Quantifying the Effects of Deuterium Substitution on Phospholipid Mixing in Bilayer Membranes. A Nearest-Neighbor Recognition Investigation

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Abstract: Nearest-neighbor recognition measurements have established that the effects of deuterium substitution on phospholipid mixing are exceedingly small. Thus, the mixing behavior of an exchangeable phospholipid bearing two stearoyl chains with a homologue containing two myristoyl chains in gel-fluid bilayers, fluid bilayers, cholesterol-rich fluid bilayers, and gel-fluid bilayers that have been enriched with cholesterol correspond to a difference in the free energy of mixing that is less than 2.2 cal/mol of hydrogen in all cases. These findings provide the strongest evidence to date in support of the use of deuterated phospholipids as "nonperturbing" probes for structural and dynamic studies of bilayer membranes.

Introduction

Deuterated phospholipids have been widely used to probe the structure and the dynamic properties of bilayer membranes.[1-5] The main assumption in all such studies has been that the replacement of hydrogen by deuterium is nonperturbing, that is, the mixing behavior of the deuterated phospholipid is the same as its nondeuterated analogue. Early studies of the mixing behavior of deuterated (d_{62}) and nondeuterated forms of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) have provided some support for this hypothesis.[6] Thus, an analysis of mixtures of these two lipids by differential scanning calorimetry led to the conclusion that these lipids "mix very nearly ideally". [6] In contrast, recent studies of the mixing behavior of DPPC with 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), at the air–water interface, have indicated that significant differences exist when DPPC is replaced by DPPC-d_{62}.[7]

Here, we provide the first quantification of the effects of deuterium substitution on lipid mixing in bilayer membranes. Specifically, we have quantified the mixing of an exchangeable phospholipid bearing two myristoyl chains (monomers of **1**) with a longer homologue that contains two fully protiated stearoyl chains (monomers of **2**) and also with a fully deuterated analogue of **2** (**2**-d_{140}), by use of the highly sensitive nearest-

neighbor recognition (NNR) method.[8] Four different membrane states that were chosen for this study included: (i) gel-fluid bilayers, (ii) fluid bilayers, (iii) cholesterol-rich fluid bilayers, and (iv) gel-fluid bilayers that were rich in cholesterol. Gel-fluid bilayers were of interest because nearest-neighbor recognition is known to be especially strong in this case.[9] Here, nearest-neighbor recognition is driven by the formation of gel-phase domains that are rich in the higher-melting phospholipid.[9] In contrast, fluid-phase analogues have been shown to form a two-dimensional structure in which the phospholipids are ideally mixed; that is, their time-averaged lateral distribution is random. Cholesterol-rich fluid bilayers were of interest to us because of their biological relevance and also because of cholesterol's ability to fluidize the gel phase and condense the fluid phase.[10,11] In both situations, the membrane is converted to the physiologically relevant, fluid phase.[10-12] As we have shown previously, the ability of cholesterol to induce homophospholipid association results from its selective association with two or more higher melting phospholipids in such mixtures.[13,14]

In brief summary, the general features of the NNR technique can be described as follows. Two lipids of interest are converted into exchangeable dimers and allowed to undergo monomer interchange via continuous thiolate-disulfide displacement reactions. The resulting equilibrium dimer distributions are then analyzed as formal, noncovalent bonds between pairs of adjacent lipids. Thus, a bilayer that is composed of dimers made from

