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Biochimica et Biophysica Acta 1416 (1999) 135–144

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Determination by photoreduction of flip-flop kinetics of spin-labeled stearic acids across phospholipid bilayers

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Received 26 May 1998; received in revised form 2 October 1998; accepted 2 November 1998

Abstract

Spin-labeled stearic acid derivatives (*N*-DS) can be used to determine the rate at which lipid-derived drugs can cross a phospholipid bilayer (flip-flop). The flip-flop rate of *N*-DS (where $N = 5, 6, 7, 9, 10, 12, 16$), was measured using vectorial photoreduction of nitroxides to their corresponding hydroxylamine by FMN, a charged, membrane-impermeable flavin, by hydrogen atom transfer from EDTA. From the time difference in the photoreduction rates of *N*-DS located in the outer and inner half of the bilayer, the flip-flop rate of *N*-DS across the bilayer can be determined. The results show that at pH 8.0 or lower, the photoreduction of 5-DS on one side of the membrane by FMN is slower than the flip-flop rate of 5-DS across phospholipid bilayers. For 5-DS at pH 7.0, this rate is at least 33.8 ± 4.24 s or faster. Stearic acids with the spin label at different positions along the acyl chain ($N = 5, 6, 7, 9, 10, 12$) have similar flip-flop rates in the liposomes at pH 7.0 although 16-DS is slower, probably due to the inaccessibility of the nitroxide moiety to FMN. It is most likely that the fast distribution of 5-DS in cells is due to the fast movement of acidic form, but not the salt form, of 5-DS across membrane bilayers. The oxazolidine (nitroxide moiety) does not seem to affect the pK_a (~ 8.3) of stearic acid at air–water interface. Thus, *N*-DS are good probes for studying the distribution kinetics of stearic acid derivatives in biological systems.

Keywords: Membrane; Spin label; Nitroxide; Flip-flop; Phospholipid; Liposome

1. Introduction

Although *N*-doxyl stearic acids (*N*-DS) have been used as spin labels to study membrane properties for many years, their distribution in eurkaryotic cell membranes has been controversial. Early EPR studies which used *N*-DS were frequently interpreted under the assumption that *N*-DS stay only in the outer layer of plasma membranes [1,2]. This assumption was based on the observation that the 5-DS reduced by cells to the EPR-invisible hydroxylamine could be reoxidized to the EPR-active nitroxide by the membrane impermeable oxidizing agent potassium ferricyanide $K_3Fe(CN)_6$ [3]. The reoxidation of reduced nitroxides by $K_3Fe(CN)_6$ has been demonstrated in several cell types [4,5].

Nettleton and his coworkers [6] showed that 5-DS

Abbreviations: *N*-DS, *N*-doxyl stearic acids, where $N = 5, 6, 7, 9, 10, 12, 16$; EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide; CAT_1, 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; Tempol, 4-hydroxyl-2,2,6,6-tetramethyl piperidine-1-oxyl; CAT_{16}, 4-*N,N*-dimethyl-*N*-hexadecyl ammonium-2,2,6,6-tetramethyl piperidine-1-oxyl iodide; DPPC, dipalmitoyl phosphatidylcholine

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0005-2736/99/$ – see front matter
PII: S0005-2736(98)00215-6

distributed completely and rapidly to all the membranes of mouse thymus-bone marrow (TB) and Chinese hamster ovary (CHO) cell. Their experiments compared the spin–spin broadening [7–9] of 5-DS as a function of 5-DS/lipid ratio in human erythrocytes, TB and CHO cells. When 5-DS was added in increasing amounts to membranes, the interactions between 5-DS nitroxides caused the EPR line to broaden. Nettleton et al. [6] showed that the midfield line of the 5-DS spectrum showed only about one-twentieth of the spin–spin broadening which would be expected if 5-DS were assumed to stay only in the plasma membranes. Since the amount of phospholipid in the plasma membrane of these cells was only about one-twentieth of the total phospholipid in these cells, they concluded that 5-DS distributed throughout all phospholipid in cell [6]. Although Nettleton and coworkers have shown the uniform distribution of 5-DS in mammalian cells, they left the unanswered question of how 5-DS attains this fast distribution.

The phospholipids in cell membranes rotate, move laterally (in the plane of the monolayer), and flip-flop (move from one half of the bilayer to the other). Rotational diffusion ($\sim 10^8$/s) [10] and lateral diffusion ($D = 1.8 \pm 0.6 \times 10^{-8}$ cm^2/s) [8] are rapid. However, flip-flop is slower because of the energy required to move the polar head group through the hydrophobic interior of the membrane bilayer. Kornberg and McConnell showed that the half time of flip-flop for spin-labeled phosphatidylcholine was about 6.5 h at 30°C [11].

