Dissociation of Long and Very Long Chain Fatty Acids from Phospholipid Bilayers†

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ABSTRACT: Dissociation of fatty acids (FA) from and transbilayer movement (flip-flop) in small unilamellar phosphatidylcholine vesicles (SUV) were monitored by measuring the pH inside the vesicle with an entrapped water-soluble fluorophore, pyranin. With a pH gradient imposed upon SUV preloaded with FA, the rate of flip-flop of saturated very long chain FA (C20:0, C:22:0, and C24:0) was shown to be fast ($t_{1/2}$ < 1 s); previously, we showed by stopped flow measurements that flip-flop of long chain (14−18 carbons) FA is very fast [$t_{1/2}$ < 10 ms; Kamp, F., et al. (1995) *Biochemistry 34*, 11928−11937]. The rates of dissociation of FA from SUV were evaluated by incorporating FA into donor vesicles and measuring transfer to acceptor vesicles. The transfer was followed by changes in internal pH of either donor or acceptor vesicles with stopped flow (C14:0, C16:0, C17:0, C18:0, C18:1, and C18:2) or on-line (C20:0, C22:0, and C24:0) fluorescence. All FA showed a single-exponential transfer process that was slower than the lower limits established for the rate of flip-flop, with $t_{1/2}$ of dissociation ranging from 20 ms for C14:0 to 1900 s for C24:0. The pseudo-unimolecular rate constants (k_{off}) for dissociation of C14:0 to C26:0 showed a 10-fold decrease for each addition of two CH_2 groups to the acyl chain and a $\Delta(\Delta G)$ of −740 cal/CH_2. The dissociation rate constants for oleic acid (18:1) and linoleic acid (18:2) were 5 and 10 times faster, respectively, than that of C18:0. The rates of dissociation for typical dietary FA are sufficiently rapid that complex mechanisms (e.g. protein-mediated) may not be required for their desorption from biological membranes. The very slow dissociation rates for C24:0 and C26:0 may accentuate their pathological effects in diseases in which they accumulate in tissues.

Transport of FA[1] into the cytosol of cells minimally involves adsorption to the plasma membrane, passage through the lipid bilayer (transmembrane movement), and desorption (or dissociation) from the cytosolic face of the membrane. Each step likely has very different kinetics, and it has been suggested by various investigators that proteins facilitate one or more of these steps [for reviews, see Veerkamp & Maatman (1995) and Kleinfeld (1995)]. In order to understand the potentially complex mechanisms that occur in cells, the kinetics and thermodynamics of the above essential steps must be understood in simple model systems. However, it has proved difficult to monitor and quantitate the individual processes, even in protein-free phospholipid bilayers. In studies of transfer of FA from bilayers to acceptors (vesicles or albumin), the combined processes of desorption and transbilayer movement have been measured by fluorescence methods (Doody et al., 1980; Daniels et al., 1985; Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). Because of the structural simplicity of FA, these assays have relied on fluorescently labeled FA, except for Daniels et al. (1985), who used the intrinsic fluorescence of albumin to monitor FA transfer. For physiological FA, the transbilayer movement step was evaluated to be faster than dissociation, on the basis of the observation of only a single kinetic process upon movement of >90% of the FA from donors to acceptors (Daniels et al., 1985). In contrast, kinetic measurements of fluorescently labeled (anthroyloxy) FA led to the suggestion that transbilayer movement is slower than dissociation (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993).

The movement of FA across phospholipid bilayers has recently been monitored by a protocol that more directly measures this specific step. As opposed to using fluorescently labeled FA, the strategy for this fluorescence measurement was to use a trapped fluorophore (pyranin) to measure pH in the aqueous inner volume of the vesicle after addition of naturally occurring FA (Kamp et al., 1995; Kamp & Hamilton, 1992, 1993). The change in fluorescence depends on the dissociation of H^+ from FA in the inner leaflet of the vesicle, which is much faster than desorption of the FA molecule from the interface. The adsorption of FA to bilayers is rapid under appropriate conditions, and transbilayer movement (flip-flop) can be monitored independently from the potentially slower step of desorption. Results from this new approach showed that flip-flop of FA is an extremely fast first-order process (Kamp et al., 1995). The $t_{1/2}$ values of all FA studied, which represented the common dietary FA, were <10 ms in SUV and <35 ms in large unilamellar vesicles (LUV).