(1) Reviews: (a) Casal, H. L.; Mantsch, H. H. *Biochim. Biophys. Acta* **1984**, *779*, 381. (b) Levin, I. W. In *Advances in Infrared and Raman Spectroscopy*; Clark, R. J. H., Hester, R. E., Eds.; Wiley: Heyden, 1984; Vol. 11, p 1. (c) Mendelsohn, R.; Mantsch, H. H. In *Progress in Protein–Lipid Interactions*; Watts, A., de Pont, J. J. H. H. M., Eds.; Elsevier: Amsterdam; Vol. 2, p 103. (d) Dluhy, R. A.; Stephens, S. M.; Widayati, S.; Williams, A. D. *Spectrochim. Acta* **1995**, *51A*, 1413.
(2) Rana, F. R.; Mautone, A. J.; Dluhy, R. A. *Biochemistry* **1993**, *32*, 3169.
(3) Pastrana-Rios, B.; Flach, C. R.; Brauner, J. W.; Mautone, A. J.; Mendelsohn, R. *Biochemistry* **1994**, *33*, 5121.
(4) Seelig, J.; Waespe-Sarcevioc, N. *Biochemistry* **1978**, *17*, 3310.
(5) Leonard, A.; Escrive, C.; Laguerre, M.; Pebay-Peyroula, E.; Neri, W.; Pott, T.; Katsaras, J.; Dufourc, E. J. *Langmuir* **2001**, *17*, 2019.
(6) Klump, H. H.; Gaber, B. P.; Peticolas, W. L.; Yager, P. *Thermochim. Acta* **1981**, *48*, 361.
(7) Baldyga, D. D.; Dluhy, R. A. *Chem. Phys. Lipids* **1998**, *96*, 81.

(8) Davidson, S. K. M.; Regen, S. L. *Chem. Rev.* **1997**, *97*, 1269.
(9) Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 9828.
(10) Gennis, R. B. *Biomembranes: Molecular Structure and Function*; Springer-Verlag: New York, 1989.
(11) Rubenstein, J. L. R.; Smith, B. A.; McConnell, H. M. *Proc. Natl. Acad. Sci., U. S. A.* **1979**, *76*, 15.
(12) Ipsen, J. H.; Karlstrom, G.; Mouritsen, O. G.; Wennerstrom, H.; Zuckermann, M. J. *Biochim. Biophys. Acta* **1987**, *905*, 162.
(13) Sugahara, M.; Uragami, M.; Regen, S. L. *J. Am. Chem. Soc.* **2002**, *124*, 4253.
(14) Sugahara, M.; Uragami, M.; Regen, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 13040.

A and **B** monomers may be treated as an equilibrium mixture of homodimers (**AA** and **BB**) and heterodimer (**AB**) according to eqs 1 and 2, where K is the equilibrium constant. If **A** and **B** are randomly distributed, then the observed dimer distribution is statistical. In other words, if an equimolar amount of **A** and **B** were present, the mole ratio of **AA/AB/BB** would be 1/2/1, and the equilibrium constant would be equal to 4. If a thermodynamic preference for heteroassociations existed, this would be reflected by a value of K that is greater than 4. In contrast, favored homoassociations would be indicated by a value of K that is less than 4. As previously discussed, although the NNR method involves the use of exchangeable dimers, it provides thermodynamic information that relates to nearest-neighbor interactions between *individual* lipid monomers.[8]

Experimental Section

All phosphoethanolamines, which were used as precursors for the requisite dimers, were obtained from commercial sources (Avanti Polar Lipids) and used without further purification. The syntheses of **1**−**3** have previously been reported.[9]

Nearest-Neighbor Recognition Measurements. In a typical liposome preparation, a test tube was charged with a chloroform solution that contained 0.3 μmol of **1**, 0.3 μmol of **2**, and 0.48 μmol of cholesterol (29% cholesterol). The chloroform was then evaporated by passing a stream of argon over the solution. The lipid mixture was then dissolved in 100 μL of chloroform and diluted with 270 μL of diisopropyl ether. Subsequent addition of 35 μL of 3.3 mM tris-HCl buffer (3.3 mM tris-HCl, 50 mM NaCl, 0.67 mM NaN$_3$, and 0.33 mM EDTA, pH 7.4) produced an emulsion. After the emulsion was sonicated for 3 min, using a mild (bath-type) sonicator, the organic phase was removed by gentle evaporation at 60 °C, resulting in a white gel at the bottom of the test tube. The gel was then collapsed by vigorous vortex mixing for 15−45 min, and 2.0 mL of additional 10 mM tris-HCl buffer (10 mM tris-HCl, 150 mM NaCl, 2 mM NaN$_3$, and 1 mM EDTA, pH 7.4) was added dropwise with vortex mixing. The dispersion was then degassed with an aspirator for 5 min, and the residual traces of organic solvent were removed by dialysis (Spectra/Por Membrane, MWCO 6000−8000) under an argon atmosphere, using two 200 mL portions of degassed 10 mM tris-HCl buffer (pH 7.4) over the course of 18 h. Large unilamellar vesicles formed under these conditions were typically 1000 nm in diameter (dynamic light scattering).