In order for *N*-DS nitroxides to distribute throughout all cell membranes, they would have to leave the outer half of the plasma membrane, where they first come in contact with the cell with current labeling procedures, and then distribute to other membranes by flipping across the plasma membranes and exchanging into other membranes through contact of these membranes with the inner surface of the plasma membrane [12]. This flip-flop motion is the only type of motion that allows molecules to cross and equilibrate between the two halves of a membrane bilayer.

In the work presented here, we measured the flip-flop rate of *N*-DS spin labels in artificial phospholipid bilayers at temperatures well below both the pre- and main phase transition. Under these conditions, the hydrocarbon region of the bilayer would be in a liquid crystal state and would be much less fluid than the hydrocarbon region of naturally occurring cell membranes. The liquid–crystal state represents a lower limit of fluidity. Our reasoning is that if flip-flop rates are rapid under these conditions, they would be at least as rapid in more fluid membranes. Our findings show that the flip-flop rates of *N*-DS in this model system are fast enough to account for the observed distribution of these labels in cell systems.

2. Materials and methods

2.1. Chemicals

All chemicals were reagent grade or better. Solutions were made using deionized water which had been redistilled in a Corning Mega-pure water distillation system.

Nitroxides were purchased from Molecular Probes (Eugene, OR). CAT_{16} was a kind gift from Dr. Rolf Mehlhorn (University of California, Berkeley, CA). FMN was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide (NaOH), citric acid, orthoboric acid, potassium phosphate dibasic (K_2HPO_4) and potassium phosphate monobasic (KH_2PO_4) were purchased from Fisher Scientific (Pittsburgh, PA). EDTA was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Sephadex G-25 beads for gel filtration were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). DPPC was purchased from Avanti Polar Lipids (Alabaster, AL). The purity of phospholipids was higher than 99% (Avanti, personal communication) and were used without further purification. Phospholipids were stored in chloroform below −20°C and were not used if the solution showed any coloration.

2.2. Preparation of solutions

Nitroxide solutions were made using either ethanol or water as the solvent depending upon the solubility of the nitroxide. The nitroxide stock solutions were made initially to a concentration of about 10 mM and the actual concentrations of the stock solutions were determined by comparison with a solution of Tempol of known concentration. Nitroxide solutions

were stored at −20°C to avoid thermal decomposition.

FMN was prepared daily as a stock solution at 10 mM. FMN with 1 mM EDTA was dissolved in either deionized water or phosphate buffer. The FMN stock solution was held in 2-ml brown vials in order to avoid any photodecomposition and was stored at −20°C until use. The concentration of EDTA used in a typical experiment was 1.0 mM. This concentration maximized the photoreduction rate (data not shown).

In order to maintain a constant ionic composition at different pH values, we used citrate–phosphate–borate buffer [13] made up as two stock solutions which were mixed to give the final pH.

2.3. Preparation of liposomes

The liposomes were made by high-pressure extrusion [14] using a stainless-steel extruder (Lipex, Canada). In a typical preparation, 50 mg of DPPC in chloroform was placed in a 50-ml round-bottom flask. In some cases, FMN (0.1 mM), EDTA (1 mM) and/or CAT_1 (1 mM) were also added. The chloroform was removed by rotary evaporation at 50°C for 3 h. Two ml of buffer solution was added to the dried lipid and the flask was then placed on the rotary evaporator and rotated at 50°C at atmospheric pressure under nitrogen until the lipid was dispersed in the buffer solution. The lipid–buffer solution was then frozen in liquid nitrogen and thawed in water at 50°C for a total of five times. The lipid–buffer mixture was then extruded at 55°C through a pair of 0.1-μm Costar Nucleopore filters (Cambridge, MA). When FMN was present inside the liposomes, the liposomes were filtered through Sephadex G-25 beads to remove exogenous FMN and were used immediately. The size distribution of the liposomes prepared by this method is 60–100 nm [14]. The trapped volume of these liposomes corresponds to 1.5 l/mol which is characteristic of unilamellar liposomes with an approximate diameter of 100 nm [15].

In experiments where the flip-flop rates of 5-DS were measured at various pH values, liposomes were made in citrate–phosphate–borate buffer solutions. At pH ≥10.0, extrusion proceeded very slowly. This problem was solved by reversing the upper polycarbonate filter after the first run. For experiments where the flip-flop rates of *N*-DS ($N = 5$, 6, 7, 9, 10, 12, 16) across liposome membrane were measured, 50 mM phosphate buffer at pH 7.0 was used for liposome preparation instead.