The same fluorescence approach can be adapted to measure transfer of FA from phospholipid vesicles (Zhang & Hamilton, 1995; Kamp et al., 1995). With knowledge of the rate of flip-flop, the kinetics of FA dissociation can be described quantitatively. In this study, we have monitored

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[1] Abbreviations: BSA, bovine serum albumin; DMPC, dimyristoylphosphatidylcholine; FA, fatty acids; LUV, large unilamellar vesicles; PC, phosphatidylcholine; SUV, small unilamellar vesicles; VLCFA, very long chain fatty acids.

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the transfer of FA with chain lengths of 14−24 carbons from phospholipid donor vesicles to acceptor vesicles with trapped pyranin. The very long chain (≥20 carbons) saturated FA (VLCFA) are less common than the shorter chain acids but are biological markers for inherited disorders of FA metabolism such as adrenoleukodystrophy (Moser & Moser, 1989; Ho et al., 1995). Because of the extremely low solubility of VLCFA in water, a modified procedure of the pyranin assay was used to measure the rate of flip-flop. For all FA tested, we show that the rate of dissociation is strongly dependent on the chain length of the FA and is much slower than the rate of flip-flop.

MATERIALS AND METHODS

Chemicals. Myristic acid (C14:0), palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) were from Nu Chek Prep, Inc. (Elysian, MN) (>99% pure). Bovine serum albumin (BSA, fraction V) was from Sigma (St. Louis, MO). Eicosanoic acid (C20:0), docosanoic acid (C22:0), tetracosanoic acid (C24:0), hexacosanoic acid (C26:0), and hydroxylpiperazineethanesulfonic acid (HEPES) were from Aldrich Chemical Co. (Milwaukee, WI). Egg phosphatidylcholine (PC) was from Avanti Polar Lipid, Inc. (Alabaster, AL). Sephadex G-25 was from Pharmacia Biotech (Picataway, NJ). Hydroxypyrenetrisulfonic acid trisodium salt (pyranin) was from Eastman Kodak Co. (Rochester, NY).

Preparation of Small Unilamellar Vesicles (SUV). Pyranin, a water-soluble, pH-sensitive fluorescent molecule, was trapped inside well-sealed small unilamellar egg PC vesicles (SUV) with 25 or 100 mM HEPES buffer (pH 7.4), as described in Kamp & Hamilton (1992). VLCFA were incorporated into egg PC vesicles by cosonication as follows. VLCFA and egg PC were comixed in the desired amount in chloroform/methanol, and the solvent was removed under vacuum. The thin film was warmed by immersing the flask in hot water (∼50 °C) and warmed buffer added to the flask for hydration of the sample. Sonication was performed as before (Kamp & Hamilton 1992, 1993; Kamp et al., 1995).

Preparation of Large Unilamellar Vesicles (LUV). LUV composed of egg PC were prepared by the extrusion method as described previously; such preparations have been characterized by electron microscopy and NMR spectroscopy to show that the majority of vesicles are unilamellar with a *d* of ∼1000 Å (Kamp et al., 1995). The desired FA was added in ethanol solution after preparation of LUV. The final PC concentrations in SUV and LUV samples used for data collection were determined by the Bartlett method (Bartlett, 1959).

On-Line Fluorescence Measurements. Fluorescence measurements for VLCFA were carried out on a Hitachi F2000 fluorometer with a stirred cuvette at 23−25 °C unless otherwise stated. External pH (pH_{out}) was measured with a pH minielectrode and internal pH (pH_{in}) by the pyranin fluorescence (excitation wavelength, 455 nm; emission wavelength, 509 nm). The relation between pH_{in} and pyranin fluorescence was calibrated as described (Kamp & Hamilton, 1992). Transfer of VLCFA to acceptor vesicles was monitored by the fluorescence of pyranin contained either in the vesicles with VLCFA or in acceptor vesicles without VLCFA. An aliquot of the vesicles containing pyranin was first added to 2.0 mL of HEPES/KOH buffer (pH 7.40) to establish fluorescence, and acceptor or donor vesicles without pyranin were subsequently added. The donor/acceptor PC molar ratio was varied from 0.57 to 4.57. In general, the concentration of the donor vesicles was 2−3 times higher than that of the acceptor vesicles. In some experiments, BSA was used as a FA acceptor. The response time of the pH_{in} measurement (1−2 s) was limited by the mixing in the cuvette.

Stopped Flow Fluorescence Measurements. Stopped flow fluorescence measurements were performed for FA with ≤18 carbons with a KinTek Inc. spectrophotometer operating at a constant temperature of 25 °C. The dead time of this instrument was ∼10 ms. One syringe contained egg PC vesicles with FA, and the other syringe contained egg PC vesicles with pyranin and no added FA. Alternatively, one syringe contained egg PC vesicles with pyranin and the FA, and the other syringe contained egg PC vesicles or BSA. The probe was excited at 445 nm and the fluorescence intensity monitored at 510 nm using a 1 in. filter band-pass via a National Instruments analog/digital interface. For each sample, at least 10 successive runs were made in order to ensure reproducibility. The individual runs were averaged to improve signal to noise ratios and for calculation of rate constants.

