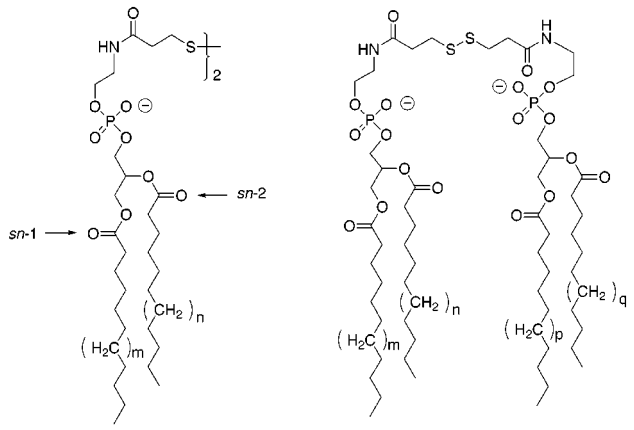
The Importance of Acyl Chain Placement on Phospholipid Mixing in the Physiologically Relevant Fluid Phase

Michihiro Sugahara, Maki Uragami, Nobuya Tokutake, and Steven L. Regen*

Department of Chemistry and Zettlemoyer Center for Surface Studies, Lehigh University Bethlehem, Pennsylvania 18015

Received November 21, 2000

In this paper, we show that the mixing behavior of phospholipids, bearing two acyl chains that differ in length by two methylene groups, depends on the positioning of the chains. Specifically, we show that the monomer units of **1a** and **1b**, having their shorter chains attached to the *sn*-1 position, form *nonideal* mixtures in cholesterol-rich, fluid bilayers. In sharp contrast, monomers of **2a** and **2b**, which have their shorter chains attached to the *sn*-2 position, yield *ideal* mixtures. These findings represent the first experimental evidence that acyl chain placement can influence the mixing properties of phospholipids in the physiologically relevant fluid phase. Moreover, they lend strong support for the putative "skewed tuning fork" conformation of phospholipids.



Phospholipids are the major building blocks for all biological membranes.[1,2] At present, such molecules are presumed to favor a conformation in which the glycerol backbone lies perpendicular to the membrane surface, thereby creating a skewed tuning fork-type of arrangement (Chart 1)[3]. If two identical acyl chains are attached to the glycerol linkage, one then expects the *sn*-1 chain to extend deeper into the bilayer than the *sn*-2 chain. Unambiguous evidence for such a conformation has previously been obtained for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine dihydrate [(DMPC)·$2H_2O$] in the *crystalline* state, based on X-ray analysis.[4] Although NMR studies of fluid phospholipid bilayers have yielded

Chart 1

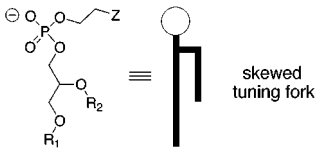
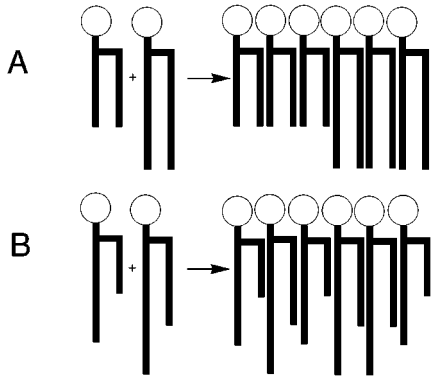


Chart 2



results that are consistent with this conformation, its predominance in the fluid phase is less certain.[3,5,6]

We have posited that nearest-neighbor recognition (NNR) in fluid bilayer mixtures of two phospholipids of unequal chain lengths is the result of maximizing chain−chain interactions by aligning the longer acyl chains both intramolecularly and intermolecularly.[7] This premise can be conclusively tested, experimentally, if the skewed tuning fork conformation does persist in the fluid phase. Indeed, in that case, the chain−chain interactions of a phospholipid comprising two acyl groups of equal length should be further increased by moving one methylene group from the *sn*-1 chain to the *sn*-2 chain. Conversely, moving a methylene group from the *sn*-2 chain to the *sn*-1 chain should result in a decrease of chain−chain interactions. Thus, if chain−chain interactions are, in fact, solely responsible for nearest-neighbor recognition, then a mixture of phospholipids having acyl chains with *m* and *n* carbons in the *sn*-2 position, and $m - 2$ and $n - 2$ carbons, respectively, in the *sn*-1 position, should show a higher degree of nearest-neighbor recognition than a mixture of phospholipids in which the position of the *sn*-1 and *sn*-2 chains is reversed. A stylized illustration of the former is shown in Chart 2A; the latter is depicted in Chart 2B.