2.4. Dialysis of liposomes

Dialysis of the liposomes removes any residual molecules from the external aqueous phase. The dialysis tubing was a 10 cm Spectra/Por membrane tubing (VWR) which was previously soaked in the appropriate buffer solution for 1 h. After placing the liposomes in the tubing, the tubing was sealed at both ends. Then, the tubing containing the liposomes was placed in a 250-fold excess of buffer solution identical to that used to make the liposomes. The buffer solution was stirred at 0°C and changed at least once after 12 h.

2.5. Spin labeling of liposomes

Spin labels were incorporated into the liposomes by two methods. In the first, an appropriate amount of *N*-DS (to make a molar ratio of *N*-DS/DPPC = 1:100) was placed into the bottom of a 1.8-ml microcentrifuge tube. The solvent was removed under a stream of nitrogen gas to give a thin film of nitroxide on the walls of the tube. Then, 100 μl of liposomes were added to the tube and vortexed with a vortex mixer (Fisher) at a setting of 6 for 2-min at intervals of 1 s on and 1 s off.

For some experiments, nitroxides were added with the lipids during the preparation of liposomes in which the ratio between *N*-DS and DPPC was the same as method 1 (1:100). For example, for pH measurement of flip-flop rate, 5-DS was added during liposome preparation to ensure that 5-DS distributed to both sides of the bilayer. No difference in results was observed between these two labeling methods.

2.6. Preparation of samples for photoreduction

Samples were prepared under a light intensity not exceeding 50 lux to prevent premature photoreduction of the nitroxides. FMN (1.75 μl of 10 mM) was added to a sample of 100 μl of liposomes, and followed with 98.25 μl of buffer solution containing

1 mM EDTA. The final concentration of FMN was 0.1 mM because the trapped volume of the liposomes is typically 12.5% of the total sample volume (determined by comparing integrated EPR spectral intensities of CAT_1 before and after dialysis of the liposomes) so that 25 μl of the buffer was trapped inside the liposomes and inaccessible to FMN.

2.7. Deoxygenation of samples

Sample temperature was maintained by a Varian variable temperature controller using nitrogen as the working gas before and during the photroreduction step. Samples were placed in a Teflon tube 4 cm long with a diameter of 1 mm (Zeus Industries, Raritan, NJ) which was inserted into a 5 mm EPR tube open at both ends. The Teflon tube allowed removal of oxygen from the sample to eliminate photoreduction of oxygen prior to nitroxide reduction. Each sample was deoxygenated for 5 min prior to photoreduction measurements.

2.8. EPR measurements

EPR measurements were made on a Varian E4 EPR spectrometer (Varian Associates, Palo Alto, CA). The front cover of the cavity (TE102) was removed for in situ sample illumination. Peak height was monitored by setting the field center of the spectrometer to coincide with the peak of the midfield line. In a typical experiment, the FMN would start reducing the nitroxides upon illumination and the EPR signal would decay as nitroxides were photoreduced to their corresponding EPR-invisible hydroxylamines. All experiments were performed between 22°C and 25°C, well below the pre- and main phase transition temperature of these lipids (33°C and 41°C, respectively [16,17]).

2.9. Photoreduction

Light from a light source of 150 W (Ealing Electro-Optics, Watford, UK) was guided through a Plexiglas light pipe 13.5 cm long wrapped with aluminum foil. A rectangular half-circular lens was placed in front of Plexiglas pipe to focus the circular light into a line which fell directly on the sample in the EPR cavity [18]. Most of the photoreduction experiments were done at an EPR scan time of 300 s. The selection of scan time depended upon experimental need.

Photoreduction of FMN

$$\mathrm{FMN}(i\text{-}ox) \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} \mathrm{FMN}(i\text{-}red) \qquad \mathrm{FMN}(o\text{-}ox) \underset{K_{-2}}{\overset{K_{+2}}{\rightleftharpoons}} \mathrm{FMN}(o\text{-}red)$$

Reduction of N-DS by FMN

$$\mathrm{FMN}(o\text{-}red) + \mathrm{N\text{-}DS}(o\text{-}ox) \underset{K_{-3}}{\overset{K_{+3}}{\rightleftharpoons}} \mathrm{FMN}(o\text{-}ox) + \mathrm{N\text{-}DS}(o\text{-}red)$$

$$\mathrm{FMN}(i\text{-}red) + \mathrm{N\text{-}DS}(i\text{-}ox) \underset{K_{-4}}{\overset{K_{+4}}{\rightleftharpoons}} \mathrm{FMN}(i\text{-}ox) + \mathrm{N\text{-}DS}(i\text{-}red)$$