Thiolate-disulfide interchange reactions were initiated, after the dispersions had equilibrated at the desired temperature, by increasing the pH to 8.5 (addition of ca. 10 μL of 1.0 M NaOH) and injecting 54 μL of a tris-buffer solution of 15.1 mM *threo*-dithiothreitol (0.82 μmol) and 24 μL of a tris-buffer solution of 0.42 μM monensin (10.2 pmol) with brief vortex mixing. All dispersions were maintained under an argon atmosphere throughout the course of the interchange reactions. Aliquots (0.30 mL) were withdrawn as a function of time and quenched with 80 μL of 30 mM HCl (final pH 5.0). After removal of water under reduced pressure, the residue was triturated with 2 mL of chloroform and centrifuged. The chloroform layer was then removed under reduced pressure to yield a clear film, which was, subsequently, dissolved in 10 μL of chloroform and 90 μL of the mobile phase that was used for HPLC analysis. Under the conditions used, the extent of reduction of the lipid dimers to thiol monomers by dithiothreitol was less than 10%. All

Chart 1

1 **2** **3**

$$\text{AA} + \text{BB} \xrightleftharpoons{K} 2\,\text{AB} \qquad (1)$$
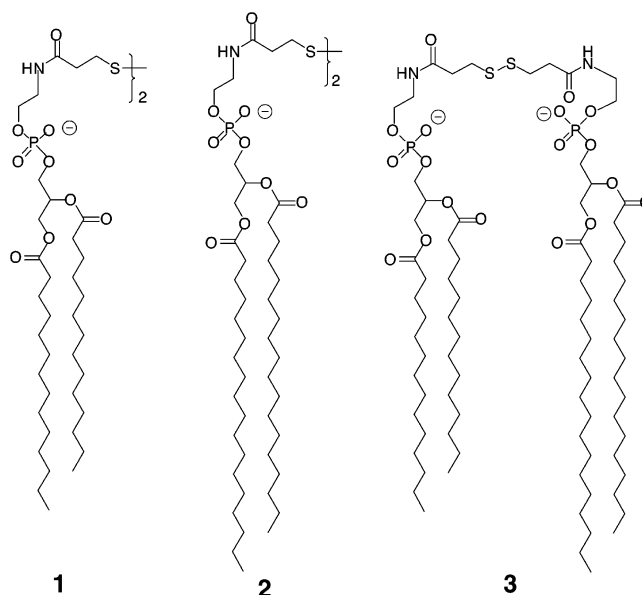
$$K = [\text{AB}]^2/([\text{AA}][\text{BB}]) \qquad (2)$$

exchange reactions that were carried out at 30.5 °C reached an equilibrium point after ca. 12 h; at 60 °C, equilibrium was reached after ca. 3 h.

Analysis of Dimer Distributions by High-Performance Liquid Chromatography. Mixtures of lipid dimers were analyzed by HPLC using a Beckman Ultrasphere C18 reverse-phase column (4.6 × 250 mm, 5 μm particle size). The flow rate was 0.9 mL/min, and the column temperature was maintained at 31.0 °C. Peaks were monitored at 205 nm by use of a Waters 996 photodiode array detector. Data were collected and processed using a Millennium workstation (Waters Corp., Milford, MA). The mobile phases that were used in these analyses were composed of 10 mM tetrabutylammonium acetate (TBA) in denatured ethanol/water/hexane in volume ratios of 81/12/7, 77/13/10, and 83/11/6 (v/v/v).

