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Phospholipid Flip-flop Modulated by Transmembrane Peptides WALP and Melittin

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Abstract

Select transmembrane proteins found in biogenic membranes are known to facilitate rapid bidirectional flip-flop of lipids between the membrane leaflets, while others have no little or no effect. The particular characteristics which determine the extent to which a protein will facilitate flip-flop are still unknown. To determine if the relative polarity of the transmembrane protein segment influences its capacity for facilitation of flip-flop, we have studied lipid flip-flop dynamics for bilayers containing the peptides WALP₂₃ and melittin. WALP₂₃ is used as a model hydrophobic peptide, while melittin consists of both hydrophobic and hydrophilic residues. Sum-frequency vibrational spectroscopy (SFVS) was used to characterize the bilayers and determine the kinetics of flip-flop for the lipid component, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), within the mixed bilayers. The kinetics data were utilized to determine the activation thermodynamics for DSPC flip-flop in the presence of the peptides. Melittin was found to significantly reduce the free energy barrier to DSPC flip-flop when incorporated into the bilayer at 1 mol%, while incorporation of WALP₂₃ at the same concentration led to a more modest reduction of the free energy barrier. The possible mechanisms by which these peptides facilitate flip-flop are analyzed and discussed in terms of the observed activation thermodynamics.

Keywords

SFVS; flip-flop; melittin; WALP; thermodynamics

Introduction

Lipid transmembrane diffusion (flip-flop) is of great interest to the biophysical community. Lipids synthesized on the cytosolic leaflet of the endoplasmic reticulum (ER), must be capable of moving across this membrane rapidly in order to support uniform growth of the cell.(Devaux, 1993; Kol et al., 2002; Pomorski and Menon, 2006) Likewise, lipids arriving at the plasma membrane (PM) must also distribute appropriately across the bilayer into the appropriate leaflet, via flip-flop.(Bretscher, 1972) Though the importance of flip-flop in cell growth and maintenance of membrane asymmetry is well known, there is little consensus on the exact mechanisms by which the cell regulates lipid flip-flop.

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Membrane proteins have long been hypothesized to play a role in regulating the flip-flop of lipids in various cellular membranes. Bretscher proposed a flip-flop mediating enzyme as a possible mechanism for maintenance of the asymmetric lipid distribution he observed across the leaflets of erythrocyte membranes.(Bretscher, 1973) Around the same time, Kornberg and McConnell demonstrated very slow spontaneous flipping of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*n*,*n*-dimethyl-*n*-(29,29,69,69-tetramethyl-49-piperidyl) (TEMPO-DPPC) in protein-free vesicles, suggesting that the spontaneous movement of lipids was not rapid enough to meet the demands of cell growth in its own right.(Kornberg and McConnell, 1971) This evidence supported the idea that proteins must be required for adequate flip-flop.

Since that time, ATP-dependent transfer of amino-phospholipids in erythrocyte plasma membranes has been demonstrated using spin-labeled lipid probe molecules.(Balasubramanian and Gupta, 1996; Seigneuret and Devaux, 1984) Attempts to isolate a particular ATP-dependent “flippase” from the protein fraction have been met with only limited success, although several candidate proteins were identified.(Devaux and Zachowski, 1994) While this may explain the movement of aminophospholipids by directed movement, there does not appear to be a mechanism for specific, metabolic, energy-dependent flipping of phosphocholine or sphingomyelin lipids in the plasma membrane.(Meer et al., 2008) For these species, spontaneous flip-flop may still be an important mechanism of transmembrane redistribution.

Unlike the plasma membrane where lipids are asymmetrically distributed and spontaneous flip-flop is slow ($t_{1/2} \sim$ days for phosphatidylcholine at 5°C)(Seigneuret and Devaux, 1984), the endoplasmic reticulum exhibits rapid flip-flop of all lipid types ($t_{1/2} \sim$ 25 seconds at 10°C) and a symmetric distribution of lipids across the membrane.(Buton et al., 1996; Menon et al., 2000) While there has been some progress in the attempt to identify plasma membrane flippases, study of flip-flop in biogenic membranes, such as the ER membrane, has revealed important mechanistic differences for flip-flop in these two cellular environments. The most notable difference being the apparent absence of a “flippase” similar to those proposed for the PM. Flip-flop in the ER is known to be protein dependent, but is not energy (ATP) dependent or headgroup selective.(Gummadi and Menon, 2002; Herrmann et al., 1990; Menon et al., 2000) Proteins in the ER are thought to facilitate spontaneous diffusion of lipids between the inner and outer membrane leaflets, but do not transfer them against their concentration gradient.

The role of ER membrane proteins in the promotion of flip-flop may be clearly demonstrated by incorporating extracted ER protein fractions into pure lipid systems. The incorporation of these ER membrane fractions into liposomes induces rapid lipid flip-flop, while protein-free liposomes exhibit slow lipid flip-flop.(Pomorski and Menon, 2006) When membranes containing ER-protein fractions were subjected to treatment with protease or *N*-ethylmaleimide (NEM), inhibition of flip-flop was minimal.(Bishop and Bell, 1985; Hrafnisdottir et al., 1997; Huijbregts et al., 1998) However, when this treatment was applied to detergent solubilized ER proteins prior to incorporation into the membrane, inhibition of facilitated flip-flop was achieved.(Chang et al., 2004) These results suggest that facilitated flip-flop in the ER is due to proteins which have their active portions buried in the membrane and therefore inaccessible to proteases or NEM.(Pomorski and Menon, 2006) Another possibility is that transmembrane protein regions are able to maintain their activity when exposed regions of the protein are degraded by protein modifying agents.(Pomorski and Menon, 2006) This also supports the hypothesis that it may be the transmembrane regions of proteins which are responsible for facilitating flip-flop. Fractionation of solubilized ER membrane constituents prior to reconstitution demonstrated that only certain populations of ER membrane proteins are capable of facilitating flip-flop, while some of the isolated peptides showed little or no effect.(Backer and Dawidowicz, 1987; Chang et al., 2004; Gummadi and Menon, 2002; Hrafnisdottir and Menon, 2002; Menon et al., 2000) This finding indicates that specific protein

characteristics are required for protein facilitated flip-flop, although these characteristics have not yet been definitively characterized.

In an effort to explain the mechanism of action by which peptides facilitate flip-flop, several hypotheses have been proposed. These hypotheses generally suppose that transmembrane helices disrupt the membrane lipid packing or provide a low energy surface for the diffusion of lipids across the membrane without requiring the consumption of ATP or other energy source.(Fattal et al., 1994; Kol et al., 2001) Flip-flop by relatively hydrophobic peptides are most often thought to rely on a perturbation-type mechanism(Kol et al., 2001; Kol et al., 2003), while hydrophilic/amphiphilic peptides are thought to facilitate flip-flop by providing polar residues that stabilize the lipid headgroup during transfer across the hydrophobic core. In some cases, hydrophilic peptides are thought to form water filled pores which permit rapid transmembrane diffusion.(Fattal et al., 1994) In support of these theories, Fattal et al have demonstrated that hydrophilic pore-forming peptides are capable of facilitating flip-flop in synthetic membranes, while hydrophobic transmembrane peptides have little or no effect on the rate of lipid transfer.(Fattal et al., 1994) Although their work was aimed specifically at determining the role of pore formation in flip-flop, their results also show a correlation between polarity of the peptide and the degree to which it enhances flip-flop. This lends further support to the idea that the relative hydrophobicity / hydrophilicity of a particular transmembrane peptide or protein segment may be a possible determinant of its capacity to act as a flip-flop facilitator.

Our research focuses on the determination of the thermodynamics of peptide mediated flip-flop in order to gain insight into the possible mechanisms by which transmembrane helices induce the spontaneous flipping of lipids across the lipid bilayer. This work expands upon our studies of the kinetics and thermodynamics of lipids in homogenous(Anglin et al., 2008) and binary lipid mixtures(Anglin and Conboy, 2008a) and a previous work, in which we demonstrated enhanced flip-flop in lipid bilayers containing the dimeric transmembrane peptide gramicidin A.(Anglin et al., 2007)

In this study, we investigate the synthetic hydrophobic transmembrane peptide, WALP₂₃, and an amphiphilic transmembrane peptide, melittin, in order to assess their effect on the spontaneous transmembrane transfer of native lipids in planar-supported lipid bilayers (PSLBs). The extent of lipid transfer between the two leaflets of the bilayer is tracked using sum-frequency vibrational spectroscopy (SFVS), which allows for label free detection of the native species, thereby avoiding the error associated with the use of fluorescent or slip-labeled probe molecules.(Liu and Conboy, 2005b) The kinetics and thermodynamics of peptide mediated flip-flop can be readily determined by SFVS investigation of peptide doped PSLBs, and provide insight into the mechanism by which peptides enhance the spontaneous movement of lipids.

Methods for the measurement of flip-flop

To date, the majority of studies which investigate flip-flop rely on the use of chemically modified probes, which are known to exhibit altered kinetics of translocation relative to the native species.(Liu and Conboy, 2005b) Numerous membrane models have been utilized, and the choice of model may also influence the observed rate of flip-flop. For instance, it has been shown that small unilamellar vesicles (SUVs) have unequal rates of inward and outward translocation, due to their high degree of curvature.(Wimley and Thompson, 1990) Packing of phospholipids is often unequal in the inner and outer leaflets of SUVs, which could explain some of the discrepancies in the measured rates. The larger radius of curvature found in large unilamellar vesicles improves on this problem, but care must be taken with the method of preparation to ensure that such vesicles are not multi-lamellar in structure.(Cherney et al., 2006)

In order to circumvent many of these complications, our studies make use of PSLBs prepared by the Langmuir-Blodgett (LB) and Langmuir-Schaeffer (LS) deposition method as a model membrane system. The planar geometry of PSLBs avoids the complications that accompany highly curved membranes. The LB and LS methods of preparing PSLBs also provides the ability to control the composition of each leaflet of the bilayer independently, something which is not possible with liposomal models. This method also allows for manipulation of the lateral surface pressure (Π) during deposition and can thereby specify the molecular packing in the film. The ability to prepare films with such high fidelity of pressure and composition is the primary advantage of PSLBs in this application and is essential for the detailed study of lipid flip-flop. In combination with SFVS, an accurate determination of the thermodynamics of lipid flip-flop is possible, something which has eluded investigation until recently.