Flip-flop of N-DS across membrane bilayer

$$\mathrm{N\text{-}DS}(i\text{-}ox) \underset{K_{-5}}{\overset{K_{+5}}{\rightleftharpoons}} \mathrm{N\text{-}DS}(o\text{-}ox) \qquad \mathrm{N\text{-}DS}(i\text{-}red) \underset{K_{-6}}{\overset{K_{+6}}{\rightleftharpoons}} \mathrm{N\text{-}DS}(o\text{-}red)$$

Fig. 1. Mechanism descriptor. The abbreviations, i, o, ox, and red represent inside, outside, oxidized, and reduced, respectively.

2.10. Computer software

Computer software and hardware for data acquisition and manipulation was a commercial package from Scientific Software Services, Bloomington, IL. Decay rates were calculated from EPR decay curves on a VAXStation 3100 using KINSIM [19] which implements Euler's method and Gear's method [20] of numerical integration for solving the instantaneous concentrations of the products and reactants. The mechanism we used for simulation of flip-flop kinetics is shown in Fig. 1. K_1 is the rate of photoreduction of flavin inside the liposomes and K_2 is the rate of photoreduction of flavin outside the liposomes. K_3 is the reduction of the *N*-DS nitroxide in the outer half of the bilayer and K_4 is the reduction of the nitroxide in the inner half of the bilayer. K_5 is the flip rate of *N*-DS nitroxide from inner leaflet to the out leaflet and K_6 is the reverse. We assumed that $K_1 = K_2$, $K_3 = K_4$, and $K_5 = K_6$. The general order of magnitude of K_1 and K_2 were assumed to be about 10^2-times faster than K_3 through K_6. K_3 and K_4 were determined by measuring reduction rates of the *N*-DS nitroxides with FMN and EDTA on both sides of the liposome membranes. Thus, only K_5 and K_6, which measure the flip-flop rates, were determined by the simulation.

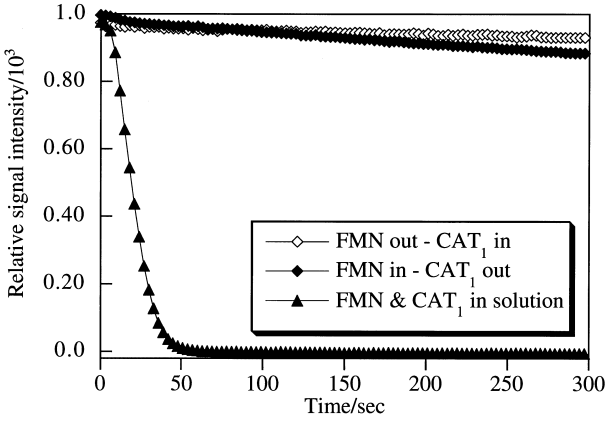

Fig. 2. The photoreduction of CAT_1 inside or outside DPPC liposomes and in solution vs. time. Spectrometer settings were 3387.6 gauss field center, 5.0 gauss modulation amplitude, and 5.0 mW power. CAT_1 was trapped inside the DPPC liposomes during the liposome preparation and external CAT_1 was removed through dialysis. The CAT_1 concentration inside liposomes after 24-h dialysis was 0.24 mM. The FMN concentration outside the DPPC liposomes was 0.1 mM.

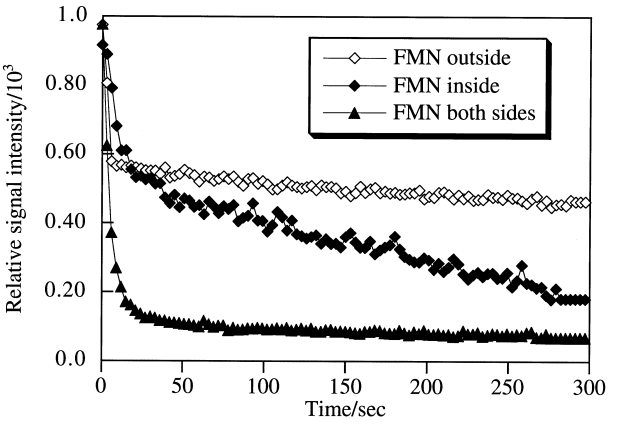

Fig. 3. The photoreduction of CAT_{16} inside, outside or on both sides of DPPC liposomes vs. time. Spectrometer settings were 3384 gauss field center, 5.0 gauss modulation amplitude, and 5.0 mW power. Liposomes were prepared as described in the text. The molar ratio between CAT_{16} and DPPC in liposomes was 1:100. The exogenous FMN in the sample, where FMN was trapped inside liposomes, was removed by Sephadex G-25 beads. The FMN concentration outside the DPPC liposomes was 0.1 mM.

