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Spectroscopic Studies of Peptide-Membrane Interactions

Kathryn Blakey Dupont
University of Pennsylvania, kabsmith@mail.med.upenn.edu

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Spectroscopic Studies of Peptide-Membrane Interactions

Abstract
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SPECTROSCOPIC STUDIES OF PEPTIDE-MEMBRANE INTERACTIONS

Kathryn Blakey Dupont

A DISSERTATION

in

Biochemistry and Molecular Biophysics

Presented to the Faculties of the University of Pennsylvania in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy 2013

Supervisor of Dissertation

________________________

Feng Gai, Ph.D.
Professor of Chemistry

Graduate Group Chairperson

________________________

Kathryn M. Ferguson, Ph.D.
Associate Professor of Physiology

Dissertation Committee

Heinrich Roder, Ph.D. (Committee Chair)
Professor of Biochemistry and Biophysics

Tobias Baumgart, Ph.D.
Associate Professor of Chemistry

Sergei Vinogradov, Ph.D.
Professor of Biochemistry and Biophysics

Jeffery G. Saven, Ph.D.
Associate Professor of Chemistry

Casey Londergan, Ph.D.
Assistant Professor of Chemistry, Haverford College

E. James Petersson, Ph.D.
Assistant Professor of Chemistry
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DEDICATION

This work is dedicated in memory of my Grandmother.
ACKNOWLEDGMENTS

"All the variety, all the charm, all the beauty of life are made up of light and shade."
- Stepan Arkadych in Anna Karenina, by Leo Tolstoy

First, I would like to express my deepest gratitude to Professor Feng Gai for mentoring me and challenging me. I am grateful to have the opportunity to work with him and learn from him, and appreciate the impact that he has made on me as a scientist.

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ABSTRACT

SPECTROSCOPIC STUDIES OF PEPTIDE-MEMBRANE INTERACTIONS

Kathryn Blakey Dupont

Feng Gai, Ph.D.

Understanding the structure-dynamics-function relationship is a fundamental motivation for studying how proteins fold. Over the past several decades, significant progress has been made in elucidating the folding energy landscapes and dynamics of soluble, globular proteins. In contrast, the folding kinetics and mechanisms of membrane proteins are much less studied and understood, due in part to the fact that they reside in the heterogeneous and complex membrane environment. To provide new mechanistic insights into membrane protein folding, herein we studied the folding kinetics of the influenza hemagglutinin fusion peptide (HAfp), which folds into a representative helix-turn-helix structure in model membranes. Our stopped-flow fluorescence and fluorescence resonance energy transfer (FRET) kinetics, obtained at different peptide-to-lipid ratios, support a parallel mechanism for membrane-peptide binding, wherein folding can occur either before or after membrane binding, but prior to membrane insertion. Thus, this result underscores the importance of the water-membrane interfacial region in mediating the process of folding, at least for short peptides. In turn, the association of the peptide to the interfacial region could induce local and global structural changes in the membrane. To help better characterize peptide-induced membrane structural changes as well as how cell penetrating peptides translocate across membranes, the second portion of this thesis was devoted to method development. Using two antimicrobial peptides and a
cell penetrating peptide as examples, we showed that diffusion measurements via fluorescence correlation spectroscopy (FCS), can be used to ‘image’ peptide-induced lipid domain formation in model membranes and to elucidate the mechanism of peptide translocation.
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CHAPTER 1

Introduction

1.1 General Motivation

Membrane proteins play essential roles in many fundamental biological activities. In comparison to soluble proteins, we know relatively little about the structural details and folding of membrane proteins in their natural environment. This is due to the fact that membrane proteins are difficult to isolate and are often insoluble in aqueous environments. As such, thoughtful simplification of these systems provides the molecular details of protein-membrane interactions and folding. By isolating specific variables that could influence membrane protein folding, such as peptide sequence, membrane composition and structure, we can build a better understanding of these mechanisms. This thesis highlights several projects that are aimed towards understanding the kinetic mechanisms of model membrane-peptide systems using fluorescence spectroscopic methods.

1.2 Membrane Proteins

Proteins are made up of conjugated, chiral, chemically distinct building blocks known as amino acids. The distinct characteristics of each of the twenty naturally occurring amino acids arise from the structure, size, and chemical properties of the functional R side chain. The amino and carbonyl groups of each amino acid are integral to the formation of intra-peptide hydrogen bonds responsible for folding and stabilization of a protein’s overall conformation, as well as interactions with the environment and other biomolecules. The seemingly infinite number of combinations of amino acids, in addition to the three-dimensional structures and chemical modifications that occur in
nature, makes it possible for proteins to perform such a wide variety of functions in different biochemical environments. It is important to note that protein conformations are dynamic. The structure of a protein is not merely dependent on its primary structure, but also on the conditions under which it is placed. Therefore, it is not surprising that although much progress towards a better understanding of protein folding and structure has been made, many questions still remain.

Membrane-associated proteins comprise approximately 30% of encoded genes in the genomes of several organisms, including humans (Wallin and Von Heijne, 1998). They play essential roles in cell signaling (Cho and Stahelin, 2005), exocytosis and endocytosis (Okamoto et al., 1998), and host-pathogen interactions (Wiley and Skehel, 1987). In eukaryotic cells, membrane proteins are often co-translationally assembled in the endoplasmic reticulum membrane with the assistance of chaperone proteins, such as Sec61 (Molecular Biology of The Cell, pg. 593-613). Diseases like cystic fibrosis are caused by misfolding and spontaneous mutation of membrane proteins (Sanders and Myers, 2004). There is some evidence that destabilizing interactions with the membrane interfacial region may compound the effects of α-synuclein misfolding in Parkinson’s disease (Lee et al., 2002; Jo et al., 2002). Although many membrane proteins exist only within the membrane, there are many proteins that must partition from an aqueous environment to the membrane in order to function, such as viral fusion peptides, and antimicrobial peptides (AMPs). Antimicrobial peptides are essential components of the innate immune response in most living organisms, and function by destabilizing the membrane structure of invading pathogens (Leontiadou et al., 2006; Matsuzaki, 1999; Arouri et al., 2009). Since their discovery in the latter half of the 20th century (Zasloff,
AMPs have not only served as natural model systems with which to study peptide-membrane interactions, but also as templates for the design of potent antimicrobials (Rotem and Mor, 2009). Understanding the structure/function relationship of important membrane proteins is not only necessary for a complete scientific understanding of protein folding, but also for effective drug design and delivery.

As illustrated in Figure 1.1, a thermodynamic cycle for membrane protein folding has been developed based on thermodynamic measurements of partitioning, folding, and oligomerization (Almeida et al., 2012; Popot and Engelman, 2000; Popot and Engelman, 1990; White and Wimley, 1999). It is important to note that the thermodynamic cycle is based on the folding of naturally-occurring and designed α-helical proteins. Of course, the physiological relevance of this thermodynamic cycle is limited also by its application to proteins that do not require additional machinery or chaperones to partition into the membrane and fold. The partitioning and folding of an α-helix into the interfacial region of a membrane, and subsequent insertion and oligomerization, can be measured by fluorescence spectroscopy and oriented circular dichroism spectroscopy (Ladokhin et al., 2000; Ladokhin and White, 2004; Ladokhin and White 1999) as well as isothermal titration calorimetry (Ladokhin an White 2004). Several naturally occurring (Ladokhin and White, 1999; Almeida et al., 2012; Yandek et al., 2008; Schäfer et al., 2011) and designed peptide systems (Ladokhin and White, 2004) have been studied in model membranes in order to quantify the thermodynamic steps of folding.

The partitioning of an unfolded peptide into the membrane environment is driven partially by the unfavorable interactions between hydrophobic side chains and water, and
by the favorable partitioning of hydrophobic peptide sequences into the hydrophobic bilayer and interactions between aromatic residue side chains and the interfacial region of the membrane. The folding of the protein backbone to form intra-helical hydrogen bonds is favored, especially in the low dielectric environment of the membrane (Li and Deber, 1993; White et al., 2001). A systematic review of experimentally-determined folding energies of α-helical proteins concluded that the Gibbs free energy of the concerted partitioning and folding steps of a helical protein is approximately -0.4 kcal/mol per residue (Almeida et al., 2012). The insertion of transmembrane helices across the bilayer is characterized by the length of the helix, the number of ionizable and polar side chains in the peptide sequence (e.g., Cys, Ser, Thr, His) and the placement of aromatic side chains (i.e., Trp, Tyr, Phe) within the interfacial region of the membrane. Thus, the transmembrane orientation of a given protein is determined by both the primary sequence of the protein and direct interactions with the lipid membrane like hydrophobic mismatch, membrane curvature, and membrane composition (Ladokhin and White, 2004; Dumas et al., 1999; Constantinescu and Lafleur, 2004). The stabilization of oligomeric protein species within membranes is driven by several factors that are also dependent on protein primary structure, including inter-helical hydrogen bonding (Lee and Im, 2008), van der Waals interactions between hydrophobic side chains (Zhang et al., 2009; Lemmon et al., 1994), and interactions between polar and charged side chains (Choma et al., 2000; Zhou et al., 2001). Finally, while the partitioning of folded, oligomeric species into the membrane is rare, it has been observed in certain cases (Oesterhelt et al., 2000). The thermodynamic cycle of folding for a simple alpha-helical motif can be pieced
together from thoughtful reduction of each thermodynamic step in isolation. However, the kinetic mechanisms underlying these steps are more difficult to quantitatively assess.

While the kinetics of folding events of soluble protein motifs have been extensively explored (Vu et al., 2004; Hagen et al., 1996; Du and Gai, 2006; Eaton et al., 2000), the microscopic kinetic folding events in membrane proteins are still relatively unstudied (Kruetzberger and Pokorny, 2012; Constantinescu and Lafleur, 2004; Yandek et al., 2008; Lorch and Booth, 2004). The molecular level interactions between proteins and lipids in the heterogeneous membrane environment are difficult to assess through conventional ensemble methods. Elucidation of the physical principles and kinetics of membrane protein folding has implications beyond folding in membranes. The membrane has been shown to provide an environment which favors specific folded motifs, and intra- and interhelical peptide interactions (Popot and Engelman, 2000). In turn, the folding of a peptide or protein, or partitioning into a specific membrane domain has been demonstrated to relieve line tension between membrane domains (García-Sáez and Schwille, 2010; Almeida et al., 2005; Trejo and Amar, 2011; Lee and Im, 2008; Bowie, 2011). The underlying kinetics and thermodynamics of these processes are applicable to understanding the induced folding of intrinsically disordered proteins.

1.3 Lipid Membrane Structure and Dynamics

Lipids, like proteins, sugars, and nucleic acids, are essential biomacromolecules for the survival and function of biological organisms. Fatty acid lipid molecules contain a polar head group region and one to three hydrocarbon chains. The carbon chains range in number, length, and level of saturation, which contribute to their size, shape, and transition temperature. Unsaturated fatty acid chains exist as *cis* or *trans* isomers, which
ultimately affect the size and shape of the chains. The polar head group region can vary by size, shape, polarity, and charge, as well as functional groups containing oxygen, nitrogen, halogens, or phosphate. Fatty acids spontaneously organize into micelles and continuous bilayers in aqueous solvent because of their amphipathic nature. Lipid membrane bilayers are characterized by a hydrophobic core, composed of the inward facing hydrocarbon tails, flanked on either side by the polar head groups that make up the interfacial regions (Figure 1.1).

Membranes vary in thickness, curvature, and rigidity, as determined by their chemical composition. For example, bacterial membranes tend to contain phosphatidylglycerol, the head group of which carries a negative charge, and a high content of branched-chain fatty acids, which increase the fluidity of the membrane (Cho and Salton, 1966; Dowhan, 1997). In contrast, eukaryotic cell membranes contain zwitterionic phospholipids and sterols, which reduce the fluidity of the membrane (Devaux, 1990). Membrane composition differs between compartments of eukaryotic cells, for example, bis(monoacylglycero)phosphate is found in late endosomes and lysosomes, but is not found in other organelle membranes (Kobayashi et al., 2002; van Meer et al., 2008). Often, the physical characteristics of a membrane can drive interactions with specific proteins (Epand et al., 2008b). For example, AMPs exhibit selectivity for bacterial membranes, specifically, because of the charge and relative fluidity of bacterial membranes in comparison to eukaryotic membranes (Matsuzaki, 2009; Epand et al., 2006; Epand et al., 2008b).

The membrane bilayer restricts protein dynamics and concentration, and provides a heterogeneous environment. Thus, it is necessary to understand the kinetics and
molecular details involved in membrane and protein interactions, including the consequences of these associations on both protein and membrane structure and dynamics.

1.4 Methods for Probing Membrane Protein Structure and Dynamics

1.4.1 Fluorescence

Spectroscopy is a powerful tool for physical characterization of biological molecules. Spectroscopic methods, such as ultraviolet-visible spectroscopy and fluorescence spectroscopy, are used to measure the absorbance or emission of light from a chromophore. In this thesis, ensemble, single-molecule, time-independent, and time-resolved fluorescence spectroscopic methods will be discussed in detail.

A luminescent substance emits light during relaxation from an electronically excited state. Luminescent substances include small molecules, such as tryptophan or quinine; large proteins, like green fluorescent protein; or even nanomaterials, such as quantum dots. Luminescence is a result of absorption of energy from exposure to light of a particular wavelength. Before absorption, electrons occupy a ground state ($S_0$), as illustrated in the Jablonski diagram in Figure 1.2. The energy absorbed excites one electron in ground state orbital to a higher electronic singlet state, $S_1$. This absorption process, represented in purple, occurs almost instantaneously ($10^{-15}$ s$^{-1}$), before any physical displacement of the nuclei can occur. Excited electrons can occupy the higher vibrational levels of $S_1$ or even $S_2$ excited states, but in most cases relax to the lowest level vibrational $S_1$ state quickly (within $10^{-12}$ s$^{-1}$) through a process called internal conversion. Fluorescence occurs when relaxation of this electron from the excited singlet state to the ground state, occupied by a ground state orbital electron of the opposite spin,
results in the emission of a photon. If the electron in the excited state orbital is in the same spin orientation as the ground state electron, then the relaxation process is forbidden. The electron relaxes to a lower-energy triplet (T₁) state through a process of intersystem crossing, before relaxing to the ground state and emitting a photon. This process is called phosphorescence and occurs on relatively longer time scales than fluorescence emission, (i.e., longer than 1 ms).

Experimental methods that utilize the photo-physical properties of fluorescent molecules are ubiquitous in biochemical and biophysical research. Technological advances in optics, spectroscopy, detection, and imaging have made it possible to probe the mechanisms of biological processes to a high level of temporal and spatial resolution. In addition, the discovery and development of fluorescent spectroscopic probes have widened the range of possible applications to biochemical systems. Some fluorescence spectroscopic methods have single-molecule sensitivity, and can be used in complex in vivo systems without disruption of the target organism. In this thesis, we will explore the application of ensemble, time-resolved, and single-molecule fluorescence spectroscopy to study the kinetics of membrane protein folding and dynamics.

1.4.2 Fluorescence Spectroscopic Probes

The appropriate probes must be used in order to successfully dissect the kinetics and dynamics of protein folding events. The right combination of fluorescent probes can identify major folding steps in a mechanism. Proteins contain few intrinsic fluorophores that exhibit a high quantum yield and are sensitive to changes in the environment (i.e., solvent-ion effects). One characteristic of fluorophores that must be taken into account during experimental design is the Stoke’s shift. The Stoke’s shift manifests as a
difference in the excitation and emission maxima, the latter of which is shifted to lower energy due to vibrational relaxation of the fluorophore and loss of energy to its immediate environment (e.g., water).

The most commonly used intrinsic fluorophore is tryptophan, a naturally-occurring amino acid that contains an indole ring. The quantum yield of tryptophan is 0.13, higher than that of phenylalanine by a factor of 5.4 and more responsive to the environment than tyrosine (Lakowicz, 2006). Tryptophan is commonly found in the interface-associated regions of membrane proteins (Ridder et al., 2000). In addition, tryptophan fluorescence intensity and spectrum profile is sensitive to the polarity of the environment. In the case of tryptophan, a solvated indole ring emission spectrum contains a maximum at 355 nm, whereas the emission spectrum of a desolvated/buried indole ring contains a maximum at 335 nm. Excitation of tryptophan at 290 nm induces a dipole moment in the molecule, and the reorganization of water molecules around the indole ring in response to the induced dipole moment further dissipates the energy of the excited tryptophan molecule. Thus, in water, the Stoke’s shift of tryptophan is greater than in hydrophobic environments. The quantum yield of tryptophan is lowered in aqueous solvents, primarily because of quenching by water. Because of these properties, tryptophan has been used for decades as a reporter of folding of solubilized proteins and membrane-protein association.

While tryptophan is an excellent reporter of protein folding and stability, a variety of spectroscopic probes are often needed to fully understand complex folding mechanisms and protein conformational dynamics. However, one must be thoughtful in choosing the proper probe that will report on the question at hand without disturbing the
structural or functional integrity of the protein. Fluorescent proteins and small-molecule fluorophores can easily be conjugated to a protein of interest for in vivo studies (Tsien, 1998), but with the caveat that the presence of the fluorescent probe may bias the target protein’s structure or function. Thus, novel amino acids and small molecules, like p-cyanophenylalanine (Phe$_{CN}$) (Tucker et al., 2005; Rogers et al., 2010) are excellent for use with fluorescent spectroscopic methods. p-cyanophenylalanine is a non-natural amino acid and a useful spectroscopic probe for protein folding and dynamics (Figure 1.3) (Tucker et al., 2004; Tucker et al., 2005; Tucker et al., 2006a; Tucker et al., 2006b). It exhibits a quantum yield that is five times larger than that of phenylalanine in water (Tucker et al., 2005; Serrano et al., 2010).