Measurement of phospholipid flip-flop kinetics by SFVS

SFVS is a coherent nonlinear optical technique which is surface specific in nature. The application of SFVS to the measurement of membrane dynamics is now well established. (Chen et al., 2007a; Chen et al., 2007b; Liu and Conboy, 2004a; Liu and Conboy, 2004b; Liu and Conboy, 2005a; Liu and Conboy, 2005b; Liu and Conboy, 2007) Its strength as a technique for studying lipid translocation phenomena lies in its ability to probe the vibrational resonances of the molecules at the interface in a surface selective fashion, while also being sensitive to the local symmetry of the membrane. This has led to novel applications in the measurement of transmembrane dynamics (Anglin et al., 2007; Liu and Conboy, 2004a; Liu and Conboy, 2005b) as well as lipid phase behavior. (Liu and Conboy, 2007)

A number of excellent sources are available for review regarding the physical principles underlying SFVS, and only a brief discussion will be presented here. (Miranda and Shen, 1999; Shen, 1984) Experimentally, SFVS involves overlapping a fixed frequency visible and a tunable infrared laser source at the sample of interest, where photons are produced at the sum of the input frequencies.

$$\omega_{sum} = \omega_{vis} + \omega_{IR} \quad (1)$$

The intensity, I_{SFVS} , of the light generated at ω_{sum} is proportional to the square of the second-order nonlinear susceptibility tensor ($\chi^{(2)}$) which describes the response of the molecules comprising the interface to the input optical fields:

$$I_{SFVS} \propto |\chi^{(2)}|^2. \quad (2)$$

The susceptibility tensor $\chi^{(2)}$ may be expressed in terms of a resonant $\chi_R^{(2)}$ and nonresonant contribution $\chi_{NR}^{(2)}$ according to:

$$\chi^{(2)} = \chi_R^{(2)} + \chi_{NR}^{(2)}. \quad (3)$$

The resonant contribution, given as:

$$\chi_R^{(2)} = \sum_v \frac{N \langle A_i M_{jk} \rangle}{\omega_v - \omega_{IR} - i\Gamma_v}, \quad (4)$$

depends upon the infrared (A_i) and Raman (M_{jk}) transition probabilities, as well as the number of molecules at the interface (N), the frequency of the vibrational mode (ω_v), and the linewidth of the v^{th} transition (Γ_v). Because of the coherent nature of the process, SFVS is sensitive to the relative phase or orientation of the vibrational transitions that are probed. Mathematically, this is indicated in Equation 4 using the bra ($\langle$) and ket ($\rangle$) notation to indicate the ensemble orientational average of A_i and M_{jk} . Unlike linear spectroscopic methods, SFVS transitions with opposite phase will destructively interfere with one another and the signal will be reduced. This symmetry constraint restricts the SFVS process to media which lack inversion symmetry. This leads to the well known surface specificity of the process, as the interface of two dissimilar media provide the necessary break in symmetry for generation of SFVS signal.

Within a phospholipid bilayer, the relative orientation of molecules comprising the proximal and distal leaflets will also influence the observed SFVS signal, as transitions with opposite orientation within the bilayer will interfere destructively. This can be illustrated by considering the symmetric stretching vibration of the terminal methyl group ($\text{CH}_3 \nu_s$) on each phospholipid alkyl chain. For a symmetric bilayer of a single lipid species, the $\text{CH}_3 \nu_s$ transitions from the inner and outer membrane leaflets will be directly opposed to one another and will destructively interfere. This effect is demonstrated in Figure 1A (gray dash), which shows the SFVS response for a symmetric bilayer of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC). If the lipids comprising one monolayer are replaced with their deuterated analogs, the vibrational transitions of the terminal groups in each leaflet will lie at different frequencies and no interference will occur. This leads to large signal for asymmetric bilayers, as illustrated in Figure 1 A, where a spectrum of an asymmetric bilayer of DSPC and 1,2-distearoyl-*sn*-D70-glycero-3-phosphocholine (DSPC-d₇₀) is presented (black dash).

Figure 1 illustrates the ability of SFVS to distinguish between symmetric and asymmetric bilayers. This feature of SFVS can also be used to measure the rate of flip-flop in asymmetric bilayers composed of proteated and deuterated lipid species. As flip-flop occurs in an initially asymmetric bilayer, the proteated and deuterated species will mix across the bilayer leaflets, thereby reducing the asymmetry and concomitantly reducing the SFVS signal. The bilayer will eventually reach a state where the distribution of proteated and deuterated species in each leaflet is identical and a minimum in the SFVS signal is reached. The signal from these symmetric bilayers composed of isotopic mixtures is indistinguishable from those of pure symmetric bilayers. (Liu and Conboy, 2005a; Liu and Conboy, 2005b) By tracking the decrease in SFVS signal with time as flip-flop occurs, it is possible to determine the rate constant for lipid flip-flop.

The rate expression relating the SFVS intensity of the $\text{CH}_3 \nu_s$, (I_{CH_3}), to the rate constant for flip-flop (k) is given by: (Liu and Conboy, 2005b)

$$I_{\text{CH}_3}(t) = I_{\text{Max}} e^{(-4kt)} + I_0 \quad (7)$$

where I_{Max} represents the initial maximum intensity and I_0 represents the baseline offset to the signal. Full derivation of the rate expression is given elsewhere. (Liu and Conboy, 2004a; Liu and Conboy, 2005b) SFVS signal decays are collected for asymmetric lipid bilayers at a number of temperatures and pressures and the rate constants for decay determined by fitting with Equation 7.

For the study described here, this approach is used to measure the rate of lipid flip-flop as a function of peptide composition and concentration in the membrane under a variety of conditions. The dependence of the flip-flop rate on temperature and pressure is used to characterize the thermodynamic barrier to flip-flop using basic transition state theory, revealing

new information about the physical and chemical factors which govern lipid translocation in the presence of transmembrane peptides.

Thermodynamics of Lipid Flip-flop

By characterizing the energetic barrier to flip-flop, it is possible to gain insight into the mechanism of transmembrane lipid diffusion and how the presence of peptides alters the transition state energy landscape. We have presented a complete description of the activation thermodynamics for DSPC flip-flop elsewhere, and details of the approach may be found in reference (Anglin et al., 2008). Briefly, by collecting both pressure and temperature dependent kinetics of lipid flip-flop, it is possible to accurately determine the complete set of thermodynamic parameters that govern the net free energy barrier ($\Delta G^\ddagger$). Such an analysis provides the Arrhenius activation energy (E_a), the entropy of activation ($\Delta S^\ddagger$) and the area of activation ($\Delta a^\ddagger$), which is related to the work required to reach the transition state ($\Pi \Delta a^\ddagger$). The influence of each of these terms on the net free energy barrier is given by:

$$\Delta G^\ddagger = E_a + \Pi \Delta a^\ddagger - RT - T \Delta S^\ddagger. \quad (8)$$

Equation 8 is related to the rate constant for flip-flop (k) by:

$$k = \frac{k_B T}{h} e^{\left(\frac{-\Delta G^\ddagger}{RT} \right)} \quad (9)$$

where k_B is Boltzmann's constant, h is Planck's constant, and R is the gas constant.

The free energy barrier may be analyzed in terms of both an entropic contribution given as $-T \Delta S^\ddagger$, and an enthalpic contribution given as:

$$\Delta H^\ddagger = E_a + \Pi \Delta a^\ddagger - RT. \quad (10)$$

The Arrhenius activation energy, E_a , is determined from an Arrhenius plot of the data, where $\ln(k)$ is plotted as a function of inverse temperature as:

$$\ln(k) = \ln(A) - \frac{E_a}{RT}. \quad (11)$$

The Arrhenius pre-exponential factor (A) may also be determined from the intercept of the Arrhenius plot, and is essential to the determination of the activation entropy, which is discussed below. (Anglin et al., 2008)

For flip-flop at a given temperature, the change in rate as a function of lateral surface pressure may be used to calculate the area of activation ($\Delta a^\ddagger$) according to: (Anglin and Conboy, 2008b)

$$-RT \left(\frac{\partial \ln(k)}{\partial \Pi} \right)_T = \Delta a^\ddagger. \quad (12)$$

The activation area represents the increase in mean molecular area between the ground state and transition state geometry for a lipid undergoing translocation. The energy, as work, required

for this local expansion of the membrane is given as $\Pi\Delta a^\ddagger$. The calculated activation area and Arrhenius pre-exponential factor may further be used to determine the activation entropy for flip-flop according to: (Anglin et al., 2008)

$$\Delta S^\ddagger = R \left(\ln \left(\frac{Ah}{k_B T} \right) + \left(\frac{\Pi\Delta a^\ddagger}{RT} \right) - 1 \right) \quad (13)$$

where R is the gas constant, k_B is Boltzman's constant, and h is Planck's constant. The contribution of the activation entropy to the total free energy barrier is given as $-T\Delta S^\ddagger$.

The alteration of the activation thermodynamics for DSPC flip-flop upon incorporation of WALP₂₃ or melittin were used here to provide important clues about the mechanism of lipid flip-flop in the presence of these transmembrane peptides.

Transmembrane Peptides WALP₂₃ and Melittin

WALP₂₃ is a 2.48kDa model hydrophobic peptide with the sequence Gly-Trp-Trp-Leu-Ala-Leu-Ala-Leu-Ala-Leu-Ala-Leu-Ala-Leu-Ala-Leu-Trp-Trp-Ala. (Killian et al., 1996; Planque et al., 1999) Melittin is a 2.85 kDa hemolytic peptide isolated from bee venom with the sequence Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH₂. (Habermann and Jentsch, 1967) Both WALP₂₃ and melittin form stable α -helices when intercalated into lipid bilayers (Conboy and Kriech, 2003; Killian et al., 1996; Petrache et al., 2002; Planque et al., 1999; Smith et al., 1994; Strandberg et al., 2004; Toraya et al., 2004; Yang et al., 2001), and are similar in size. WALP₂₃ is one of a family of ideal hydrophobic peptides, which are often used in studies of hydrophobic mismatch by pairing peptides of differing helical length to bilayers of varying thickness. (Killian et al., 1996; Petrache et al., 2002; Planque et al., 1999; Strandberg et al., 2004) The 23-residue WALP peptide was selected in this case because it is well matched to the thickness of a *gel* phase DSPC bilayer. (Killian et al., 1996) WALP₂₃ is known to adopt a stable transmembrane orientation perpendicular to the membrane surface, with the tryptophan residues anchoring the peptide terminus in the interfacial region. (Killian et al., 1996; Planque et al., 1999; Strandberg et al., 2004)

Melittin is known to adopt an α -helical structure with a slight kink between α -helical segments, due to the proline at position 14. (Smith et al., 1994) Like WALP₂₃, melittin may adopt a transmembrane orientation (Conboy and Kriech, 2003; Smith et al., 1994; Weaver et al., 1992; Yang et al., 2001), but can also form surface associated α -helices which are parallel to the membrane surface. (Chen et al., 2007a; Chen et al., 2007b; Sharon et al., 1999) The orientation that is determined depends strongly upon the method of preparation and degree of hydration of the sample. A recent SFVS study utilizing PSLBs has successfully shown that bilayers exposed to melittin in solution contain both surface associated and transmembrane melittin helices, in a roughly 3-1 ratio. (Chen et al., 2007b) However, in cases where the lipid and peptide are pre-mixed in suitable solvent prior to preparation of the mixed bilayer systems the orientation of peptide is primarily perpendicular to the membrane surface. (Conboy and Kriech, 2003; Smith et al., 1994; Toraya et al., 2004; Yang et al., 2001) Our method of preparation is consistent with the latter case, and should produce bilayers containing membrane bound melittin in a transmembrane orientation.

As discussed above, one of the significant differences between WALP₂₃ and melittin is the relative hydrophobicity or hydrophilicity of the residues that comprise each peptide. While WALP₂₃ consists entirely of hydrophobic residues, melittin contains ~10 polar residues and bears a net charge of +5 to +6 under physiological conditions. (Sessa et al., 1969; Strömstedt et al., 2007) The majority of the polar residues in melittin are located near the C terminus.

(Sessa et al., 1969; Smith et al., 1994) Thus the peptide consists of two linked segments, where one is highly charged and the other is relatively non-polar. Melittin and WALP₂₃ are ideal for evaluation of the effect of peptide polarity on the capacity for facilitation of lipid flip-flop, as they are similar in size and secondary structure but have very different surface charge and hydrophobicity in neutral solutions.