Data Analysis. Fluorescence data were saved as ASCII files, and the program ORIGIN was used to analyze the data. The data were fitted to a single-exponential decay, since the fitting to double-exponential decay did not change the χ^2 significantly (χ^2 for single-exponential/ fits χ^2 for double-exponential fits = 1.00 ± 0.08).

RESULTS

Flip-Flop of VLCFA. Prior to measurement of the dissociation of VLCFA from phospholipid bilayers, it was essential to determine the rate of transmembrane movement (flip-flop) of these FA. We first attempted to monitor flip-flop of VLCFA by addition of the K^+ soap or ethanolic solutions of the acid, as done before with long chain FA (Kamp & Hamilton, 1992, 1993; Kamp et al., 1995). Only the 20-carbon acid was soluble (by visual inspection) at room temperature at the desired concentration (∼10 mM). Addition of this acid to vesicles with pyranin yielded a slow ($t_{1/2}$ ∼ 1 min) decrease in pyranin fluorescence. Addition of longer chain VLCFA, which were only partially soluble, yielded slower and inconsistent decreases in fluorescence. It was inferred that the rate being measured was that of dissolution of the acid from aggregates in solution and not that of flip-flop.

An alternative way of measuring transbilayer movement is incorporate the FA into the phospholipid bilayer by cosonication of the FA and PC and then change the pH of the outer buffer. Since H^+ leak is slow (Kamp & Hamilton, 1992, 1993), changes in the internal pH reflect the rate of redistribution of the FA between the bilayer leaflets. Increasing pH_{out} changes the ionization equilibrium of FA in the outer leaflet in favor of the ionized species and results in a net movement of un-ionized FA from the inner to the outer leaflet (Hope & Cullis, 1987); the subsequent altered ionization equilibrium of FA in the inner leaflet leads to an increase in pH_{in}. This protocol was used recently to measure the flip-flop of (anthroyloxy)stearic acid without complications arising from the very low solubility of this FA (Kamp et al., 1995).

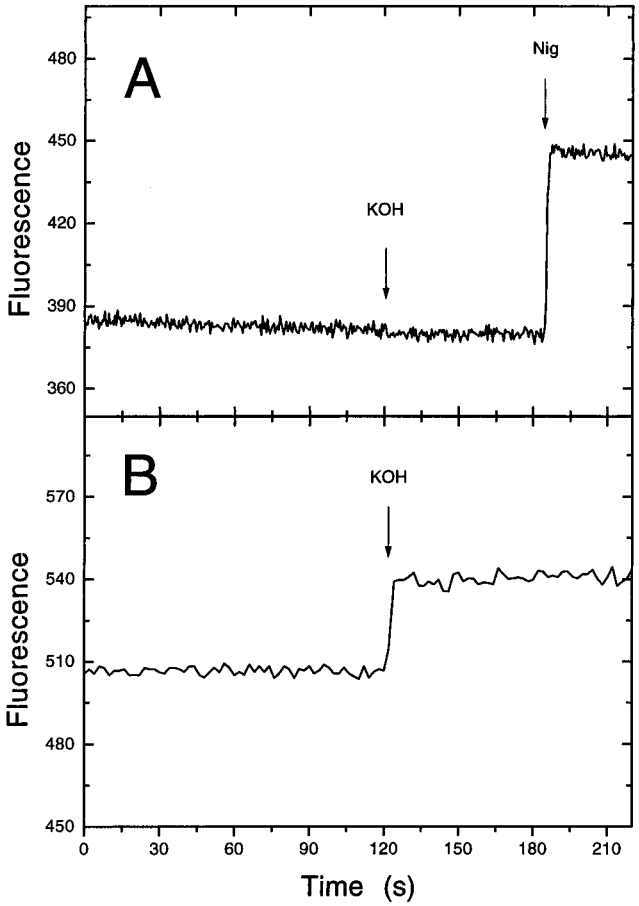

FIGURE 1: Flip-flop of VLCFA in SUV with pyranin. (A) Control experiments for flip-flop. PC vesicles (0.39 mM PC) with entrapped pyranin suspended in 2.0 mL of buffer [100 mM HEPES (pH 7.4)]. Addition of KOH increased the external pH to 7.5 but had no effect on the pyranin fluorescence (pH_{in}). Addition of nigericin permeabilized the vesicles to protons instantaneously to make pH_{in} equal pH_{out}. (B) Flip-flop of VLCFA in SUV (0.97 mM) with 4 mol % cosonicated C20:0. Imposing a pH gradient of 0.48 unit by adding KOH caused the pyranin fluorescence (pH_{in}) to increase instantaneously because of rapid net movement of some of the VLCFA to the external leaflet of the SUV. Fluorescence units are arbitrary.