The primary aim of the work described herein was to determine whether acyl chain placement can, in fact, influence the mixing properties of phospholipids in the fluid phase. To probe this question, we sought to quantify nearest-neighbor interactions between phospholipids of the type that are illustrated in Chart 2. With this goal in mind, exchangeable phospholipid dimers (**1a**, **1b**, **1c**, **2a**, **2b**, and **2c**) were synthesized, and the mixing properties of each monomeric unit then analyzed by use of the nearest-neighbor recognition (NNR) method.[7−10] Thus, vesicles were formed from 1/1 molar mixtures of the homodimers (**1a**/**1b** and **2a**/**2b**), and the monomer units allowed to equilibrate via thiolate−disulfide interchange. To confirm that an equilibrium

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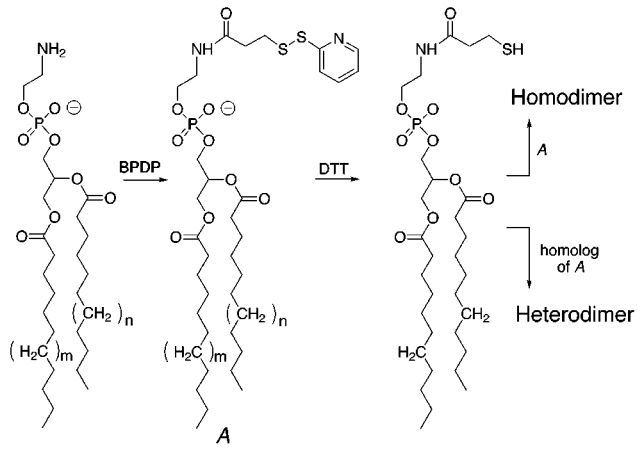
10.1021/ja005811h CCC: $20.00
Published on Web 02/24/2001

Scheme 1



point has been reached, product mixtures were also generated from vesicles that originally contained the corresponding heterodimers (**1c** and **2c**). As noted previously, equilibrium mixtures that are found to be statistical reflect ideal mixing.[7] In those cases where homodimers are found to be in excess (i.e., NNR exists), and when this excess can be reduced or eliminated by the presence of a nonexchangeable phospholipid that functions as a mixing agent, lateral heterogeneity is indicated.[9]

Each of the phospholipids, **1a**, **1b**, **1c**, **2a**, **2b**, and **2c**, were synthesized from the corresponding phosphatidylethanolamines (PEs, Avanti Polar Lipids) according to the reaction sequence that is shown in Scheme 1.[11] Thus, acylation of an appropriate PE with *N*-[*O*-1,2,3-benzotriazin-4(3*H*)one-yl]-3-(2-pyridyldithio)-propionate (BPDP) followed by reduction with dithiothreitol (DTT) and coupling with its precursor afforded the desired homodimer; coupling with a homologue of the activated thiol monomer, *A*, afforded the corresponding heterodimer.[12] Large unilamellar vesicles were prepared via reverse-phase evaporation methods. Experimental procedures that were used in synthesizing the lipids, forming vesicles, initiating the thiolate−disulfide interchange reaction, and analyzing dimer distributions by HPLC were similar to those previously described.[7,9,13]

A summary of our principal findings is shown in Table 1. In the absence of cholesterol, monomer units of **1a** and **1b**, and also those of **2a** and **2b**, formed ideal mixtures, i.e., statistical mixtures of dimers were observed (entries 1 and 2, respectively). When 29 mol % cholesterol was included in the bilayer, monomers of **2a** and **2b** maintained ideal mixing (entry 3). In sharp contrast, inclusion of 29 mol % cholesterol in membranes composed of exhangeable monomers of **1a** and **1b** resulted in signficant nearest-neighbor recognition (entry 4). When a similar NNR experiment was carried out in cholesterol-rich membranes made from **1a** and **1b**, in which 50 mol % of the exchangeable phospholipid was replaced with 1,2-dipentadecanoyl-*sn*-glycerol-3-phosphocholine (a nonexchangeable phospholipid having an intermediate number of carbons in its acyl chains), a statistical mixture of dimers was found (entry 5).