Materials and Methods

Synthetic melittin was obtained from Sigma-Adrich (St. Louis, MO) and was used without further purification. In these studies, synthetic melittin was used in order to ensure the absence of phospholipase A₂ or other contaminants commonly encountered in natural melittin isolated from bee venom. WALP₂₃ (95% purity) was ordered from Sigma-Genosys (The Woodlands, TX), and used as received. Spectral grade chloroform and methanol were obtained from Mallinckrodt (Phillipsburg, NJ) and Sigma-Adrich (St. Louis, MO) respectively and were used as received. Nanopure water (Barnstead Thermolyne, Dubuque, IA) with a minimum resistivity of 18.2 MΩ-cm was used for all sample preparation. D₂O was purchased from Cambridge Isotope laboratories (Andover, MA) and passed through a 0.20μm syringe filter prior to use.

DSPC, DSPC-d₇₀, and 1,2-disearoyl-D70-sn-glycero-3-phosphocholine-1,1,2,2-D₄-N,N,N-trimethyl-D9 (DSPC-d₈₃) were purchased from Avanti Polar Lipids (Alabaster, AL). All lipid and protein samples were dissolved in 65:35:8 (CHCl₃:MeOH:H₂O) to a concentration of 1mg/mL. The peptide and lipid solutions were mixed to achieve peptide:lipid molar ratios of 1:99 and 2:98 for WALP₂₃ and 0.5:99.5 and 1:99 for melittin. These peptide:lipid ratios are in keeping with other literature on the promotion of lipid flip-flop by transmembrane peptides (0.25-3 mol% for hydrophobic peptides and 0.001-3mol% for melittin)(Anglin et al., 2007; Fattal et al., 1994; Kol et al., 2001; Kol et al., 2003), permitting direct and relevant comparison of this work to those studies. It is also worth noting that the melittin-lipid ratios used here are at or below the threshold for formation of stable pores (peptide to lipid ratios of ~ 1:100 - 1:70) as reported for phosphocholine derived bilayers.(Bogaart et al., 2008; Lee et al., 2008; Matsuzaki et al., 1997) However, the pore formation threshold is known to vary significantly with membrane composition and phase(Bogaart et al., 2008; Strömstedt et al., 2007).

Hemicylindrical fused silica prisms were utilized as the substrates for bilayer preparation. Prior to bilayer deposition, the prisms were kept in a 70% H₂SO₄:30% H₂O₂ solution (Pirahna solution), for several hours or overnight. (Note: This solution is a strong oxidizer and reacts violently with organic materials. Appropriate safety precautions such as acid resistant gloves and proper shielding should be used during handling.) The prisms were then rinsed with copious amounts of Nanopure water (minimum resistivity 18.2MΩcm), dried, and plasma cleaned in an argon plasma for a minimum of two minutes.

PSLBs were constructed by spreading the lipid:peptide solutions at the air-water interface of a Langmuir-Blodgett trough (KSV Instruments; Helsinki, Finland). After allowing for the evaporation of solvent (typically 15 minutes) the barriers of the trough were compressed to the desired surface pressure, typically 30mN/m, and maintained at that pressure for a minimum of 10 minutes to allow for further equilibration of the monolayer. After lipid equilibration, the prism was slowly withdrawn vertically through the interface, resulting in deposition of a monolayer of the phospholipid-peptide mixture (LB layer). A new monolayer of the phospholipid-peptide mixture was then prepared and compressed at the trough interface as before. After allowing for equilibration, the second monolayer was assembled by pushing the prism through the interface horizontally (LS deposition), thereby completing assembly of the bilayer. The sample was maintained in an aqueous environment from this point forward.

Asymmetric bilayers were prepared for study by SFVS. This entails preparing a LB layer consisting of a DSPC+peptide monolayer, followed by preparation of the LS layer consisting of deuterated DSPC-d₇₀+peptide. In order to ensure that the order of preparation did not alter the observed kinetic behavior, asymmetric films were prepared in which DSPC was placed in the distal layer and DSPC-d₇₀ in the proximal. Changing the deposition order for the bilayers allows the rate of flip-flop away from and toward the substrate to be measured independently. Any differences in the rates observed for these two deposition orders would be indicative of a perturbation by, or interaction with, the substrate.

PSLBs were assembled into a custom Teflon flow cell and flushed with D₂O in order to avoid spectral interference between the lipid vibrational modes (2800 – 3000 cm⁻¹) and the O-H stretching modes of water at the interface. SFVS spectra were collected by scanning the input infrared frequencies in 2 cm⁻¹ steps with the SFVS intensity integrated at each step for 3 seconds (30 laser pulses). The instrument was then returned to 2875 cm⁻¹ in order to observe the change in the terminal methyl symmetric stretch (CH₃ ν_s) intensity over time so that the kinetics of lipid flip-flop could be determined.

Temperature control for the sample cell was provided by means of a thermoelectric peltier heating unit (TE Technologies, Traverse City, MI.; TE-127-1.0-2.5, TE-36-25-RS232) and circulating water bath (Thermo Haake, P1-C35P) incorporated into the back of the Teflon sample cell. The heating unit was controlled by feedback from a sealed thermistor (TE Technologies, PN: MP 2444) located in the solution of the sample cell in close proximity to the prism surface. This allows for rapid heating and stable temperature control after the set temperature was reached. The SFVS signal was monitored over the course of the heating step, however the data obtained during the initial heating prior to reaching stable temperature was not used in the determination of the rate of translocation. Typically, stable temperatures were reached within 4 minutes and could be reliably maintained for hours. This initial time period required to reach a stable temperature limits the range of kinetics which can be measured by this method, as the decay of signal must occur on a time scale longer than the thermal equilibration period in order to be accurately measured.

Attenuated total-internal reflection Fourier-transform infrared (ATR-FTIR) spectroscopy was used to verify incorporation of the peptides into the bilayer. For the ATR-FTIR studies a germanium ATR crystal was cleaned by repeated washings with isopropanol and methanol, followed by thorough rinsing with Nanopure H₂O. The germanium crystal was then plasma cleaned in an argon plasma for two minutes, followed by annealing at 300-310°C for thirty minutes in air. The annealing step ensures the formation of a stable oxide layer on the germanium (Hayashi et al., 1990), thereby providing a suitable hydrophilic surface for the deposition of the bilayer. (Cha et al., 2006)

Following preparation of the ATR crystal, a symmetric bilayer was prepared on the surface by the LB/LS method as described above. A proteated matrix was used for both the LB and LS depositions. After deposition of the bilayer, the sample was carefully transferred to a customized ATR sample holder (Pike technologies, Madison, WI) which was subsequently flushed with D₂O. The cell was then placed into a Perkin Elmer Spectrum One FTIR spectrometer equipped with a dry nitrogen purge and a liquid nitrogen cooled MCT detector. A minimum of 256 scans were collected and averaged for each sample and solvent combination. Several (typically 3) of such spectra were collected and averaged for each peptide-phospholipid bilayer sample. The spectra were baseline corrected for averaging and presentation.

Results and discussion

Spectral characterization of WALP₂₃+DSPC and Melittin+DSPC bilayers

SFVS spectra were collected over the C-H stretching region from 2750 cm⁻¹ to 3100 cm⁻¹ for DSPC bilayers containing WALP₂₃ or melittin. Comparison of these spectra with those from pure DSPC bilayers provides a measure of the contribution to the SFVS signal from the peptide alone. It is important to characterize the spectral contributions of the peptide prior to measuring the flip-flop kinetics, as interference between the peptide and lipid would seriously complicate such measurements and might require the use of a more complex fitting expression.

The SFVS spectrum of an asymmetric DSPC:DSPC-d₇₀ bilayer containing 1 mol% WALP₂₃ is shown in Figure 1A (solid black). This spectrum is quite similar to that obtained from an asymmetric DSPC:DSPC-d₇₀ bilayer without peptide (Figure 1A, black dash). Symmetric bilayers of 1:1 DSPC:DSPC-d₇₀ were also prepared both with and without 1% WALP₂₃ and are presented in Figure 1A. In the absence of peptide, there is complete cancellation of the vibrational contributions from DSPC and no spectral features are observed. As the symmetric bilayers containing 1% WALP₂₃ or melittin have little or no spectral features over the frequency range examined, this suggests that there is little contribution to the SFVS spectrum from the peptide. Upon closer inspection, a small peak near 2950 cm⁻¹ can be observed for the symmetric bilayers containing the peptide that is not observed in the absence of WALP₂₃, yet this peak is extremely small in relation to the intensity obtained from an asymmetric phospholipid film. However, overlap of the C-H vibrational frequencies for the peptide and lipids may disguise any contribution from WALP₂₃, and spectral interference between the lipid and peptide may also contribute to the lack of signal. In order to better evaluate the contribution from the peptide alone, SFVS spectra were collected for bilayers consisting of completely perdeuterated DSPC-d₈₃ containing 2% WALP₂₃. The perdeuterated lipids have no vibrational resonances in the spectral region examined, therefore any signal observed can be attributed directly to the peptide. The spectrum of WALP₂₃ in DSPC-d₈₃ is shown in Figure 1A (Inset). A small peak is observed near 2975 cm⁻¹, however the response from the peptide is negligible in comparison to the signal from the asymmetric lipid bilayer. From this data, it can be concluded that the peptide has little effect on the SFVS signal and will not interfere with the measurement of the DSPC flip-flop kinetics at 2875 cm⁻¹. Similar results also are obtained for DSPC bilayers containing melittin, as seen in Figure 1B which shows a representative SFVS spectrum for an asymmetric DSPC:DSPC-d₇₀ bilayer containing 1.0% melittin (black) and a symmetric 1:1 DSPC:DSPC-d₇₀ bilayer containing 1.0% melittin (solid gray). No significant differences are observed between these spectra and those for pure DSPC. Figure 1B also shows the spectrum of a symmetric DSPC-d₇₀ bilayer containing 1% melittin (gray dash). As for WALP₂₃, the response from melittin in the deuterated bilayer was negligible, indicating that melittin does not interfere with the SFVS response of the lipids and that the SFVS signal decays can be directly related to the kinetics of flip-flop according to Equation 7.

