Facile Lipid Flip-Flop in a Phospholipid Bilayer Induced by Gramicidin A Measured by Sum-Frequency Vibrational Spectroscopy

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ABSTRACT The first direct experimental evidence that gramicidin A (gA), a transmembrane peptide, facilitates the translocation of unlabeled lipids in a phospholipid bilayer was obtained with sum-frequency vibrational spectroscopy (SFVS). SFVS was used to investigate the effect of gA on lipid flip-flop in a planar 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) lipid bilayer. The kinetics of lipid translocation were determined by an analysis of the SFVS intensity versus time at different temperatures in the presence of 2 mol % gA. The rate constants of DSPC flip-flop increase from 2 to 10 times relative to the pure DSPC system. The results indicate that facial lipid exchange can be induced by a hydrophobic transmembrane helix. The increase in lipid flip-flop rates is correlated to an increase in the gauche content of the lipid tails. The results suggest that membrane defects induced by the presence of integral membrane proteins may play a large role in modulating the rate of lipid flip-flop.

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It has been hypothesized that lipid transbilayer migration is a protein-mediated process. However, the mechanism of action for such a putative protein ''flipase'' or ''flopase'' has yet to be definitively identified. One possibility is that the translocation of glycerophospholipids in membranes is not governed by proteins directly but rather by membrane defects, or heterogeneities that are introduced by transmembrane helices (1). Although a number of defect-mediated mechanisms have been proposed for peptide induced flip-flop, little direct evidence linking defects to flip-flop has been presented (2). Our work aims to address both the structural and dynamic effects of a preincorporated transmembrane peptide on lipid flip-flop.

One such peptide that is known to form a stable transmembrane helix is gramicidin A (gA). The peptide consists of a hydrophobic linear *N*-acylated pentadecapeptide with the sequence: HCO-X-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$, (where $X =$ valine or isoleucine) (3). As an integral membrane peptide, gA forms a well-ordered β-helix dimeric structure in phospholipid bilayers, in which opposing monomer units in each leaflet of the bilayer assemble to form an ion channel, Fig. 1 (4,5).

The peptide-induced lipid flip-flop of labeled-lipid species has been measured in phospholipid vesicles (6). The results of these studies indicate that the presence of transmembrane helices may facilitate the translocation of phospholipids (7). However, we recently reported that the chemical modification of lipid species with a fluorescent or spin-labeled probe can significantly alter the intrinsic rate of lipid translocation (8). The extent to which integral membrane proteins affect the flip-flop rate of native unlabeled lipid species is unknown.

We report here the first direct experimental evidence, to our knowledge, that a transmembrane peptide, such as gA, can facilitate the translocation of unlabeled lipids in a phospholipid bilayer. Sum-frequency vibrational spectroscopy (SFVS) was used to investigate the effect of gA on lipid flip-flop in a planar 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) lipid bilayer supported on a fused silica substrate. The kinetics of lipid translocation were determined by an analysis of the SFVS intensity versus time at different temperatures in the presence of gA. The advantage of using SFVS is that it provides a direct measurement of lipid flip-flop without the need for a fluorescent or spin-labeled lipid probe (9). Experimentally, SFVS is performed by combining a visible and tunable IR laser source at a surface where they generate a third photon at the sum of their respective frequencies. A sum-frequency spectrum can be obtained by tuning the IR frequency through the vibrational resonance of the molecules comprising the interface and measuring the resulting SF intensity. One of the key advantages of SFVS over Raman or IR is that the technique is interface specific and more importantly is exquisitely sensitive to the molecular arrangement of the molecules comprising the interface (9).

For the studies described here, an asymmetric DSPC/DSPC-d_{70} bilayer containing 2 mol % gA was constructed using the Langmuir-Blodgett/Langmuir-Schaefer (LB/LS) method. Fig. 1 shows the SFVS spectrum of 2 mol % gA in a DSPC/DSPC-d_{70} bilayer in the C-H stretching region (2750–3100 cm^{-1}) recorded at 23°C before lipid inversion. The resonances at 2849 cm^{-1} and 2876 cm^{-1} are assigned as the CH_2 symmetric stretch (ν_s) and CH_3 ν_s, respectively. The peak centered at 2942 cm^{-1} is a combination of two resonances: the CH_3 Fermi resonance (FR) at 2936 cm^{-1} and the CH_3 asymmetric stretch (ν_{as}) at 2960 cm^{-1}. The shoulder

