both types of measurements, the time course of transfer was well described by a single exponential with a rate constant of 0.0077 s^{-1}. Although Kamp et al. (1995) ascribed this to the rate of 12-AO-stearate dissociation, this value is virtually identical to the value of the *slow* component (0.007 s^{-1}) that we observed in our previous studies of 12-AO-stearate transfer between SUV and attributed to flip-flop (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). As acknowledged by Kamp et al. (1995), because their measurements could detect only the rate-limiting step, the faster (0.04 s^{-1}) component we observed in the SUV transfer studies and attributed to the off-step, could not have been detected. Thus the results of the intervesicle transfer studies of Kamp et al. (1995), in fact, provide strong support for slow flip-flop of the long chain AOFA.

Quenching of pyranine-containing vesicles provides a more direct method for assessing AOFA flip-flop than intervesicle transfer. In their studies, Kamp et al. (1995) mixed AOFA and vesicles using a pipette to add an ethanolic solution of the AOFA directly to a cuvette containing the pyranine vesicles, rather than by stopped-flow mixing, as in the present study. Their results showed that pyranine fluorescence was quenched within the time required for these measurements. Kamp et al. (1995) concluded, therefore, that, in contrast to our previous intervesicle exchange results (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993), flip-flop was not rate limiting for transmembrane transport of the AOFA. However, as discussed in the Results, the present studies demonstrate that addition of ethanol alone to pyranine-containing vesicles results in a reduction in pyranine fluorescence (Figure 5). In contrast to FA, ethanol should not quench pyranine fluorescence by releasing H^+. This raises the possibility that direct addition of ethanol perturbs

bilayer structure and that this perturbation may promote the rapid quenching of pyranine fluorescence when an ethanolic solution of AOFA is added directly to these vesicles. Consistent with this mechanism, previous studies have shown that (1) ethanol interacts directly with phospholipids, suggesting that this interaction increases membrane disorder (Barry & Gawrisch, 1994), (2) direct addition of ethanol to membranes increases rates of phospholipid dissociation (Slater et al., 1993), and (3) ethanol increases proton permeability across phospholipid membranes (Zeng et al., 1993).

In contrast to the rapid flip-flop observed upon direct ethanol addition, stopped-flow mixing of AOFA and pyranine vesicles, which would be expected to resolve the very rapid quenching of pyranine fluorescence observed in direct ethanol mixing measurements, and was not done in the study of Kamp et al. (1995), reveals a very slow rather than a fast rate of pyranine quenching (Figure 2). Moreover, this slow rate of pyranine fluorescence quenching occurs at the same time that, AOFA binding to the vesicles, as monitored by the increase in AOFA fluorescence, is extremely rapid (Figure 2). Thus, these results, as well as those obtained using CF-containing vesicles, are consistent with flip-flop as the rate-limiting step for transbilayer AOFA transfer and with the rate constants found previously in intervesicle transfer measurements (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). We conclude, therefore, that flip-flop is rate limiting and slow ($\tau > 50$ s) for long chain AOFA transport across lipid bilayer membranes.

Short Chain AOFA and the Nature of the Barrier for Flip-Flop. Although definitive understanding of the mechanism of flip-flop is lacking, evidence from studies using small molecules and molecular dynamics simulations suggest that the rate of flip-flop should decrease sharply with increasing FA size (Stein, 1986; Bassolino-Klimas et al., 1993; Kleinfeld, 1995). Thus, it was surprising that Kamp et al. (1995) found that flip-flop rates were, within a factor of 2, independent of FA structure and chain length for the C-12−C-18 native and the C-18 AOFA. In contrast, the results shown in Figure 4 indicate more than 200-fold faster flip-flop rates for an 11 carbon as compared to a 16 carbon AOFA. This is also consistent with our previous results for the intervesicle transfer of the 11 and 12 carbon AOFA, although actual k_{ff} values were not resolved in Storch and Kleinfeld (1986). Comparison of the binding results for the 11 and 16 carbon AOFA reveals an approximately 1000-fold faster rate of binding for the shorter chain AOFA. We estimate, however, that the on rate *constants* for the short and long chain AOFA are quite similar and consistent with a diffusion limited process. The observed 10−20 ms time constant for 11-AOUD (Figure 3) is about the value expected for a diffusion-limited interaction between 100 μM SUV and 10 μM AOFA monomers. The much slower rate observed for AO-palmitate is likely a consequence of the much lower monomeric concentration of the 16 carbon AOFA. Thus, in Figure 2b, we see that about half of the added 0.5 μM 2-AOPA is in monomer form and binding of this concentration to the larger LUV occurs with a time constant of 4 s, also about the expected rate for a diffusion-limited process. Thus, while the intrinsic rates of binding to the vesicles are similar, the rates of flip-flop for the shorter AOFA are significantly faster than for longer chain AOFA.

Although one would expect much faster flip-flop rates for the neutral than the ionized form of the FA (Kamp et al., 1993), we had previously suggested that the ionized AOFA might undergo more rapid flip-flop than the neutral species (Storch & Kleinfeld, 1986). It now appears that this was erroneous. As discussed elsewhere (Kleinfeld, 1995), this conclusion was based on SUV studies that were done at high pH to ensure complete ionization of the membrane-bound AOFA. We failed to realize, however, that the low fraction of inner leaflet AOFA in these SUV obscured the relatively subtle difference between the amount transferred at the end point of these measurements (15 min) and the actual equilibrium value. Studies in progress using LUV clearly show that, compared to the neutral species, the charged form of 2-AOPA exhibits much slower ($\tau \geq 5$ h) flip-flop (K. Im, A. M. Kleinfeld, and J. Storch, unpublished observations), consistent with the results for native FA (Kamp et al., 1993). Results in the present study are for the neutral species of FA because at pH 7.5 about half of the membrane-bound AOFA is protonated (Storch & Kleinfeld, 1986; Kamp et al., 1993) and the much slower ($\tau \geq 5$ h) movement of the charged AOFA would not be detected in the time frame of these measurements (<500 s).

Native FA. The flip-flop rate constants reported by Kamp et al. (1995) for the 1000 Å LUV ($\sim$15 s^{-1}) are more than 5000-fold greater than those we observe for the long chain AOFA in these same vesicles. We have also carried out measurements of the transport of long chain native FA across lipid membranes using the fluorescent probe of free fatty acids ADIFAB, to monitor the movement of native FA from the outer to the inner aqueous phase (Kleinfeld, 1995; Kleinfeld et al., 1997). These studies also indicate faster flip-flop rates for the native as compared to the AOFA. However, our results for the 1000 Å LUV yield k_{ff} values that are 10−30-fold smaller than Kamp et al. (1995) and indicate that flip-flop is the rate-limiting step in transbilayer transport. Most importantly, these results also indicate that the rate of flip-flop is a sensitive function of vesicle diameter, yielding flip-flop times >10 s ($k_{ff} < 0.1$ s^{-1}) for 2000 Å diameter vesicles (Kleinfeld, 1995; Kleinfeld et al., 1997). Furthermore, in preliminary studies of human red cells, using the ADIFAB methodology, we find that transport of long chain native FA occurs more rapidly (<1 s) than in lipid vesicles (Kleinfeld & Chu, 1993), raising the possibility that protein-mediated transport in biological membranes may be necessary because spontaneous transport through the lipid phase is insufficient. Thus, although the magnitude of AO and native FA flip-flop rates are substantially different, flip-flop across the lipid phase of membranes appears to be rate limiting for both types of FA.

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