The lack of SFVS intensity from the peptide may result from one of two possible scenarios: either the peptides are present in the bilayer in an arrangement that leads to cancellation of transition dipoles with opposite orientation or the peptides are not successfully incorporated into the bilayer at all. It is possible to determine if the peptides are indeed present in the bilayer by using a linear spectroscopic method which is not affected by the orientation or symmetry of the sample. Accordingly, ATR-FTIR was used to detect the presence of peptide in PSLBs prepared on germanium substrates. An ATR-FTIR spectrum of a symmetric DSPC bilayer containing 2 mol% WALP₂₃ is shown in Figure 2B. The CH₂ scissoring and lipid carbonyl stretching modes are visible at 1462 cm⁻¹ and 1734 cm⁻¹ respectively, confirming the presence of the lipid bilayer. (Tamm and Tatulian, 1997) An additional peak is observed at 1650 cm⁻¹ which corresponds to the amide-I vibrational mode of WALP₂₃. The observation of the amide

I band indicates that the peptide is present in the lipid bilayer. (Bellamy, 1975) It should also be noted that the frequency of the amine-I band is sensitive to the secondary structure of the peptide. The frequency observed here (1650 cm^{-1}) is consistent with an α -helical structure for WALP₂₃. (Bellamy, 1975) Similar results are observed for DSPC bilayers containing melittin. The ATR-FTIR spectrum of a 1% melittin DSPC bilayer is shown in Figure 2C. The amide I absorbance visible in the spectrum (1650 cm^{-1}) indicates the presence of the peptide in the bilayer film and is consistent with previous ATR-FTIR results. (Axelsen et al., 1995; Frey and Tamm, 1991; Sharon et al., 1999) As with WALP₂₃, the frequency of the amide-I vibrational mode for melittin is consistent with an α -helical secondary structure. (Bellamy, 1975; Chen et al., 2007b; Flach et al., 1996; Sharon et al., 1999) For comparison, the ATR-FTIR spectrum of a DSPC bilayer without peptide is shown in Figure 2A, in which case the amide band at 1650 cm^{-1} is clearly absent. The ATR data provide strong evidence that WALP₂₃ and melittin are successfully incorporated into the bilayer during the LB/LS bilayer preparation and that they adopt a primarily α -helical structure in the bilayer. It can therefore be inferred that the low SFVS signal is not due to absence of the peptide, but most likely results from a symmetric arrangement of peptides that leads to effective cancellation of the vibrational transition dipoles for the ensemble.

The most probable structure which is consistent with the ATR and SFVS spectral results is a symmetric arrangement of α -helices with equal numbers oriented toward the proximal or distal leaflets (Figure 3). Such an arrangement would lead to effective cancellation of the SFVS signal, due to the symmetric arrangement of peptides with respect to the plane of the bilayer and is also consistent with the secondary structure of the peptides determined from the ATR data. This arrangement of peptides might be expected, *a-priori*, based on the method of preparation used to create the peptide doped lipid bilayers. For peptides such as WALP₂₃, which are symmetric, there is little to suggest that the C- or N-terminus is preferentially directed toward or away from the water interface during LB or LS deposition. Thus the distribution of WALP₂₃ peptides in the resulting bilayer should be random with respect to which peptide terminus resides on the proximal or distal sides of the bilayer. It is known that this peptide prefers to adopt a transmembrane orientation (Killian et al., 1996), and is therefore likely to exist in the bilayer as transmembrane helices with random “up” or “down” orientations with respect to the substrate. For melittin, the C-terminus is slightly more polar than the N-terminus, and may preferentially orient toward the lipid-water interface of the LB trough during deposition. However, if the peptide is incorporated into the LB layer with a preferred orientation, this effect will be negated upon deposition of the second leaflet of the bilayer, as the LS layer is deposited in the opposite orientation. Thus, even if the peptide adopted a nonrandom distribution in the monolayer, the resulting bilayer would still contain equal numbers of peptide oriented “up” (from the LB deposition) and “down” (from the LS deposition). In either case, a symmetric arrangement of peptides would result in the bilayer. For the case of melittin, it is also possible to form a surface bound state in which the peptide lies parallel to the bilayer plane rather than inserted into the bilayer along the surface normal. (Bogaart et al., 2008; Chen et al., 2007a; Chen et al., 2007b) Such a structure is generally found for systems where melittin is introduced into solution and allowed to adsorb to the membrane. Membranes which are prepared by direct incorporation of melittin during the LB/LS deposition (such as those employed here) most often result in only the transbilayer arrangement (Smith et al., 1994; Toraya et al., 2004; Yang et al., 2001), as noted above. Chen et al. examined melittin binding to anionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) bilayers and noted that the surface bound state of melittin disrupts the lipid packing and produces an increase in the SFVS response of the lipids in the bilayer due to symmetry breaking. (Chen et al., 2007a) This effect was observed in symmetric bilayers and isotopically asymmetric bilayers when melittin was bound parallel to the surface. The fact that such behavior is not observed in the present study suggests that the melittin is in the more stable transmembrane arrangement. Although the absolute arrangement of the peptides cannot be deduced from this data alone, the

structures proposed here are consistent with both the ATR and SFVS spectral data shown above. For the purposes of our study, the more important point is that there is little to no spectral interference between the lipids and peptides. Because the peptides appear to be spectroscopically silent by SFVS, the lipid vibrational resonances may be directly utilized to track the membrane asymmetry and measure DSPC flip-flop kinetics without complication due to peptide spectral contributions.

Perturbation of DSPC Flip-flop Kinetics by WALP₂₃ and Melittin

The kinetics of DSPC flip-flop were measured in the presence of WALP₂₃ and melittin in order to characterize the ability of these peptides to facilitate and enhance spontaneous flip-flop. The purpose of these measurements was twofold. First, the kinetics of DSPC flip-flop was studied as a function of peptide concentration in order to determine if WALP₂₃ and melittin perturb the kinetics of lipid flip-flop in a concentration dependent manner. This provides a measure of the “potency” of the peptide as a facilitator of lipid flip-flop. Second, the kinetics of DSPC flip-flop were measured as a function of membrane lateral pressure and temperature for bilayers containing WALP₂₃ or melittin at the same lipid:peptide mole ratio (1%), in order to fully characterize the thermodynamic barrier to DSPC flip-flop in the presence of these two peptides. The thermodynamic data offer insight into the nature of the interactions between lipid and peptide underlying the observed changes in the kinetics of flip-flop. For this step, it is important that identical concentrations be utilized so that the relative effect of the peptides is examined under conditions where peptide identity is the only variable. This data may then be utilized to evaluate the different mechanisms by which WALP₂₃ and melittin influence the rate of DSPC flip-flop.

Flip-Flop Kinetics: Concentration Dependence

SFVS decays for 30mN/m DSPC bilayers containing 1% and 2% WALP₂₃ were measured at temperatures ranging from 35.0 to 46.0°C. Representative decays are shown in Figure 4A as a function of peptide concentration. In all cases, the data were well fit using Equation 7. From the fit to the decays, the rate constant k , and the half-life for flip-flop, given as $t_{(1/2)} = \ln(2)/2k$, were determined and are summarized in Table 1. For bilayers containing 1% WALP₂₃, flip-flop was more rapid than is found for pure DSPC. At 46°C, the half life for flip-flop in the presence of 1% WALP₂₃ was only 19.40 ± 0.02 minutes, while the half-life of pure DSPC is at this same temperature is calculated to be 109 ± 12 minutes. (Liu and Conboy, 2005b) The calculated SFVS decay for a pure DSPC bilayer at 46.0 °C is also shown in Figure 4A. Increasing the concentration of WALP₂₃ to 2% led to further increases in the rate of flip-flop relative to bilayers containing 1% WALP₂₃, with a half-life for DSPC flip-flop of 8.26 ± 0.05 min at 46.0°C. The difference in DSPC flip-flop kinetics for bilayers containing 1% WALP₂₃ and 2% WALP₂₃ was more pronounced at higher temperatures and diminished as the temperature decreased, although bilayers containing 2% WALP₂₃ had consistently higher flip-flop rates over the temperature range examined. The variation in kinetic behavior for DSPC bilayers containing 0%, 1%, and 2% WALP₂₃ is illustrated graphically in the Arrhenius plots shown in Figure 5A.

We also determined the effect of melittin on the flip-flop kinetics of DSPC. Decays were collected for 30mN/m DSPC bilayers containing 0.5% or 1% melittin at temperatures ranging from 25.0°C to 40.0°C. Representative decays for these bilayers are presented in Figure 4B as a function of temperature and peptide concentration. The decays were well fit by Equation 7 and the rate constants and half-life for DSPC flip-flop in the presence of melittin are summarized in Table 2. Melittin is found to facilitate DSPC flip-flop in a concentration dependent manner, consistent with other literature results. (Fattal et al., 1994) The half life for DSPC flip-flop at 32.0°C is 86.1 ± 0.2 minutes in the presence of 0.5% melittin and 14.1 ± 0.1 minutes in the presence of 1% melittin, compared to a calculated half life of 66 ± 6 hours for

pure DSPC. The Arrhenius plots for flip-flop of DSPC in the presence of 0%, 0.5%, and 1% melittin are provided in Figure 5B in order to graphically illustrate the effect of melittin on DSPC flip-flop. It is also worth noting that the effect of melittin on the rate of DSPC flip-flop is considerably greater than that of WALP₂₃. At 40.0°C, a melittin concentration of 0.5% decreased the half-life for flip-flop to 8.69 ± 0.03 minutes, while a concentration of 2% WALP₂₃, the highest studied, has a longer half-life of 45.9 ± 0.3 minutes (The half-life for pure DSPC is 8.2 ± 0.7 hours at the same temperature).

Direct comparison of the effect of WALP₂₃ and melittin at the same concentration (1%) requires extrapolation of the data, as DSPC flip-flop in the presence of 1% melittin becomes too rapid to directly measure at the temperatures used in the study of DSPC + 1% WALP₂₃. The highest temperature examined for the 30 mN/m DSPC + 1% melittin bilayers was 34.0 °C. At this temperature, the half-life for DSPC flip-flop in the presence of 1% melittin is a mere 9.0 ± 0.1 minutes. For comparison, the half-life for flip-flop of DSPC in the presence of 1% WALP₂₃ is calculated to be 5.7 hours and that for pure DSPC is calculated to be 1.6 ± 0.1 days when extrapolated to this lower temperature. The Arrhenius plots for DSPC flip-flop containing 1% WALP₂₃ and 1% melittin are presented in Figure 6, along with the data for pure DSPC, in order to illustrate the relative effects of these peptides on DSPC flip-flop. Examination of the data in Figure 6 reveals that DSPC flip-flop has a greater sensitivity to melittin concentration than to WALP₂₃ concentration. This effect is readily seen in the Arrhenius plots for each species (Figure 5), where the data for 0.5% and 1% melittin are shifted well away from one another while 1% and 2% WALP₂₃ differ only slightly.

It is also important to note that the order of deposition does not appear to affect the measured rate of flip-flop. Bilayers containing 0.5% melittin, 1% melittin, or 1% WALP₂₃ were prepared from DSPC:DSPC-d₇₀ or DSPC-d₇₀:DSPC (LB layer:LS layer), and the kinetics of decay measured for each deposition order. The rate constants for each deposition order are summarized in Figures 5A and 5B, where the DSPC:DSPC-d₇₀ bilayers are shown as solid symbols and the DSPC-d₇₀:DSPC bilayers as open symbols. Bilayers prepared with the proteated lipids in the distal leaflet are consistent with the trend observed for those with the proteated lipids in the proximal leaflet. This finding is consistent with previous work from our lab, where we have shown that the rate of flip-flop is independent of deposition order for a wide range of membrane compositions. (Anglin and Conboy, 2008a; Anglin and Conboy, 2008b; Anglin et al., 2008; Liu and Conboy, 2005b)

Flip-Flop Kinetics: Pressure Dependence

The lateral pressure dependence of DSPC flip-flop kinetics was also investigated for the peptide-lipid systems. Knowledge of the lateral pressure dependence for the rate of flip-flop allows for calculation of the area of activation and activation entropy, thereby providing a detailed description of the activation thermodynamics for phospholipid flipping in the presence of a peptide. In the preceding section, a pressure of 30mN/m was used for both WALP₂₃- and melittin-containing bilayers, as this pressure is thought to be most representative of biological systems. (Marsh, 1996) In this portion of the study, bilayers containing 1% WALP₂₃ were prepared at lower pressure, as flip-flop is faster for these systems at lower pressure. This permitted study of WALP₂₃ facilitated flip-flop at lower temperatures closer to the physiologically relevant value of 37.0°C. However, bilayers containing 1% melittin could not be studied at lower pressure as flip-flop in these bilayers is already quite rapid. Further acceleration of the rate would severely limit the temperature range over which these bilayers could be reliably studied. By instead choosing a higher pressure for the bilayers containing 1% melittin this problem can be avoided, as the rate of flip-flop is reduced at higher membrane pressures. This allows the pressure dependent kinetics of DSPC flip-flop to be studied in the presence of melittin over a more suitable temperature range while providing sufficient variation

in pressure to accurately determine the pressure dependent thermodynamic variables. As noted in previous publications (Anglin and Conboy, 2008b), the absolute choice of pressure does not affect the calculation of the pressure dependent transition state thermodynamics we wish to study, as the activation area depends only the relative difference in pressure and the respective difference in rates.