1.4.3 Fluorescence Resonance Energy Transfer

Phe$_{CN}$ can also excite Trp via fluorescence resonance energy transfer (FRET), which can be used to report on protein folding processes (Tucker et al, 2005; Tucker et al., 2006a). Resonance energy transfer (RET) occurs between two fluorophores, if the emission spectrum of the donor fluorophore overlaps the absorption spectrum of the acceptor fluorophore (Figure 1.3). RET occurs without emission of a photon and the rate of RET is dependent on the degree of spectral overlap, the photophysical characteristics of the donor, the relative dipole orientation of and distance between the donor and acceptor molecules. FRET has many applications for studying biochemical processes, including protein folding and protein-protein interactions. FRET is commonly used to assess the thermodynamics and kinetics of protein-ligand interactions (Granier et al., 2007; Tamrazi et al., 2002), protein conformational changes (Tucker et al., 2005), and association of proteins in membranes (Wang et al., 1988; Lear et al., 2004; White and
Wimley, 1999; You et al., 2005), both in vivo (Nagai et al., 2000) and in vitro (Tamrazi et al., 2002; Tang et al., 2008; Tucker et al., 2005; Rogers et al., 2010). By thoughtfully choosing the donor and acceptor molecules, and labeling the protein(s) of interest without compromising their structural integrity, one can use FRET to study the spatial and temporal changes in protein conformation and interactions. The rate of transfer ($k_T$) for a donor and acceptor separated by a distance ($r$) is

$$k_T(r) = \frac{Q_D \kappa^2}{\tau_D n^4} \left( \frac{10000 \, \text{cm}^2}{128 \pi N_A} \right) \int_0^\infty F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda$$  \hspace{1cm} (1.1)

where $Q_D$ is the quantum yield of the donor, $\kappa^2$ is an orientation factor describing the relative orientation of the transition dipole of the donor and acceptor, $\tau_D$ is the lifetime of the donor in the absence of the acceptor, $N_A$ is Avogadro’s number, and $n$ is the refraction index of the medium. The integral is the overlap integral, which describes the extent of spectral overlap. $F_D(\lambda)$ is the normalized fluorescence intensity of the donor for a range of wavelengths $\lambda$ to $\lambda + \Delta \lambda$, and $\epsilon_A$ is the extinction coefficient of the acceptor as a function of wavelength. To illustrate further the dependence of rate of transfer on distance, the rate of transfer can be summarized as,

$$k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6$$  \hspace{1cm} (1.2)

where $r$ is the distance between the donor and acceptor molecules, and $\tau_D$ is the decay time of the donor in the absence of the acceptor. $R_0$ is the Förster distance, which is the distance at which the donor and acceptor exhibit a 50% transfer efficiency. This distance is important to consider when designing an experiment, because it defines a workable range for assessing the distance between the two fluorophores. Most biologically relevant FRET pairs have Förster distances between 20 Å and 90 Å (Lakowicz, 2006),
and the distance can be calculated from the photophysical properties of the pair, much like the rate of transfer.

$$R_0^6 = \frac{900(Ln10)^2Q_D}{128\pi^5N^4} \int_0^\infty F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda \quad (1.3)$$

The Förster distance for the PheCN-Trp FRET pair is 16 Å, which is a reasonable distance for assessing conformational changes in small proteins and peptides (Tucker et al., 2005). Figure 1.3 contains the excitation and emission spectra for PheCN and Trp (Tucker et al., 2006a). PheCN can also be used in conjunction with additional novel amino acid fluorophores, like 5-hydroxytryptophan and 7-azatryptophan to design multi-step FRET systems (Rogers et al., 2010).

### 1.4.4 Stopped-Flow Fluorescence Spectroscopy

Stopped-flow fluorescence spectroscopy is a powerful tool for measuring time-dependent changes in fluorescence signal that arise due to chemical processes. First developed in the 1940’s, this time-resolved technique for measuring the kinetics of chemical reactions has proved to be an invaluable method for the study of protein folding (Roder et al., 2006) and protein-membrane interactions (Tucker et al., 2006b; Tang and Gai, 2008; Tang et al., 2009). The instrument consists of two or more reservoirs for initial reagents, which are mixed rapidly and passed through a cuvette, which is exposed to light of the appropriate wavelength for exciting the fluorophore of interest. After the cuvette is filled, the flow is stopped in order to trap the reactants in the cuvette. The emitted light is collected and filtered to remove any stray excitation light, and detected using a photomultiplier tube (PMT).

The instrument used here consisted of a Fluorolog 3.10 spectrophotometer, a SFM-300 BioLogic stopped-flow mixing apparatus, and photomultiplier tube, connected
by home-built optics. The incident light was tuned to excite Trp or PheCN at 290 nm and 240 nm, respectively. The emitted light was collected perpendicular to the incident beam path and directed through a 320 nm long-pass filter to remove stray excitation light. The current amplification was controlled by a SR750 current amplifier, and digitized to output voltage using Gagescope software. The set up is illustrated in Figure 1.4.

It is essential to calibrate the instrument before each set of measurements to ensure the collection of high-quality data. Two measurements, the dead time and valve lead time, were critical for accurate data collection and analysis. The dead time is determined by the specifications of the instrument set up, including the size of the cuvette used, the volume of the shot, and the flow rate. In short, the dead time is the time between the initiation of the flow and the collection of the first data points, and ideally would be as small as possible to observe kinetics at short timescales. In our experiments, a μFC-08 quartz cuvette with a pathlength of 0.8 mm was used to minimize the dead time, which was calculated to be between 0.5 and 1.0 ms. Measurement and calculation of the deadtime was performed according to previously published protocols (Tang, Ph.D. Dissertation, 2008; Peterman, 1979) using fluorescence quenching of N-acetyl-tryptophanamide (NATA), a water-soluble tryptophan derivative, by N-bromosuccinimide (NBS). The valve lead time is measured in order to synchronize the closing of the valve lead in front of the stop with the stop of the motors controlling the flow of reagent. A valve lead time that is too short or too long could cause cavitations in the cuvette and flow line, contamination of the reaction with aged solution, and prevent the flow from reaching a steady state before data collection begins. The valve lead time
is typically 2-3 ms, and is measured and corrected (if necessary) in order to create a reproducible stop.

1.4.5 Diffusion Theory

Diffusion is a fundamental physical phenomenon exhibited by all molecules. Diffusion is the movement of particles down a force gradient, resulting in translational motion of the particles. It is essential for simple chemical reactions and complex biological processes. Several methods can be used to measure diffusion dynamics of molecules, including FCS (Magde et al., 1972), fluorescence anisotropy, and fluorescence recovery after photobleaching (FRAP) (Jacobson et al., 1976). The measurement of rotational and translational diffusion is fundamental to the understanding of how molecules interact with their environment. Diffusion of spherical particles moving in three dimensions, subject only to Brownian motion, can be described by the Stokes-Einstein equation (1905),

\[ D_T = \frac{k_B T}{(c \pi \eta R)} \quad (1.4) \]

where \( k_B T \) is thermal energy, \( \eta \) is solvent viscosity, \( R \) is the hydrodynamic radius of the diffusing particle, and \( c \) describes the boundary conditions between the solvent and particle.

In membranes, this simple expression for diffusion is no longer appropriate. For Brownian motion within a two-dimensional plane (i.e., a membrane) the mean square displacement (MSD) is defined by

\[ MSD = 4D_T t \quad (1.5) \]

where \( t \) is time and \( D_T \) is the translational diffusion coefficient. The translational diffusion coefficient,
The Saffman-Delbrück approximation withstood initial experimental tests of the relationship between the radius of the protein inclusion and the observed diffusion coefficient. However, further experimental and computational testing of this approximation (Guigas and Weiss, 2006; Hughes et al., 1981, Gambin 2006; Petrov and Schwille, 2008) indicated that the translational diffusion coefficient in 2 dimensions is more dependent on the radius of the inclusion. The model proposed by Hughes, Pailthorpe, and White argued that \( D_T \sim \frac{1}{a} \) (Hughes et al., 1981). Further, restrictions were placed on how far each scaling can estimate the diffusion coefficient. Guigas
argued that the HPW model only accurately calculated $D_T \sim \frac{1}{a}$ for protein inclusions below a critical radius of approximately 7 nm, $a_c \approx \frac{h \eta m}{2 \eta}$, above which the relation, $D_T \sim \frac{1}{a^2}$, applies. A recent systematic investigation assessing the dependency of the diffusion coefficient on protein inclusion radius for proteins with radii ranging from 0.5 nm to 5 nm in GUVs and under very dilute (P:L = 1:3,000,000) to very concentrated (P:L = 1:20) surface densities, concluded that the measured diffusion coefficient depends weakly on the radius, as suggested by the Saffman-Delbrück approximation (Ramadurai et al., 2009). The Saffman-Delbrück approximation, although based on the simplification of the membrane as a planar sheet in the hydrodynamic continuum model (Figure 1.5), is accurate for describing the diffusion of a wide range of transmembrane proteins in model membranes. However, the exact specifications for the height and viscosity of the membrane must be critically assessed (Ramadurai et al., 2009; Saffman and Delbrück, 1975), as discussed in Chapters 3 and 4.

1.4.6 Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) is a method with which to measure spatial and temporal fluctuations in signal intensity to reveal concentrations, reaction rate constants, and diffusion coefficients of fluorescent biomolecules. The measured fluctuations of fluorescence intensity reflect changes in diffusion, rotation, intra- and interparticle interactions, or any physical change in the probe molecule or its environment. The sensitivity of this method allows for the temporal measurement and autocorrelation of spontaneous changes in fluorescent signal intensity of particles in nanomolar concentrations. In addition, the sensitivity of FCS allows for measurement of
changes in signal intensity on the nanosecond timescale without triggering some
perturbation from equilibrium, like temperature-jump methods. These two factors, the
reduction of probe concentration and high temporal resolution under equilibrium
conditions make FCS a powerful tool for use in the study of biological processes.

FCS was first used by Magde, \textit{et al.} to measure the diffusion and dynamics of
DNA and ethidium bromide binding (Magde \textit{et al.}, 1972; Elson and Magde, 1974). Since
that first demonstration, the technique has been applied to a growing number of systems
of increasing complexity, including diffusion of fluorescent biomolecules in live cells
(Brock \textit{et al.}, 1998; Hess and Webb, 2002; Elson, 2011). With increased complexity, the
process of autocorrelation and eventually cross-correlation algorithms were modified to
make sense of signal fluctuations arising from 3D and 2D diffusion, dynamics, rotation,
and account for artifacts (Rigler \textit{et al.}, 1993; Hess and Webb, 2002).

The instrumentation involved in FCS is comprised of three basic components, as
seen in Figure 1.6, and is based on the experimental realization of the technique by Elson
and Magde (1974). First, a light source in the form of a laser is set to a specific
wavelength and used to excite the reporter fluorophore. In the work presented here, the
laser was set to 514 nm, an appropriate wavelength for excitation of the Texas Red and
tetramethylrhodamine dyes. This light is filtered and the beam is expanded, before it is
directed into the microscope. The second component is a confocal microscope, in which
a dichroic mirror is used to reflect the excitation light, and then filter the emitted light
after passing through the objective and sample. A confocal microscope is used to reduce
the size of the volume of sample illuminated by the incident light to approximately 1
femtoliter. The excitation beam is directed to the objective via reflection with a dichroic
mirror, which also filters the emitted light before it is directed to the pinhole. The 100x oil-immersion objective has a high numerical aperture. After passing through the dichroic mirror, the emitted light is then directed through a 50 µm pinhole, which defines the size of the confocal volume (Rigler et al., 1993; Hess and Webb, 2002). The detection and autocorrelation components comprise the third components of the instrument. As shown in Figure 1.6, a beam-splitter is used to split the emitted light and direct it to two avalanche photodiode detectors. A single detector may generate two electronic pulses after collecting a single photon, a process called “afterpulsing” (Krichevsky and Bonnet, 2002). The two electronic pulses are correlated at short lag time intervals. This results in distortion of the overall correlation function due to the presence of a strong correlation peak for lag times below 1 µs. Thus, two detectors are cross-correlated to eliminate the problem of afterpulsing.

FCS is considered a single-molecule technique, as it can measure changes in particle number within the confocal volume at very low concentrations of fluorophore. The following discussion of the derivation of the autocorrelation function of fluorescence fluctuations is based on several previously published derivations (Elson and Magde, 1974; Schwille and Haustein, *Biophys. Text. Online*; Krichevsky and Bonnet, 2002). Assuming that the intrinsic photophysical properties of the fluorophore are constant over the course of the experiment, then the autocorrelation function of fluorescence fluctuations reflects the changes in the number of fluorophores within the confocal volume as a result of three-dimensional translational diffusion. Assuming that the fluorophore concentration is sufficiently low (i.e., nanomolar range), the probability of $n$ fluorophores occupying the confocal volume at a given time is described by a Poisson distribution,
\[ P(n, N) = \frac{N^n}{n!} e^{-N} \quad (1.8) \]

where \( N \) is the average number of fluorescent molecules in the confocal volume. This results in the normalization of the correlation function by \( N \), in which \( G(0) \) (defined below) is proportional to \( \frac{1}{N} \). However, to estimate the average number of particles, we must know the effective confocal volume and the particle concentration, as

\[ N = CV_{eff} \quad (1.9). \]

If, for example, the diffusion of a fluorophore in the confocal volume is slower than the duration of \( \tau \), then the intensities measured at time \( t \) and \( (t + \tau) \) will be similar in magnitude, and thus correlated. If diffusion of the fluorophore in the confocal volume is faster than the duration of \( \tau \), then intensities measured at times \( t \) and \( (t + \tau) \) will not be correlated.

The autocorrelation device calculates the correlation between two intensity measurements as deviations from the average fluorescence intensity as a function of time between those measurements. A fluctuation in fluorescence intensity, \( F \), at any time, \( t \), can be expressed as the variance in fluorescence intensity,

\[ \delta F(t) = \langle F \rangle - F(t) \quad (1.10). \]

This can also be applied to the variance measured at a time between measurements (or lag time), \( \tau \), as

\[ \delta F(\tau) = \langle F \rangle - F(t + \tau) \quad (1.11) \]

where \( t \) is the time that the first variance was calculated. The fluorescence correlation \((G(\tau))\) measured as a function of a given lag time \((\tau)\), is the product of the fluorescence...
intensity fluctuations at each time \( (t \text{ and } t + \tau) \) normalized by the squared average fluorescence intensity,

\[
G(\tau) = \frac{\langle \delta F(t)\delta F(t+\tau) \rangle}{\langle F \rangle^2} \tag{1.12}
\]

The correlation is calculated as a function of many lag times \( (\tau) \). Equation 1.12 can be rewritten as

\[
G(\tau) = \frac{\langle \delta F(0)\delta F(\tau) \rangle}{\langle F \rangle^2} \tag{1.13}
\]

Equations 1.12 and 1.13 are the autocorrelation functions of fluorescence fluctuation. Depending on the experimental design, the intensity fluctuations may arise from changes in particle brightness, due to a chemical reaction, and simple diffusion by Brownian motion. For the purposes of this thesis, we will pursue the simplest case, in which the fluorescence fluctuations arise from diffusion of particles in and out of the confocal volume. The variance in fluorescence intensity arising from the fluctuations in particle concentration \( (\delta C(\vec{r}, t)) \) is

\[
\delta F(t) = \kappa \int I(\vec{r})\delta (\sigma qC(\vec{r}, t))d^3\vec{r} \tag{1.14}
\]

where \( r \) is the position vector and \( t \) is time. The fluctuations are determined by the overall detection efficiency of the instrument \( (\kappa) \) and the intensity profile of the excitation beam \( (I(\vec{r})) \). \( I(\vec{r}) \) is proportional to the “spatial collection efficiency” determined by the pinhole and objective (Schwille and Haustein, *Biophys. Text. Online*) and the intensity profile of excitation light \( (I_{ex}(\vec{r})) \). The fluctuations in the absorption cross-section \( (\sigma) \) and quantum yield \( (q) \) are unique to each fluorophore. The measured fluorescence intensity fluctuations and the autocorrelation function of fluorescence fluctuation depend on the assumption that the intensity profile of the excitation light is approximated by a
three-dimensional Gaussian function. This affects the excitation and collection efficiency and intensity of light observed within the confocal volume. The confocal volume is ellipsoidal, the surface of which is not sharply defined, with an intensity maximum \( I_0 \) located at the center of the volume. The light intensity profile as a function of position within the beam is

\[
I_{ex}(\vec{r}) = I_0 \exp \left[ -\frac{2(x^2+y^2)}{w_y^2} - \frac{2z^2}{w_z^2} \right] \quad (1.15)
\]

in which \( w \) is the size of the beam radius where \( I(\vec{r}) = I_0 e^{-2} \) in the x, y, and z directions, where x and y are perpendicular to the beam path, and z is parallel to the beam path.

From this assumption the effective confocal volume \( (V_{\text{eff}}) \) is

\[
V_{\text{eff}} = \frac{3}{2} w_x^2 w_y w_z \quad (1.16)
\]

We can simplify equation 1.13 by separating out the fluctuation of terms that are dependent on the photophysics of the fluorescent molecule. Since we only consider the fluorescence fluctuations to be a result of changes in the local concentration of the fluorophore, and not any fluctuation in the number of photons emitted by each molecule, then these remain constant over the experiment. By substituting equation 1.14 into equation 1.13 and only considering diffusion of the particles, we see that

\[
G(\tau) = \frac{\iint I(\vec{r}) I(\vec{r}) \delta C(\vec{r}, 0) \delta C(\vec{r}, \tau) d^3\vec{r} d^3\vec{r}'}{\langle C \rangle \int I(\vec{r}) d^3\vec{r}^2} \quad (1.17)
\]

The \( \langle \delta C(\vec{r}, 0) \delta C(\vec{r}', \tau) \rangle \) term is the number density autocorrelation term and is

\[
\langle \delta C(\vec{r}, 0) \delta C(\vec{r}', \tau) \rangle = \langle C \rangle \frac{1}{(4\pi D\tau)^{\frac{3}{2}}} \exp \left( -\frac{(\vec{r}-\vec{r})^2}{4D\tau} \right) \quad (1.18)
\]
where $D$ is the diffusion coefficient intrinsic to the fluorescent particle regardless of the instrumentation. Substitution of equations 1.15, 1.16, and 1.18 into equation 1.17 and calculation of integrals yields

$$G(\tau) = \left( \frac{1}{V_{\text{eff}}(C)} \right) \left( \frac{1}{1+\frac{\tau}{\tau_D}} \right) \left( \frac{1}{\sqrt{1+\omega^2 \cdot \frac{\tau}{\tau_D}}} \right) \quad (1.19)$$

The first term can be simplified with equation 1.9, to $\frac{1}{N}$. For three-dimensional diffusion described in equation 1.19, the omega term describes the aspect ratio of the confocal volume as $\omega = \frac{w_z}{w_{xy}}$. The lateral diffusion time ($\tau_D$) is proportional to the intrinsic diffusion coefficient for a given molecule ($D_T$) and the aspect ratio ($\omega$) by

$$\tau_D = \frac{\omega^2}{4D_T} \quad (1.20)$$

In two dimensions, such as diffusion of a membrane-bound fluorophore in a lipid bilayer, the autocorrelation function of fluorescence fluctuations is

$$G(\tau) = \left( \frac{1}{N} \right) \left( \frac{1}{1+\frac{\tau}{\tau_D}} \right) \quad (1.21)$$

Equations 1.19 and 1.21 are applied to fit autocorrelation functions to collected data and thus extract diffusion times, as illustrated in Figure 1.7. But equations 1.19 and 1.21 are only appropriate under the constraint that the particles in the sample are sufficiently dilute and that all intensity fluctuations measured are due only to changes in local concentration of fluorophores within the confocal volume. For real systems, it is necessary to account for the photophysics of the target fluorophore. Loss or interruption of emission is commonly caused by transition to the first excited triplet state. The occurrence of this transition is often caused by the high power of the laser excitation or
simply by the photophysical properties of a given fluorophore. The process of triplet
state excitation and emission is illustrated in the Jablonski diagram (Figure 1.2). Because
the relaxation of the fluorophore from the triplet state is forbidden, the lifetime of
emission is delayed and long in comparison to fluorescence emission. This delayed
relaxation process causes “blinking” of the fluorophore. The triplet state relaxation
kinetics can be described by an exponential decay function

\[ X_{\text{triplet}}(\tau) = 1 - T + T \cdot e^{-\frac{\tau}{\tau_{\text{triplet}}}} \] (1.23)

and then factored into the overall \( G(\tau) \) function. This simple multiplication of the two
types of dynamics can only be done if the triplet state kinetics do not affect the diffusion
coefficient of the fluorophore. Finally, the autocorrelation functions of fluorescence
fluctuation in two and three dimensions, respectively, are

\[ G(\tau) = \left( \frac{1}{N} \right) \left( \frac{1}{1 + \frac{T}{\tau_D}} \right) \left( 1 - T + T \cdot e^{-\frac{\tau}{\tau_{\text{triplet}}}} \right) \] (1.24)

and

\[ G(\tau) = \left( \frac{1}{N} \right) \left( \frac{1}{1 + \frac{T}{\tau_D}} \right) \left( \frac{1}{\sqrt{1 + \omega^2 \cdot \frac{T}{\tau_D}}} \right) \left( 1 - T + T \cdot e^{-\frac{\tau}{\tau_{\text{triplet}}}} \right). \] (1.25)