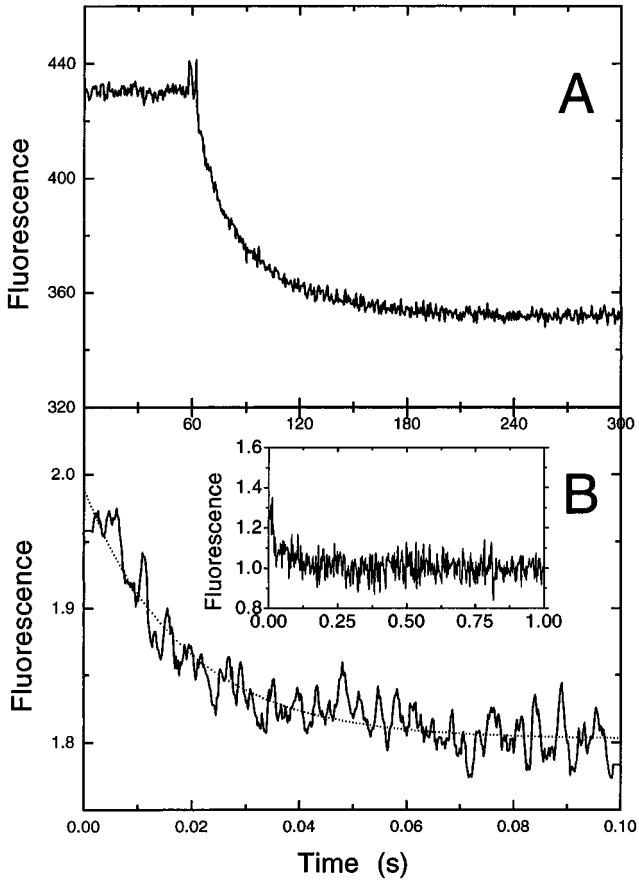

FIGURE 2: Transfer of long chain FA from donor vesicles with cosonicated FA to acceptor vesicles trapped pyranin. (A) On-line fluorescence measurement of transfer of C20:0. Acceptor SUV (0.34 mM) with trapped pyranin in 2 mL of buffer [100 mM HEPES (pH 7.4)]. At $t = 60$ s, 40 μL of donor SUV with 4 mol % C20:0 was added. The final concentration of donor SUV vesicles was 0.71 mM phospholipid. The time resolution of the on-line fluorescence measurement was 2 s. (B) Stopped flow fluorescence measurement of the transfer of C14:0. Equal volumes (100 μL) of donor SUV (1.14 mM) with 4 mol % C14:0 and acceptor SUV (0.75 mM) with trapped pyranin. The inset shows the same experiment as panel B shown for a longer time interval and demonstrates that equilibrium was reached with 200 ms.

Figure 1 illustrates the measurement of flip-flop of arichidic acid (C20:0) in PC vesicles by the above protocol. In the control experiment (panel A), PC vesicles contained no added FA, and no effect on the fluorescence of entrapped pyranin was seen following addition of 50 μL of 1.0 N KOH, as expected. Furthermore, addition of nigericin, which makes the membrane permeable to protons, caused a large instantaneous increase in fluorescence, demonstrating that a pH gradient had been established (panel A). This showed that proton leak and the potential effect of small amounts of FA present in SUV (Kamp & Hamilton, 1992) were both negligible. It also demonstrated the absence of any external pyranin. On the other hand, PC vesicles with 4 mol % C20:0 showed an immediate increase in fluorescence (corresponding to an increase in pH of 0.48) upon addition of 50 μL of 1.0 N KOH (panel B). This result indicates rapid equilibration of some of the VLCFA to the external leaflet in response to the imposed pH gradient. The experiment of Figure 1B was repeated for C22:0, C24:0, and C26:0 at the same level of FA in SUV as for C20:0 (4 mol %). An instantaneous increase in fluorescence ($t_{1/2} < 1$ s) was also found for these FA. These experiments demonstrate rapid flip-flop of VLCFA in SUV comprised of egg PC. In our previous studies with long chain FA, we showed that there is no dependence (less than a factor of 2) of the flip-flop rate either on the concentration (0.5−26 mol %) or on the chain length of long chain FA in vesicles (Kamp & Hamilton, 1993; Kamp et al., 1995).