DSPC bilayers containing 1% WALP₂₃ were studied at 20mN/m, over a temperature range from 34.0 to 41.0°C. A strong pressure dependence was observed for the rate of DSPC flip-flop in bilayers containing 1% WALP₂₃. The half-life for flipping at 36°C decreased from 195.0 ± 0.1 minutes to 32.1 ± 0.1 minutes for deposition pressures of 30mN/m and 20mN/m respectively Figure 7 (A). DSPC bilayers containing 1% melittin were studied at 40mN/m for temperatures ranging from 30.0 to 37.0°C. Representative decays measured at 30.0°C are shown in Figure 7B for bilayers containing 1% melittin prepared at 30mN/m and 40mN/m. Slower flip-flop is clearly observed for the bilayers prepared at 40mN/m, with the half-life for flip-flop at 30.0°C increasing from 25.4 ± 0.1 min to 160.1 ± 0.4 min for bilayers at 30mN/m and 40mN/m respectively. The pressure dependent kinetics of flip-flop for DSPC+WALP₂₃ and DSPC+melittin bilayers are summarized in Tables 3 and 4 respectively. Taken alone, the kinetic data for 20mN/m and 40mN/m bilayers would not provide much insight into the facilitation of flip-flop by WALP₂₃ or melittin. However, this data is exceedingly useful when used in conjunction with the 30mN/m data to evaluate the pressure-dependent transition state thermodynamics, which are considered below.

Thermodynamics of WALP₂₃ and Melittin Facilitated DSPC Flip-Flop

The pressure and temperature dependent kinetic data presented above can be used to characterize the transition state thermodynamics for DSPC flip-flop in the presence of WALP₂₃ or melittin. The contributions to the total free energy barrier from enthalpy, entropy, and work can be evaluated individually for each bilayer-peptide combination. These provide essential clues regarding the chemical and physical interactions between the lipid and peptide which ultimately lead to a reduction in the flip-flop free energy barrier.

Activation Area and Work Term—The activation area is determined from the pressure dependence of the flip-flop rate, and is indicative of the degree of spatial rearrangement required for a lipid molecule as it moves from the ground state to the flip-flop transition state. Specifically, the activation area provides a measure of the local expansion of the film required to accommodate a lipid undergoing flip-flop. From the data presented in Table 1, we determine an activation area of $\Delta a^\ddagger = 74 \pm 4 \text{ \AA}^2/\text{molecule}$ at 37.0°C for DSPC bilayers containing 1% WALP₂₃. The corresponding activation area for pure DSPC bilayers is $61 \pm 5 \text{ \AA}^2/\text{molecule}$ at the same temperature. (Anglin et al., 2008) This indicates that the area change involved in reaching the transition state for flip-flop is greater in the presence of WALP₂₃ than it is in a film of pure DSPC. It is not possible, however, to determine from the thermodynamic data if the increase in $\Delta a^\ddagger$ is due to a decreased area per molecule in the ground state (local ordering induced by the peptide) or an increased area per lipid in the activated state. Intuitively, ordering of the ground state is not likely in this case. The lipid molecules in pure *gel* phase DSPC bilayers are highly ordered, and the presence of WALP₂₃ is not likely to increase this ordering beyond the *all-trans* structure that dominates the pure bilayer. Molecular dynamics simulation of lipid-WALP systems suggest that the bulky tryptophan residues on the peptide termini disorder the membrane at the lipid-water interface due to molecular crowding. (Petrache et al., 2002) In light of these facts, it would seem more likely that the increase in activation area does not correspond to an ordered ground state, but to a larger total transition state area for DSPC+1% WALP₂₃ than for pure DSPC. Unlike WALP₂₃, melittin does not appear to significantly alter the activation area for DSPC flip-flop. At 37.0 °C the activation area for DSPC bilayers containing 1% melittin is $57 \pm 1 \text{ \AA}^2/\text{molecule}$, compared to $61 \pm 5 \text{ \AA}^2/\text{molecule}$ for the pure

DSPC bilayer.(Anglin et al., 2008) This suggests that molecular packing or geometric constraints are not a significant factor in the facilitation of DSPC flip-flop by melittin.

The activation area may be used to calculate the work required for a lipid to reach the transition state. At 37.0°C and 30mN/m the work term, $\Pi\Delta a^\ddagger$, for DSPC flip-flop in the presence of WALP₂₃ is 13.4 ± 0.7 kJ/mol. This is a slightly higher work term than is observed for pure DSPC (11.0 ± 0.9 kJ/mol).(Anglin et al., 2008) The higher work term simply represents the additional energetic cost associated with the larger expansion required to reach the transition state (larger $\Delta a^\ddagger$) when WALP₂₃ is present in the bilayer. For DSPC bilayers containing melittin, the work term is calculated to be 10.3 ± 0.2 kJ/mol at 37.0°C, similar to the value determined for pure DSPC. The activation area and work terms suggest that perturbation of local molecular packing may be an important aspect of the facilitation of DSPC flip-flop by WALP₂₃, but is considerably less important for melittin. However, it should also be noted that the elevated work term for WALP₂₃ represents an increase in the barrier to flip-flop. Taken alone, this would lead to a decrease in flip-flop kinetics. Yet, DSPC flip-flop is more rapid in the presence of WALP₂₃, indicating that the total energy barrier is actually decreased by addition of the peptide. Therefore, both WALP₂₃ and melittin must also alter the activation enthalpy or activation entropy for flip-flop in such a way as to reduce the total energy barrier and enhance the rate of flipping. These additional contributions to the energy barrier are considered in turn.

Activation Enthalpy—The activation enthalpy, $\Delta H^\ddagger$, is the largest contributor to the total free energy barrier for flip-flop of pure DSPC.(Anglin et al., 2008) We can examine the activation enthalpy for DSPC flip-flop in the presence of WALP₂₃ and melittin in order to determine to what extent the facilitation of flip-flop by these peptides is driven by changes in the enthalpic configuration of the flip-flop ground and transition states. Numerous enthalpic interactions such as lipid-solvent hydrogen bonding, electrostatic interactions, and van der Waals interactions are present in the ground state arrangement of the bilayer. Many of these favorable stabilizing interactions are lost or diminished as the lipid molecule adopts the transition state configuration, leading to a large enthalpic barrier for the process. By evaluating $\Delta H^\ddagger$ in the presence of WALP₂₃ and melittin, it is possible to determine whether the peptides provide alternative stabilizing interactions or otherwise reduce the enthalpic barrier and thereby increase the rate of flip-flop. The total activation enthalpy can be calculated from the work term and the Arrhenius activation energy, E_a , according to Equation 10.

From the Arrhenius plots shown in Figure 6, we calculate an E_a of 196 ± 1 kJ/mol for 30mN/m DSPC+ 1% WALP₂₃, which is somewhat lower than the value of 228 ± 28 kJ/mol measured for pure DSPC under the same conditions. We only report the E_a value for bilayers prepared at 30mN/m, as this is considered to be the most physiologically relevant pressure. For 30mN/m DSPC bilayers containing 1% melittin, we measure an E_a of 232.3 ± 1.2 kJ/mol using the data shown in Figure 6. The Arrhenius activation energy in the presence of melittin is indistinguishable from that for pure DSPC (228 ± 28 kJ/mol).

The Arrhenius activation energy and activation area were used to calculate the total activation enthalpy. For DSPC+ 1% WALP₂₃ at 30mN/m and 37.0°C, $\Delta H^\ddagger$ is 207 ± 2 kJ/mol, while for DSPC+1% melittin $\Delta H^\ddagger$ is 240 ± 2 kJ/mol. For comparison, the activation enthalpy for pure DSPC is 237 ± 28 kJ/mol under the same conditions. The nearly identical $\Delta H^\ddagger$ values for flip-flop of DSPC and DSPC containing 1% melittin indicates that the charged residues of the peptide do not act to reduce the enthalpic penalty of moving the polar headgroup through the hydrocarbon membrane core. This result is somewhat surprising, as the previously proposed mechanisms for melittin facilitated flip-flop imply a significant reduction in the enthalpic barrier to flip-flop.(Fattal et al., 1994) Our finding indicates that the lipids headgroup must still pass through the hydrophobic core without the benefit of low-energy stabilizing interactions

with water or the polar residues of the peptide. This would argue against the presence of any interactions between the lipid headgroup and the polar residues of the peptide, which could stabilize the transition state and reduce the enthalpic barrier to flip-flop. Alternatively, such stabilizing interactions may exist but be limited to the C terminus of the peptide where the majority of the charged residues are located. In such a scenario, association to the polar residues of the peptide would facilitate only partial penetration of the lipid headgroup into the membrane core, effectively reducing the thickness of the non-polar region of the bilayer. In this case, the enthalpic barrier related to forcing the polar headgroup into the hydrocarbon core would still be present; however the activation entropy would be significantly affected.

Unlike melittin, WALP₂₃ does significantly alter the activation enthalpy. The decrease in $\Delta H^\ddagger$ induced by WALP₂₃ indicates that the peptide has somewhat relaxed the enthalpic barrier restricting lipid molecules from adopting a transition state configuration. This could result from either a disruption of the attractive interactions in the ground state (e.g. Van der Waals interactions between lipid chains) or by the presence of a stabilizing interaction between the peptide and the lipid at the flip-flop transition state. Stabilizing interactions in the lipid flip-flop transition state are unlikely, as WALP₂₃ is a hydrophobic peptide which does not possess the polar groups on its surface which are necessary to stabilize the polar headgroup within the membrane interior. It is more likely that WALP₂₃, by disrupting the packing and order of the lipids in the film, disrupts the Van der Waals forces between the lipids in the ground state. Such a scenario is consistent both with the decrease in activation enthalpy and increase in activation area observed for DSPC flip-flop in the presence of WALP₂₃.

Activation Entropy—The final contribution to the free energy barrier to flip-flop is the activation entropy, which represents the difference in entropy between lipid molecules in the flip-flop transition state and ground state configurations. The potential sources of entropy in the lipid bilayer system are distinct from the enthalpic contributions described above, and the relative contributions of enthalpy and entropy are indicative of the nature of the chemical and physical interactions governing the flip-flop process. The most significant differences in the entropy of the ground and transition states for DSPC flip-flop are thought to be related to the changes in lipid alkyl chain order and solvent ordering near the lipid headgroup as flip-flop occurs. The degree to which the peptide influences these specific physical and chemical properties of the membrane can be determined from their influence on $\Delta S^\ddagger$ for lipid flip-flop relative to pure lipid bilayers.