1.5 Thesis Outline

Membrane protein folding processes are complex and membrane proteins are
often difficult to synthesize or express, which makes them a challenge to study in isolated
systems. However, thoughtful experimental design and analysis can provide insight into
the kinetics of membrane protein folding. The overall goal of this work is to provide
insight into the kinetic mechanisms of folding and activity of membrane proteins using
novel spectroscopic tools.
Chapter 2 focuses on the development of a mechanism for membrane binding and folding of the Hemagglutinin fusion peptide (HAFp), using stopped-flow fluorescence spectroscopy in conjunction with a novel PheCN-Trp FRET pair. Chapters 3 and 4 reveal a model for the activity of two antimicrobial peptides (AMPs) in two model membrane systems. Chapter 3 contains a proof-of-principle study in which diffusion is used as a probe for structural changes in model membranes as a result of AMP association. Chapter 4 contains evidence that AMPs induce domain formation in lipid membranes and act as “fences” to stabilize the domains. In chapter 5, diffusion measured by fluorescence correlation spectroscopy is used to measure translocation of cell-penetrating peptides (CPPs) across model endosomal membranes. Here, the effects of pH, membrane composition, and charge distribution are discussed in detail in reference to this physical phenomenon.
Figure 1.1. Thermodynamic cycle for α-helical peptide association and folding in a membrane environment. Modified from Almeida et al., 2012; Popot and Engelman, 2000.
Figure 1.2. Jablonski diagram illustrating the processes of electronic excitation through absorption, and relaxation processes of internal conversion, intersystem crossing, fluorescence and phosphorescence. This figure is modified from that published in *Principles of Fluorescence Spectroscopy* (Lakowicz, 2006).
**Figure 1.3.** (Top) Chemical structures of p-cyanophenylalanine (PheCN) and tryptophan (Trp) amino acids. (Bottom) Absorbance (purple) and fluorescence emission intensity (green) spectra of 20 µM PheCN (solid) and Trp (dashed) in water. The wavelength of excitation was 240 nm and 290 nm for PheCN and Trp, respectively. This figure is modified from that published in Tucker et al., 2005.
Figure 1.4. Stopped-flow fluorescence spectroscopy set up. Path of incident light to sample is traced from the Xenon lamp source to the photomultiplier tube (PMT). Note: Sizes of objects in figure are not to scale.
Figure 1.5. The hydrodynamic membrane model, as presented in by P. G. Saffmann and M. Delbrück. The membrane is represented as a plane in the x and y dimensions, with a height of h, viscosity of $\mu$, and surrounding environment with viscosity of $\mu'$. The object has a height h, a radius of a, and moves a distance of r, with rotational motion, $\Theta$. Figure obtained from Saffman, P. G. and Delbruck, M. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3111-3113.
Figure 1.6. Fluorescence Correlation Spectroscopy (FCS) setup. Path of incident light to sample is traced in dark green. Path of fluorescence emission is shown in neon green. Abbreviations are: L, lens; M, mirror; P, pinhole.
Note: Sizes of objects in figure are not to scale.
Figure 1.7. Method of data collection using fluorescence correlation spectroscopy (FCS). The diffusion of molecules (red) through the confocal volume (green oval) is measured as changes in fluorescence intensity over time. This is then correlated to give the correlation ($G(\tau)$) as a function of the lag time between measurements ($\tau$). Correlation data is fit to a two- (or three-) dimensional autocorrelation function of fluorescence fluctuations to extract diffusion times of fluorescent components in the sample ($\tau_D$).
CHAPTER 2

Kinetic Measurements Provide New Insights into the Folding Mechanisms of Fusion Peptides and Interactions with Model Membranes

The helical hairpin is a ubiquitous structural motif in proteins, but the folding kinetics and dynamics of this structural element are not well understood. The influenza hemagglutinin fusion peptide (HAnp) is a 23-residue helical hairpin located at the N-terminus of the influenza hemagglutinin fusion protein, which is essential for viral entry into a host cell during infection. It is known that, during infection, acidification of the environment inside the endosome results in a radical conformational change in the hemagglutinin protein, exposing the fusion peptide to the host endosomal membrane. Herein, we propose a self-consistent model for peptide binding and folding in zwitterionic and anionic membrane environments, as well as the conformational response of the peptide to environmental acidification.

2.1. Introduction

The helical hairpin, or helix-turn-helix (HTH) motif is a common structural feature found in proteins. It plays important structural and functional roles in globular and integral membrane proteins. The HTH motif is highly conserved among DNA and RNA binding domains (Dong et al., 2004) and found in many proteins involved in gene regulation, including gene repressors (Kelly and Yanofsky, 1985), DNA repair proteins (Yang et al., 2002), and transcription factors (Aravind et al., 2005 and references within). In viruses, the HTH motif is found in DNA integrase (Cai et al., 1997), and replication
origin proteins (Deb and Deb, 1991). The simplest form of this motif is two antiparallel α-helices separated by a short linker of approximately four residues in length. However, more than two helices may be involved (Aravind et al., 2005) and the turn region may contain additional residues, known as “wings” (Dong et al., 2004). The HTH motif is also common in protein-protein interfaces/ligand binding (Johnson et al., 1998) and in integral membrane proteins (Hargrave et al., 1982; Nagy and Turner, 2007).

The hairpin, whether involving two soluble β-strands or two α-helices, is a ubiquitous motif found in antimicrobial peptides (Venanzi et al., 2006; Subasinghage et al., 2008), toxins (Barnham et al., 1997), peptide hormones (Waegele and Gai, 2010; Neumoin et al., 2007), and viral fusion peptides (Fritz et al., 2011). The mechanisms of β-hairpin folding have been investigated (Culik et al., 2012; Du et al., 2004); however, the mechanisms of α-helical hairpin folding are still relatively unknown. Understanding the mechanism of folding of a helical hairpin peptide is the next logical step in complexity from monomeric helices (Tang and Gai, 2008; Tucker et al., 2006b; Polozov et al., 1998) and α-helical dimers (Tang et al., 2009). The mechanisms of folding of soluble helical hairpin peptides that have been studied suggest that the entropic cost of forming the turn structure is integral to the folding of the HTH conformation (Du and Gai, 2006; Nagy and Turner, 2007). However, many helical hairpin peptides fold on the surface of a membrane, the folding mechanisms of which have not been studied. Membrane proteins are often difficult to synthesize and isolate outside of the membrane environment due to their hydrophobic nature. Understanding the mechanisms of how these peptides fold in a membrane environment is important because over 30% of open reading frames in the human genome encode for integral membrane proteins (Wallin and
Von Heijne, 1998), of which many contain the HTH motif (Hargrave et al., 2011; Nagy and Turner, 2007). The HTH motif is ubiquitous in nature, with over 1100 identified sequences characterized in this structural family. Interestingly, the sequence divergence and functional diversity of HTH motifs are vast (Rosinski and Atchley, 1999). Thus, the HTH motif plays a crucial role in understanding how protein folding occurs within the context of a lipid membrane. For this study, we chose the hemagglutinin fusion peptide (HAfp) as a model for helical hairpin folding in a membrane.

HAfp is a highly conserved N-terminal region of the trimeric HA2 hemagglutinin glycoprotein of the influenza virus (Maeda et al., 1981; White et al., 1982). The C-terminus of each hemagglutinin domain is anchored to the viral membrane by a transmembrane helix. During endocytosis of the viral particle, the endosome interior becomes more acidic, triggering a structural change in the hemagglutinin protein and exposing the N-terminal fusion peptide to the host endosomal membrane (Maeda et al., 1981; White et al., 1982; Wilson et al., 1981). This region is essential for facilitating viral-host cell fusion in the endosome, which is an early and critical step in viral infection. Fusion of the two membranes releases the contents of the virus into cytosol of the host. While X-ray crystallographic structures of hemagglutinin exist (Cross et al., 2009; Bizebard et al., 1995; Bullough et al., 1994; Chen et al., 1999; Wilson et al., 1981), the constructs include neither the full N-terminal fusion peptide nor C-terminal transmembrane domain. The hemagglutinin fusion peptide alone is structurally responsive to changes in pH (Rafalski et al., 1991; Epand et al., 1992; Han et al., 2001; Lorieau et al., 2012a; Lorieau et al., 2012b) and can even induce fusion of model membranes without the presence of the full hemagglutinin protein (Haque et al., 2011).
The mechanism of membrane fusion has been studied extensively using various constructs and mutants of HAfp (Rafalski et al., 1991; Epand et al., 1992; Haque et al., 2011; Lear and DeGrado, 1987). Unfortunately, HAfp is not readily soluble in aqueous solvents (Rafalski et al., 1991). Therefore, care must be taken when choosing a specific variant of the sequence to study in order to accurately understand the folding mechanism and activity of this fusion peptide.

A number of views of HAfp structure and function have emerged based on a series of NMR structures of this protein segment in DPC micelles published over the last 13 years (Han et al., 2001; Lorieau et al., 2010; Chang et al., 2000). Our understanding of the structural response to changes in pH and the overall orientation of the peptide with respect to the membrane is evolving. In a 2001 *Nature* paper, Tamm and coworkers published two NMR structures of HAfp with a lysine-rich C-terminal tail, at pH 7.4 and pH 5, suggesting that a conformational change in HAfp is responsible for its fusion activity at low pH. Specifically, when bound to DPC micelles at pH 7.4, the six C-terminal residues of HAfp do not fully form a helix and the peptide forms an inverted V on the surface of the membrane. At pH 5, the C-terminal 3_{10}-helix forms and the repositioning of Asp 19 and Glu15 within the C-terminal helix at the water-membrane interface allows for deeper insertion of the peptide into the membrane. In 2010, Bax and coworkers (Lorieau et al., 2010; Lorieau et al., 2011) published the structure of a slightly longer (23-residue) HAfp construct containing a C-terminal lysine-rich tail in DPC micelles, and argued that the peptide exists only as one major conformation, specifically a tight helical hairpin oriented nearly parallel to the membrane surface. More recently, Bax and coworkers (Lorieau et al., 2012a; Lorieau et al., 2012b) investigated the structure, at
low pH in DPC micelles, of another variant of the HAfp construct that included a C-terminal lysine-rich tail. Under acidic conditions, the peptide sampled three conformational populations: tight hairpin, “L-shaped”, and “extended”. Most of the peptides adopted a tight helical hairpin, consistent with their previous results, while the L-shaped and extended structures comprise only 20% of the total population, with exchange rates for opening of approximately $\tau_{ex} = 28 \pm 1 \, \mu s$ (Lorieau et al., 2012b). One major concern with several of the previous NMR studies is the effect of the presence of a C-terminal lysine-rich tail, which was added to the native sequence to improve solubility. While there is evidence to suggest that the host-guest peptide method does not affect the structure of HAfp variants (Han and Tamm, 2000a; Han and Tamm, 2000b), these positive charges are placed close to the aspartic and glutamic acid residue side chains in the C-terminal helix, which may affect the extent of insertion into the bilayer. In order to fully understand the fusion activity of HAfp, it is necessary to look at time- and pH-dependent structural changes of the peptide on lipid bilayers in the absence of these non-native residues. The low concentrations required for fluorescence spectroscopic measurements allows us to use peptides that do not contain lysine tags. Thus, the caveats associated with having a positively charged C-terminus are eliminated. Unfortunately, a caveat with our stopped-flow set up is that we are limited to a time resolution of a few hundred microseconds, too long to resolve the conformational changes reported by Lorieau et al. (2012b). Nevertheless, by studying the kinetics and dynamics of HAfp-membrane interactions using stopped-flow fluorescence spectroscopy methods, we believe that we can lend some insight to the conformational dynamics of this peptide in a model membrane environment.
Stopped-flow fluorescence spectroscopy is a powerful tool for resolving the kinetics of chemical processes that occur on a time scale longer than one millisecond (Roder et al., 2006 and references within). Our laboratory (Tucker et al., 2005; Tang and Gai, 2008; Tang et al., 2009; Tucker et al., 2006a) has used stopped-flow fluorescence spectroscopy to identify the rates and mechanisms of folding of small peptides both in solution and lipid membranes. With this technique, we can monitor the changes in fluorescence of the intrinsic fluorophore, tryptophan (Trp), over the course of the binding and folding process. By monitoring the rates over a wide range of peptide and lipid concentrations, we can rule out many of the possible mechanisms of binding and folding of HAfp. This method of analysis was recently employed by Kreutzberger and Pokorny to elucidate the mechanism of Lysette-26 binding to model membranes (2012). Our laboratory has studied non-native fluorophores, which can be used as FRET pairs with Trp (Tucker et al., 2005; Tucker et al., 2006b; Rogers et al., 2010), such as $p$-cyano-phenylalanine ($\text{Phe}_{\text{CN}}$). $\text{Phe}_{\text{CN}}$ not only has an increased quantum yield in comparison to phenylalanine, but can also act as a donor fluorophore to Trp (Tucker et al., 2005; Tucker et al., 2006b). The efficiency of this energy transfer is proportional to the distance between the donor and acceptor fluorophores. As shown by Tucker et al. in a 2005 study of folding of the amphipathic peptide Mastoparan X, the $\text{Phe}_{\text{CN}}$-Trp FRET pair has a Förster distance of 16 Å, making it ideal for monitoring changes in relatively short separation distances (Tucker et al., 2005). $\text{Phe}_{\text{CN}}$ is easily incorporated into a peptide during solid-phase peptide synthesis and the CN group causes minimal change in the size and polarity of the phenylalanine side chain. $p$-cyano-phenylalanine can be selectively excited at 240 nm and the indirect excitation of Trp via FRET allows us to monitor
conformational changes in the peptide. In addition, PheCN is also sensitive to changes in solvent environment (Tucker et al., 2006a). Using the PheCN-Trp FRET pair in conjunction with Trp fluorescence we are able to dissect folding and membrane binding events (Tang and Gai, 2008; Tang et al., 2009; Tucker et al., 2006b).

Herein, we used stopped-flow fluorescence spectroscopy in conjunction with the novel PheCN-Trp FRET pair to elucidate the mechanism of folding of a membrane-bound helical hairpin peptide, HAfp. Specifically, two sequences

HAfpwt: GLFGAIAGFIENGWEGMIDGWYG-CNH

F3HAfp: GL-FCN-GAIAGFIENGWEGMIDGWYG-CNH2

were studied spectroscopically. The underlined portions of the sequences denote the N- and C-terminal helices, as estimated from the literature (Han et al., 2001; Lorieau et al., 2010; Lorieau et al., 2011). 100% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and mixed 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPC/POPG, 3/1) lipids were used to form large unilamellar vesicles (LUVs). The goal of this study is to provide a mechanism of membrane binding and folding of this helical hairpin model peptide, as well as provide insight into pH-dependent conformational changes. To dissect the kinetics of binding and folding, we monitored Trp fluorescence emission as a result of the direct and indirect (via FRET) excitation. Three well-separated rate constants were extracted from this data, two of which were dependent on lipid concentration, suggesting that HAfp binds to LUVs via two parallel binding pathways and a third slow insertion step. However, global numerical fitting the data to a parallel mechanism model using KinTek software resulted in a revision of the model to include an off-pathway aggregation step in
solution under relatively high P/L ratio conditions (Scheme 2.1). The FRET data provided evidence suggesting that the folding steps occur during both parallel binding events, and favor folding in solution before binding. Upon lowering the pH of pre-equilibrated HAfp and LUVs, we found that the Trp residues become more sequestered from solvent, but that there is negligible structural rearrangement. Our study also highlights the importance of critical analysis of kinetic data under multiple binding conditions in identifying complex membrane protein folding mechanisms.

2.2 Experimental Methods

2.2.1 Materials

The two peptides, HAfpwt (GLFGAIAGFIENGWEGMIDGWYG-NH$_2$) and F3HAfp (GLF$_{CN}$GAIAGFIENGWEGMIDGWYG-NH$_2$) were synthesized using Fmoc chemistry on a PS3 solid-phase peptide synthesizer (Protein Technologies, Inc., AZ) on NOVAPEG Rink amide resin (EMD Novabiochem, MA). The peptides were cleaved from the resin using a cleavage cocktail (95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsaline) and precipitated using cold ethyl ether. The peptides were purified using reverse-phase High-Performance Liquid Chromatography (HPLC) using a modified set of methods described by Rafalski et al. (1991). Because of the hydrophobic nature of the peptides, they were dissolved in dimethylsulfoxide (DMSO) prior to injection onto the C18 column. They were eluted from the column using a linear mixture of two solvents: 10% acetonitrile (ACN) in 90% water with 10 mM ammonium acetate, pH 6.6; and 10% water in 90% ACN with 10 mM ammonium acetate, pH 6.6. Peptide identity was confirmed using Matrix-Assisted Laser Desorption Ionization (MALDI) spectroscopy. The concentration of the peptide was calculated from the Trp/Tyr
absorption of 280 nm light (HAfpwt $\varepsilon_{280} = 12490 \, \text{M}^{-1} \, \text{cm}^{-1}$; F3HAfp $\varepsilon_{280} = 14430 \, \text{M}^{-1} \, \text{cm}^{-1}$) measured on a Lambda 25 UV-Vis spectrometer (Perkin-Elmer, MA). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2.2 Peptide Preparation

Due to their high hydrophobicity, lyophilized HAfpwt and F3HAfp samples were dissolved in DMSO at high concentrations (1-2 mM) and diluted into aqueous solvent to the desired concentration of peptide and 0.5%-1% DMSO. Peptide DMSO stocks were stored up to two weeks at 4°C or dried for longer storage below -20°C. Both peptides aggregate in solution at low pH, and thus were always diluted into solvent with a pH of 7.4 before use.

2.2.3 Preparation of Large Unilamellar Vesicles

Large Unilamellar Vesicles (LUVs) were prepared fresh for each set of experiments and stored for no longer than one week at 4°C. Two µmoles of lipid were dried using nitrogen gas in glass vials and lyophilized overnight to evaporate any contaminants and chloroform. The lipid films were then dissolved in 50 mM PBS (pH 7.4) to a final concentration of 2 mM. The lipid solutions were frozen in an ethanol-dry ice bath, thawed in a water bath, and then vortexed, five times. The lipid solution was extruded eleven times through a 100 nm pore membrane filter at room temperature. LUVs were kept for up to one week at 4°C.

2.2.4 Equilibrium Spectroscopic Measurements

All circular dichroism (CD) measurements were collected on a Model 420 AVIV
circular dichroism spectrometer (Aviv Biomedical, Inc., NJ). CD samples were prepared in a 1 cm pathlength cuvette and contained approximately 20 µM – 40 µM HApwt or F3HAp. Because DMSO absorbs strongly in the near-UV spectrum, HApwt and F3HAp were dissolved in trifluoroethanolamine before 100x dilution into 50 mM PBS (pH 7.4). Lipid solutions were prepared as described at 2 mM in 50 mM PBS (pH 7.4). All equilibrium fluorescence measurements were collected on a Fluorolog 3.10 spectrofluorometer (Jobin Yvon Horiba, NJ) using a quartz cuvette with a 1 cm pathlength. The concentration of peptide and lipid was 2.5 µM and 250 µM, respectively. DMSO concentration was kept at 0.5 % in all control experiments, even when no peptide was present. DMSO stocks of HApwt or F3HAp were diluted in the appropriate amount of POPC or POPC/POPG (3/1) LUVs at pH 7.4 and allowed to equilibrate for a few minutes. For measurements gathered under neutral pH conditions, the equilibrated sample was then diluted by a factor of two with 50 mM PBS (pH 7.4). For measurements gathered under acidic conditions, the equilibrated sample was diluted by a factor of two with PBS at pH 2.5, resulting in a final pH of 5. During acquisition, the solutions were stirred constantly with a stir bar. Trp was excited at 290 nm and collected between 305 and 450 nm, with 1 nm resolution and averaging time of 3 seconds. Phe\textsubscript{CN} was selectively excited at 240 nm and Trp emission collected between 260 and 450 nm. At least two scans were collected for each sample.