Transfer of FA from Vesicles. The general protocol for measuring desorption of FA from PC vesicles was to monitor transfer of the desired FA from donor vesicles to acceptor vesicles or, in some cases, to albumin (BSA). Pyranin was trapped either in the donor vesicles, in which case the fluorescence *increases* as FA leave the inner leaflet of the vesicles, or in the acceptor vesicles, in which case the fluorescence *decreases* as FA flip-flop to the inner leaflet of acceptor vesicles. Changes in fluorescence reflecting these processes were observed until the system reached equilibrium.

Transfer of VLCFA (C20:0 to C24:0) from SUV. Having determined the upper time limit of the rate of transbilayer movement of VLCFA ($t_{1/2} < 1$ s), we carried out experiments to monitor the kinetics of transfer of VLCFA from SUV. Figure 2A illustrates desorption of the 20-carbon FA from PC vesicles, as measured by the pyranin fluorescence in

Table 1: Observed Time Constants (t_{obs}), Half Times ($t_{1/2}$), and Evaluated Rate Constants (k_{off}) for FA Desorption from SUV at 24 ± 1 °C

FA	t_{obs} (s)	$t_{1/2}$ (s)	k_{off} (s^{-1})
myristic (C14:0)	0.0270 ± 0.0056	0.0187	39.0
palmitic (C16:0)	0.123 ± 0.014	0.0858	8.22
margaric (C17:0)	0.485 ± 0.049	0.336	2.08
stearic (C18:0)	1.88 ± 0.30	1.30	0.548
oleic (C18:1)	0.568 ± 0.099	0.393	1.81
linoleic (C18:2)	0.0804 ± 0.0029	0.0557	12.4
eicosanoic (C20:0)	22.3 ± 1.96	15.5	0.0450
docosanoic (C22:0)	281 ± 4.03	194	0.00355
tetracosanoic (C24:0)	2720 ± 755	1890	0.000404

[a] On line fluorescence was used for C20:0 to C24:0. Stopped flow was used for all other FA. The experimental data were fitted to a single-exponential decay [$F(t) = F(\infty) + F(0)\exp(-t/t_{obs})$] to obtain the time constant t_{obs}. Values for t_{obs} are mean ±SD for at least three separate determinations and did not depend on the molar ratio of donor and acceptor SUV over the range investigated (0.57−4.57). The k_{off} was calculated as $1/t_{obs}$, and $t_{1/2}$ was calculated as t_{obs} ln 2.

acceptor PC vesicles. There was a single-exponential decrease in fluorescence over a time course of minutes ($t_{1/2}$ = 15 s). Since the transbilayer movement (flip-flop) of C20:0 was shown to occur with $t_{1/2} < 1$ s, this single-exponential process reflects desorption from the outer leaflet of donor vesicles. When transfer was monitored by adding donor vesicles with C20:0 and pyranin to PC vesicles containing no added FA or pyranin, an exponential increase in fluorescence with the same kinetics was observed (data not shown). A third protocol (also not shown), in which C20:0 was incorporated into vesicles containing pyranin and BSA was used as an acceptor, also showed a single-exponential increase in fluorescence. With the highest concentration of BSA used (20 μM), all VLCFA were extracted from the vesicles (2.1 μM/μM, BSA) and the same $t_{1/2}$ as that found with the vesicle to vesicle transfer was observed; however, lower concentrations of BSA gave lower $t_{1/2}$ values, reflecting the complicated kinetics of exchange of FA between vesicles and albumin (Daniels et al., 1985). The standard protocol chosen for subsequent studies with other FA was vesicle to vesicle transfer, in which case the kinetics are the same in both the forward and backward reactions.

Experiments monitoring the transfer of C22:0 and C24:0 from vesicles as in Figure 2A also showed single-exponential changes in pyranin fluorescence. However, the rates were considerably slower compared to that of C20:0. With elongation of the FA chain by two CH_2 groups, the time scale for each FA was about 1 order of magnitude slower. Thus, fluorescence changes for C24:0 were monitored over a time period of 6000 s ($t_{1/2} \sim$ 1890 s). For this FA, the fluorescence began to show a slow drift toward equilibrium (reflecting slow H^+ permeation) after reaching a maximal change. It was not feasible to monitor the transfer of C26:0 because the transfer was slower than proton leak. As described previously, in these experiments, the pseudo-unimolecular rate constant for dissociation (k_{off}) equals the observed rate constant ($1/t_{obs}$) of the single-exponential change in fluorescence (Kamp et al., 1995). The observed time constants (t_{obs}) and half-times for a first-order process ($t_{1/2}$) as well as the evaluated k_{off} values for VLCFA are listed in Table 1.

