Transverse Membrane Asymmetry in Model Phospholipid Bilayers: NBD-Phosphatidylethanolamine and the Separation of Flip from Flop

Robert A. Moss* and Soumendu Bhattacharya

Department of Chemistry
Rutgers, The State University of New Jersey
New Brunswick, New Jersey 08903

Biological membranes frequently feature transverse asymmetry in the distribution of constituent lipids between the outer (exo) and inner (endo) leaflets of the bilayers.[1−5] Moreover, such asymmetry is physiologically important,[4] as illustrated by the membrane chemistry of, e.g., erythrocytes, platelets, and various cellular organelles.[4,6,7] Accordingly, artificially induced lipid asymmetry has been intensively examined in synthetic and natural membranes[6−11] with both lipid distribution and the dynamics of lipid migration between the bilayer leaflets of central concern.

An important approach to constructing asymmetric lipid bilayers involves the chemical differentiation of an initially lipid-symmetric membrane. In our laboratory, lipids bearing the 4-nitro-1-(benzoyloxy)-3-benzyl group, as in the dipalmitoylphosphatidylcholine (DPPC) derivative 1, were dispersed into bilayer liposomes and then chemically differentiated by exoliposomal-specific esterolysis in a 12/6 exo/endo pH gradient.[12] The dynamics of exo/endo reequilibration by transverse bilayer lipid migration ("flip-flop") could then be examined, enabling us to relate lipid molecular structure to intraliposomal dynamics.[13]

1 (R = $C_{15}H_{31}$)

2 (R = $C_{15}H_{31}$)
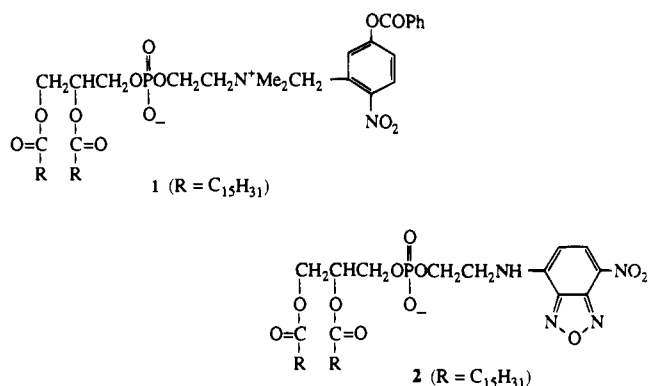
A second residue useful in the study of lipid asymmetry is 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD),[14] depicted in the headgroup-labeled dipalmitoylphosphatidylethanolamine deriva-

tive 2 (NBD-PE).[15] The strong, environmentally-sensitive fluorescence of the NBD group allows NBD-labeled lipids to be visualized within bilayer leaflets and tracked as they translocate between them.[8] Endo NBD-labeled membranes can be produced from membranes with symmetrically distributed probe lipid by out-transfer of the exo leaflet probe to bovine serum albumin[7,9,10] or "acceptor" liposomes.[11] Exo-specific labeling can be achieved by "inserting" a NBD-lipid into the outer leaflet of an unlabeled membrane using either ethanol[9,10] or "donor" liposomes[11] as NBD-lipid vectors.

Most conveniently, exoliposomal-specific reduction of the NBD nitro to an amino group by dithionite ($S_2O_4^{2-}$) destroys the NBD fluorescence, affording a basis for quantitative discrimination between exo and endo NBD probes.[6,9,10] Exo/endo lipid asymmetry can thus be directly determined, and $S_2O_4^{2-}$ reduction can be used to chemically differentiate NBD-labeled biological membranes for subsequent studies.[9,10]

Now we unite the most efficient features of these varied techniques in a study of NBD-PE probe 2 in DPPC liposomes, where dithionite reduction allows the rapid determination of exo/endo probe distribution, the generation of endo-only probe-labeled bilayer leaflets, and the first comparative dynamic analyses of *thermally-driven* endo → exo (*flip*) and exo → endo (*flop*) translocative lipid reequilibrations. A unique advantage of probe 2 is that it permits separate dynamic assessments of *both lipid flip and flop*, whereas only lipid flip is accessible with probe 1.[12] Moreover, the NBD-lipid dynamics can be readily followed by either absorbance or fluorescence, affording results that can be related to those obtained using probe lipid 1.[12]