### 2.2.5 Stopped-Flow Measurements

Stopped-flow fluorescence kinetics were measured using a partially home-built system containing a SFM-300 stopped-flow apparatus (Bio-logic, Claix, France) and excitation light generated from the Fluorolog 3.10 spectrofluorometer. The details of this
system were described previously by Tucker, et al. (Tucker et al., 2006b). A µFC-08 microcuvette with a pathlength of 0.8 cm was used to reduce the dead time of the mixing module. The dead time of the system was calculated to be between 0.5 ms and 0.75 ms (data not shown) based on the measurement of the quenching of N-acetyl tryptophanamide (NATA) fluorescence by N-bromo-succinamide (NBS) (Peterman, 1979). All stopped-flow binding experiments were performed using 50 mM PBS at pH 7.4. pH drop experiments were performed by pre-equilibrating peptide and lipid LUVs in 50 mM PBS at pH 7.4, which was then subsequently mixed with 50 mM PBS at pH 2.5. In order to reduce Phe<sub>CN</sub> quenching by chlorine, α-phosphoric acid was used to titrate PBS to pH 2.5.

2.3 Results

2.3.1 Development of a Model for Binding of HAfp to Lipid Vesicles

Direct excitation of Trp is often used to assess the solvation of the indole ring during protein folding. Because of the hydrophobic environment of the lipid membrane, this fluorophore can be employed as a reporter of protein-membrane interaction and burial of the indole ring into the membrane. Here, we used the two Trp residues present in the C-terminal helix of HAfpwt and F3HAfp as reporters of peptide burial into the membrane. In HAfpwt and F3HAfp, the solvent-exposed Trp indole ring, when excited at 290 nm, results in a Stokes shift of the emission wavelength to approximately 355 nm at both pH 5 and pH 7.4 (Figures 2.1A, 2.1B). The peak emission wavelength for Trp sequestered from solvent (i.e., embedded in the membrane) shifts to shorter wavelengths and the band increases in intensity. When HAfpwt or F3HAfp are exposed to POPC or POPC/POPG LUVs at a 1:100 peptide-to-lipid ratio, the emission spectra exhibit a shift
and increase in intensity (Figures 2.1A, 2.1B). The peak maximum of the emission spectrum is centered at a slightly shorter wavelength under acidic conditions than the peak maximum observed under neutral conditions. Interestingly, the peak maximum intensity is increased to a greater extent in the presence of POPC LUVs than POPC/POPG LUVs. This may indicate a larger population of bound peptide to zwitterionic lipids, and that the presence of anionic lipid head groups may repel the negatively charged protein under neutral conditions. In addition, circular dichroism measurements were performed to ensure that both HAfpwt and F3HAfp formed alpha-helical structures when exposed to LUVs. These results are shown in Figure 2.2, and indicate that the wild type and mutant HAfp molecules form alpha-helical structures in the presence of LUVs, but that in solution, they contain no discernable structure.

We used the mutant F3HAfp peptide, which contained a $p$-cyano-phenylalanine residue in the place of a phenylalanine residue at position 3, to assess structural changes in the peptide, particularly the distance between the N- and C-terminal helices. Phe$_{\text{CN}}$ is demonstrated to function as a FRET donor in a Phe$_{\text{CN}}$ – Trp FRET pair. The exact distances between the Phe$_{\text{CN}}$ phenyl ring and the two Trp indole rings cannot be discerned; however, a qualitative argument for the overall distance between these two FRET pairs can be made. When we selectively excite the $p$-cyano-phenylalanine ring, we expect to see both the $p$-cyano-phenylalanine fluorescence emission peak centered around 300 nm as well as the Trp emission peak as a result of FRET between the Phe$_{\text{CN}}$ and Trp (Figure 2.3A). In the presence of LUVs (Figure 2.3B), the emission spectrum contains a single peak maximum around 245 nm. The noise observed in Figures 2.3A and 2.3B is likely due to the presence of 0.5% DMSO, which we attempted to subtract by subtracting
spectra of buffer containing 0.5% DMSO. In the processed spectra it is clear that there is a shift in the emission peak maximum of Trp as well as an increase in intensity. In Figure 2.3B, we subtracted spectra of LUVs in buffer containing 0.5% DMSO, but any presence of vesicles with a diameter of 240 nm could scatter some of the incident light, making this subtraction difficult. This is the likely culprit of the slanted baseline and differences in the peak intensities for each condition in Figure 2.3B. Nevertheless, these data indicate that the PheCN and Trp fluorophores are being brought closer together upon peptide binding to the membrane, indicating that F3HAfp is at least partially folding into a helical hairpin.

Since we observed a distinct difference in the spectral features of Trp emission after direct excitation in response to binding, pH, and membrane composition, we hypothesized that these changes would occur on a time scale measurable by stopped-flow fluorescence spectroscopy. The stopped-flow experiments were designed to address three questions that would elucidate the mechanistic details of the binding and folding processes, and are discussed in the following paragraphs.

First, we wanted to address the binding process from the perspective of the sequestration of Trp residues into the membrane. We directly excited the two Trp residues at 290 nm and monitored the emission intensity above 320 nm. A 5 µM solution of HAfpwt or F3HAfp (1% DMSO) in 50 mM PBS (pH 7.4) was mixed in a 1:1 (vol:vol) ratio with solutions of 200 µM, 500 µM, 600 µM, 800 µM, 1000 µM, 1200 µM, 1400 µM, or 1500 µM lipid in LUV form in 50 mM PBS (pH 7.4, 1% DMSO). Thus, the initial concentrations of peptide and lipid were diluted by a factor of two after mixing. The reactions were monitored for 520 ms to 1 s after the stop, in order to collect the
maximum amount of data points during the fast phases of binding. The increase in voltage corresponds to an increase in fluorescence signal, due to the amplification of the signal in the preamplifier and photomultiplier tube detector. These experiments were repeated twice using HAfpwt mixed with POPC or POPC/POPG (3/1) lipid compositions. All experiments were also repeated with F3HAfp for comparison of the mutant to the wild type fusion peptide.

Second, we wanted to designate which steps during the binding process involved folding. We indirectly excited the Trp residues via FRET through the donor fluorophore, PheCN, at 240 nm. During the process of folding the distance between the two arms of the hairpin would decrease, thus increasing the FRET efficiency between the donor and acceptor fluorophores, leading to an increased signal observed in Trp emission. In these experiments, a 5 µM solution of F3HAfp (1% DMSO) in 50 mM PBS (pH 7.4) was mixed in a 1:1 (vol:vol) ratio with solutions of 200 µM, 500 µM, 600 µM, 800 µM, 1000 µM, 1200 µM, 1400 µM, or 1500 µM lipid in LUV form in 50 mM PBS (pH 7.4, 1% DMSO). The fluorescence signal from indirectly exciting the Trp residues via FRET was collected in succession with signal due to direct excitation for consistency.

Third, we wanted to provide some insight into any changes in structure or membrane integration upon acidification. We chose to use the longer sequence variant, which was used by Lorieau et al. (Lorieau et al., 2010; 2011; 2012a; 2012b), because it features a C-terminal Trp residue and has been synthesized in the past without the need for a lysine tail (Lear and DeGrado, 1987; Rafalski et al., 1991). The kinetic measurements, designated as “pH drop” experiments, were designed to provide a new view of this folding process. For our mixing experiments, 5 µM HAfpwt or F3HAfp was
pre-equilibrated in 50 mM PBS (pH 7.4, 1% DMSO) with POPC or POPC/POPG (3/1) at 1:100, 1:200, and 1:300 (peptide: lipid) ratios. The pre-equilibrated peptide and lipid solution was then mixed in a 1:1 (vol:vol) ratio with 50 mM PBS at pH 2.5. The final pH after mixing was 5 and the total concentration of peptide and lipid was reduced by a factor of two. Control experiments in which solutions of the peptide and LUVs were mixed with PBS at pH 7.4 were also performed to capture any changes in fluorescence signal due to dilution. pH “jump” experiments were not performed because of possible peptide aggregation under long-term storage (>5 minutes) in acidic conditions. No changes in signal over time were observed during pH drop experiments using excitation of Trp via FRET (data not shown). Figure 2.11 contains three pH drop kinetic traces of 1:100, 1:200, and 1:300 (peptide: lipid) solutions of HAfpwt and POPC LUVs.

Stopped-flow kinetics were analyzed using two methods. We first performed a least-squares fit of the data using a triple exponential fit with the equation,

$$ F(t) = B + A_1 \cdot \exp(-k_1 \cdot t) + A_2 \cdot \exp(-k_2 \cdot t) + A_3 \cdot \exp(-k_3 \cdot t). $$  (1)

This resulted in the calculation of three separated kinetic steps and their relative amplitudes. A triple exponential resulted in the best fit and lowest $\chi^2$ for all data sets. Figure 2.4 shows the $\chi^2$ values and residuals resulting from a fit of a single trace to a single, double, and triple exponential function. The extracted rate constants were used as an initial analysis of the dependence of the rate on lipid concentration, as well as starting values for analysis using KinTek software (Johnson et al., 2009). The relative amplitudes provide information on which kinetic step contributes the greatest change in fluorescence signal. Figure 2.5 contains rates plotted as a function of lipid or peptide concentration from experiments in which HAfpwt was mixed with POPC LUVs. Under all conditions,
the two fastest rates both showed dependence on lipid concentrations, indicating that
these two steps followed pseudo-first order or second order kinetics. The third, and
slowest, step, did not exhibit consistent dependence on lipid concentration and the
relative amplitude of this step accounted for only 10-20% of the total signal. In
experiments in which Trp was directly excited at 290 nm, the amplitudes were used to
estimate which kinetic step involved the greatest amount of Trp burial in the membrane.
In experiments in which Trp was indirectly excited via FRET at 240 nm, the steps
containing the largest relative amplitude were designated as major folding steps (Tang et
al., 2009; Tucker et al., 2006b; Tang and Gai, 2008). Although the information derived
from this level of analysis is useful, it cannot be directly applied to any physical model.
To apply the data to a physical binding and folding mechanism, it is necessary to use
software with which the user can globally simulate and compare models to the data.

We used KinTek to simulate and globally fit each set of kinetic data. From the
simple exponential fit of the data, we hypothesized that one of two models (Scheme 2.1)
could explain the observation of two fast rate constants that show dependence on lipid
concentration and one slow, concentration independent rate constant. The first, or
Parallel Model I features a thermodynamic equilibrium of two soluble peptide species (P_a
and P_b), each of which binds to the membrane in parallel steps (M.P_a and M.P_b). The
bound species quickly interchanges between the two bounds states. Once on the
membrane, the peptide slowly continues to fold or embed into the membrane to a final
state (M.P_b*). The second, or Sequential Model, features a single unbound species (P)
that binds to the membrane (M.P) and subsequently dimerizes (M.P_2). After
dimerization, further folding or insertion of the peptide into the membrane results in
generation of a final state (M.P$_2^*$). Discrimination between these two models is not simple. Both models fit the data “by-eye” almost equally as well, as shown Figure 2.6, with comparable $\chi^2$/DoF values. However, the real test for each model is the simulation of the signal (i.e., quantum yield) of each species, which we refer to as the “optical response”. We expect that the relative increase in signal over the course of the stopped-flow experiment should mimic the relative increase in area under the curve in the equilibrium spectra. The relative increase in signal, based on the equilibrium spectra and the kinetic traces for mixing 5 $\mu$M HAfpwt with 500 $\mu$M POPC lipid (LUVs) can be found in Table 2.1. These values are compared to the individual responses for each species and their populations from the simulation of each of the models. The optical responses and $\chi^2$/DoF values for HAfpwt and F3HAfp binding are listed in Tables 2.2 and 2.3, respectively. Although these results favor the Sequential Model as a self-consistent model to describe the binding processes of HAfp, the exponential fit results do not.

The Sequential Model results in the calculation of optical responses (data not shown) that are within reason in comparison to the predicted values and a reasonable fit (Figure 2.6). However, referring back to the exponential fit analysis of the data poses an additional quandary. If the rates calculated from the exponential fit to the data are assigned to each of the three kinetic steps in the Sequential Model, then one would assume that the two fastest, concentration-dependent rates would account for steps involving membrane binding and peptide oligomerization on the membrane surface. As shown in Figure 2.5, the fastest rate has a second order rate constant of approximately 5.0E5-1.0E6 M$^{-1}$s$^{-1}$. The second rate shows a second order rate constant of approximately 1.0E5 M$^{-1}$s$^{-1}$. These two rates are consistent with the binding rates of
other small alpha-helical peptides, such as Magainin 2, anti-\(\alpha_{\text{IIb}}\), and pHLIP, which exhibit second order binding rate constants of 2.75E5 M\(^{-1}\)s\(^{-1}\), 3.4E6 M\(^{-1}\)s\(^{-1}\), and 2.5E5 M\(^{-1}\)s\(^{-1}\), respectively (Tang and Gai, 2008; Tang et al., 2009; Tucker et al., 2006b). The third rate, which shows no significant dependence on lipid concentration, exhibits a rate of approximately 5 s\(^{-1}\), consistent with other membrane-associated \(\alpha\)-helical peptides, including diacylglycerol kinase (Lorch and Booth, 2004) and pHLIP (Tang and Gai, 2008). While these observed macroscopic rates and corresponding amplitudes cannot be directly assigned to physical processes without further numerical analysis, they serve as a starting point for building possible mechanisms.

However, closer investigation of the Sequential Model in light of the trends observed by exponential fitting (Figure 2.5) suggest that this model does not accurately describe the observed trend in rate as a function of both lipid and peptide concentration. Suppose that rate 1 is assigned to the process of HAfp binding to the surface of membrane vesicles. A higher concentration of lipid in solution would result in faster observed rates due to the increased concentration of binding sites for HAfp. Similarly, higher concentrations of peptide would also increase the observed rates. We observe that under increasingly concentrated solutions of lipid, there is an increase in rate 1. We observe little or no dependence of rate 1 on HAfp concentration. Now suppose that rate 2 is assigned to the process of oligomerization on the surface of membrane vesicles. Increased lipid concentration, and thus increased number of lipid vesicles, would result in a dilution of the peptides among an increased number of vesicles available for association. Since the increase in lipid concentration would result in a dilution of the peptide on the surface of each vesicle, we would expect to see a decrease in the rate as a
function of lipid concentration. In addition, increasing peptide concentration would result in an increase in the second rate observed due to an increase in the density of peptides on the surface of the lipid vesicles. It is clear in our data that an increase in lipid concentration leads to an increase in the second rate, and that an increase in peptide concentration results in a slight decrease or no change in the second rate. Taken together, these trends associated with the second rate do not support the Sequential Model. These trends would support a parallel binding model, at least with respect to changes in lipid concentration. Furthermore, the slight inverse relationship between the first and second rates and the peptide concentration would suggest that there is a competing reaction at high peptide concentrations, thus reducing the observed rates of binding. Thus, we arrive at a model that can explain the physical phenomena that we observe, in which binding occurs through a parallel mechanism, but includes a potentially competing aggregation step in solution, and is accounted for under high P/L ratio conditions (Scheme 2.1). This is certainly possible, since HAfp monomers are known to aggregate in solution and even form beta-sheet structures (Dubovskii et al., 2000; Han et al. 2000b; Lear and DeGrado 1987; Rafalski et al. 1991). In addition, equilibrium fluorescence measurements of HAfpwt and F3HAfp in PBS at pH 5 show decreased intensity (Figures 2.1A, 2.1B, and 2.3A), suggesting that there is some self-quenching of the Trp fluorophores from aggregation in solution. In summary, a new model was designed to account for the observed exponential fit results as well as improve the numerical fit, which is discussed further below.

The numerical fit results, shown in Figures 2.6.C, 2.6.D, 2.7, and 2.8 and summarized in Tables 2.4 and 2.5, show that the $\chi^2$/DoF and rates calculated provide a
self-consistent model to describe the observed fluorescence kinetics. The responses are also well within the limits imposed by the equilibrium measurements (Tables 2.1, 2.2, and 2.3). Parallel Model I (Scheme 2.1) was used to globally fit to data sets in which the lipid concentration varied, and Parallel Model II was used to globally fit to data sets in which the peptide concentration varied. Data from FRET experiments were fit to the Parallel Model I using a constrained set of rate constants taken from the Parallel Model I fit to directly excited Trp fluorescence kinetics.

The global numerical fitting process required the following assumptions to sufficiently test Parallel Models I and II. The three observable kinetic steps were assigned initially from the results of the exponential fit to the data. Specifically, these were the two binding processes and the slow insertion step. In order to provide flexibility to our model, the two species in solution (P_a and P_b) were assumed to be in rapid equilibrium. The equilibrium FRET spectra (Figure 2.3) suggest that the Trp and Phe_{CN} fluorophores are separated, and thus if a second population exists in solution, it is relatively small. Therefore, we set the P_a to P_b equilibrium constant to approximately 0.01, with forward and backwards rates much larger than other rates in the model (Tables 2.4 and 2.5). The two species M.P_a and M.P_b were also assumed to achieve rapid equilibrium, with an equilibrium constant that favored the formation of the M.P_b species (Tables 2.4 and 2.5). The initial rates for binding and insertion were taken directly from the exponential fitting results and allowed to fluctuate within the constraints of the tested models. For example, due to the assumption that the species P_b is not highly populated, we assume that the faster of the two binding rates would be associated with the process of species P_b binding to the membrane (M) to compete with the binding process of species
Because the overall change in signal is positive, indicating an overall increase in Trp emission, the membrane bound species were initially set to values larger than those of \( P_a \) and \( P_b \).

The aggregation step explicitly written in Parallel Model II was only employed for fitting data sets that included a concentration series with changing peptide concentration. This added step improved the fit and optical responses calculated for these data sets. The \( P_{agg} \) species was represented in the model as a dimer, but this constraint was only placed as a way to simplify the aggregation process. The resulting \( P_{agg} \) species also was assumed to have a low optical response due to fluorescence quenching of Trp upon aggregation, and was considered to be a “dark state” that is not significantly populated.

2.3.2 Effect of Lipid Composition on Binding and Folding of HAfp

The sequence of the X-31 strain of HAfp carries a -3 charge at pH 7.4. We hypothesized that in the presence of negatively charged membranes, HAfp binding rates would be reduced due to electrostatic repulsion. In both exponential and numerical fitting results, we did not observe significant changes in the binding rates of the HAfp and F3HAfp peptides. The exponential fitting results (not shown) indicate that the second order rate constants associated with rates \( k_{ona} \) and \( k_{onb} \) for POPC/POPG (3/1) vesicle binding are within one order of magnitude to the binding to POPC vesicles (Figures 2.6.B, 2.7, and 2.8; Tables 2.4 and 2.5). The effects of anionic lipids on HAfp binding and folding are inconclusive with the present data set. Equilibrium measurements (Figure 2.1.B) would suggest that more peptide is bound, or that the Trp indole rings are more sequestered from solvent in the presence of zwitterionic membranes than anionic
membranes. Taken together, these results are inconclusive as to the effect of the presence of negative charge on binding and folding. Future studies involving higher concentrations of anionic phospholipids may address this issue.