Using Equation 13, the activation entropy for DSPC flip-flop at 37.0°C in the presence of 1% WALP₂₃ and 1% melittin is calculated to be 336 ± 2 J/mol K and 476 ± 1 J/mol K respectively. For comparison, the activation entropy for pure DSPC flip-flop was previously determined to be 418 ± 84 J/mol K under the same conditions. The activation entropy for the peptide doped bilayers is significantly different from the pure lipid system, with WALP₂₃ reducing the activation entropy and melittin significantly increasing the activation entropy. The entropic contribution to the free energy barrier, given as $-T\Delta S^\ddagger$, is calculated at 37.0 °C to be -104.0 ± 0.5 for DSPC bilayers containing WALP₂₃, and -147.6 ± 0.3 kJ/mol for those containing melittin; compared to -130 ± 26 kJ/mol for pure DSPC. Note that the entropy contributes to the total free energy with opposite sign than the enthalpic term. Therefore, a larger activation entropy term is more favorable while a smaller activation entropy term represents a reduced entropic driving force.

WALP₂₃ is found to clearly reduce the favorable entropic contribution to the transition state. Again, it should be noted that $\Delta S^\ddagger$ represents only a difference in entropy for the ground and transition states without providing any measure of the absolute entropy of either state. For the activation entropy to decrease, the transition state must possess a lower entropic configuration (corresponding to greater ordering at the transition state) or the ground state must possess

greater entropy (corresponding to less order in the ground state) relative to the pure lipid system. Increased ordering of the transition state by the peptide is unlikely, particularly given the larger activation area for DSPC flip-flop in the presence of WALP₂₃ (recall that the increase in $\Delta a^\ddagger$ was attributed to an increase in the transition state area, rather than a decrease in ground state molecular area). A larger transition state area should lead to a decrease in transition state order and an increase in the entropy of the transition state. It is possible to evaluate the effect of the increase in $\Delta a^\ddagger$ on $T\Delta S^\ddagger$ using Equation 13. The increase in $\Delta a^\ddagger$ when WALP₂₃ is present in the bilayer is ($\Delta(\Delta a^\ddagger) = 13 \text{ \AA}^2/\text{molecule}$), which corresponds to an increase in $T\Delta S^\ddagger$ of $\sim 3 \text{ kJ/mol}$ relative to the pure DSPC bilayer. This effect is minor relative to the large decrease in entropy which is observed ($\sim 26 \text{ kJ/mol}$), suggesting that the majority of the change in $\Delta S^\ddagger$ is not due to changes in the lipid packing (lipid alkyl chain order). Instead, the change in entropy is likely due to alteration of the lipid solvent interactions with the phosphocholine headgroup.

Melittin has the opposite effect on the activation entropy for DSPC flip-flop. There is a far greater change in entropy for lipids undergoing flip-flop in the presence of melittin than is observed for DSPC alone. In order for the activation entropy to increase, the ground state must become more ordered (suggesting local ordering near the peptide) or the transition state must become more disordered (greater perturbation of the transition state by the peptide). A recent AFM study of melittin defects in *gel* phase phosphocholine bilayers indicates that the lipids in the vicinity of melittin are more disordered than the bulk lipids comprising the membrane, and resemble *liquid* phase lipids. (Oliyanyk et al., 2007) This finding would suggest that a higher entropy transition state is more likely than a low entropy ground state. However, significant alteration of lipid structure might be expected to alter the activation area and work term as well, which is not observed. Thus, a simple defect mediated mechanism for flip-flop does not sufficiently describe the action of melittin in the bilayer. Another possible mechanism discussed earlier, in which the peptide facilitates partial penetration of the lipid along the polar end of the peptide, could be interpreted as a reduction in the hydrophobic thickness experienced by the lipid headgroup as it flips across the bilayer. In a previous study, we examined the flip-flop activation thermodynamics for a series of saturated phosphocholines of varying chain length, which effectively varied the bilayer thickness while maintaining constant headgroup chemistry. In that study, the membrane thickness was correlated strongly to the activation entropy, with decreasing membrane thickness corresponding to an increase in the entropic driving force for flip-flop. Accordingly, the activation entropy measured for DSPC flip-flop in the presence melittin is certainly consistent with an effective reduction in the bilayer thickness. Further exploration of the total activation thermodynamics as a function of lipid alkyl chain length may address this possibility.

Gibbs Free Energy Barrier $\Delta G^\ddagger$ —The transition state thermodynamics for DSPC flip-flop in the presence of 1% WALP₂₃ and melittin can be summarized by considering the Gibbs free energy, $\Delta G^\ddagger$ (Equation 8), which includes all the energetic contributions discussed above. At 37.0°C, we calculate a $\Delta G^\ddagger$ for DSPC flip-flop of $103 \pm 1 \text{ kJ/mol}$ in the presence of 1% WALP₂₃, and $92.4 \pm 0.1 \text{ kJ/mol}$ in the presence of 1% melittin. For comparison, $\Delta G^\ddagger = 107 \pm 1 \text{ kJ/mol}$ for pure DSPC under the same conditions. This result nicely illustrates the relative effects of these peptides, as the inclusion of 1% WALP₂₃ reduces the energy barrier by only 4 kJ/mol, while 1% melittin reduces $\Delta G^\ddagger$ by 14.6 kJ/mol. However, it should be noted that even relatively small changes in $\Delta G^\ddagger$ correspond to significant changes in the rate of flip-flop. For instance, the 4 kJ/mol reduction in $\Delta G^\ddagger$ for DSPC flip-flop in the presence of WALP₂₃ at 37.0°C corresponds to a roughly twenty-fold increase in the rate constant for flip-flop relative to pure DSPC (Calculated using Equation 9). For WALP₂₃, it is also interesting to note that the small change in $\Delta G^\ddagger$ for DSPC flip-flop is the result of much larger perturbations to the entropy and enthalpy of the bilayer. The inclusion of WALP₂₃ significantly reduces the enthalpic energy barrier to flip-flop relative to pure DSPC ($\Delta(\Delta H^\ddagger) = -30 \text{ kJ/mol}$), but simultaneously reduces the entropic driving force ($\Delta(T\Delta S^\ddagger) = -26 \text{ kJ/mol}$) as well. Because

these two terms contribute to $\Delta G^\ddagger$ with opposite sign, the changes in the total free energy barrier are much smaller than the changes in the individual components. For bilayers containing 1% melittin, the lower free energy barrier to flip-flop (relative to pure DSPC) is due almost entirely to the larger entropic driving force for bilayers containing 1% melittin ($\Delta(T\Delta S^\ddagger) = +18$ kJ/mol), while $\Delta H^\ddagger$ remains essentially constant ($\Delta(\Delta H^\ddagger) = +3$ kJ/mol).

It is also possible to calculate $\Delta G^\ddagger$ directly from the kinetic data using Equation 9, although this is substantially less informative than the analysis of $\Delta a^\ddagger$, $\Delta H^\ddagger$, and $\Delta S^\ddagger$ in addition to $\Delta G^\ddagger$. This approach was used to determine $\Delta G^\ddagger$ for those peptide concentrations where pressure dependent kinetics were not measured. The $\Delta G^\ddagger$ for DSPC flip-flop was found to decrease almost linearly with peptide concentration for both WALP₂₃ and melittin (Figure 8), with a far greater sensitivity to melittin concentration compared to WALP₂₃. Peptides which perturb flip-flop according to a pore forming mechanism are not expected to exhibit such linear concentration dependence (Fattal et al., 1994), thus the linear dependence on concentration suggests that pore formation is not occurring for melittin (or WALP₂₃) at these membrane concentrations. A linear dependence on peptide concentration would instead indicate that the peptides act as monomers within the bilayer. (Kol et al., 2001) Over the concentration range examined, the dependence appears linear with peptide concentration; although additional concentrations would be required to definitively establish this fact. However, this was not the primary goal of this study. Instead, we have focused on determining the effect of these peptides on the factors contributing to the net free energy barrier $\Delta G^\ddagger$. Analysis of $\Delta G^\ddagger$ alone would not fully reveal the extent to which WALP₂₃ and melittin perturb the bilayer. Analysis of the area, enthalpy, and entropy of activation provides far greater detail and insight into the nature of the chemical and physical interactions between peptide and lipid than is obtained simply from comparison of the kinetics or $\Delta G^\ddagger$ for flip-flop. Based on the thermodynamic data presented here, it is clear not only that melittin induces more rapid lipid flip-flop than WALP₂₃, but that the mechanism by which melittin alters the flip-flop behavior of DSPC is fundamentally different from that of WALP₂₃. In the sections that follow, we will discuss possible mechanisms for peptide facilitated flip-flop in light of the transition state thermodynamics presented here.

Possible Mechanisms for WALP₂₃ and Melittin Facilitated DSPC Flip-Flop

WALP₂₃—The transition state thermodynamic data for facilitated lipid flip-flop by WALP₂₃ and melittin yield a great deal of information regarding the effect of these peptides on the flip-flop transition state and the bilayer properties which govern flip-flop. This information may be used to critically assess the various potential mechanisms for peptide facilitated flip-flop, in order to determine which theories are most consistent with the current findings. Figure 9 illustrates several of the proposed mechanisms by which transmembrane peptides may facilitate flip-flop, including perturbation mediated methods (Figure 9A and 9C), and diffusion of the polar lipid headgroup along a polar peptide surface (Figure 9B). Note that while both WALP₂₃ and melittin are theoretically capable of participating in defect mediated process (Figures 9A, 9C), only melittin possesses the polar residues required for the process illustrated in Figure 9B. An alternative hypothesis supposes that flip-flop is facilitated by the presence of transmembrane pores (Figure 10). This hypothesis supposes that lipid molecules partially line the pore interior and may diffuse through the pore structure while the lipid headgroup is maintained in a polar environment (Figure 10A). Alternatively fluctuations of the peptides lining the pore create spatial defects which promote lipid flip-flop (Figure 10B). The mechanism by which a pore could facilitate flip-flop depends on whether the pore is best described by a toroidal pore or barrel-stave model (Figures 10A, 10B).