Small, "symmetrically-labeled" unilamellar coliposomes were prepared from $CHCl_3$-evaporated films of DPPC and 2 in molar ratios of 7:1 (for absorbance studies) or 99:1 (for fluorescence studies). Small coliposomes were chosen to permit direct comparisons with previous studies.[12] Dry films were suspended in pH 7.4, 0.01 M HEPES buffer (containing 0.01 M NaCl) by vortexing at 55 °C. Repetitive extrusion[16] through two stacked 200 nm Nucleopore polycarbonate membranes (2×) and two stacked 50 nm membranes (8×) at 55 °C afforded liposomal solutions which were cooled to 25 °C and diluted to 350 μM DPPC and 50 μM 2 (for absorbance) or 9.9 μM DPPC and 0.1 μM 2 (for fluorescence) by addition of HEPES buffer−NaCl.

The extruded coliposomes had a hydrodynamic diameter of 345 Å by dynamic light scattering (Ar laser, 488 nm, 90° scattering angle), within the anticipated range for small unilamellar liposomes.[17] Their gel to liquid crystal phase transition temperature (T_c) was 40 °C, as determined by differential scanning calorimetry, identical to that of 7:1 DPPC/1 coliposomes.[12] The T_c value falls in the range reported for pure DPPC liposomes (40.6−43 °C),[18] suggesting no disturbance in packing or thermal stability within the coliposomes due to the probe lipids.

The distribution of exo leaflet/endo leaflet 2 was determined with both absorbance and fluorescence measurements.[19] In the former case, the absorbance of 2 was measured at 450 nm: 20 μL of 1 M $Na_2S_2O_4$ solution in 1 M pH 10 Tris buffer was added to 2 mL of liposome solution (concentrations as above), and the absorbance was remeasured after 90−120 s. The absorbance decrease was ascribed to exo leaflet 2 that had been

(1) Gennis, R. B. *Biomembranes: Molecular Structure and Function*; Springer Verlag: New York, 1989; in particular, p 151 ff.

(2) Jain, M. K. *Introduction to Biological Membranes*, 2nd ed.; Wiley: New York, 1988; in particular, p 105 ff.

(3) Fendler, J. H. *Membrane Mimetic Chemistry*; Wiley: New York, 1982; p 113 ff.

(4) Devaux, P. F.; Zachowski, A. *Chem. Phys. Lipids* 1994, 73, 107. Zachowski, A.; Devaux, P. F. *Comments Mol. Cell. Biophys.* 1989, 6, 63.

(5) Op den Kamp, J. A. F. *Annu. Rev. Biochem.* 1979, 48, 47.

(6) Balch, C.; Morris, R.; Brooks, E.; Sleight, R. G. *Chem. Phys. Lipids* 1994, 70, 205.

(7) Colleau, M.; Hervé, P.; Fellmann, P.; Devaux, P. F. *Chem. Phys. Lipids* 1991, 57, 29.

(8) Chattopadhyay, A. *Chem. Phys. Lipids* 1990, 53, 1.

(9) McIntyre, J. C.; Sleight, R. G. *Biochemistry* 1991, 30, 11819.

(10) Nolan, J. P.; Magargee, S. F.; Posner, R. G.; Hammerstedt, R. H. *Biochemistry* 1995, 34, 3907.

(11) Fattal, E.; Nir, S.; Parente, R. A.; Szoka, F. C., Jr. *Biochemistry* 1994, 33, 6721.

(12) Moss, R. A.; Okumura, Y. *J. Am. Chem. Soc.* 1992, 114, 1750.

(13) Moss, R. A. *Pure Appl. Chem.* 1994, 66, 851 and references therein.

(14) See ref 8 for a review.

(15) NBD-PE is available from Avanti Polar Lipids, Inc., Alabaster, AL.

(16) Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. *Biochim. Biophys. Acta* 1985, 812, 55. Mayer, L. D.; Hope, M. J.; Cullis, P. R. *Biochim. Biophys. Acta* 1986, 858, 161.

(17) See ref 2, p 90. The 99:1 DPPC/2 coliposomes had d = 360 Å, whereas the average diameter of sonicated 7:1 coliposomes of DPPC and 1 was 420 Å.[12]

(18) Marsh, D. *CRC Handbook of Lipid Bilayers*; CRC Press: Boca Raton, FL, 1990; pp 139−140.