2.3.3 FRET Measurements Reveal Conformational Changes of F3HAfp

We employed a novel FRET pair, PheCN-Trp, to probe changes in distance between the N-terminal helix and the C-terminal helix of a mutant HAfp molecule, F3HAfp. PheCN contains a cyano group in the para position of the phenyl ring of phenylalanine, and exhibits an increased quantum yield relative to Phe (Tucker et al., 2006a). The excitation band of Trp overlaps the emission band of PheCN, and both fluorophores can be excited selectively, making them a useful FRET pair (Tucker et al., 2005; 2006a; 2006b). The Förster radius of the pair is measured to be 16Å (Tucker et al., 2005; Tucker et al., 2006a), which is a useful reference distance for probing the conformational changes of small proteins. The F3HAfp molecule contains a PheCN in place of the Phe residue in the third position of the sequence. This serves as a FRET donor to the two Trp residues, located at positions 14 and 20, both of which are located in the C-terminal helix region of HAfp. We selectively excited PheCN at 240 nm, and then probed Trp emission as a result of indirect excitation via FRET. One caveat with using the PheCN donor fluorophore is the solvent-sensitive nature of the emission intensity. Specifically, the overall emission intensity of PheCN and FRET are reduced in a hydrophobic environment (Serrano et al., 2010). The physical processes occurring during HAfp-membrane interactions are the same, regardless of the incident light and subsequent fluorophore excitation. Thus, the kinetics observed will be the same for experiments in which Trp was directly and indirectly excited (via FRET). However, the
relative amplitudes and the optical responses associated with each rate and species may change, depending on which steps in the mechanism involve folding (Tucker et al., 2005; Tang and Gai, 2008; Tang et al., 2009).

Exponential fitting of the FRET data requires only a double exponential function, indicating that out of the three observable kinetic steps extracted from fitting Trp fluorescence data, two contain FRET responses. The Parallel Model fit both sets of data well, and the optical response ratios for species $P_a$, $P_b$, $M.P_a$, $M.P_b$, and $M.P_b*$ are 1.00, 1.23, 1.47, 1.10, 1.11, respectively (Table 2.3). The rate constants observed in the global numerical fit of both sets of data are expected to correspond to observable rates calculated from the exponential fit. To address this, the observed rate for each POPC concentration in the series and the peptide species of interest ($P_a$ or $P_b$) was estimated from the calculated rate constants (Table 2.5 and Figure 2.9). One assumption based on the equilibrium constant for the $P_a$ and $P_b$ equilibrium determined the effective concentrations of $P_a$ and $P_b$ to be 2.5 µM and 0.025 µM, respectively. In addition, low P/L ratios (i.e., below 2.5 µM/500 µM) were used to approach conditions exhibiting pseudo-first order kinetics. Both the Trp fluorescence and FRET data were then fit to the same set of estimated observable rates, and the amplitudes were allowed to vary. Comparison of the resulting amplitudes shows that the event that exhibits the largest changes in FRET signal, and thus identifying the event in which most of the observed folding occurs, is linked to the $P_b$ binding arm of the parallel binding events (Scheme 2.1). The average amplitude for the rates associated with $P_a$ binding, $P_b$ binding, and $M.P_b$ insertion after direct excitation are $28.4 \pm 3.33\%$, $64.1 \pm 3.35\%$, and $7.45 \pm 2.98\%$, respectively (Figure 2.10). The FRET data fitting to rates associated with $P_a$ binding and
Pb binding are 25.4 ± 7.90% and 74.6 ± 7.9%, respectively. The average increase in amplitude associated with the Pb binding event is 10.4% ± 4.76. Taken together, the folding events of HAfp occur during the parallel binding processes, specifically through the Pb binding pathway. This evidence suggests that initial structural changes that occur in solution facilitate binding and folding on the membrane surface. This and the optical response ratios extracted from the global numerical fitting method support the hypothesis that there are multiple possible conformations of HAfp in thermodynamic equilibrium in solution, that are not observable by ensemble fluorescence techniques.

2.3.4 pH Drop Measurements Indicate Changes in Insertion Depth of Trp

In order to identify changes in HAfp binding and folding as a function of pH, we utilized a “pH drop” method. The pH was dropped rapidly upon mixing a pre-equilibrated solution of peptide and lipid LUVs at pH 7.4 in a 1:1 ratio with pH 2.5 phosphate-buffered saline (PBS). The resulting solution contained vesicles and peptide diluted by a factor of two at a lowered pH between 4 and 5. Trp was directly excited at 290 nm or indirectly via FRET at 240 nm. The results using HAfpwt are shown in Figure 2.11; however, when Trp was excited indirectly via FRET in the F3HAfp mutant, no changes in fluorescence signal as a function of time were observed. The change in emission intensity from Trp was not observed using our method for simple dilution of the pre-equilibrated peptide-lipid mixture with pH 7.4 PBS (data not shown), suggesting that the increase in Trp emission is due to changes in the environment around these residues resulting from the acidification of the environment.

An exponential fit to the data (Figure 2.9) resulted in a good fit to the data ($\chi^2$) for a double exponential function. This indicates that there are two, well separated kinetic
steps observable from this data. One of these is lipid concentration-dependent and has a second order rate constant of approximately 2.3E5 M$^{-1}$s$^{-1}$, comparable to that of a binding event. The protonation of charged side chains under acidic conditions results in more peptides interacting with the membrane, and/or the bound peptides inserting deeper into the membrane. This is corroborated by the measurement of the $K_d$ of a 20 residue HAfp by a physical separation method, in which the $K_d$ of HAfp binding to POPC LUVs at pH 7.4 and 5 is measured to be 1.4 µM and 0.8 µM, respectively (Rafalski et al., 1991). It is possible that the increase in Trp fluorescence upon acidification is a result of more HAfp molecules binding to the surface of POPC vesicles.

Previously published studies on the effects of side chain burial into membranes on $pK_a$ of glutamic acids indicate that the $pK_a$ of these side chains are increased upon burial into a membrane environment. Protonation of these residues results in possible deeper burial of these residues, which are located in the C-terminal half of the peptide. The $pK_a$s of isolated glutamic acid and aspartic acid side chains in aqueous solvent are 4.2 and 3.8, respectively. However, the $pK_a$s of Glu11 and Glu15 in a 20-residue, glutamic acid-rich analog of HAfp were calculated to be 5.6 once the peptide was bound to SDS or DPC micelles, suggesting that the protonation and hydrogen bonding capabilities of these two residues drives pH-dependent fusion (Hsu et al., 2002; Dubovskii et al., 2000). Taken together, these data suggest that the acidification of peptide-LUV mixtures results in increased binding and/or insertion of the peptides into the membrane. A future test of this would be to make a mutant in which the two glutamic acid residues are changed to glutamine and the aspartic acid residue is changed to asparagine. This peptide is currently being synthesized and studied to determine if this process abolishes the change
in Trp environment at pH 5. In addition, exposing a solution of HAfp and LUVs to solubilized or membrane-associated Trp quenching molecules, like N-bromosuccinimide and acrylamide, may provide a method of measuring the burial of Trp indole rings inside the LUV bilayer as a function of pH.

2.4 Discussion

While the understanding of how alpha-helical peptides bind and fold in membranes has advanced over the past decade, the folding mechanisms of more complex structures in membranes are still unknown. Even in soluble proteins, the folding mechanism of the helix-turn-helix motif is not well defined. Herein, we used a viral fusion peptide as a model for studying the folding mechanism of the helix-turn-helix motif within the membrane environment. We used stopped-flow fluorescence spectroscopy to monitor changes in fluorescence emission of two Trp residues in HAfp to dissect the binding mechanism of HAfp to model membranes. We used this same method in conjunction with a HAfp mutant containing a novel PheCN-Trp fluorescence resonance energy transfer (FRET) pair to identify major folding events that occur during the binding process. Using analytical and non-linear regression fitting software, we determined a self-consistent model for the mechanism of folding for a membrane-associated helix-turn-helix peptide.

Our approach used two peptides: HAfpwt, which is the 23-residue N-terminal segment of the X-31 strain of the influenza virus; and F3HAfp, a mutant in which the Phe at the third position is replaced with the novel PheCN fluorophore. Changes in Trp fluorescence in the presence of lipid vesicles indicate burial of the two C-terminal Trp residues into the hydrophobic membrane. By collecting both equilibrium fluorescence
spectra and time-resolved fluorescence signal under varying conditions, we can limit the possible mechanisms available for folding and binding. For example, by globally fitting data in which the concentration of the lipid is varied from 1:40 to 1:300 (peptide: lipid), we can identify concentration-dependent kinetic steps. From the original analytical fit, we proposed two possible models (Scheme 2.1): the Parallel Model I, in which the two concentration-dependent rates correspond with parallel binding steps followed by a slower step; and a Sequential Model, in which the two concentration-dependent rates correspond to a binding and oligomerization step, followed by a slower step. However, critical examination of the results of exponential fitting to the data revealed physical phenomena that could not be described by oligomerization. With this information, we were able to modify the Parallel Model I, which described the physical phenomena, with an optional reversible aggregation step in solution (Scheme 2.1). This allowed us to fit all data sets successfully, formulating a self-consistent model with which to describe the binding and folding of this helical hairpin.

2.5 Conclusions

Our systematic investigation into the HAfp molecule reveals a self-consistent model for binding and folding to model membranes. To our knowledge, this is the first study of membrane helical hairpin folding kinetics. HAfp was chosen as a model because of its ability to fold as a single unit in the presence of membranes. It would be interesting to investigate further the effects of using a longer segment of the protein, including the linker region between the HAfp and the rest of the HA protein, because it has been suggested to play a role in fusion (Lorieau et al., 2010). In addition, exploring these events within the context of the full HA protein would be ideal. The experimental
and analytical methods used here are certainly necessary for the study of other membrane-protein systems and the mechanism proposed here may serve as a starting point for other helical hairpin studies. In addition, the results of this study have broader implications for the kinetic mechanisms that drive folding of intrinsically disordered proteins in specific environments.
Table 2.1. Ratios of increase in fluorescence intensity for samples containing 2.5 µM HAfpwt or F3HAfp and 250 µM POPC or POPC/POPG (3/1) LUVs. Steady-state ratios were calculated from the integral area of Trp fluorescence spectra in the absence and presence of LUVs. Stopped-flow ratios were calculated from the average of the first five data points and the last five data points. These values were used for evaluating the optical responses calculated from the numerical fit of the data to each model.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>290 nm</th>
<th>240 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POPC</td>
<td>POPC/POPG</td>
</tr>
<tr>
<td>Steady-State Fluorescence: Ratio of Integral Area (&gt;320 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAfpwt</td>
<td>1.76</td>
<td>1.52</td>
</tr>
<tr>
<td>F3HAfp</td>
<td>2.83</td>
<td>2.09</td>
</tr>
<tr>
<td>Stopped-Flow Fluorescence: Ratio of Fluorescence Intensity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAfpwt</td>
<td>1.40</td>
<td>1.32</td>
</tr>
<tr>
<td>F3HAfp</td>
<td>1.73</td>
<td>1.63</td>
</tr>
</tbody>
</table>
Table 2.2. Optical response and $\chi^2$/DoF values for each fluorescent species from the Parallel Model (top) I or (bottom) II fit. Values were calculated from global numerical fitting of each data set, wherein HAfpwt was mixed with either POPC or POPC/POPG (3/1) LUVs at pH 7.4. Values in red were constrained during the fitting process.

<table>
<thead>
<tr>
<th></th>
<th>2.5 µM HAfpwt + (100 µM – 750 µM) POPC LUVs</th>
<th>2.5 µM HAfpwt + (100 µM – 750 µM) POPC/POPG (3/1) LUVs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Species</td>
<td>Value</td>
</tr>
<tr>
<td><strong>Parallel I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P_a</td>
<td></td>
<td>0.165</td>
</tr>
<tr>
<td>P_b</td>
<td></td>
<td>0.165</td>
</tr>
<tr>
<td>M.P_a</td>
<td></td>
<td>0.444</td>
</tr>
<tr>
<td>M.P_b</td>
<td></td>
<td>0.284</td>
</tr>
<tr>
<td>M.P_b*</td>
<td></td>
<td>0.329</td>
</tr>
<tr>
<td>$\chi^2$/DoF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Parallel II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.25 µM – 5.5 µM) HAfpwt + 250 µM POPC LUVs</td>
<td>(1.25 µM – 5.5 µM) HAfpwt + 250 µM POPC/POPG (3/1) LUVs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Value</td>
</tr>
<tr>
<td></td>
<td>P_a</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>P_b</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>P_agg</td>
<td>7.00E-04</td>
</tr>
<tr>
<td></td>
<td>M.P_a</td>
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</tr>
<tr>
<td></td>
<td>M.P_b</td>
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</tr>
<tr>
<td></td>
<td>M.P_b*</td>
<td>0.041</td>
</tr>
<tr>
<td>$\chi^2$/DoF</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. Optical response and $\chi^2$/DoF values for each fluorescent species from the Parallel Model (top) I or (bottom) II fit. Values were calculated from global numerical fitting of each data set, wherein F3HAfp was mixed with either POPC or POPC/POPG (3/1) LUVs at pH 7.4. Values in red were constrained during the fitting process.

<table>
<thead>
<tr>
<th>Species</th>
<th>Value</th>
<th>Std. Error</th>
<th>Ratio (xx/Pa)</th>
<th>Value</th>
<th>Std. Error</th>
<th>Ratio (xx/Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parallel I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pa</td>
<td>0.043</td>
<td>ND</td>
<td>1.00</td>
<td>0.055</td>
<td>2.49E-04</td>
<td>1.00</td>
</tr>
<tr>
<td>Pb</td>
<td>0.043</td>
<td>ND</td>
<td>1.00</td>
<td>0.055</td>
<td>2.49E-04</td>
<td>1.00</td>
</tr>
<tr>
<td>M.Pa</td>
<td>0.13</td>
<td>3.19E-03</td>
<td>3.02</td>
<td>0.001</td>
<td>x</td>
<td>0.01</td>
</tr>
<tr>
<td>M.Pb</td>
<td>0.065</td>
<td>ND</td>
<td>1.51</td>
<td>0.310</td>
<td>5.77E-01</td>
<td>5.68</td>
</tr>
<tr>
<td>M.Pb*</td>
<td>0.07</td>
<td>ND</td>
<td>1.63</td>
<td>0.109</td>
<td>1.52E-01</td>
<td>1.99</td>
</tr>
<tr>
<td>$\chi^2$/DoF</td>
<td>2.30</td>
<td></td>
<td></td>
<td>6.97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Parallel II | | | | | | |
| Pa | 0.058 | 4.57E-04 | 0.24 | 0.025 | 5.66E-05 | 1.00 |
| Pb | 0.058 | 4.57E-04 | 0.24 | 0.025 | 5.66E-05 | 1.00 |
| Pagg | 3.61E-04 | x | 1.48E-03 | 0.075 | 0.026 | 3.00 |
| M.Pa | 0.083 | 1.77 | 0.34 | 0.044 | 1.80E-03 | 1.76 |
| M.Pb | 0.128 | 0.452 | 0.52 | 0.055 | 4.50E-03 | 2.20 |
| M.Pb* | 0.134 | 2.11E-02 | 0.55 | 0.025 | 5.66E-05 | 1.00 |
| $\chi^2$/DoF | 6.70 | | | 1.56 | | |
Table 2.4. Rates calculated for each step in each of the Parallel Model (top) I or (bottom) II fit. Values were calculated from global numerical fitting of each data set, wherein HAfpwt was mixed with either POPC or POPC/POPG (3/1) LUVs at pH 7.4. Equilibrium constants in blue were constrained during the fitting process.

<table>
<thead>
<tr>
<th>Step</th>
<th>Rate</th>
<th>Value</th>
<th>Std. Error</th>
<th>$K_{eq}$</th>
<th>Value</th>
<th>Std. Error</th>
<th>$K_{eq}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_a = P_b$</td>
<td>$k_h$</td>
<td>1.23E+10</td>
<td>1.16E+12</td>
<td>0.01</td>
<td>2.23E+12</td>
<td>1.37E+12</td>
<td>0.01</td>
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<tr>
<td></td>
<td>$k_a$</td>
<td>1.24E+12</td>
<td>-</td>
<td>0.130E-03</td>
<td>8.70E-02</td>
<td>5.79E-03</td>
<td>9.93E-04</td>
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<td>$k_{rea}$</td>
<td>0.905</td>
<td>0.949</td>
<td>1.30E-03</td>
<td>8.76E-01</td>
<td>8.76</td>
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<tr>
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<td>$k_{effa}$</td>
<td>73.2</td>
<td>23.2</td>
<td>1.44</td>
<td>12.7</td>
<td>6.70E-03</td>
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<tr>
<td>$P_b + M = M.P_b$</td>
<td>$k_{orb}$</td>
<td>3.62</td>
<td>1.8</td>
<td>3.85</td>
<td>0.994</td>
<td>0.938</td>
<td>2.31</td>
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<td>$k_{softb}$</td>
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<td>1.25</td>
<td>1.44</td>
<td>11.8</td>
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<td>$k_{sta}$</td>
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<td>5.00</td>
<td>0.036</td>
<td>0.029</td>
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<tr>
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<td>0.2642</td>
<td>5.00</td>
<td>3.92E-03</td>
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<td>$k_{nmb}$</td>
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<td>$\chi^2$/DoF</td>
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<td></td>
<td></td>
<td></td>
<td>1.62</td>
<td>1.39</td>
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<table>
<thead>
<tr>
<th>Step</th>
<th>Rate</th>
<th>Value</th>
<th>Std. Error</th>
<th>$K_{eq}$</th>
<th>Value</th>
<th>Std. Error</th>
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<tbody>
<tr>
<td>$P_a = P_a$</td>
<td>$k_{agg}$</td>
<td>0.405</td>
<td>0.219</td>
<td>0.052</td>
<td>1.21</td>
<td>0.217</td>
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<tr>
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<td>7.86</td>
<td>1.80</td>
<td>0.01</td>
<td>8.90E+09</td>
<td>1.56E+10</td>
<td>0.01</td>
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<tr>
<td>$P_a$</td>
<td>$k_3$</td>
<td>9.11E+04</td>
<td>6.49E+08</td>
<td>0.01</td>
<td>8.90E+11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$k_4$</td>
<td>9.11E+06</td>
<td>-</td>
<td>0.01</td>
<td>8.90E+09</td>
<td>1.56E+10</td>
<td>0.01</td>
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<tr>
<td>$P_a + M = M.P_a$</td>
<td>$k_{ona}$</td>
<td>0.154</td>
<td>0.257</td>
<td>0.988</td>
<td>0.033</td>
<td>0.054</td>
<td>5.67E-03</td>
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<tr>
<td></td>
<td>$k_{effa}$</td>
<td>0.156</td>
<td>1.11E+03</td>
<td>6.44E-03</td>
<td>50.3</td>
<td>15.7</td>
<td>0.640</td>
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<tr>
<td>$P_b + M = M.P_b$</td>
<td>$k_{orb}$</td>
<td>200.00</td>
<td>36.8</td>
<td>2.99</td>
<td>18.4</td>
<td>20.5</td>
<td>2.16</td>
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<td>$k_{offb}$</td>
<td>43.2</td>
<td>395</td>
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<td>78.6</td>
<td>29.1</td>
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<td>$M.P_a = M.P_b$</td>
<td>$k_{nab}$</td>
<td>621</td>
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<td>1.30</td>
<td>0.427</td>
<td>3.67</td>
</tr>
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<td>$k_{nma}$</td>
<td>208</td>
<td>1.35E+03</td>
<td>4.98</td>
<td>0.354</td>
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<tr>
<td>$M.P_b = M.P_b^{*}$</td>
<td>$k_{nmb}{^*}$</td>
<td>121</td>
<td>414</td>
<td>4.98</td>
<td>1.30</td>
<td>0.427</td>
<td>3.67</td>
</tr>
<tr>
<td></td>
<td>$k_{nmb}$</td>
<td>24.3</td>
<td>-</td>
<td>4.98</td>
<td>-</td>
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Table 2.5. Rates calculated for each step in each of the Parallel Model (top) I or (bottom) II fit. Values were calculated from global numerical fitting of each data set, wherein F3HAfp was mixed with either POPC or POPC/POPG (3/1) LUVs at pH 7.4. Equilibrium constants in blue were constrained during the fitting process.