For WALP₂₃, a pore-mediated mechanism such as that shown in Figure 10 may be rejected as it has no known propensity for pore formation or aggregation. (Kol et al., 2001; Kol et al.,

2003) Given the structure of the peptide, defect-mediated facilitation of flip-flop appears to be the most likely possibility. The thermodynamic analysis of WALP₂₃ facilitated DSPC flip-flop reveals the surprising finding that the activation enthalpy is significantly reduced by the presence of the peptide. The reduction in $\Delta H^\ddagger$ is not likely to result from favorable association of the lipid with the peptide at the transition state because of the nonpolar nature of the peptide. Instead, the reduced $\Delta H^\ddagger$ likely reflects a reduction in the favorable ground state enthalpy via disruption of the van der Waals interactions between lipid molecules and/or a reduction in the headgroup-solvent interactions due to the presence of the tryptophan moieties at the interface. The activation area for DSPC in the presence WALP₂₃ also suggests that the peptide disorders the transition state, relative to the pure lipid. This evidence is consistent with a defect mediated mechanism for flip-flop in which the peptide alters the lipid packing, thereby promoting flip-flop of the lipid molecules. The reduction in the activation entropy term is consistent with perturbation of the ground state of the bilayer and an increase in ground state entropy. However, the reduction in $\Delta H^\ddagger$ cannot be explained based on alteration of lipid packing and alkyl chain order alone. This suggests that the entropy of the solvent around the lipid headgroup must also be considered. As noted above, the tryptophan moieties on the peptide termini are thought to lead to steric crowding at the bilayer interface and can disrupt the packing of the lipids in the vicinity of the peptide. (Petrache et al., 2002) It is also possible that these moieties alter the solvent structure for neighboring lipid species in their immediate surroundings. We recently observed that the structure of the solvent surrounding the lipid headgroup plays a significant role in determining the thermodynamic barrier to flip-flop. (Anglin and Conboy, 2008a) For DSPC, an ordered clathrate-like water structure surrounds the choline moiety when the lipid is in the ground state. (Murzyn et al., 2006) As the choline portion of the headgroup enters the membrane core, these structured water molecules are released from their hydrophobic interactions with the choline group, which leads to a significant change in entropy. (Anglin and Conboy, 2008a) Association of the choline moiety of the lipid headgroup with tryptophan residues of the peptide or with hydrophobic portions of the peptide at the solvent-lipid interface may provide an alternative to the clathrate structure, thereby reducing the entropy difference between ground and transition states. This possible scenario is consistent with the observed thermodynamic parameters, but the exact solvent structure for the lipids undergoing flip-flop is not known at this time. However, it is clear that the reduced activation entropy cannot be explained by changes in alkyl chain order alone, and that the entropy of the solvent must also be affected by the presence of WALP₂₃. All of these observations are consistent with a perturbation mediated mechanism, with the caveat that the perturbation of the bilayer by WALP₂₃ extends to both the lipid and the solvent associated with the choline headgroup.

Melittin—Because melittin is more hydrophilic than WALP₂₃, it is expected to instead facilitate flip-flop by providing a polar surface along which the lipid headgroup may pass as it traverses the bilayer, thereby reducing the energetic penalty for this process. Specifically, Fattal et al. suggested that a pore-mediated mechanism is responsible for melittin induced lipid flip-flop. (Fattal et al., 1994) If a torroidal pore model is assumed, the lipid molecules would line the pore with their headgroups oriented toward the water pore traversing the membrane (See Figure 10). In such a structure, the headgroups do not need to pass through the nonpolar lipid tails, as the movement of lipids from one leaflet to another would actually occur via lateral diffusion around the edge of the pore. The enthalpic barrier for such a process would be quite low, nearly equivalent to the barrier to lateral membrane diffusion within a single bilayer leaflet (20-65 kJ/mol). (Filippov et al., 2004) This model is not consistent with the thermodynamics presented here, as melittin does not significantly reduce the enthalpic barrier to DSPC flip-flop. Fattal et al. also discussed mechanisms by which a pore conforming to the barrel-stave model (Figure 10B) may facilitate flip-flop. However, this mechanism is not relevant to the peptides studied here, as melittin appears to conform only to the torroidal pore structure (Allende et al., 2005; Yang et al., 2001; Zemel et al., 2003), and WALP₂₃ lacks the capacity

for pore formation. Moreover, the linear dependence of $\Delta G^\ddagger$ on peptide concentration (for both WALP₂₃ and melittin) indicates that these peptides promote flip-flop as monomers rather than oligomers. (Fattal et al., 1994; Kol et al., 2001) Thus while it is possible that pore-forming oligomeric structures may exist in the membrane under certain conditions, they do not appear to play a role in the facilitation of flip-flop for the membranes studied here. Notably, the facilitation of flip-flop by melittin appears to be a purely entropically driven process, which does not appear to be consistent with any of the currently proposed models. An alternative mechanism for the promotion of flip-flop by melittin was presented above which is more consistent with the measured activation thermodynamics. This hypothesis supposed that melittin may enhance the rate of DSPC flip-flop by reducing the effective membrane thickness by partially facilitating the penetration of the lipid headgroup into the bilayer. Additional support for this model is found in the literature where the amphipathic peptide Alamethicin is reported to lead to membrane thinning while hydration and van der Waals forces remain unaffected. (Pabst et al., 2007) The thinning effect observed in the case of Alamethicin was also implicated with an alteration of the entropic configuration of the system.

Comparison with Previous Results

Fattal and co-workers were able to demonstrate that melittin induces rapid flip-flop of fluorescently labeled lipids in vesicles, even at very low peptide-lipid ratios (0.001-1%). (Fattal et al., 1994) Our results for melittin facilitated flip-flop corroborate the finding that melittin promotes rapid lipid flip-flop, although our thermodynamic results do not point to a pore-mediated mechanism. However, Fattal et al. found that hydrophobic peptides, such as gramicidin and vanillomycin do not induce more rapid flipping in phospholipid vesicles. (Fattal et al., 1994) A subsequent study using SFVS revealed that gramicidin is capable of speeding flip-flop in PSLBs when incorporated at a 2% peptide to lipid ratio (1% as dimer), although the effect was less pronounced at temperatures of 37°C and below. (Anglin et al., 2007) Our current study, which examined the effect of WALP₂₃, also indicates that hydrophobic peptides may facilitate lipid flip-flop. One possible explanation for this discrepancy is the use of chemically modified probes in earlier studies, which have markedly slower flip-flop relative to the unlabeled species that may mask the subtle effects of the peptide. Regardless of the absolute agreement between flip-flop kinetics measured by SFVS and other methods, the relative differences between flipping in the presence or absence of peptide should hold true. This is certainly the case, with our observation that facilitated flipping by melittin is far more rapid than that induced by its hydrophobic counterpart. Our thermodynamic results also suggest that pore formation is not essential to the facilitation of flip-flop by transmembrane peptides, but do not rule out such a possibility as an additional mechanism for facilitation of flip-flop.

Kol et al. have also investigated the effect of WALP₂₃ and related peptides on lipid flip-flop in *liquid* phase vesicles using the short chained fluorescent lipid analogs C₆NBDX, where X=PC, PE, PG, or PS for phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine respectively. (Kol et al., 2001; Kol et al., 2003) In their findings, the rate of facilitated flip-flop by WALP₂₃ was generally quite slow and depended on the lipid composition of the bilayers. (Kol et al., 2003) Flip-flop of phosphocholine derived lipids was slowest among the lipid types examined, where the rate of flipping was almost negligible at 25°C. For charged lipid derivatives, the rate of facilitated flip-flop by WALP₂₃ was more rapid, but still occurred on the time scale of hours for most lipid species. (Kol et al., 2003) Given the lower temperatures used in these studies, the rate of flipping is still consistent with our results presented above, but direct comparison is somewhat difficult due to the difference in methodologies and lipid phase. It should be noted however, that Kol et al. observed only minor perturbation of flip-flop by WALP₂₃, and a more significant perturbation of flip-flop by the slightly more polar KALP₂₃, which contains lysine flanking residues rather than tryptophans. (Kol et al., 2001) This result agrees qualitatively with our finding that the

charged, cationic peptide melittin induces more rapid flipping of lipids than the neutral hydrophobic WALP₂₃. This also supports the hypothesis that the flanking residues strongly influence the flip-flop dynamics by perturbation of the lipid and solvent structure in the interfacial region. Although these authors did not evaluate the thermodynamics for flip-flop of the probe molecules, they investigated the dependence of the flip-flop rate on the concentration of peptide and their results indicate that the peptide acts as a monomer. This provides further evidence that WALP₂₃ facilitates flip-flop according to a defect mediated mechanism, without the requirement for aggregation.

Conclusions

Our results have demonstrated the ability of the peptides WALP₂₃ and melittin to facilitate spontaneous bidirectional flipping of DSPC in model bilayers. The polar peptide melittin had a more profound effect on the rate of flipping than WALP₂₃ which is hydrophobic. Both peptides were also found to alter the free energy barrier to flip-flop in a dose dependent fashion. The thermodynamics of DSPC flip-flop in the presence of these peptides suggests subtle differences in the mechanisms by which they facilitate flip-flop. For WALP₂₃, structural perturbation of the membrane appears to be the most likely mechanism. Unlike WALP₂₃, the facilitation of flip-flop by melittin appears to be strictly entropy driven. Although a single mechanism for facilitated DSPC flip-flop by melittin may not be identified from this data alone, we can propose several mechanisms which are consistent with the data. These include partial facilitation of headgroup insertion into one leaflet of the bilayer, resulting in an effective decrease in the membrane thickness experienced by the lipid molecule undergoing flip-flop.

A common critique of this method is that model bilayers are poor models for cellular membranes as they contain relatively few membrane components. Although PSLBs and other model membrane systems lack the complexity and diversity found *in vivo*, they provide an essential research tool in that they allow individual variables to be addressed and characterized in a controlled fashion. Our aim is to determine how membrane peptides alter the inherent rate of flip-flop in PSLBs, rather than to quantify the exact rate of flip-flop *in vivo*. The mechanistic insight gained from such studies is certainly relevant to cellular systems as well. The use of PSLBs does present the distinct advantage of allowing the preparation of an asymmetric distribution of lipids without requiring chemical treatment or the use of modified probes, something which is not possible with *in-vivo* measurements.

Our results corroborate the finding that the presence of transmembrane proteins may induce rapid flipping, and that such peptides may play an important role in the redistribution of phospholipids in cellular membranes. The thermodynamic results presented here are a complement to the current body of work which seeks to describe the role of transmembrane proteins in the regulation of lipid flip-flop. It is our hope that this work will provide important insight into the mechanisms by which such peptides affect the distribution of lipids in cellular membranes.

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Figure 1.

SFVS spectra for DSPC bilayers containing WALP₂₃ or melittin. (A) SFVS spectra for symmetric (gray dash) and asymmetric (black dash) bilayers of DSPC:DSPC-d₇₀. The solid lines represent the spectra for DSPC:DSPC-d₇₀ containing 1% WALP₂₃ with either an asymmetric (black) or symmetric (gray) distribution of lipid species. Inset: Magnified region of the SFVS spectral response for a symmetric DSPC-d₈₃ bilayer containing 1% WALP₂₃. Although there is a weak signal near 2975 cm⁻¹ when the peptide is incorporated, no measurable effect can be found at 2875 cm⁻¹, where decay measurements are performed. This permits direct tracking of the lipid asymmetry at 2875 cm⁻¹ without interference from the peptide. (B) SFVS spectrum of an asymmetric DSPC:DSPC-d₇₀ bilayer with 1% melittin in each layer (black). A SFVS spectrum of a symmetric bilayer consisting of 1:1 DSPC:DSPC-d₇₀ with 1% melittin is also shown (solid gray). A SFVS spectrum of a symmetric DSPC-d₇₀+1% melittin bilayer was obtained (gray dash) in order to determine if there was any spectral contribution from the peptide. Inset: Magnified spectrum of a DSPC-d₇₀+1% melittin bilayer. All spectra were collected at 25°C. Spectra are offset for clarity.

Figure 2.

ATR-FTIR spectra for symmetric bilayers consisting of DSPC (A), DSPC + 2% WALP₂₃ (B), or DSPC + 1% melittin (C). The peaks are assigned to the lipid carbonyl stretch (1734 cm⁻¹), the amide I vibrational mode from WALP₂₃ (1648 cm⁻¹), and the methylene scissoring mode from DSPC (1462 cm⁻¹). (Tamm and Tatulian, 1997) The presence of an amide I band in the spectra for (B) and (C) indicates the presence of melittin or WALP₂₃ in the bilayer. All spectra were baseline corrected prior to fitting.