(19) See ref 9 for typical absorbance and fluorescence spectra of NBD-labeled lipids.
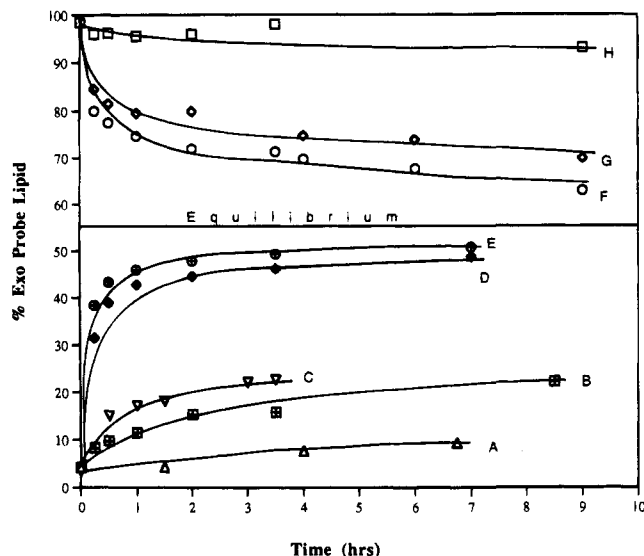
Communications to the Editor

J. Am. Chem. Soc., Vol. 117, No. 33, 1995 8689

Figure 1. Percent of probe lipid **2** in the exo leaflet of surface-differentiated 1:7 coliposomes of **2**/DPPC vs time of incubation at various incubation temperatures. Lower panel: flip (endo → exo) of lipid **2**, starting with endo **2**. Upper panel: flop (exo → endo) of lipid **2**, starting with exo **2**. Curve C represents 1:7 coliposomes of **1**/DPPC at 45 °C; the other curves are for **2**/DPPC at (A) 25, (B) 35, (D) 45, (E) 55, (F) 55, (G) 45, and (H) 35 °C.

reduced by $S_2O_4{}^{2-}$; reduction of the NBD nitro to an amino group largely destroys absorbance above 400 nm.[9] Heating the coliposome/dithionite solution to 65 °C for 30 min then led to the loss of the residual 450 nm absorbance, which was attributed to endo NBD probe lipids. The exo/endo probe ratio was found to be 56:44 (±2%), very similar to the ratio (55:45−60:40) observed with DPPC/**1** coliposomes.[12]

Similar experiments carried out with the 99:1 DPPC/**2** coliposomes using fluorescence monitoring (excitation 460 nm, observation 531 nm) gave an exo/endo probe distribution of 66:34 (±2%). Apparently, the greater proportion of probe lipid used in the absorption experiment has a modest effect on the exo/endo distribution of **2** in the coliposome.

Reequilibration of lipid **2**, initiated from the endoliposomal leaflet (flip), could be allowed after exoliposomal reduction of 56:44 exo/endo **2**/DPPC coliposomes: 200 μL of 1 M $Na_2S_2O_4$ in 1 M pH 10 Tris buffer was added to 20 mL of a 1:7 **2**/DPPC coliposome solution in pH 7.4 HEPES buffer, 0.01 M NaCl, prepared as described above, and the exoliposomal reduction of **2** was followed by absorbance at 450 nm. After 4−5 min, excess dithionite was removed by four or more successive dilution/washing/filtration cycles in an Amicon stirred ultrafiltration cell, fitted with a PM-30 filter that retained the liposomes.[20] This procedure afforded surface-differentiated DPPC coliposomes that were labeled with **2** only in their endoliposomal leaflets.

Aliquots of this solution were incubated at 25, 35, 45, or 55 °C for various periods, cooled to 25 °C, and assayed for the exo/endo distribution of **2**. The results of these thermally-driven lipid redistributions are illustrated in the lower panel of Figure 1, where the extent of "flipped" (exo) **2** is seen to increase with both the temperature and the duration of incubation.[21] There is an obvious "jump" in the net rate of lipid flip when the

temperature exceeds the T_c (40 °C) of the coliposomes. Flip is relatively slow in gel phase coliposomes at 25 and 35 °C but much more rapid at 45 and 55 °C, where the liposomes are in the more fluid liquid crystal state. Related behavior has been observed for the flip of endoliposomal **1** in **1**/DPPC coliposomes.[12,22]