Transfer of Long Chain FA (C14:0 to C18:0, C18:1, and C18:2) from SUV. In the above fluorescence experiments the response time of the pH in measurement was limited by the mixing time in the cuvette (∼1 s). Stopped flow experiments were performed to measure desorption of FA with <20 carbons because of the expected faster time frames for transfer of these FA (Daniels et al., 1985). Figure 2B shows the time-dependent fluorescence change in acceptor vesicles with entrapped pyranin upon addition of donor vesicles containing myristic acid (C14:0). The decrease in fluorescence follows a single-exponential decay with a rate constant $1/t_{obs}$ of 39 s^{-1}. The inset of Figure 2B shows the same experiment as in panel B followed over a longer (10 times) time course to demonstrate that the single-exponential decrease reaches an equilibrium value and that no slow component of the decay in fluorescence is observed. Since the flip-flop of C14:0 in egg PC SUV has been shown to be very fast ($t_{1/2} <$ 10 ms; Kamp et al., 1995), the single-exponential reflects only the kinetics of dissociation. As in the case of VLCFA assay, the pseudo-unimolecular rate constant of dissociation (k_{off}) is equivalent to the rate constant of the observed single-exponential decrease in fluorescence (Kamp et al., 1995).

Using the same protocol as described for C14:0, the transfer of C16:0, C17:0, and C18:0 was monitored. The rate of transfer decreased with increasing chain length in the saturated series of FA. The kinetics of transfer of oleic acid (C18:1) and linoleic acid (C18:2) were also measured to compare their rates with that of stearic acid (C18:0). With addition of each double bond, the transfer rate increased significantly; C18:1 was ∼5 times and C18:2 ∼10 times faster than C18:0. Table 1 also summarizes t_{obs}, $t_{1/2}$, and k_{off} values for the six FA measured by stopped flow fluorescence.

Transfer of Long Chain FA (C16:0, C18:0, and C18:1) from LUV. To assess the influence of PC vesicle curvature on the rates of FA desorption, experiments with C16:0, C18:0, and C18:1 were performed with LUV. Donor LUV were pre-equilibrated with FA (by addition in ethanol solution), and acceptor LUV contained pyranin. First-order kinetics were also observed, and the calculated k_{off} values were similar (within 33%) to those for the corresponding experiments with SUV. We conclude that bilayer curvature of PC vesicles does not make a large difference in the desorption rates of long chain FA. This result is different from an earlier report (Kleinfeld & Storch, 1993) that showed about 1 order of magnitude difference between the rate of desorption of 12-(9-anthroyloxy)stearic acid from small versus from large vesicles. A possible reason for this discrepancy is that the physiological FA used in the present study interact differently with the phospholipid bilayer than do the fluorescently labeled FA .

Dependence of Dissociation on Temperature. The effect of temperature (10−45 °C) on the dissociation rate of C20:0 from SUV was studied to obtain thermodynamic constants for dissociation. Experimental conditions were similar to those described above for the dissociation studies of C20:0 at room temperature. Notably, donor vesicles contained the FA, and acceptor vesicles contained trapped pyranin. A single-exponential decrease in fluorescence of the acceptor vesicles was observed for each temperature, and k_{off} was evaluated from the measured rate constants. Figure 3 shows an Arrhenius plot of $1/k_{off}$ versus $1/T$. A linear relationship was observed (r = 0.998), and the thermodynamic parameters of the dissociation (ΔE_a = 8.98 kcal/mol, $\Delta H^{\ddagger}$ = 8.39

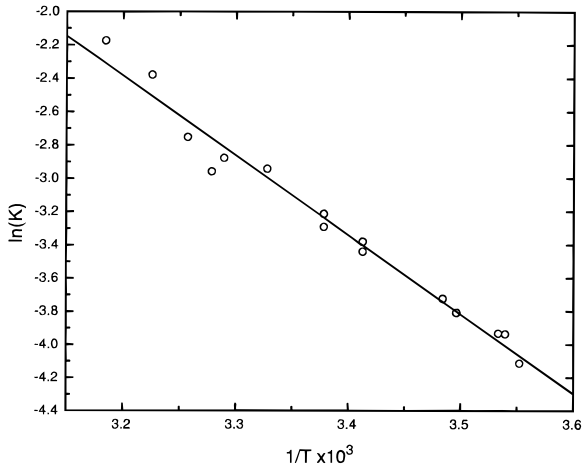

FIGURE 3: Temperature dependence (10 − 45 °C) of the transfer of C20:0 from donor vesicles (SUV) with 4 mol % cosonicated C20:0 to acceptor vesicles with trapped pyranin. After mixing, the PC concentration of donor vesicles was 0.71 mM and that of acceptor vesicles was 0.34 mM. The Arrhenius plot was obtained by repeating the experiment of Figure 2A at various temperatures.