Figure 3.

Illustration of a lipid bilayer containing transmembrane helices with equal populations oriented along the surface normal with either the N- or C-terminus oriented away from the substrate. Peptides arranged in this manner with symmetry about the bilayer normal will possess transition dipoles with opposite orientation (on average) and should produce little SFVS signal.

Figure 4.

SFVS decays illustrating flip-flop as a function of peptide concentration. (A) Representative SFVS decays for 30mN/m DSPC in the presence of 1% WALP₂₃ (dash) or 2% WALP₂₃ (solid) obtained at 46.0°C. (B) SFVS decays for 30mN/m DSPC bilayers containing 0.5% melittin (dash) or 1.0% melittin (solid) obtained at 32.0°C. SFVS decays for pure DSPC bilayers are calculated from previous data (Anglin et al., 2008) and are shown for comparison in each plot (dash-dotted lines). All decays are normalized to facilitate comparison. The data are shown in gray while the black lines represent the fits to the data according to Equation 7.

Figure 5.

(A) Arrhenius plots for DSPC (circles), and DSPC containing 1% WALP23 (diamonds) or 2% WALP23 (downward triangles). (B) Arrhenius plots for DSPC (circles), and DSPC containing 0.5% melittin (upward triangles) and 1.0% melittin (squares). The data points for pure DSPC were obtained from a previous publication.(Anglin et al., 2008) The data for the 1% WALP₂₃, 1.0% melittin, and 0.5% melittin bilayers consist of bilayers prepared with proteated lipids in both the proximal leaflet (closed symbols) and distal leaflet (open symbols). Bilayers prepared with proteated lipids in the proximal leaflet appear to follow the same trend as those prepared in with proteated lipids in the distal leaflet, suggesting that the substrate has little or no influence on lipid flip-flop.

Figure 6.

Comparison of the Arrhenius behavior for pure DSPC bilayers (closed circles) and bilayers containing either 1% WALP₂₃ (open diamonds), or 1% melittin (closed triangles). The data for pure DSPC were obtained from an earlier publication.(Anglin et al., 2008)

Figure 7.

(A) Representative SFVS decays obtained at 36.0°C for 1% WALP₂₃-DSPC bilayers at 20mN/m (solid) and 30mN/m (dash). (B) SFVS decays obtained at 30.0°C for DSPC bilayers containing 1.0 % melittin at 30mN/m (solid) or 40mN/m (dash). The data are shown in gray while the black lines represent the fits to the data according to Equation 7. The decays are normalized for presentation.

Figure 8.

Variation in the activation free energy for DSPC flip-flop at 37.0°C as a function of melittin concentration (triangles) and WALP₂₃ concentration (squares). All data refer to bilayers prepared at 30mN/m. The free energy of activation for pure DSPC flip-flop was determined in a previous publication.(Anglin et al., 2008)

Figure 9.

Illustration of several hypothesized mechanisms for facilitation of flip-flop by membrane spanning peptides. The helical peptides are color coded according to the general arrangement of their polar (red) or nonpolar residues (light brown). (A) Perturbation of the local lipid packing by a hydrophobic peptide. The peptide is thought to disrupt the order of the lipids in its vicinity (green) relative to the well ordered lipids found in the bulk of the membrane (light red or blue). (B) Association of a polar lipid headgroup with polar residues of the peptide (red), promoting flip-flop by providing a hydrophilic path through the membrane interior. (C) Peptides with polar residues may also participate in a perturbation mediated mechanism similar to that shown in (A), however the hydrophobic peptides cannot facilitate flip-flop by the mechanism hypothesized in (B).

Figure 10.

An illustration of pore structures conforming to the torroidal pore model (A) and barrel-stave model (B). Lipid flip-flop through a torroidal pore structure is thought to occur via lateral diffusion of the lipid around the interior of the core without requiring the lipid headgroup to pass through the membrane interior.(Fattal et al., 1994) It is also thought that the barrel-stave model may facilitate flip-flop. In the case of a barrel-stave pore, fluctuation of the monomers is thought to produce packing defects which would permit more facile flip-flop.(Fattal et al., 1994)

Kinetics of DSPC flip-flop for 30mN/m bilayers containing WALP₂₃. The kinetic data for the pure DSPC bilayers (0% WALP₂₃) were taken from a previous publication. (Anglin et al., 2008)

Table 1

30mN/m DSPC: DSPC-d ₇₀ + WALP ₂₃						
Temp (°C)	1% WALP ₂₃		2% WALP ₂₃		0% WALP ₂₃	
	k (s ⁻¹) × 10 ⁵	$t_{1/2}$ (min)	k (s ⁻¹) × 10 ⁵	$t_{1/2}$ (min)	$t_{1/2}$ (hours)	
35	-	-	2.08 ± 0.01	278 ± 1	30 ± 3	
36	2.96 ± 0.01	195.0 ± 0.1	-	-	23 ± 2	
37	3.64 ± 0.01	159.7 ± 0.2	-	-	18 ± 2	
40	6.82 ± 0.01	84.7 ± 0.1	12.58 ± 0.8	45.9 ± 0.3	8.2 ± 0.7	
43	17.79 ± 0.04	32.5 ± 0.1	-	-	3.8 ± 0.3	
44	-	-	21.5 ± 0.1	26.8 ± 0.2	3.0 ± 0.3	
46	29.78 ± 0.04	19.40 ± 0.02	69.9 ± 0.5	8.28 ± 0.06	1.8 ± 0.2	

Table 2

Kinetics of DSPC flip-flop for 30mN/m bilayers containing melittin. The kinetic data for the pure DSPC bilayers (0% melittin) were calculated from previous published data. (Anglin et al., 2008)

30mN/m DSPC:DSPC-d ₇₀ + Melittin							
Temp (°C)	0.5% Melittin		1% Melittin		0% Melittin		
	k (s ⁻¹) × 10 ⁵	$t_{1/2}$ (min)	k (s ⁻¹) × 10 ⁵	$t_{1/2}$ (min)	$t_{1/2}$ (min)	$t_{1/2}$ (hours)	
25	-	-	4.77 ± 0.01	121 ± 0.2	450 ± 40		
30	-	-	22.71 ± 0.05	25.4 ± 0.1	110 ± 10		
31	5.81 ± 0.01	99.4 ± 0.2	-	-	87 ± 8		
32	6.71 ± 0.02	86.1 ± 0.2	41.0 ± 0.2	14.1 ± 0.1	66 ± 6		
33	-	-	-	-	51 ± 5		
34	7.26 ± 0.01	79.6 ± 0.1	67.0 ± 0.5	8.63 ± 0.07	39 ± 3		
35	12.59 ± 0.04	45.9 ± 0.2	-	-	30 ± 3		
35	18.97 ± 0.07	30.4 ± 0.1	-	-			
37	12.12 ± 0.02	47.66 ± 0.07	-	-	18 ± 2		
38	47.9 ± 0.2	12.06 ± 0.06	-	-	14 ± 1		
40	66.5 ± 0.2	8.69 ± 0.03	-	-	8.2 ± 0.8		

Pressure dependent flip-flop kinetics for DSPC+1% WALP₂₃. The half life of decay (*t*_{1/2}) is also shown. All errors are ± one standard deviation. The kinetics of flip-flop for pure DSPC bilayers were calculated from previously published data.(Anglin et al., 2008)

Table 3

DSPC:DSPC-d70 + 1% WALP ₂₃						DSPC:DSPC-d70	
30 mN/m			20 mN/m			30 mN/m	
Temp (°C)	<i>k</i> (s ⁻¹) × 10 ⁵	<i>t</i> _{1/2} (min)	Temp (°C)	<i>k</i> (s ⁻¹) × 10 ⁵	<i>t</i> _{1/2} (min)	Temp (°C)	<i>t</i> _{1/2} (hours)
36	2.96 ± 0.01	195.0 ± 0.1	34	4.72 ± 0.01	122.5 ± 0.1	36	23 ± 2
37	3.64 ± 0.01	159.7 ± 0.2	34	4.19 ± 0.01	137.8 ± 0.1	37	18 ± 2
40	6.82 ± 0.01	84.7 ± 0.1	36	17.98 ± 0.05	32.1 ± 0.1	40	8.2 ± 0.7
43	17.79 ± 0.04	32.5 ± 0.1	39	48.6 ± 0.1	11.89 ± 0.02	43	3.8 ± 0.3
46	29.78 ± 0.04	19.40 ± 0.02	41	72.5 ± 0.3	7.97 ± 0.03	46	1.8 ± 0.2

Summary of the pressure dependent flip-flop kinetics for DSPC+1% melittin as a function of temperature. The kinetics of flip-flop for pure DSPC bilayers at 30mN/m are provided for comparison.(Anglin et al., 2008) All errors are $\pm$ one standard deviation.

Table 4

Temp (°C)	DSPC: DSPC-d70 + 1% Melittin				DSPC: DSPC-d70	
	30mN/m		40mN/m		30mN/m	
	k (s^{-1}) $\times 10^6$	$t_{1/2}$ (min)	k (s^{-1}) $\times 10^6$	$t_{1/2}$ (min)	k (s^{-1}) $\times 10^6$	$t_{1/2}$ (days)
25.0	47.73 $\pm$ 0.08	121 $\pm$ 0.2	-	-	0.21 $\pm$ 0.02	18 $\pm$ 2
30.0	227.1 $\pm$ 0.5	25.4 $\pm$ 0.1	36.1 $\pm$ 0.1	160.1 $\pm$ 0.4	0.85 $\pm$ 0.08	4.7 $\pm$ 0.4
32.0	410 $\pm$ 2	14.1 $\pm$ 0.1	78.4 $\pm$ 0.5	73.7 $\pm$ 0.5	1.5 $\pm$ 0.1	2.8 $\pm$ 0.2
34.0	670 $\pm$ 5	8.63 $\pm$ 0.07	-	-	2.6 $\pm$ 0.2	1.6 $\pm$ 0.1
37.0	-	-	480 $\pm$ 4	12.0 $\pm$ 0.1	-	-

A summary of the activation thermodynamics for DSPC with 1% WALP₂₃, 1% melittin, or without peptide. The thermodynamic data for DSPC are taken from an earlier publication.(Anglin et al., 2008) All errors are reported to ± one standard deviation.

Table 5

	$\Delta a^\ddagger$ ($\text{\AA}^2/\text{molecule}$)	$\Pi\Delta a^\ddagger$ (kJ/mol)	E_a (kJ/mol)	$\Delta S^\ddagger$ (J/mol K)	$T\Delta S^\ddagger$ (kJ/mol)	$\Delta G^\ddagger$ (kJ/mol)
DSPC	61 ± 5	11.0 ± 0.9	228 ± 28	418 ± 84	130 ± 26	107 ± 1
DSPC + 1% WALP ₂₃	74 ± 4	13.4 ± 0.7	196 ± 1	336 ± 2	104.0 ± 0.5	103 ± 1
DSPC + 1% Melittin	57 ± 1	10.3 ± 0.2	232 ± 1	476 ± 1	147.6 ± 0.3	92.4 ± 0.1