2.11. Statistical analysis

The results are expressed as mean ± standard deviation (S.D.) of independent measurements.

3. Results

To determine whether FMN crosses liposome membranes, a control experiment was performed in which the membrane impermeable nitroxide CAT_1 was trapped inside liposomes [21]. When FMN and EDTA were added on the outside of the liposomes, illumination of the sample did not result in reduction of the CAT_1 inside the liposome (Fig. 2). A similar experiment was performed in which FMN and EDTA were trapped inside the liposomes and CAT_1 was placed outside. Again, upon illumination, CAT_1 was not reduced. In aqueous solution without liposomes, CAT_1 can be photoreduced by FMN in about 3.36 s [22]. These results show that FMN cannot cross liposome membranes over a time period of at least 300 s.

To determine if FMN reduces only nitroxides on the half of the bilayer which is in contact with FMN, a control experiment was performed in which lipo-

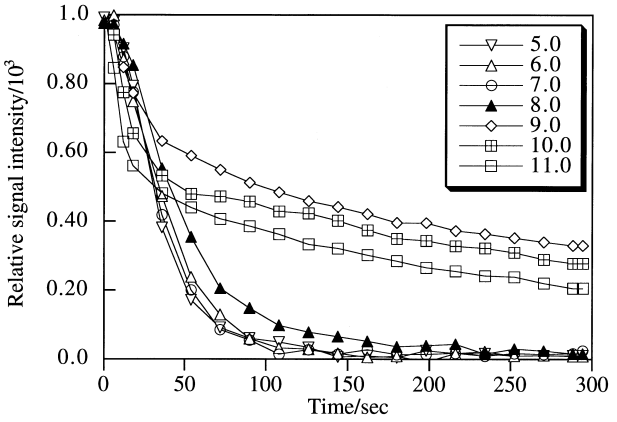

Fig. 4. Photoreduction curves of 5-DS in DPPC liposomes at various pH (5.0–11.0) vs. time. Spectrometer settings were 3324 gauss field center, 5.0 gauss modulation amplitude, and 5.0 mW power. The DPPC liposomes were spin-labeled with 5-DS during preparation and the molar ratio between 5-DS and DPPC was 1:100. The liposome concentration was 25 mg/ml. The FMN concentration outside liposomes was 0.1 mM. N_2 was used as the perfusing gas. The total sample volume was 200 μl.

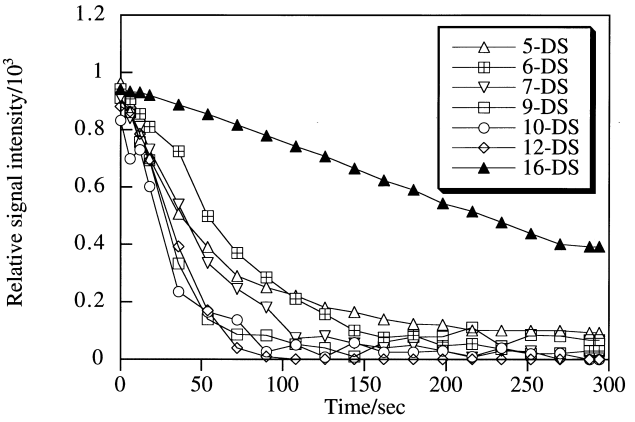

Fig. 5. Photoreduction curves of *N*-DS in DPPC liposomes at pH 7.0 vs. time. Spectrometer settings were 3240 gauss field center, 5.0 gauss modulation amplitude, and 5.0 mW power. The DPPC liposomes were spin-labeled with *N*-DS during preparation and the molar ratio between 5-DS and DPPC was 1:100. The liposome concentration was 25 mg/ml. The FMN concentration outside liposomes was 0.1 mM. N_2 was used as the perfusing gas. The total sample volume was 200 μl.

somes were spin-labeled with CAT_{16}. Castle and Hubbell [23] showed that it took about 580 min for CAT_9 to flip across phospholipid vesicle. It seems reasonable to assume that CAT_{16} has a low flip-flop rate as well. When the liposomes labeled with CAT_{16} were illuminated in the presence of FMN and EDTA outside the liposomes, only half of the signal was reduced (Fig. 3). When FMN and EDTA were present on both sides of the bilayer, all the CAT_{16} was reduced instantaneously. This shows that FMN can only reduce the nitroxide with which it is in contact.