The appearance of nearest-neighbor recognition in cholesterol-rich bilayers containing monomers of **1a** and **1b**, but not in membranes made from **2a** and **2b**, establishes not only that the skewed tuning fork conformation persists in the fluid phase, but also that nearest-neighbor recognition is due only to chain−chain interactions, as previously postulated. The elimination of this recognition by the presence of 1,2-dipentadecanoyl-*sn*-glycerol-3-phosphocholine further indicates that lateral heterogeneity exists in the absence of this diluent. Finally, the ability of cholesterol to induce nearest-neighbor recognition in **1a**/**1b**, and also in exchangeble phospholipids that contain two identical acyl chains (previously reported), is a likely consequence of its condensing effect on the membrane.[14] Thus, stronger van der Waals forces appear to be needed before the lipids are able to recognize like-nearest-neighbors.[9,12,15,16]

Table 1. Equilibrium Heterodimer/Homodimer Ratios[a]

entry	exchangeable monomers[b]	cholesterol (mol %)	heterodimer[c] / homodimer
1	12-14/16-18	0	1.97 ± 0.05
2	14-12/18-16	0	2.00 ± 0.03
3	14-12/18-16	29	2.00 ± 0.03
4	12-14/16-18	29	1.72 ± 0.03
5[d]	12-14/16-18	29	1.97 ± 0.04

[a] All thiolate-disulfide interchange reactions were carried out at 60 °C; equilibrium was reached within 2 h, starting from either a 1/1 mixture of homodimers or pure heterodimer. [b] Monomers of **1a** (12−14), **1b** (16−18), **2a** (14−12), and **2b** (18−16). [c] Molar ratio of heterodimer to each homodimer ± one standard deviation. [d] Replacement of 50 mol % of the exchangeable phospholipid with 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine.

From a biological point of view, the results presented herein raise the intriguing possibility that nature has designed phospholipids with particular attention being paid to the *location* of the acyl chains. Thus, it is conceivable that acyl chain positioning helps to control the two-dimensional organization of biomembranes. As noted by other investigators, such organization may well have important consequences on some of the most fundamental of cellular processes, e.g., membrane fusion, transport, cell surface recognition, and signal transduction.[18−20]

Acknowledgment. We are grateful to the National Institutes of Health (PHS Grant GM56149) for support of this research.

Supporting Information Available: Spectral data (^{1}H, ^{13}C, ^{31}P NMR, IR, HRMS) and R_f values for **1a**, **1b**, **1c**, **2a**, **2b**, and **2c** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA005811H

(11) Analysis of multilamellar vesicles made from **1a**, **1b**, **2a**, and **2b** by high-sensitivity differential scanning calorimetry (*hs*-DSC) revealed gel to liquid-crystalline phase transition temperatures equalling 10.9°, 48.8°, <7.5°, and 42.7 °C, respectively. A 1/1 molar mixture of **1a**/**1b** exhibited two broad endotherms, one appearing at 10.4 °C and a second appearing at 42.8 °C. Inclusion of 29 mol % of cholesterol into bilayers made from a 1/1 molar mixture of **1a**/**1b** led to the disappearance of the lower melting endotherm, and the appearance of one very broad endotherm at 37.1 °C. All *hs*-DSC data were recorded with a Microcal MC-2 equipped with DA-2 data acquisition and analysis software, Northampton, MA.

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(15) Based on phase diagrams that have been developed for analogous membranes (i.e., 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine/cholesterol and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine/cholesterol), one would expect that 29 mol % of cholesterol in the systems reported herein would place these membranes in what has been termed the liquid ordered/liquid disordered coexistence region, which is a region in which many biological membranes are thought to exist: (a) Almeida, P. F. F.; Vaz, W. L.; Thompson, T. E. *Biochemistry* **1992**, *31*, 6739. (b) Sankaram, M. B.; Thompson, T. E. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8686.

(16) Because of the lack of significant curvature in large unilamellar vesicles, which are formed via reverse-evaporation methods, the inner and outer monolayer leaflet is expected to have the same composition. Previous experiments for systems closely related to the ones reported herein have demonstrated this to be the case.[8,17]

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