Results and Discussion

The syntheses of **1** and **2**, and the corresponding heterodimer (**3**), have previously been reported (Chart 1).[9] Deuterated analogues of **2** and **3** (i.e., **2**-d_{140} and **3**-d_{70}) were prepared using similar procedures, starting from commercially available 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-d_{70}. As noted earlier, the gel- to liquid-crystalline-phase transition temperatures for **1**, **2** and **3** are 22.7, 55.4, and 33.9 °C, respectively;[9] **2**-d_{140} shows a melting temperature of 49.4 °C.

Using experimental procedures similar to those previously described, NNR experiments were carried out with the above four bilayer states using fully protiated and fully deuterated exchangeable monomers of **2**. To ensure that product mixtures were thermodynamically controlled, liposomes were prepared using equimolar mixtures of **1** and **2**, as well as from **3**. Convergence of both data sets was excellent in all cases.

Table 1. Influence of Deuterium Substitution on K^a

starting phospholipid(s)	cholesterol^a(mol %)	temp (°C)	K^b
1 + **2** or **3**	0	30.5	0.52 ± 0.07
1 + **2**-d_{140} or **3**-d_{70}	0	30.5	0.62 ± 0.13
1 + **2** or **3**	0	60.0	3.92 ± 0.16
1 + **2**-d_{140} or **3**-d_{70}	0	60.0	4.00 ± 0.16
1 + **2** or **3**	29	30.5	1.30 ± 0.14
1 + **2**-d_{140} or **3**-d_{70}	29	30.5	1.32 ± 0.10
1 + **2** or **3**	29	60.0	2.02 ± 0.14
1 + **2**-d_{140} or **3**-d_{70}	29	60.0	2.02 ± 0.17

^{a} Mol % of cholesterol is based on total lipid that is present, where each phospholipid "counts" as two lipid molecules. ^{b} Equilibrium constants were calculated from eq 2.

Apparent equilibrium constants, K, that are reported in Table 1 are based on both sets of experiments.

As shown in Table 1, the effects of deuterium substitution in each of these systems fall within the limits of experimental error. On the basis of the high-sensitivity of this method, we can assign *upper limits* to the perturbing influence that deuterium substitution has on such mixing. Thus, for gel-fluid bilayers at 30.5°C, the *minimum* value of K is $0.52 - 0.07 = 0.45$, corresponding to a $\Delta G°$ of mixing of 482 cal for 2 mol of dimer. For the deuterated analogue, the *maximum* value of K is $0.62 + 0.13 = 0.75$, corresponding to $\Delta G° = 173$ cal for 2 mol of dimer, the difference being $482 - 173 = 309$ cal for 2 mol of dimer. Since there is 1 molar equiv of dimer **2** involved in this equilibrium, this corresponds to $309/2 = 155$ cal/mol of exchangeable phospholipid. In other words, replacement of the 70 hydrogen atoms in each monomer of **2** with deuterium perturbs its mixing with monomers of **1** by less than 2.2 cal/mol of hydrogen atoms. By a similar analysis, and on the basis of the error limits in cholesterol-rich fluid bilayers at 60 °C, the effect of deuterium substitution on lipid mixing is less than 0.7 cal/mol of hydrogen atoms. For gel-fluid bilayers that were enriched in cholesterol at 30.5 °C, the effect is less than 0.9 cal/mol of hydrogen atoms. Finally, in the case of fluid bilayers that are devoid of cholesterol (60°C), the effect is less than 1.0 cal/mol of hydrogen atoms.

Conclusions

Nearest-neighbor recognition measurements have demonstrated that the perturbing influence that deuterium substitution has on the mixing behavior of two exchangeable phospholipids in gel-fluid bilayers, in fluid bilayers, in cholesterol-rich fluid bilayers, and in gel-fluid bilayers that are enriched in cholesterol is exceedingly small, corresponding to a difference in the free energy of mixing that is less than 2.2 cal/mol of hydrogen in all cases. These findings provide the strongest evidence to date in support of the use of deuterated phospholipids as "nonperturbing" probes for structural and dynamic studies of bilayer membranes.