<table>
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<th>Step</th>
<th>Rate</th>
<th>Value</th>
<th>Std. Error</th>
<th>$K_{eq}$</th>
<th>Value</th>
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Scheme 2.1. Parallel Model II, Parallel Model I, and Sequential Models for binding and folding of HAfp to LUVs, as discussed in the text.
Figure 2.1. Equilibrium fluorescence spectra of Trp emission after direct excitation at 290 nm. (A) 2.5 µM HAfpwt or (B) 2.5 µM F3HAfp samples were equilibrated at pH 7.4 (blue) and pH 5 (red) in PBS (dotted lines), with 250 µM POPC (LUVs) (solid lines), or with 250 µM POPC/POPG (3/1) (LUVs) (xxx lines).
Figure 2.2. Equilibrium circular dichroism spectra of HAfpwt (solid lines) and F3HAfp (circles) in pH 7.4 PBS (black), pH 7.4 POPC LUVs (blue), and pH 5 POPC LUVs (red). The final concentrations for HAfpwt and F3HAfp were estimated to be between 20 and 40 µM and the final POPC concentration was estimated to be 2 mM.
Figure 2.3. Equilibrium fluorescence spectra of Trp emission after indirect excitation at 240 nm. 2.5 µM F3HAfp samples were equilibrated at pH 7.4 (blue) and pH 5 (red) in (A) PBS (dotted lines), (B) with 250 µM POPC (LUVs) (solid lines), or with 250 µM POPC/POPG (3/1) (LUVs) (xxx lines).
Figure 2.4. Trp emission intensity after direct excitation at 290 nm, plotted as a function of time after mixing at pH 7.4 (Blue traces). Final concentrations of HAfpwt and POPC (LUVs) were 2.5 µM and 750 µM, respectively. Black lines indicate single (bottom), double (middle), and triple (top) exponential analytical fits to the data. Residuals for each fit are shown as individual traces. \( \chi^2 \) values for the single, double, and triple exponential fits are 0.15, 0.087, and 0.068, respectively. Traces have been offset for clarity.
Figure 2.5. Rates extracted from triple exponential fit to stopped-flow kinetics and plotted as a function of POPC (A, B, C) or HAfpwt (D, E, F) concentration. For all experiments, the pH was 7.4 and Trp was excited directly at 290 nm and emitted light was collected above 320 nm. For experiments in which POPC concentration was varied, the final HAfpwt concentration was 2.5 µM. For experiments in which HAfpwt concentration was varied, the final POPC concentration was 250 µM.
Figure 2.6. Stopped-flow fluorescence kinetics of Trp emission after direct excitation at 290 nm. Data shown in left column (A, C, colored lines) result from mixing peptide and LUVs to final concentrations of 2.5 µM HAfpwt and 100 µM, 250 µM, 300 µM, 400 µM, 500 µM, 600 µM, 700 µM, and 750 µM POPC. Data shown in right column (B, D, colored lines) result from mixing peptide and LUVs to final concentrations of 250 µM POPC and 1.25 µM, 1.5 µM, 2.0 µM, 2.5 µM, 3.0 µM, 3.5 µM, 4.0 µM, 4.5 µM, and 5.0 µM HAfpwt. All traces are spaced for clarity. Black lines indicate fitting to the Sequential (A, B), Parallel I (C), and Parallel II (D) models.
Figure 2.7. Stopped-flow fluorescence kinetics of Trp emission after direct excitation at 290 nm. Data shown on left (A, colored lines) result from mixing peptide and LUVs to final concentrations of 2.5 µM HApwt and 100 µM, 250 µM, 300 µM, 400 µM, 500 µM, 600 µM, 700 µM, and 750 µM POPC/POPG (3/1). Data shown on right (B, colored lines) result from mixing peptide and LUVs to final concentrations of 250 µM POPC and 1.25 µM, 1.5 µM, 2.0 µM, 2.5 µM, 3.0 µM, 3.5 µM, 4.0 µM, 4.5 µM, 5.0 µM, and 5.5 µM HApwt. All traces are spaced for clarity. Black lines indicate fitting to the (A) Parallel I and (B) Parallel II models.
**Figure 2.8.** Stopped-flow fluorescence kinetics of Trp emission after direct excitation at 290 nm. (A) Data shown (colored lines) result from mixing peptide and LUVs to final concentrations of 2.5 µM F3HAfp and 100 µM, 250 µM, 300 µM, 400 µM, 500 µM, 600 µM, 700 µM, and 750 µM POPC. (B) Data shown (colored lines) result from mixing peptide and LUVs to final concentrations of 250 µM POPC and 1.25 µM, 1.5 µM, 2.0 µM, 2.5 µM, 3.0 µM, 3.5 µM, 4.0 µM, and 5.5 µM F3HAfp. (C) Data shown (colored lines) result from mixing peptide and LUVs to final concentrations of 2.5 µM F3HAfp and 100 µM, 250 µM, 300 µM, 400 µM, 500 µM, 600 µM, 700 µM, and 750 µM POPC/POPG (3/1). (D) Data shown (colored lines) result from mixing peptide and LUVs to final concentrations of 250 µM POPC/POPG (3/1) and 1.25 µM, 1.5 µM, 2.0 µM, 3.0 µM, 3.5 µM, 4.0 µM, 5.0 µM, and 5.5 µM F3HAfp. All traces are spaced for clarity. Black lines indicate fitting to the (A, C) Parallel I, and (B, D) Parallel II models.
Figure 2.9. Stopped-flow fluorescence kinetics of Trp emission after mixing peptide and POPC (LUVs) to final concentrations of 2.5 μM F3HAfp and 100 μM, 250 μM, 300 μM, 400 μM, 500 μM, 600 μM, 700 μM, and 750 μM POPC. Trp was excited (A) directly at 290 nm or (B) indirectly via FRET at 240 nm. All traces are spaced for clarity. Black lines indicate numerical fit to Parallel Model I.
Figure 2.10. Kinetics of Trp fluorescence emission as a function of time after (A) direct and (B) indirect excitation via FRET. F3HAfp was mixed with POPC to a final concentration of 2.5 µM peptide and 500 µM (light blue), 600 µM (dark blue), 700 µM (purple), and 750 µM (pink) lipid. Estimated exponential equations to fit the data, as detailed in the text, are shown in black. Relative amplitudes associated with rates 1 (grey), 2 (black), and 3 (white) calculated from results of fitting to Trp fluorescence kinetics after (C) direct and (D) indirect excitation via FRET.
Figure 2.11. (A) Stopped-flow fluorescence kinetics of Trp emission after mixing pre-equilibrated HAfpwt and POPC (LUVs) with PBS (pH 2.5) to a final pH of 5. The final concentration of HAfpwt was 2.5 µM and the final concentration of POPC was 250 µM (red), 500 µM (teal), and 750 µM (purple). Trp was excited directly at 290 nm. Best fit was obtained with a double exponential function (black). Extracted rates (B and C) are plotted as a function of POPC concentration.
CHAPTER 3

Diffusion as a Probe of the Heterogeneity of Antimicrobial Peptide-Membrane Interactions

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Many antimicrobial peptides (AMPs) function by forming various oligomeric structures and/or pores upon binding to bacterial membranes. Because such peptide aggregates are capable of inducing membrane thinning and membrane permeabilization, it is expected that AMP-binding would also affect the diffusivity or mobility of the lipid molecules in the membrane. Herein, we show that measurements of the diffusion times of individual lipids through a confocal volume via fluorescence correlation spectroscopy (FCS) provide a sensitive means to probe the underlying AMP-membrane interactions. In particular, results obtained with two well-studied AMPs, magainin 2 and mastoparan X, and two model membranes indicate that this method is capable of revealing structural information, especially the heterogeneity of the peptide-membrane system, that is otherwise difficult to obtain using common ensemble methods. Moreover, because of the high sensitivity of FCS, this method allows examination of the effect of AMPs on the membrane structure at very low peptide/lipid ratios.

3.1. Introduction

Peptide-membrane interactions play a key role in many biological activities and functions. For example, the innate immune system of many organisms uses antimicrobial peptides (AMPs) as a line of defense against invading bacteria. This arises because the binding and insertion of an AMP can disrupt the structural integrity of the targeted cell membrane (Dathe and Wieprecht, 1999; Sato and Feix, 2006). Therefore, numerous studies have been devoted to the understanding of the thermodynamics, kinetics, and mechanism of AMP-membrane interactions. Previous studies have provided invaluable insights into the mode of AMP action; however, most of them have drawn their conclusions based on probing certain physical properties of the peptide monomers and oligomers, or the release of a foreign molecule, rather than the lipid (Matsuzaki et al., 1995; Matsuzaki et al., 1996a; Gregory et al., 2009; Matsuzaki et al., 1989; Duclohier et al., 1989). Thus, it would be advantageous to devise a method that can directly assess the response of the membrane lipids to peptide binding. Herein, we show that the mobility of individual lipid molecules in the membrane provides a sensitive means to monitor how AMP binding affects the membrane structure, as a previous nuclear magnetic resonance (NMR) study (Picard et al., 1998) has indicated that the diffusion behaviors of a lipid in a supported membrane could be significantly altered by the presence of an AMP.

The lateral diffusion of lipids in the membrane system of interest is probed via fluorescence correlation spectroscopy (FCS), which is a commonly used technique for measuring the diffusion time of fluorescent molecules through a small confocal volume (Magde et al., 1974). For a given confocal microscopic setup and solvent condition, the characteristic diffusion time of a diffusing species is related to its size (Magde et al.,
1974). Thus, FCS has been used to investigate the conformational distribution or heterogeneity of unfolded proteins (Sherman et al., 2008; Guo et al., 2008) and lateral organization in model membranes (Chiantia et al., 2009; Bacia et al., 2004; Kahya and Schwille, 2006; Chen et al., 2006). Similarly, we expect that FCS can be used to reveal the heterogeneity underlying AMP-membrane interactions. To test this expectation, we use FCS to measure how binding of two well-studied AMPs, magainin 2 (hereafter referred to as mag2) and mastoparan X (referred to hereafter as mpX), to model membranes affects the diffusive motion of a small number of fluorescently labeled lipids in the membrane (Chiantia et al., 2009; Kahya and Schwille, 2006; Chen et al., 2006).

Mag2 is a 23-amino acid AMP isolated from the African clawed frog, *Xenopus laevis* (Zasloff, 1987), while mpX is a 15-residue peptide toxin found in the venom of *Vespa xanthoptera* (Hirai et al., 1979). Both peptides are unstructured in aqueous solution but fold into an α-helical conformation upon association with membranes (Matsuzaki et al., 1994; Matsuzaki et al., 1996b; Ludtke et al., 1996; Ludtke et al., 1995). It has been suggested that both peptides disrupt the integrity of the targeted membrane by forming short-lived toroidal pores (Matsuzaki et al., 1995; Matsuzaki et al., 1996a; Matsuzaki et al., 1994; Matsuzaki et al., 1996b; Ludke et al., 1996). However, depending on the peptide/lipid ratio, the molecularity of the toroidal pores may vary (Matsuzaki et al., 1996b; Ludtke et al., 1995; Matsuzaki et al., 1998; Tamba and Yamazaki, 2009). In addition, it has been shown that asymmetric binding of these AMPs to a membrane results in local expansion of the headgroup region and thinning of the membrane in the immediate vicinity of the peptide (Ludtke et al., 1995; Zhao et al., 2001; Lee et al., 2005; Lee et al., 2008; Bouvrais et al., 2008; Khandelia et al., 2008; Salnikov et al., 2009) and
that the orientation and aggregation states of the peptide are determined by local destabilization of the membrane due to the bound peptide/lipid ratio (Matsuzaki et al., 1994; Ludtke et al., 1996; Lee et al., 2005; Huang, 2000; Huang, 2009). Hence, mag2 and mpX constitute good model systems to test the aforementioned notion that diffusion measurements could provide useful insight into the mechanism of AMP action, as the latter is expected to modulate the mobility of lipids in the targeted membrane. Indeed, using solid state NMR spectroscopy, Picard et al. have shown that in the presence of a large amount of melittin (peptide/lipid ratio of 1/20) the lateral diffusion of dipalmitoylphosphatidylcholine (DPPC) lipids deposited on silica beads is slowed and the distribution of the diffusion constants is broadened (Picard et al., 1998). Our results further show that the characteristic diffusion times of the lipids in an AMP-bound membrane are distributed over a wide range of timescales, depending on the peptide/lipid ratio, the composition of the membrane, and the sequence of AMP. Taken together, these results not only demonstrate the sensitivity and applicability of the current method in the study of AMP-membrane interactions, but they also provide new insight into the mechanism of AMP action.

3.2 Experimental Methods

3.2.1 Materials

All materials were used as received. Fmoc-protected amino acids were purchased from Advanced Chem Tech (Louisville, KY). Mag2 and mpX were synthesized using the standard fluoren-9-ylmethoxycarbonyl (Fmoc)-based solid-phase method on a PS3 peptide synthesizer (Protein Technologies, MA), purified by reverse-phase chromatography, and verified by matrix-assisted laser desorption ionization (MALDI)
mass spectroscopy. Phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3[phospho-rac(1-glycerol)] (sodium salt) (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red® 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (referred to hereafter as TR-DHPE) was purchased from Molecular Probe (Eugene, OR).

3.2.2 Preparation of Giant Unilamellar Vesicles

Giant unilamellar vesicles (GUVs) were prepared by the standard method of electroswelling (Mathivet et al., 1996) using a custom-made closed perfusion chamber and indium-tin-oxide (ITO) coated slides (Delta-Technologies, Stillwater, MN) as electrodes. Briefly, 1 μmole/mL POPC or POPC/POPG (3/1, mol/mol) lipid mixture solution was prepared in chloroform. TR-DHPE (0.002%) was then added to the solution; 100 μL of the lipid solution in chloroform was deposited onto an ITO slide. After evaporation of the solvent, the electroswelling chamber was assembled from two lipid-coated ITO slides separated by a rubber spacer and filled with 100 mM sucrose buffer. A voltage of 1.2 V/mm at a frequency of 5 Hz was applied to the system for 2 hours while the sample was incubated at 60 °C.

3.2.3 Sample Preparation for FCS Measurements

Before each FCS experiment, an aliquot (10 μL) of a stock peptide solution at the appropriate concentration (0.2 nM – 20 μM) was mixed with an aliquot (10 μL) of a GUV suspension to achieve a desired final bulk peptide concentration (0.1 nM – 10 μM). Ten microliters of the peptide-GUV solution was then added to a custom-made, closed chamber where the GUVs were allowed to settle for at least one hour on the bottom of
the coverslip. All the FCS traces were collected with GUVs that remained stationary over the course of the experiment. For all the peptide-GUV solutions (containing 25 mM phosphate buffered saline and 50 mM sucrose buffer), the total lipid concentration is the same. Thus, the peptide/lipid ratio for these solutions is proportional to the peptide concentration. Specifically, the peptide/lipid ratios were estimated to be 1/250000, 1/25000, 1/2500, and 1/250 for the 1 nM, 10 nM, 100 nM, and 1 µM peptide solutions, respectively.

3.2.4 FCS Setup and Data Analysis

The detail of the FCS apparatus has been described elsewhere (Chowdhury et al., 2007). In the current study, each FCS curve was obtained by correlating the fluorescence signal for a duration of 40 s. For a given peptide concentration within a set of experiments, more than 250 FCS traces were collected from 25 different GUVs within the apical region of each individual GUV. Due to the large number of GUVs required to compose a full distribution, traces were taken over a few days within several different chambers. In addition, all of the FCS curves were carefully examined and those that were affected by membrane undulations/drifting movements were rejected. Collections of distributions taken from different GUVs and chambers were cross-examined to ensure reproducibility of the results. The remaining FCS autocorrelation curves were fit to the following equation,
\[ G(\tau) = \left( \sum_{i=1}^{n} \frac{1}{N} \left( \frac{f_i}{1 + \frac{\tau}{\tau_D^i}} \right) \right) \times \left( \frac{1 - T + T \cdot e^{-\frac{\tau}{\tau_{triplet}}}}{1 - T} \right) \]  

(3.1)

where \( \tau_D^i \) represents the characteristic diffusion time constant of species \( i \), \( N \) represents the number of fluorescent molecules in the confocal volume, \( f_i \) represents the fraction of diffusion component \( i \), \( \tau_{triplet} \) is the triplet lifetime of the fluorophore and \( T \) represents the corresponding triplet amplitude. It was found that most FCS curves could be adequately fit by a single diffusion component (i.e., \( n = 1 \)), and only a small number of FCS curves required a second component (i.e., \( n = 2 \)) in order to yield a satisfactory fit. For each experimental condition, the resultant \( \tau_D \) values were compiled and presented in a distribution format using a bin size of 200 µs.

3.3 Results

In order to eliminate any potential effects of the cover slip on the diffusion behavior of the lipids in the peptide-bound membranes, all the FCS measurements were performed by placing the focus of the excitation laser beam near the center of the apical region of the GUVs which contain a very small amount of a tracer fluorescently labeled lipid (i.e., 0.002% TR-DHPE). In addition, these GUVs have a diameter of approximately 50-150 µm and remain static on the timescale of the FCS experiments. While occasional thermal fluctuations (Bacia et al., 2004; Kahya and Schwille, 2006; Chen et al., 2006; Milon et al., 2003) of the lipid bilayer in the confocal volume and movement of the GUV induce additional components in the FCS curve, these motions occur on a timescale much
longer than the characteristic diffusion time of the lipids (Kahya and Schwille, 2006) and therefore are not included in the subsequent data analysis and discussion.

### 3.3.1 Lipid Diffusion Times in Peptide-Free GUVs

In order to determine the effect of AMPs on the mobility of lipid molecules in the membrane of interest, a reference point must be established. Thus, we first measured the characteristic diffusion time (i.e., $\tau_D$) of the tracer lipid in the membrane of POPC GUVs without the presence of any AMPs. As shown (Figure 3.1), the diffusion times obtained from repeating measurements are distributed around 1.2 ms (such distribution is referred to hereafter as $\tau_D$-distribution), which is in agreement with the result of Kahya et al. (2006). These results are consistent throughout each batch of GUVs, and were compared to ensure reproducibility. While this distribution of diffusion times must arise from the heterogeneous environment experienced by individual lipids in the membrane, due likely to transient interactions between lipids (Bacia et al., 2004; Kahya and Schwille, 2006), its narrow width nevertheless suggests that the degree of heterogeneity in this case is small. As expected (Figure 3.1), for GUVs consisting of binary mixtures of POPC and POPG (3/1, mol/mol) the diffusion heterogeneity of the tracer lipid is slightly increased.