Photoreduction curves of 5-DS across DPPC liposome membranes at pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 are shown in Fig. 4 and the flip-flop rates are given in Table 1. Below pH 8.0, only a single decay rate was observed while at pH 9.0 and above, biphasic curves were obtained. It is most likely that at pH 9.0 and above, the initial decay rate results from the photoreduction of the nitroxide in the outer half of the bilayer while the second decay rate results from flip-flop motion of nitroxide across liposome membranes. From these data, it is clear that the flip-flop rates decrease dramatically at pH 9.0 and above.

Photoreduction curves of *N*-DS ($N = 5$, 6, 7, 9, 10, 12 and 16) by external FMN at pH 7.0 are shown in Fig. 5. In all cases, the decay rate is a single exponential which suggests that the flip-flop rate of *N*-DS are as fast or faster than the photoreduction rate.

Table 1
The flip-flop rate of 5-DS in DPPC liposomes at different pH values

pH	Half-time of 5-DS photoreduction in liposomes	Half-time of 5-DS flip-flop from inner to outer bilayer	Number of measurements
5.0	28.5 ± 3.05*	28.5 ± 3.0*	4
6.0	40.8 ± 11.3*	40.8 ± 11.3*	4
7.0	33.8 ± 4.24*	33.8 ± 4.24*	4
8.0	47.0 ± 7.24*	47.0 ± 7.24*	4
9.0	15.1 ± 2.54	200 ± 64.0	4
10.0	9.80 ± 1.68	292 ± 118	4
11.0	8.55 ± 0.21	262 ± 132	4

All times are reported as mean ± S.D. in seconds. *Denotes that rates of flip-flop and photoreduction cannot be resolved from each other.

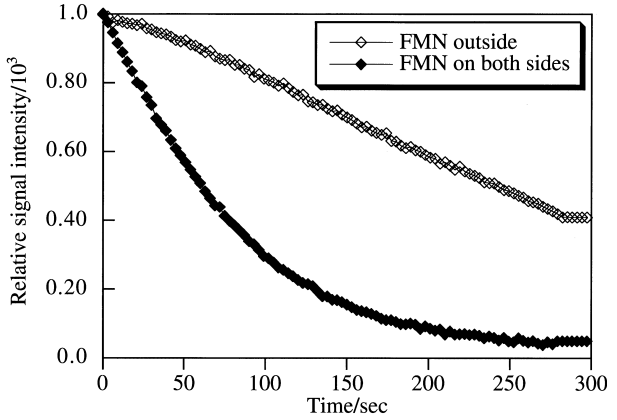

Fig. 6. Photoreduction curves of 16-DS in DPPC liposomes at pH 7.0 with FMN outside or on both sides vs. time. Spectrometer settings were 3240 gauss field center, 5.0 gauss modulation amplitude, and 5.0 mW power. The DPPC liposomes were spin-labeled with 16-DS during preparation and the molar ratio between 16-DS and DPPC was 1:100. The liposome concentration was 25 mg/ml. The FMN concentration outside liposomes was 0.1 mM. N_2 was used as the perfusing gas. The total sample volume was 200 μl.

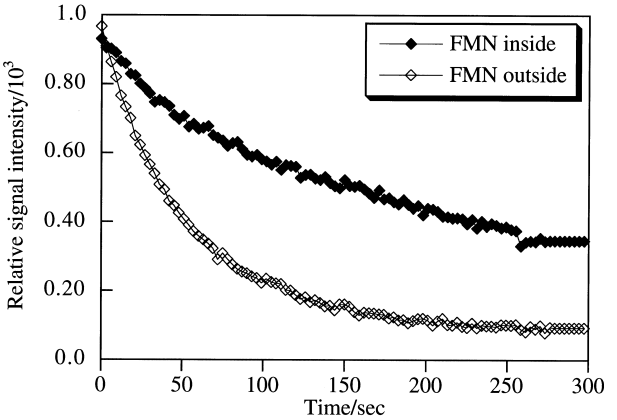

Fig. 7. Photoreduction curves of 5-DS in DPPC liposomes at pH 7.0 with FMN inside or outside vs. time. Spectrometer settings were 3240 gauss field center, 5.0 gauss modulation amplitude, and 5.0 mW power. The DPPC liposomes were spin-labeled with 16-DS during preparation and the molar ratio between 5-DS and DPPC was 1:100. The liposome concentration was 25 mg/ml. The FMN concentration outside liposomes was 0.1 mM. The exogenous FMN in the sample, where FMN was trapped inside liposomes, was removed by Sephadex G-25 beads. N_2 was used as the perfusing gas. The total sample volume was 200 μl.