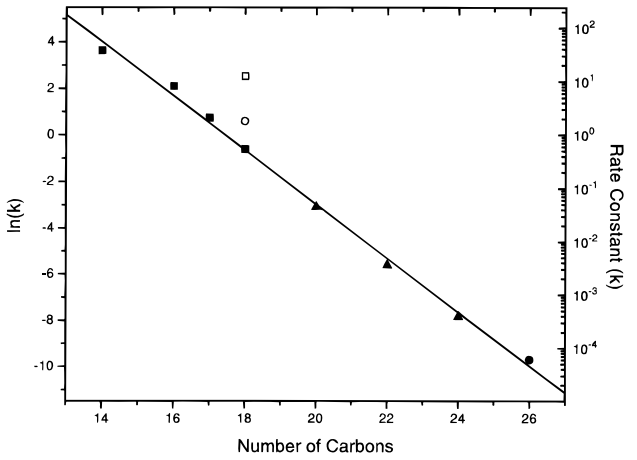

FIGURE 4: Plot of ln(k_{off}) at 24 ± 1 °C versus FA chain length. Solid symbols are for saturated FA with chain lengths of 14−26 carbons. The k_{off} values for C14:0, C16:0, C17:0, and C18:0 (solid squares), C18:1 (open circle), and C18:2 (open square) were obtained by transfer studies using stopped flow fluorescence; the k_{off} values for C20:0, C22:0, and C24:0 (solid triangles) were obtained by on-line fluorometry. The k_{off} for C26:0 (solid circle) was previously measured by ^{13}C NMR methods (Ho et al., 1995).

kcal/mol, $\Delta S^{\ddagger} = -36.6$ cal mol^{-1} K^{-1}, $\Delta G^{\ddagger} = 19.3$ kcal/mol, and $K_{eq} = 7.10 \times 10^{-15}$) were calculated using conventional equations (Doody et al., 1980; Daniels et al., 1985).

DISCUSSION

Long chain FA and VLCFA exhibit rapid equilibration (flip-flop) between the leaflets of a phospholipid bilayer in small unilamellar vesicles, as shown in this and previous studies (Kamp & Hamilton, 1992, 1993; Kamp et al., 1995). The analysis of the dissociation rates was simplified by these fast flip-flop rates. Because of the wide range of the rate constants for FA with different chain lengths, the dissociation rates of the saturated series from C14:0 to C26:0 were measured by three different methods: stopped flow fluorescence, on-line fluorescence, and NMR spectroscopy. The $t_{1/2}$ values for the long chain FA C14:0, C16:0, and C18:0 were in a range of milliseconds to seconds (Table 1) and were in excellent quantitative agreement with the rates reported by Daniels et al. (1985) for desorption of the same FA from dimyristoylphosphatidylcholine (DMPC) vesicles. In the present study, we were able to attribute the rate process exclusively to desorption because of our independent measurements of flip-flop. VLCFA desorbed much more slowly from SUV than did normal dietary FA. The $t_{1/2}$ values for C20:0, C22:0, and C24:0 were in a range of seconds to hours, and the rates were conveniently measured by on-line fluorescence. The desorption of C26:0 from SUV was too slow to measure with fluorescence and was determined instead by ^{13}C NMR (Ho et al., 1995).

The measured rate constants (Table 1, Figure 4) represent the pseudo-unimolecular rate constant for dissociation of FA from the donor vesicle. Figure 4 graphically summarizes the rate constants compiled from the three different types of measurements. The plot of k_{off} versus chain length of saturated FA shows a precise exponential (log linear) trend, with an about 10-fold slower rate of dissociation for the addition of each two CH_2 groups. Addition of one double bond was equivalent to shortening the alkyl chain by one CH_2 group. The striking trend in kinetics with FA chain length supports the hypothesis that the transfer of FA between vesicles occurs by a first-order dissociation process of FA monomers rather than by a collision-mediated mechanism. Our results are consistent with, and lend further support to, the models of Daniels et al. (1985) for FA transfer between SUV and BSA and of Doody et al. (1980) for transfer of pyrene-labeled FA between vesicles.