### 3.3.2 Lipid Diffusion Times in Mag2-Bound GUVs

As shown (Figure 3.2), addition of mag2 to the solution containing POPC/POPG GUVs significantly alters the $\tau_D$ distribution of the fluorescent tracer lipid. While the variability in GUV samples (e.g., size) could cause the underlying $\tau_D$ distribution to broaden, the fact that the diffusion times obtained with a single GUV show a broad distribution indicates that the lipid diffusion indeed becomes more heterogeneous in the
presence of mag2. It is evident that the shape of the $\tau_D$ distribution depends on the AMP concentration, indicating that measurements of lipid diffusion could provide a convenient yet sensitive means to probe the underlying changes in the membrane structure in response to association with an AMP, even at a very low peptide/lipid ratio. While other studies (Matsuzaki et al., 1996b; Ludtke et al., 1995; Matsuzaki et al., 1998; Zhao et al., 2001; Lee et al., 2005; Bouvrais et al., 2008; Khandelia et al., 2008; Salnikov et al., 2009; Wieprecht et al., 1999; Munster, 2002; Hallock et al., 2003) have suggested that peptide-binding would perturb the lipid dynamics, to the best of our knowledge, the current study represents the first systematic investigation of how interaction with an AMP affects the mobility of the lipids in the membrane.

Previous studies have shown that membrane-bound AMPs form transmembrane pores when the bulk peptide/lipid ratio reaches a critical threshold, which is in the range of 1:300 to 1:10 for mag2, depending on the membrane composition (Matsuzaki et al., 1995; Matsuzaki et al., 1998). For the GUV samples used in the current study, the lipid concentration was estimated to be about 250 $\mu$M using a phosphorus assay (Bartlett, 1959). Thus, for the 1 $\mu$M peptide sample the peptide/lipid ratio was estimated to be approximately 1/250, which is within the range of the critical threshold for pore formation for mag2 in POPG bilayers (Matsuzaki et al., 1998). Therefore, those very slow diffusing species (i.e., those with a $\tau_D$ of 20 ms or longer) observed at 1 $\mu$M peptide concentration may indicate pore formation. Nevertheless, it is apparent that even at very low peptide concentrations (e.g., 1 and 10 nM), in which no pores are expected to form, the corresponding $\tau_D$ distributions are drastically different from that measured in the
absence of mag2, suggesting that pore formation is not the main underlying cause of the
decrease in lipid mobility. In addition, results obtained at relatively high concentrations
of mag2 (i.e., 100 nM and 1 µM) clearly show that some lipids even diffuse faster than
those in the unperturbed GUV membranes (Figure 3.3).

Since the activity of AMPs depends on the membrane composition (Gregory et al., 2009), we further studied how mag2 affects the mobility of lipids in GUVs composed
of POPC. As shown (Figure 3.4), the resultant \( \tau_D \)-distributions are less heterogeneous in
comparison to those obtained with POPC/POPG GUVs, consistent with the fact that
mag2 has a weaker affinity towards zwitterionic membranes (Gregory et al., 2009;
Wieprecht et al., 1997).

3.3.3 Lipid Diffusion Times in MpX-Bound GUVs

To further substantiate the notion that lipid diffusion is a useful probe of the
AMP-membrane interactions, we also measured the \( \tau_D \) distributions of the tracer
fluorescent lipid in both POPC and POPC/POPG GUVs with the presence of another
AMP, mpX. As expected, binding of mpX to these model membranes also causes the \( \tau_D \)
distribution to broaden (Figures 3.5 and 3.6). Compared to those obtained with mag2,
however, the effects of mpX on the lipid mobility are clearly different. For example, even
at a mpX concentration of 10 µM, the very fast-diffusing species observed in the case of
mag2 is not present (Figure 3.7). Thus, these results further demonstrate the sensitivity
of the current method.
3.4. Discussion

Because of their biological significance, the AMP-membrane interactions have been the subject of extensive studies. However, most ensemble methods employed in previous studies are unable to reveal the intrinsic heterogeneity associated with such interactions and also lack the ability to probe the perturbation to the membrane structure at low peptide/lipid ratios. On the other hand, techniques based on FCS, which is able to measure lipid dynamics in GUVs at the pseudo-single-molecule level (Chantia et al., 2009; Bacia et al., 2004; Kahya and Schwille, 2006; Chen et al., 2006), are suitable to reveal the effect of AMPs on membranes that might be difficult to determine by other methods. Herein, we show that the mobility of the lipid molecules is a sensitive probe of the effect of AMPs on the structural integrity of the targeted membrane for a wide range of peptide/lipid ratios.

3.4.1 Effect of Peptide Concentration

Previous studies have shown that the activity of mag2 and mpX depends on the local peptide concentration in the membrane (Tamba and Yamazaki, 2009). Huang and coworkers (Huang, 2000) have proposed a two-state-like model to understand this dependence wherein the S state, which is inactive, is populated at relatively low peptide concentrations. While it is generally believed that in the S state the peptides form monomeric α-helices and lie on the surface of the membrane (Huang, 2000), it has been difficult to characterize the changes in the membrane structure induced by adsorption of a relatively small number of peptides. For both mag2 and mpX, our results show that even in the S state these peptides can induce significant changes in the diffusion dynamics and hence the packing of the lipids.
Recently, a vesicle fluctuation analysis study (Bouvrais et al., 2008) indicated that the membrane bending rigidity of POPC GUVs decreases drastically upon interaction with mag2, even at a very low peptide surface coverage. Consistent with this picture, our results show that at 1 nM mag2 (Figure 3.2), where the bulk peptide/lipid ratio is estimated to be around 1/250000, the $\tau_D$ distribution of TR-DHPE obtained in POPC/POPG GUVs is notably different from that obtained in the absence of any peptides, with the appearance of slower-diffusing species. As expected, increasing the peptide concentration increases the population of the lipids whose diffusion dynamics are affected. The latter is evidenced by the appearance of separate peaks in the $\tau_D$ distribution, as well as the increased width of the distribution. Under the current experimental conditions, mag2 and mpX are not expected to induce any micellization or disintegration of the GUVs (Tamba and Yamazaki, 2005), which was confirmed by visual inspection of the GUVs through a microscope eyepiece. Thus, the resulting distributions in $\tau_D$ manifest peptide-induced structural and/or organizational changes in the membrane instead of membrane destruction. Consistent with this picture, a recent molecular dynamics (MD) simulation study (Leontiadou et al., 2006) showed that asymmetrical binding of a related AMP, magainin H2, to a DPPC lipid bilayer creates a local tension in the membrane and asymmetric perturbation of the lipid order.

However, achieving a quantitative interpretation of such $\tau_D$ distributions is difficult, as they present a ‘chaotic-like’ picture regarding the AMP-membrane interactions, especially those obtained at relatively high peptide concentrations. For example, the $\tau_D$-distributions obtained at 100 nM and 1 µM mag2 are exceedingly broad.
and consist of several peaks (Figure 3.2). Since the diffusivity of a membrane species (i.e., lipid or lipid-solvated peptide monomer or oligomer in the current case) depends on several factors (Vaz et al., 1985; Liu et al., 1997), there are two possible interpretations for why the diffusion time of the tracer lipids shows a distribution. First, the decreased lipid diffusion time in AMP-bound membranes could be due to the formation of stable or transient peptide-lipid clusters. It is known that membrane-bound AMPs can form various peptide species (Matsuzaki et al., 1994; Schumann et al., 1997; Hara et al., 2001; Matsuzaki, 1999), such as monomer, dimer, oligomer, and transient or stable pores. Thus, it is probable that some lipids that are ‘solvating’ such peptide species could diffuse together with the peptide species and, as a result, show a decrease in their mobility due to an increase in their effective size (i.e., the size that determines the diffusion coefficient of the peptide-lipid cluster). In this case, the widespread distribution of the diffusion times is a direct manifestation of the existence of a large number of stable or transient peptide-lipid clusters of different sizes. While this interpretation is to some extent attractive, it has limitations.

Theoretical treatments of two-dimensional diffusion in membranes based on both the free volume and continuum models are available (Vaz et al., 1985; Liu et al., 1997). For example, Saffman and Delbrück (1975) have shown that the diffusion coefficient of a transmembrane-bound protein is proportional to the logarithm of the reciprocal of its radius. While the applicability of the Saffman and Delbrück (SD) model has been shown to have a size limit (Guigas and Weiss, 2006), we used it to estimate the sizes of the slow-diffusing species observed in our case with the assumption that the diffusing entity corresponds to a cluster formed by lipid and peptide molecules. On the basis of the SD
relationship measured by Ramadurai et al. (Ramadurai et al., 2009), a diffusing object that has a diffusion time of 4 ms (12 ms) in our case would have an effective radius of about 7.0 nm (75 nm). While forming tightly packed peptide-lipid clusters of 7 nm is entirely possible (Zemel et al., 2004), it seems unlikely that a peptide species can recruit enough lipids to form a tightly packed cluster as large as 75 nm. Furthermore, the size of the pores formed by mag2 has been estimated to be around 2-5 nm, containing 4-7 peptides and approximately 90 lipid molecules (Sato and Feix, 2006; Ludtke et al., 1996). Thus, based on the SD model even those lipids that are trapped in the lining of a peptide pore and thus move together with the pore are expected to have a diffusion time of less than 4 ms. Taken together, these pieces of evidence suggest that we cannot simply attribute the observed $\tau_D$ distribution to the formation of oligomeric peptide species.

The second possibility is that the widespread distribution of lipid diffusion times does not reflect, at least not directly, the size distribution of peptide-lipid species or clusters. Instead, it is a manifestation of AMP-induced perturbations to the local lipid organizations or peptide-induced membrane domain formation (or long-range correlations between lipids). Domain formation has been recently proposed as an alternative mechanism of action for certain antimicrobial peptides, including mag2 (Gregory et al., 2009; Salnikov et al., 2009; Pabst et al., 2007; Epand et al., 2006; Mason et al., 2007; Jean-François et al., 2008; Arouré et al., 2009; Joanne et al., 2009; Epand and Epand, 2009a; Epand and Epand, 2009b). Using fluorescence microscopy Oreopoulos et al. have shown that AMP-induced domains in supported lipid bilayers can have very different sizes, ranging from diffraction-limited to micrometer-sized (2010), which is in accordance with our observation that in AMP-bound membranes the diffusion times of
the labeled lipids are distributed over a wide range of time scales. In addition, our observation is consistent with the study of Bacia et al. (2004), which showed that the diffusion times of a probe lipid in lipid-ordered and lipid-disordered domains can differ by an order of magnitude.

Interestingly, the $\tau_D$ distributions obtained at 100 nM and 1 $\mu$M mag2 concentrations in both types of lipid membranes clearly show the presence of a diffusion component centered at about 400 $\mu$s (Figure 3.3), which is faster than the mean diffusion time (~1 ms) of the lipids in peptide-free membranes. Since this component is absent in the $\tau_D$ distributions obtained at lower peptide concentrations, it most likely arises from the sequestration of lipids due to peptide oligomerization and/or transient pore formation, leaving loosely packed regions of lipid molecules in the membrane. This picture is consistent with the notion that AMPs can induce membrane thinning and softening (Ludtke et al., 1995; Bouvrais et al., 2008; Guigas and Weiss, 2006; Ramadurai et al., 2009; Zemel et al., 2004), resulting in an increase in the lateral surface area per lipid and also a decrease in the membrane’s bending rigidity (Bouvrais et al., 2008; Ludtke et al., 1995; Zemel et al., 2004).

### 3.4.2 Effect of Membrane Composition

It is known that the chemical and physical properties of the lipids play an important role in determining the structure and dynamics of membranes and peptide-membrane interactions. For example, many AMPs, including mag2 and mpX, interact more favorably with anionic membranes rather than zwitterionic membranes (Matsuzaki, 1999). Indeed, diffusion measurements indicate that the effect of AMPs on the mobility of the lipids in POPC membranes is different from that of POPC/POPG membranes.
(Figures 3.2-3.7). It is apparent that at the same bulk peptide concentration, the structure of the POPC membranes is less perturbed upon peptide binding. This is consistent with the fact that mag2 exhibits a >100 fold weaker binding affinity towards POPC membranes (Matsuzaki et al., 1989; Almeida and Pokorny, 2009; Yandek et al., 2009) and that electrostatic forces are important for AMP-membrane interactions (Tamba and Yamazaki, 2009; Wieprecht et al., 1997; Matsuzaki et al., 1997). In addition, the larger effect of both mag2 and mpX on POPC/POPG or negatively charged membranes corroborates the aforementioned hypothesis that the observed $\tau_D$ distribution largely reflects domain formation, as it has been shown that cationic antimicrobial agents exhibit a stronger effect on domain formation for membranes consisting of both zwitterionic and anionic lipid than for zwitterionic membranes (Epand et al., 2008) and that binding of a cationic AMP induces a greater reduction in the packing order of the acyl chains in anionic membranes than in zwitterionic membranes (Mason et al., 2006).

### 3.4.3 Effect of AMPs

To show that the peptide-induced lipid mobility change is not specific to mag2, we repeated all of the experiments with mpX. As indicated (Figure 3.5), binding of mpX to POPC/POPG membranes induces a change in the mobility of the lipid that is qualitatively similar to that observed for mag2 (Figure 3.2). However, the $\tau_D$ distributions obtained with these two peptides also show significant differences. For example, for mpX, even at the highest peptide concentration studied, the very fast diffusion component (~400 µs) observed for mag2 is not observed (Figure 3.7). This is likely due to the smaller size of mpX and also the lower number of positive charges it carries. In addition, it is apparent that mpX has a stronger effect on the diffusivity of lipids in POPC
membranes than mag2 (Figures 3.4 and 3.7), due presumably to the fact that mpX has a stronger binding affinity than mag2 toward POPC membranes (Almeida and Pokorny, 2009; Yandek et al., 2009).

In summary, the diffusion data obtained with both mag2 and mpX indicate that FCS is a versatile technique for studying AMP-induced structural changes of the targeted membrane over a wide range of peptide/lipid ratios and could be used to screen AMPs. In addition, these data indicate that, from a lipid perspective, even a relatively small number of membrane-bound peptides can induce noticeable changes in the membrane structure. While further experimental and theoretical studies are needed to establish the relation between lipid diffusivity and AMP activity, the continuous increase in the width and complexity of the $\tau_D$ distribution with an increasing peptide concentration seems to suggest that models that go beyond the “all-or-nothing” framework (Gregory et al., 2009) should be considered. Furthermore, to facilitate a more comprehensive and quantitative understanding of these results, measurements of AMP diffusivity at different peptide/lipid ratios are also needed.

### 3.5 Conclusions

We demonstrate in this study that the diffusivity of lipids in an AMP-bound membrane is a useful probe of the AMP-induced structural changes of the membrane. For both magainin 2 and mastoparan X, our results show that peptide binding can significantly alter the diffusion time ($\tau_D$) of individual lipids in the membrane through a small confocal volume, measured via fluorescence correlation spectroscopy (FCS). In particular, it is found that the lipid diffusion times obtained from repeating measurements under the same experimental conditions are distributed over a wide range of timescales,
and that the characteristics of the resultant $\tau_D$ distribution depends on the peptide/lipid ratio, the AMP sequence, and the membrane composition, which demonstrates the sensitivity and also applicability of the current method. In addition, the observed heterogeneity in lipid diffusion is attributed to AMP-induced domain formation, an aspect that has been shown to be important to AMP activities.
Figure 3.1. \( \tau_D \)-distributions of TR-DHPE in GUVs composed of POPC and POPC/POPG, as indicated.
Figure 3.2. $\tau_D$-distributions of TR-DHPE in POPC/POPG GUVs obtained at different bulk mag2 concentrations, as indicated.
Figure 3.3. Comparison of the $\tau_D$-distributions of TR-DHPE in POPC/POPG GUVs obtained with and without the presence of mag2.
Figure 3.4. $\tau_D$-distributions of TR-DHPE in POPC GUVs obtained at different bulk mag2 concentrations, as indicated.
Figure 3.5. $\tau_D$-distributions of TR-DHPE in POPC/POPG GUVs obtained at different bulk mpX concentrations, as indicated.
Peptide concentrations (e.g., 1 and 10 nM), in which no pores are expected to form, the corresponding $\tau_D$ distributions are drastically different from that measured in the absence of mag2, suggesting that pore formation is not the main underlying cause of the decrease in lipid mobility. In addition, results obtained at relatively high concentrations of mag2 (i.e., 100 nM and 1 $\mu$M) clearly show that some lipids even diffuse faster than those in the unperturbed GUV membranes (Figure 3).

Since the activity of AMPs depends on the membrane composition, we further studied how mag2 affects the mobility of lipids in GUVs composed of POPC. As shown (Figure 4), the resultant $\tau_D$ distributions are less heterogeneous in comparison to those obtained with POPC/POPG GUVs, consistent with the fact that mag2 has a weaker affinity for zwitterionic membranes.

**Lipid Diffusion Times in mpX-Bound GUVs.** To further substantiate the notion that lipid diffusion is a useful probe of AMP-membrane interactions, we also measured the $\tau_D$ distributions of the tracer fluorescent lipid in both POPC and POPC/POPG GUVs in the presence of another AMP, mpX. As expected, binding of mpX to these model membranes also causes the $\tau_D$ distribution to broaden (Figures 5 and 6). Compared to those obtained with mag2, however, the effects of mpX on the lipid mobility are clearly different. For example, even at a mpX concentration of 10 $\mu$M, the very fast-diffusing species observed in the case of mag2 is not present (Figure 7). Thus, these results further demonstrate the sensitivity of the current method.

**DISCUSSION**

Because of their biological significance, the AMP-membrane interactions have been the subject of extensive studies. However, most ensemble methods employed in previous studies are unable to reveal the intrinsic heterogeneity associated with such interactions and also lack the ability to probe the perturbation to the membrane structure at low peptide/lipid ratios. On the other hand, techniques based on FCS, which is able to measure lipid dynamics in GUVs at the pseudo-single-molecule level, are suitable for revealing the effect of AMPs on membranes that might be difficult to determine by other methods. Herein, we show that the mobility of the lipid molecules is a sensitive probe of the effect of AMPs on the structural integrity of the targeted membrane for a wide range of peptide/lipid ratios.

**Figure 3.6.** $\tau_D$-distributions of TR-DHPE in POPC GUVs obtained at different bulk mpX concentrations, as indicated.
whose diffusion dynamics are affected. The latter is evidenced by appearance of slower-diffusing species. As expected, increasing the obtained at relatively high peptide concentrations. For example, estimated to be approximately 1/25000, the that at 1 nM mag2 (Figure 2), where the bulk peptide/lipid ratio is surface coverage. Consistent with this picture, our results show drastically upon interaction with mag2, even at a very low peptide.