Translocation of **2** from exo to endo leaflets (flop) could be studied with 1:7 **2**/DPPC coliposomes that were specifically labeled in their exo leaflets. These were generated by slowly injecting 400 μL of a 5 mM ethanolic solution of **2** into a stirred solution of 0.35 mM liposomal DPPC in 40 mL of pH 7.4 HEPES buffer, 0.01 M NaCl.[9,10,23] (Immediate dithionite reduction, monitored at 450 nm, confirmed that all of the inserted probe was initially located in the exo leaflet, and fluorescence intensity measurements showed that essentially all of the added probe had been taken up.) Aliquots of the exo-labeled coliposome solution were then incubated at 35, 45, or 55 °C for various periods, cooled to 25 °C, and assayed to determine exo/endo probe lipid distributions. These results appear in the upper panel of Figure 1, where it is again clear that increasing the incubation temperature and time enhances the net transfer of probe lipid **2** from exo- to endo-liposomal loci. The flop rate also increases markedly above T_c; compare the 35 °C experiments with those run at 45 and 55 °C.

The flip and flop dynamics represented in Figure 1 reveal that both modes of probe lipid translocation tend toward the 56:44 exo/endo equilibrium distribution. Half-times for re-equilibration by flip or flop are >9 h below the T_c but are considerably less than 1 h above T_c. In the flip vs flop competitive approach to equilibrium, the endo → exo flip is the faster process,[24] as expected for "passive" flip-flop in small liposomes, where the sharper curvature of the endo leaflet surface affords less area for the relatively large probe lipid headgroups, thus favoring their transfer to the more gently curved, roomier exo leaflet surface.[6] In biological cells, however (e.g., bull sperm[10]), flip-flop is mediated by translocase or flippase enzymes; the dynamics depend on the specific lipid, and anionic phosphatidylethanolamines (e.g., **2**) may exhibit flop > flip.

Comparisons of the passive endo → exo flips of probe lipids **1** and **2** in DPPC liposomes reveal that rates are similar below the T_c (25 and 35 °C; data not shown), but that anionic **2**[25] appears to flip more rapidly than zwitterionic **1** at 45 °C (just above the T_c of 40 °C, cf., curves C and D in Figure 1). The PE > PC flip ordering would be precedented and may be due to the smaller headgroup volume, weaker hydration, and greater lipidic solubility of the former.[26] However, the labeled headgroups actually present in **1** and **2** are derivatives of PE and PC, and the labels are not identical, so the comparative dynamics of **1** vs **2** reported here must still be regarded as preliminary.

The thermally-driven, mode-dissected lipid flip-flop methodology described here should stimulate wide-ranging studies of lipid dynamics in varied model membranes.

(20) Although the coliposomes were essentially impermeant to dithionite ions at 25 °C, controls indicated significant permeation above 35 °C, so thermally-driven lipid flip had to be carried out in the absence of dithionite. The dithionite−coliposome solution at 25 °C was diluted to 50 mL with HEPES buffer, stirred, and reduced to 15 mL by filtration. It was then rediluted to 50 mL with buffer, and the entire cycle was repeated at least four times, until the filtrate tested negative for dithionite against iodine in aqueous acetone.

(21) Very similar flip behavior was observed for 99:1 DPPC/**2** coliposomes in which the exo/endo probe distribution was monitored by fluorescence.

(22) Bhattacharya, S.; Moss, R. A.; Ringsdorf, H.; Simon, J. *J. Am. Chem. Soc.* **1993**, *115*, 3812.

(23) Kremer, J. M. H.; Esker, M. W. J.; Pathmamanohan, K.; Wiersema, P. H. *Biochemistry* **1977**, *16*, 3932.

(24) This is particularly clear at short incubation times above the T_c, where the dynamics are more nearly unidirectional. Curve fits to the 55 °C data, for example, suggest that the initial flip rate is ~1.7 times greater than the initial flop rate. A referee has pointed out that the flip and flop experiments are not quite equal. In the flip study, the exo and endo leaflets each initially contain 1:7 blends of **2** and DPPC, whereas in the flop experiment, the endo leaflet has only DPPC at time zero.

(25) The pK_a's of the benzofuran (−8.4) and p-nitroaniline (1.9) part structures of **2** are sufficiently lower than the reaction pH (7.4) to ensure that **2** remains anionic (at P−O⁻).

(26) Homan, R.; Pownall, H. J. *Biochim. Biophys. Acta* **1988**, *938*, 155.