Although the actual flip-flop rate of *N*-DS cannot be determined directly, the data in Table 2 allows calculation of a minimum flip-flop rate.

For 16-DS, the photoreduction rate is very slow compared to other *N*-DS. To determine whether the slow reduction rate of 16-DS was a result of slow flip-flop or slow reduction by FMN, we made liposomes labeled with 16-DS containing FMN and EDTA on both sides of the membrane. When FMN was on both sides of the 16-DS labeled liposomes, reduction rates were faster than when FMN was only outside (Fig. 6). We concluded that the slow rate of reduction of 16-DS arises from the relative inaccessibility of the oxazolidine group on 16-DS to FMN, and this limits our ability to measure flip-flop rates of 16-DS.

In order to measure the flip-flop rate of 5-DS from outside to inside of the liposome membranes, the same experiments were carried out except that the reducing agent FMN was trapped inside the liposomes and exogenous FMN was removed by Sephadex G-25 filtration. The photoreduction curve of 5-DS in DPPC liposomes at pH 7.0 with encapsulated 0.1 mM FMN is shown in Fig. 7. Compared with the decay curve, where 5-DS in the DPPC liposomes was photoreduced by 0.1 mM FMN outside,

Table 2
The flip-flop rate of *N*-DS in DPPC liposomes at pH 7.0

N-DS: *N*=	Half-time of *N*-DS photoreduction in liposomes	Half-time of *N*-DS flip-flop across liposome membrane bilayers	Number of measurements
5	46.7 ± 7.21*	46.7 ± 7.21*	4
6	54.6 ± 9.29*	54.6 ± 9.29*	4
7	41.9 ± 6.78*	41.9 ± 6.78*	4
9	29.3 ± 1.40*	29.3 ± 1.40*	4
10	26.5 ± 2.86*	26.5 ± 2.86*	4
12	32.3 ± 5.84*	32.3 ± 5.84*	4
16	212 ± 36.4*	212 ± 36.4*	4

All times are reported as mean ± S.D. in seconds. *Denotes that rates of flip-flop and photoreduction cannot be resolved from each other.

it is apparent that the photoreduction rate of 5-DS in DPPC liposomes with 0.1 mM FMN inside is slower. The flip-flop rate of 5-DS in liposomes at pH 7.0, where higher concentrations of FMN (0.5 and 1.0 mM) were trapped inside, was approximately the same as those experiments carried out when FMN was 0.1 mM inside the liposomes (data not shown).

4. Discussion

Our results show that, at pH 8.0 or lower, the photoreduction of 5-DS on one side of the membrane by FMN is slower than the flip-flop rate of 5-DS across DPPC phospholipid bilayers. Stearic acids with the spin label at different positions along the acyl chain ($N = 5, 6, 7, 9, 10, 12$) have similar flip-flop rates. The slow photoreduction of 16-DS limits comparison of its flip-flop rate to those of the other *N*-doxyl stearic acids. The flip-flop rate of 5-DS from the outer to the inner half of the bilayer is slower than that in the opposite direction.

Below pH 8.0 the actual half time of flip-flop for 5-DS across the bilayer could not be determined directly, owing to the fact that the flip-flop rate and photoreduction rate of 5-DS in DPPC liposomes were not distinguishable individually. The half-time of reduction of 5-DS by FMN in solution is 7.9 s [22]. According to the data presented here, the half-time of reduction of 5-DS in membranes is 33.8 ± 4.24 s. Under these conditions, the flip-flop rate of 5-DS across liposome membranes is at least as fast as the photoreduction rate of 5-DS in the liposome membranes.

It is obvious that there are two different reduction processes when the liposome pH values are 9.0 or higher. This indicates a population ($\sim 50\%$) of *N*-DS which is less accessible to FMN. We interpret the fast and slow components of the photoreduction curves as arising from the photoreduction of 5-DS on one side of the bilayer by FMN and the flip-flop of 5-DS across the membrane bilayer, respectively. The changes in kinetics at pH 9.0 may be explained by considering the charge on the 5-DS. The pK_a of stearic acid at the air–water interface is 8.3 [24]. Assuming that the pK_a of 5-DS in DPPC liposomes is 8.3 and the pH values of liposomes are 8.0 and 9.0, the ratio between 5-doxyl stearate ($R-CO_2^-$) and 5-doxyl stearic acid (R-CO_2H) can be evaluated according to the Henderson–Hasselbach equation, pH = pK_a+log([Salt]/[Acid]). In the liposomes at pH 8.0, the concentration of (R-CO_2H) is greater than that of $R-CO_2^-$ ($R-CO_2^-/R-CO_2H = 1:2$). This explains the single decay kinetics of the photoreduction curve of 5-DS at pH 8.0. However, the photoreduction curve at pH 8.0 does not approach the baseline as rapidly as those at lower pH values. This is probably due to the presence of 5-doxyl stearate ($R-CO_2^-$) which has a slower flip-flop rate than 5-doxyl stearic acid (R-CO_2H).