Partitioning of saturated FA (7−22 carbons) between heptane and water was studied by Smith and Tanford (1973), who showed a linear relationship between the logarithm of the true distribution constant and the FA chain length. Moreover, a plot of 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 per CH_2 group (Tanford, 1980; Small, 1986). Assuming that $k_{off}(n) = A \exp(-n\Delta E/RT)$, where A is a constant, n is the number of carbons of the acyl chain, and ΔE is the activation energy per CH_2, the data of Figure 4 give a ΔE of −740 kcal per CH_2. When we assume that the k_{on} does not depend on the chain length, this result suggests that the kinetics of FA dissociation from SUV reflect the partitioning ($K = k_{on}/k_{off}$) of FA between the lipid bilayer and the aqueous phase. In studies of phospholipid transfer between membranes, it was shown that the rate constant of monomer dissociation of PC molecules with 12−15 carbons in each acyl chain was a logarithmic function of the chain length (Ferrell et al., 1984). As expected, the rates were much slower, ~100-fold for the same chain length of unesterified FA, and flip-flop was slower than desorption.

The dissociation rate of C20:0 also showed a linear trend with temperature when plotted as ln(k) versus 1/T (Figure 3). The calculated thermodynamic constants demonstrated that the entropy change from the ground state to the activated state is a major contributor to the energy of activation. This further supports our conclusion that the dependence of k_{off} on chain length reflects a hydrophobic effect, i.e. partitioning of FA between membranes and water. Our calculated constants for C20:0 are very similar to the values for C14:0

(Daniels et al., 1985) in DMPC SUV and for the fluorescently labeled FA, pyrene nonanic acid (Doody et al., 1980), in liquid−crystalline DMPC SUV. For the proposed mechanism of desorption, the nature of the phospholipid membrane is expected to play less of a role in FA desorption than the chain length and unsaturation of the FA. It is noteworthy that the change in k_{off} for C20:0 over the 35 °C temperature range investigated is smaller than the change in k_{off} at 24 °C for the addition of 2 CH_2 groups. On going from 24 to 37 °C, k_{off} for C20:0 increased by 1.8 (Figure 3). If the other FA studied follows the same trend, k_{off} will be about 2-fold higher at physiological temperature than the values in Table 1 and Figure 4.

C24:0 and C26:0 and other VLCFA with longer saturated chains accumulate in disorders of FA oxidation, such as adrenoleukodystrophy (Moser & Moser, 1989). Our results suggest that, independent of the type of acceptor molecules, it could take days to months for VLCFA to dissociate spontaneously from a membrane interface. The slow desorption rates of VLCFA from membranes may amplify their potentially damaging effects on membrane structure and function (Ho et al., 1996). For VLCFA, collision-mediated mechanisms could potentially enhance their rates of desorption, though no such mechanism has been established. Interestingly, it has been suggested that intracellular FA binding proteins (FABP) may facilitate desorption of normal dietary FA (Glatz et al., 1995). On the basis of our studies of FA desorption from SUV, such a role would be more useful for VLCFA than for long chain FA.

The general issue of transport of FA into cells has been intensively studied and debated. Most of the attention and controversy has been centered on the transmembrane step of transport (Cooper & Zakim, 1989; Potter et al., 1989; Storch, 1990; Kamp & Hamilton, 1992). The present study in combination with our previous work specifically on transbilayer movement of FA (Kamp & Hamilton, 1992, 1993; Kamp et al., 1995) suggests that the rate-limiting step for the entry of FA into the cytosol is more likely to be the desorption of FA from the cytosolic leaflet of the plasma membrane than transmembrane movement. Aside from a small fraction of FA that might be esterified within the inner leaflet of the plasma membrane to coenzyme A by acyl-CoA synthetase, FA must desorb from the membrane in some manner to be utilized at various sites in the cell. The rates of desorption of FA with <20 carbons are fast ($t_{1/2} < 1$ s) and may not be rate-limiting for FA metabolism. However, if spontaneous desorption is an important or primary mechanism in cells, the vastly different rates for FA with different acyl chains may influence significantly their rates of equilibration among intracellular sites. Frequently, FA must also exit cells, and FA of different structures are predicted to leave the outer leaflet of the plasma membrane at different rates, not because of differences in transmembrane movement but because of differences in desorption. The desorption rates may also influence the rates of transfer of FA among cells and possibly intercellular communication and signaling pathways modulated by FA.

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