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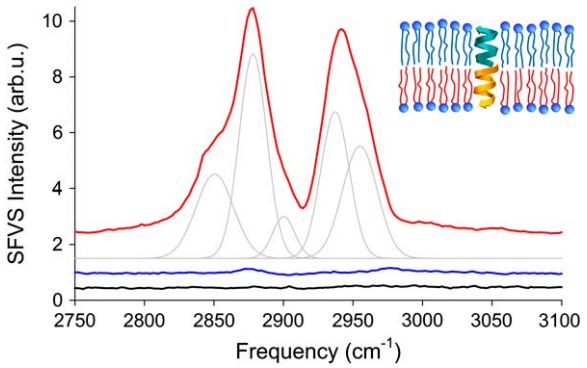

FIGURE 1 SFVS spectrum of 2 mol % gA in a DSPC/DSPC-d_{70} bilayer at 23°C (*red*) with the corresponding peaks obtained from a fit to the spectrum (*gray*). Also shown is the same bilayer at 42°C after lipid flip-flop has occurred (*blue*) in addition to the spectrum of 2 mol % gA in a symmetric DSPC-d_{83}/DSPC-d_{83} bilayer at 23°C (*black*). Spectra are offset for clarity. (*Inset*) Schematic of the dimeric structure of gA in a bilayer.

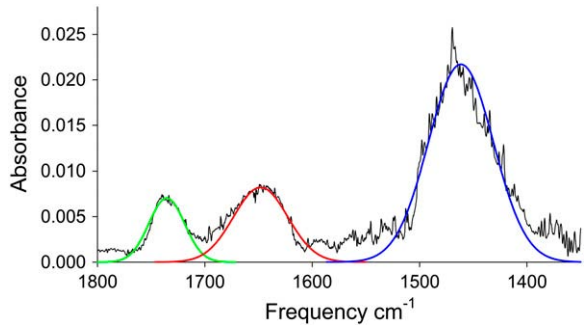

FIGURE 2 ATR-FTIR spectrum of a DSPC bilayer with 2 mol % gA. Baseline corrected data shown in black. Peaks at 1461 cm^{-1} (*blue*), 1648 cm^{-1} (*red*), and 1735 cm^{-1} (*green*) represent the lipid CH_2 scissoring, gA amide I, and lipid C-O vibrations, respectively (11,13). Spectral fits were obtained with grams/AI.

at 2903 cm^{-1} has been assigned to the CH_2 FR (10). Complete lipid transbilayer randomization was induced by elevating the temperature of the sample to 42°C for 188 min (Fig. 1). The SFVS intensities of all the peaks decrease, indicating the asymmetric bilayer has transformed to a symmetric bilayer, where the DSPC and DSPC-d_{70} are equally distributed among the top and bottom leaflets.

To determine the contribution of gA to the spectra in SFVS spectra in Fig. 1, the SFVS spectrum of 2 mol % gA in a symmetric deuterated bilayer of DSPC-d_{83}/DSPC-d_{83} was obtained and is also shown in Fig. 1. The absence of any vibrational resonances from gA indicates that there is a cancellation of the vibrational resonances associated with the peptide due to its dimeric structure (*inset* of Fig. 1). As a result of this cancellation, the dynamics of the lipids can be probed independently without spectral interference from gA.

Since SFVS is not capable of ascertaining if gA is present in the bilayer, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was used to verify that gA was successfully incorporated into the membrane. Bilayers of DSPC with 2 mol % gA were prepared on a germanium ATR crystal by the LB/LS technique. Fig. 2 shows an ATR-FTIR spectrum of a DSPC-gA bilayer in D_2O. Spectra were obtained by averaging 256 single-beam scans of the sample and subtracting the single-beam background from a clean Ge ATR crystal, obtained under the same conditions. The amide I peak at 1640 cm^{-1} is clearly visible, indicative of gA in the β-helix conformation within the bilayer (11,12). The carbonyl C-O stretch of the lipids is visible at 1740 cm^{-1}, as well as the methyl scissoring mode at 1468 cm^{-1} (13). This spectrum shows that gA was successfully incorporated into a DSPC bilayer with the proper conformation using the LB/LS technique.

For the kinetic measurements, the rate of lipid flip-flop was obtained by monitoring the intensity of the CH_3 ν_s (2876 cm^{-1}) as a function of time. The rate constants were extracted by fitting the experimental data to Eq. 1 using nonlinear least-squares regression, (8)

$$I_{CH_3\nu_s} = I_{Max}\ \exp(-4kt) + I_0, \quad (1)$$

where I_{Max} is the peak intensity, k is the rate constant of lipid flip-flop in s^{-1}, and I_0 is an offset that accounts for the nonzero SFVS background after complete lipid inversion and any baseline offset from the data acquisition equipment. Sample decay curves are shown in Fig. 3 as the natural log of intensity versus time. In all cases, the data may be fit to a single exponential. For this study, eight decay curves were collected, ranging from 37.5°C to 48.0°C. The rate constants obtained for both a pure DSPC bilayer and a DSPC bilayer containing 2 mol % gA are listed in Table 1. The rate constants of DSPC flip-flop increase from 2 to 10 times relative to the pure DSPC system over the temperature range examined when gA is incorporated in the membrane.