At pH 9.0, the ratio of ($R-CO_2^-$) to (R-CO_2H) is about 5 to 1. The photoreduction curve is now clearly biphasic which results from two different processes. The initial fast portion of the curve results from the photoreduction of FMN as described above. The later slow part of the curve can be attributed to the slower flip-flop rate of salt form of 5-DS ($R-CO_2^-$). It is very clear that there is a dramatic change in the photoreduction curves at pH values from 8.0 to 9.0. This indicates that the pK_a of 5-DS should be in this range (8.0–9.0) which verifies the results of Heikkila et al. [24] in a very different system. Although there are structural differences between stearic acid and its spin-labeled derivatives, the presence of the oxazolidine group on 5-DS does not appear to affect the pK_a of the carboxylic acid within the membrane.

Like 5-DS, when *N*-DS ($N = 6, 7, 9, 10, 12$ and 16) were incorporated into DPPC liposomes, their photoreduction rates by FMN at pH 7.0 were rate-limiting (besides 16-DS), so that the flip-flop rate could not be determined independently of reduction rate. We can, however, use this data to determine a minimum flip-flop rate.

For 16-DS, the decay curve is very slow as compared to those of the other *N*-DS. According to the results of Chen et al. [25], the nitroxide group on 16-DS can be reduced by cellular enzymes faster than that of 10-DS or 12-DS. Chen and coworkers concluded that 16-DS partitioned between the hydrophobic interior of the membranes and the aqueous compartment of their sample of TB cells and that the 16-DS in the two compartments was not in rapid equilibrium. They demonstrated the presence of 16-DS in the aqueous compartment of their sample us-

ing a broadening agent, potassium trioxalatochromate(III) [26] to broaden away the EPR signal of 16-DS in the aqueous phase of the TB cells. The discrepancy between Chen's data and those presented here might be due to two factors. First, the processes which reduce the spin labels are entirely different. In Chen's experiments, the nitroxides were reduced by cellular enzymes and some of which reside in the cell membrane, but in the present work the reducing agent is FMN which cannot enter the bilayer. Second, the time domains are different. In Chen's experiments, the nitroxides of 12-DS and 16-DS were reduced in about 20 min. However, in liposomes, 12-DS can be reduced in 1 min and 16-DS in 5 min. In the photoreduction system, the position of the nitroxide group on the fatty acid chain of 16-DS apparently places it deep in the hydrocarbon region of the lipid bilayer which might account for its relatively slow reduction by the polar FMN.

The reduction of 5-DS in DPPC liposomes with FMN inside is slower than that of 5-DS in DPPC liposomes with 0.1 mM FMN outside. This result was quite unexpected. One possible explanation of this result regards the ratio of 5-DS to FMN. The concentrations of FMN on both sides of the liposome bilayer are the same, but the amount of FMN on each side is different. This is because the aqueous volume outside the liposome exceeds inside the liposomes by a factor of four. Thus, the 5-DS in the outer half of the bilayer is exposed to three times as many molecules of FMN as the 5-DS in the inner half of the bilayer. However, we cannot exclude other interpretations of these results such as different orientation of the 5-DS molecule in the different halves of the bilayer.

In conclusion, we have shown that the *N*-doxyl stearic acid, but not the charged stearate, can move rapidly from one side of the bilayer to the other in membranes composed of DPPC. It should be noted that all present work was done with the lipids in the liquid–crystal phase (below 42°C). Thus, even in this state, the *N*-DS can move across the lipid bilayer very rapidly. Clearly, the high rate of flip-flop of *N*-DS facilitates the rapid distribution of these nitroxides across cell membranes. This implies that drugs that do not adversely affect the polarity of stearic acid could be brought rapidly into the cell by linking the drug to the acyl chain of stearic acid. Finally, the local pH could strongly influence the arrival rate of the drug in the interior of the cell.

Acknowledgements

We gratefully acknowledge support by the National Institutes of Health (GM 1R15-44365-01 to R.(P.D.)M.) and by an Illinois State University Research Grant to R.(P.D.)M. Alex Smirnov (Illinois EPR Research Center) provided helpful comments.

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