It has been suggested previously that hydrophobic peptides, such as gA, do not significantly alter the rates of flip-flop because they cannot lower the energetic barrier to lipid

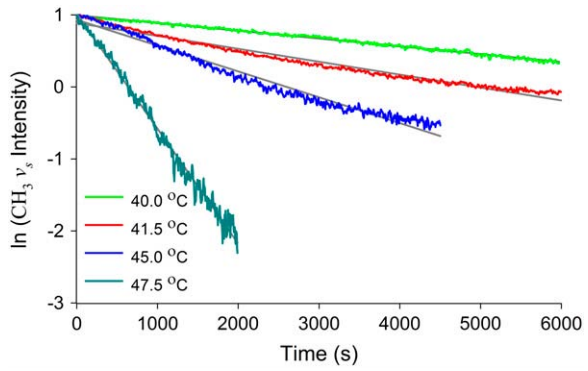

FIGURE 3 Normalized plot of the natural log of the SFVS intensity of the CH_3 ν_s (2876 cm^{-1}) versus time at 40.0, 41.5, 45.0, and 47.5°C, from top to bottom. The solid gray lines are the fits to the decays.

TABLE 1 Rates of transmembrane flip-flop for DSPC and DSPC in the presence of 2 mol % gA

Temperature (°C)	$k_{DSPC} \times 10^5$ (s^{-1})*	$k_{DSPC\ w\ 2\%\ gA} \times 10^5$ (s^{-1})
37.5 ± 0.1	0.83	1.35 ± 0.01
38.5 ± 0.1	1.07	2.71 ± 0.01
40.0 ± 0.1	1.57	4.12 ± 0.01
41.5 ± 0.1	2.29	10.5 ± 0.1
45.0 ± 0.0	5.44	15.6 ± 0.1
46.7 ± 0.2	8.23	55.1 ± 0.2
47.5 ± 0.1	9.98	31.0 ± 0.3
48.0 ± 0.1	11.3	136 ± 3

*Rate constants are extrapolated from experimental data assuming Arrhenius behavior (9).

translocation (14). It has, however, been observed that gA enhances transbilayer movement in erythrocyte membranes, which is consistent with the results observed by SFVS (9). Although it is not yet clear what drives the increase in rates upon addition of gA, one possible explanation is the introduction of membrane defects. Previous studies have indicated that gA in DSPC bilayers tends to aggregate even at 2 mol % (15). This aggregation is associated with an increase in bilayer thickness in the region of the peptide, as indicated by atomic force microscopy studies (15). Such aggregation leads to lipid defects at the boundary between the peptide and surrounding lipid matrix. The perturbation mediated flip-flop, resulting from such packing defects, should occur in the presence of membrane peptides, but has been thought to be a slow process (14).

To investigate the influence of membrane defects on lipid flip-flop, the relative *gauche* defect content in the alkyl chains of DSPC was measured. The SFVS intensity ratio of the CH_2 ν_s/CH_3 ν_s can be used to obtain a relative measure of alkyl chain defects (16). Since an all-*trans* hydrocarbon chain is locally centrosymmetric with respect to the methylene groups, little contribution from the CH_2 ν_s should be observed for a system of well-ordered (all-*trans*) hydrocarbon chains. The introduction of a *gauche* defect relaxes this local symmetry leading to an increase in the CH_2 ν_s resonance. Comparison of the CH_2 ν_s/CH_3 ν_s ratio for a DSPC/DSPC-d_{70} bilayer (0.38) (see Supplementary Material) with that obtained from a DSPC/DSPC-d_{70} bilayer with 2 mol % gA (0.57) indicates a substantial increase in the total number of *gauche* defects in the lipid alkyl chains upon the introduction of gA to the bilayer. The increase of defects in the lipid chains may account for the observed kinetics upon the addition of gA.

The results of these studies indicate that facial lipid exchange can be induced by a hydrophobic transmembrane helix. The increase in flip-flop rates is associated with an increase in the gauche content of the lipid tails. These results suggest that membrane disorder induced by the presence of integral membrane proteins may play a large role in modulating the rate of lipid flip-flop. Studies are under way to investigate the kinetic and thermodynamic effects of other transmembrane peptides, such as melittin, on lipid flip-flop. This work should give a clearer picture of the nature of protein-lipid interactions and the role of transmembrane peptides in facilitating lipid flip-flop.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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