For both mag2 and mpX, our results show that even in the S state induced by adsorption of a relatively small number of peptides. Recently, a vesicle fluctuation analysis study (27) has shown that the activity of mag2 and mpX depends on the local tension in the membrane and asymmetric perturbation of the lipid order. A related AMP, magainin H2, to a DPPC lipid bilayer creates a changes in the membrane instead of membrane destruction. Consistent with this picture, a recent molecular dynamics study (28) showed that asymmetry binding of AMP-induced domains in supported lipid bilayers can have very different sizes, ranging from diffraction-limited to micrometer-sized (29), which is in accordance with our observation.

Using fluorescence microscopy, Oreopoulos et al. have shown that AMP-bound membranes based on both the free volume and continuum models are applicable. However, the free volume model has been shown to have a size limit (30), while the continuum model has been shown to have a size limit (31). While the applicability of the Saffman and Delbruck (31) model has been shown as an alternative mechanism of action for these peptides and approximately 90 lipid molecules (32).

Furthermore, the size of the pores formed by mag2 has been estimated to be 7 nm, containing four to seven lining of a peptide pore and thus move together with the pore are probable that some lipids that are “solvating” such peptide can form various peptide species (33). However, the widespread distribution of the diffusion times is a direct manifestation of the existence of a large number of stable or transient lipid clusters. It is known that membrane-bound AMPs can form various peptide species (34). For example, Saffman and Delbruck (35) have proposed a two-state-like model to understand the diffusion of a transmembrane-bound peptide. However, this model has been shown to have limitations.

The second possibility is that the widespread distribution of diffusion times of a probe lipid (36) has been recently proposed as an alternative mechanism of action for the peptides. However, this explanation is attractive, but it has limitations. For example, the size of the diffusion time distribution of a transmembrane-bound peptide is exceedingly broad and consist of several peaks (Figure 2). Since the peptide can form various peptide species (34), we cannot simply attribute the observed spread distribution of the diffusion times to the formation of lipid clusters. Instead, it is a possible (37) that the widespread distribution of diffusion times is a direct manifestation of the existence of a large number of stable or transient lipid clusters.

**Figure 3.7.** Comparison of the $\tau_D$-distributions of TR-DHPE in POPC/POPG GUVs obtained with and without the presence of mpX.
CHAPTER 4

Diffusion as a Probe of Peptide-Induced Membrane Domain Formation

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Recently we have shown that association with an antimicrobial peptide (AMP) can drastically alter the diffusion behavior of the constituent lipids in model membranes (Smith-Dupont et al., 2010). In particular, we found that the diffusion time of a tracer fluorescent lipid through a confocal volume measured via fluorescence correlation spectroscopy (FCS) is distributed over a wide range of timescales, indicating the formation of stable and/or transient membrane species that have different mobilities. A simple estimate, however, suggested that the slow diffusing species are too large to be attributed to AMP oligomers or pores that are tightly bound to a small number of lipids. Thus, we tentatively ascribed them to membrane domains and/or clusters that possess distinctively different diffusion properties. In order to further substantiate our previous conjecture, herein we study the diffusion behavior of the membrane-bound peptide molecules using the same AMPs and model membranes. Our results show, in contrast to our previous findings, that the diffusion times of the membrane-bound peptides exhibit a much narrower distribution that is more similar to that of the lipids in peptide-free membranes. Thus, taken together, these results indicate that while AMP molecules

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prompt domain formation in membranes, they are not tightly associated with the lipid domains thus formed. Instead, they are likely located at the boundary regions separating various domains and act as mobile fences.

4.1. Introduction

The mechanism of action of antimicrobial peptides (AMPs) has been the subject of extensive studies (Brogden, 1995; Bechinger, 1997; Bechinger, 1999; Sitaram and Nagaraj, 1999; Shai, 2002; Sato and Feix, 2006; Tamba and Yamazaki, 2009). Findings from these studies have prompted the formulation of several models of membrane disruption by AMPs. For example, the ability of AMPs to disrupt the structural integrity of the targeted cell membranes has been attributed to (a) barrel-stave or/toroidal pore formation (He et al., 1996; Yang et al., 2001), (b) membrane-dissolution in a detergent-like manner (Dufourc et al., 1986; Pott and Dufourc, 1995), (c) formation of lipid-peptide domains (Latal et al., 1997; Lohner et al., 1997; Hasper et al., 2006; Jean-François et al., 2008; Epand and Epand, 2009a; Shaw et al., 2008; Epand and Epand, 2009b; Yamamoto and Tamura, 2010; Epand et al., 2010; Joanne et al., 2009; Epand et al., 2008a; Epand et al., 2008b; Arouri et al., 2009), (d) segregation of anionic lipids and zwitterionic lipids (Epand et al., 2008a; Epand et al., 2008b; Arouri et al., 2009; Polyansky et al., 2010; Epand et al., 2006), or (e) formation of non-lamellar phases (El-Jastimi and Lafleur, 1999; Staudegger et al., 2000; Yang et al., 2007). Recently, we have shown that the diffusivity of individual lipids in an AMP-bound membrane, probed via fluorescence correlation spectroscopy (FCS), provides a sensitive means to monitor how AMP binding affects the membrane’s structure and dynamics (Smith-Dupont et al., 2010), even at very
low peptide/lipid ratios. As shown (Figure 4.1), results obtained from two well-studied AMPs, magainin 2 (mag2) and mastoparan X (mpX), showed that AMP-binding can drastically alter the lipid diffusion behavior, and that at relatively high peptide/lipid ratios the lipid diffusion times through a well defined confocal volume, acquired by repeating measurements, are found to distribute over a wide range of timescales (the resultant distribution is hereafter referred to as $\tau_D$ distribution). While in our previous study we tentatively attributed the observed heterogeneity in lipid diffusion to AMP-induced domain formation (Smith-Dupont et al., 2010), which is consistent with a recent molecular simulation (Polyansky et al., 2010) as well as several experimental studies using supported lipid bilayers (Shaw et al., 2008; Oreopoulos et al., 2010) and vesicles (Yamamoto and Tamura, 2010; Epand et al., 2010; Joanne et al., 2009; Epand et al., 2008a; Epand et al., 2008b; Arouri et al., 2009; Wimley, 2010; Almeida et al., 2005), we have not ruled out other possibilities. For example, it is not clear to what extent the putative peptide oligomers and/or pores contribute to the observed distribution of lipid diffusion times.

In order to substantiate our previous conclusions and to further reveal the identity of the slowly diffusing species, herein we use FCS to measure the diffusion times of membrane-bound mag2 and mpX at various peptide/lipid ratios. The underlying premise is that if stable or transient (i.e., stable on the timescale of diffusion through the confocal volume) peptide-lipid clusters of different sizes are formed, distribution of peptide diffusion times should be, to a large extent, similar to the distribution of lipid diffusion times. On the other hand, different distribution scenarios would arise if those slowly diffusing species were composed mostly of lipids. Our results show that for both mag2
and mpX the resultant $\tau_0$ distributions are markedly different from those obtained with lipids, suggesting that AMPs prompt, but do not directly participate in the formation of lipid domains.

### 4.2. Experimental Methods

#### 4.2.1 Peptides

Mag2 (sequence: GIGKFLHASAKKWKAGFVEIMNS) and mpX (sequence: INWKGIAAMAKKLL) were synthesized using the standard fluoren-9-ylmethoxycarbonyl (Fmoc)-based solid-phase method on a PS3 peptide synthesizer (Protein Technologies, MA). The dye molecule, 5-(and 6-) carboxytetramethylrhodamine (5,6-TAMRA), was manually coupled to the N-terminus of the peptide (for mag2 one additional glycine residue was added to the N-terminus). The coupling reaction was performed prior to any cleavage reactions to minimize any undesirable couplings to sidechains or the C-terminus, according to the method of Bilgiçer and Kumar (Bilgiçer and Kumar, 2004). Prior to manual cleavage of the peptide from the resin, which was performed using a cocktail of 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsaline, the unreacted dye was washed off using N,N–dimethylformamide (DMF). The dye-labeled peptide product was further purified by reverse-phase chromatography, and verified by matrix-assisted laser desorption ionization (MALDI) mass spectroscopy. The fluorescently labeled mpX and mag2 are hereafter referred to as TMR-mpX and TMR-mag2, respectively.
4.2.2 Preparation of Giant Unilamellar Vesicles

Giant unilamellar vesicles were prepared according to an electroswelling method (Mathivet et al., 1996) and the details have been described previously (Smith-Dupont et al., 2010).

4.2.3 FCS Setup and Data Analysis

The FCS setup, sample preparation, data acquisition and analysis protocols are same as those used in our previous study (Smith-Dupont et al., 2010). Briefly, all of the FCS measurements were performed by placing the focus of the excitation laser beam near the center of the apical regions of giant unilamellar vesicles (GUVs) of approximately 10-50 µm in diameter, which remained static and intact on the timescale of the FCS measurement. Each FCS curve was obtained by correlating the fluorescence signal for a duration of 40 s and fit to a two-dimension diffusion equation to yield a characteristic diffusion time ($\tau_D$). On average, for each experimental condition (i.e., a specific bulk peptide concentration) more than 250 FCS traces were collected from approximately 25 GUVs. In addition, the final (bulk) concentration of the labeled-peptide in all of the peptide-GUV samples was kept at 1 nM, and the reported peptide concentration corresponds to the sum of the labeled and unlabeled peptide concentrations. In order to present the data in a histogram format (i.e., $\tau_D$-distribution), the experimentally determined diffusion times were binned with a bin size of 200 µs.

4.3. Results

4.3.1 Mastoparan X

As shown (Figure 4.2), the diffusion of mpX in the membrane of POPC GUVs is heterogeneous, especially at low peptide concentrations. For example, at 1 nM mpX the
The diffusion time of the peptide shows a broad distribution ranging from 600 µs to more than 10 ms with an average value of 2.2 ms. Since the peptide diffusion time in buffer (i.e., in the absence of membranes) is 82 ± 12 µs, these data indicate that the diffusivity of membrane-bound peptides is significantly smaller than that of the free peptide. What is more interesting is that the $\tau_D$ distribution of the membrane-bound peptide becomes narrower with increasing peptide concentration, which is in stark contrast with that obtained with the lipid diffusion (Figure 4.1). For example, at 1 µM peptide concentration the diffusion times are distributed between 400 µs and 3 ms, whereas under the same conditions the lipid diffusion times sample a much wider time range. In addition, the average diffusion time of the membrane-bound peptide at 1 µM is approximately 1.0 ms, which is similar to the average diffusion time (~1.2 ms) of the lipids in peptide-free POPC GUVs (Smith-Dupont et al., 2010).

As shown (Figure 4.3), the $\tau_D$ distributions of mpX obtained with POPC/POPG (3/1) GUVs qualitatively show the same behavior. In this case, the most noticeable difference is that the $\tau_D$ distribution appears to be less sensitive to the peptide concentration, namely, at peptide concentrations of ≥10 nM, the distributions are more or less the same. This presumably arises from the stronger binding affinity of cationic AMPs towards membranes containing anionic lipids (e.g., POPG). Similar to that observed in POPC GUVs, the average diffusion time of the peptide at 1 µM is about 1.2 ms.

As a control experiment, we have also measured the $\tau_D$ distribution of a mpX-GUV sample that contains a trace amount of fluorescently labeled mpX (1 nM) and lipid
(i.e., 0.002% TR-DHPE) (Smith-Dupont et al., 2010). As shown (Figure 4.4), simultaneous excitation of the fluorescent peptide and lipid results in a \( \tau_D \) distribution that is drastically different from that resulting from only the diffusion of the peptide. Taken together, these results decisively indicate that the lateral diffusion of a significant fraction of the membrane-bound peptides is independent of the diffusion of the lipid or lipid species.

**4.3.2 Magainin 2**

As shown (Figures 4.5 and 4.6), the \( \tau_D \) distributions of mag2 obtained with both POPC and POPC/POPG (3/1) GUVs also show that the translational diffusion property of the AMP is distinctly different from that of the lipid (Smith-Dupont et al., 2010). In particular, these and the data obtained with mpX all indicate that a large percentage of the membrane-bound AMP molecules have a diffusion coefficient (e.g., for the current setup of a \( \tau_D \) of 1 ms gives rise to a \( D = 1.3 \times 10^{-7} \text{ cm}^2/\text{s} \)) that is comparable to that measured for other peptides and proteins (Guo and Gai, 2010), especially at relatively high peptide concentrations (e.g., 1 \( \mu \text{M} \)) where the AMP molecules are expected to form oligomers and induce membrane domain formation. In conjunction with our previous results (Smith-Dupont et al., 2010), this finding is interesting as it suggests that the role of the AMPs is to prompt the formation of stable and/or transient lipid clusters or domains that are distinguishable by their diffusion times. Additionally, at the same bulk peptide concentration the \( \tau_D \) distribution of mag2 is measurably different from that of mpX, which is expected since the peptide sequence is known to play a key role in AMP-membrane interactions.
4.4. Discussion

Recently we demonstrated that FCS is a sensitive method to probe AMP-membrane interactions (Smith-Dupont et al., 2010). In particular, we showed that binding of an AMP can cause the diffusion times of the constituent lipids of two model membranes to significantly deviate from their intrinsic values (i.e., those measured in the absence of any peptides). In addition, we found in many cases that the diffusion times spread over a wide range of time and are too long to be ascribed to the diffusions of peptide oligomers. Thus, we tentatively attributed the observed heterogeneity in lipid diffusion to AMP-induced membrane domain formation (Smith-Dupont et al., 2010). While this interpretation is consistent with several studies (Epand and Epand, 2009a; Shaw et al., 2008; Oreopoulos et al., 2010; Epand and Epand, 2009b; Yamamoto and Tamura, 2010; Epand et al., 2010; Joanne et al., 2009; Epand et al., 2008a; Epand et al., 2008b; Arouri et al., 2009; Polyansky et al., 2010; Epand et al., 2006), our previous data in themselves do not elucidate the peptide’s role. In other words, we were unable to determine whether these slow diffusing species, monitored via a fluorescent lipid, correspond to tightly bound peptide-lipid clusters or lipid domains. In addition, a recent molecular dynamics simulation by Niemelä et al. (Niemelä et al., 2010) indicated that, in a membrane containing a transmembrane protein channel, the diffusion of the lipids could be intrinsically heterogeneous, as the lipids close to the protein are found to diffuse much slower compared to those far from the protein. Therefore, we seek to better understand our previous results by measuring the diffusion time of the AMP of interest. If the observed diffusing species consist of both AMP molecules and lipids, which are tightly bound to each other, it can be assumed that measurements of either the lipid
diffusion or peptide diffusion would result in similar \( \tau_D \) distributions. On the other hand, if the diffusion of peptide species is not slaved to the diffusion of the lipid species, a different set of results would emerge.

In particular, the corresponding \( \tau_D \) distribution is similar to that obtained with a pH low insertion peptide (pHLIP) that is bound to a membrane surface (Guo and Gai 2010). Because the diffusivity of a membrane-bound object is expected to depend on its size and orientation, as well as the strength of interaction with the surrounding lipid molecules, these results seem to be consistent with the notion that at low peptide/lipid ratios the AMPs occupy primarily the surface-bound or S state (Huang, 2000) wherein the peptide may sample an ensemble of conformations and/or orientations (Whiles et al., 2001; Tucker et al., 2004). An alternative interpretation is that this heterogeneity in peptide diffusion reflects the formation of an ensemble of transiently populated and dynamic peptide-lipid clusters, as observed in the simulation of Niemelä et al. (Niemelä et al., 2010), that have different sizes and hence different diffusion times.

In contrast, the data obtained with mag2 are less pronounced in this regard. As shown (Figures 4.5 and 4.6), in both types of membranes mag2 exhibits a \( \tau_D \) distribution that shows a high probability of occurrence within the time range of 100-600 \( \mu \)s. It is known that mpX binds to POPC membranes at least 10 times stronger than mag2 (Almeida and Pokorny 2009) and that in the entire concentration range studied mag2 is much less effective than mpX at altering the diffusivity of the lipids in POPC membranes (Smith-Dupont, et al., 2010). Thus, for POPC GUVs such fast diffusing species (i.e., those diffusing faster than the lipids) may correspond to peptides that are only weakly associated with the membrane surface. A recent single-particle tracking study has shown
that when a cell-penetrating peptide “floats” on the headgroup region of a membrane, its diffusion coefficient is much larger than that of the lipid (Ciobanasu et al., 2009). However, the results obtained with POPC/POPG GUVs are more difficult to explain. Interestingly, our previous study on the lipid diffusion in POPC/POPG GUVs indicated that mag2 causes a significant fraction of the fluorescently labeled lipids to diffuse faster than (i.e., $\tau_D \approx 400 \mu s$) the lipids in peptide-free membranes. Thus, these results provide strong evidence suggesting that a large number of mag2 molecules are located in loosely packed regions of the membrane, where the effective membrane viscosity is expected to be smaller than that of well-packed or more ordered lipid regions. This picture is consistent with the finding that transmembrane helices prefer the liquid-disordered phase in model membranes (Epand and Epand, 2009a; Shaw et al., 2008; Oreopoulos et al., 2010; Epand and Epand, 2009b; Yamamoto and Tamura, 2010; Epand et al., 2010; Joanne et al., 2009; Epand et al., 2008a; Schäfer et al., 2011).

What is more interesting is that at relatively high peptide concentrations the $\tau_D$ distribution of the membrane-bound peptide differs significantly from that of the lipid (e.g., Figure 4.4). Our previous study showed that the heterogeneity in lipid diffusion increases with increasing peptide concentration (Smith-Dupont et al., 2010), presumably due to an increased degree of membrane structural disintegration by the AMP, whereas our present study indicates that in comparison the peptide diffusion is remarkably less heterogeneous and on average much faster than the lipid diffusion. These results, taken together, provide compelling evidence suggesting that a large fraction of membrane-bound peptides, regardless of their oligomeric states, do not form stable clusters with the membrane lipids. In other words, the exceedingly wide distribution of diffusion times we
previously observed using a tracer fluorescent lipid (Smith-Dupont et al., 2010) in AMP-bound membranes is a manifestation of AMP-induced perturbations to the local lipid organization or peptide-induced membrane domain formation. Moreover, the wide $\tau_D$-distribution of the lipid suggests that the resulting membrane domains are dynamic and varied in size (Schäfer et al., 2011).

AMP-induced membrane domain formation have also been observed or suggested in other studies (Epand and Epand, 2009a; Shaw et al., 2008; Oreopoulos et al., 2010; Epand and Epand, 2009b; Yamamoto and Tamura, 2010; Epand et al., 2010; Joanne et al., 2009; Epand et al., 2008a; Epand et al., 2008b; Arouri et al., 2009; Polyansky et al., 2010; Epand et al., 2006; Epand et al., 2009; Menger et al., 2003; Epand et al., 2007). For example, a molecular dynamics simulation suggested that for membranes composed of both zwitterionic (e.g., PE) and anionic (e.g., PG) lipids, binding of an AMP can induce ordering of PG-rich domains by reducing the electrostatic repulsion between the negatively charged PG lipids (Polyansky et al., 2010). Similarly, an atomic force microscopy study (Oreopoulos et al., 2010) indicated that a cationic peptide, PFWRIRIRR-amide (PR-9), can induce domain formation by preferential interaction with the cardiolipins in supported bilayers consisting of POPE/TOCL (3/1) lipids, at a peptide/lipid ratio of 1/1. While several views have been put forward to explain peptide-induced membrane domain formation, they are all based on the notion that that domain formation is the consequence of the preferential interaction between the cationic residues in the peptide and the anionic lipid head groups, which leads to lipid segregation in membranes composed of both anionic and zwitterionic lipids (Jean-François et al., 2008; Oreopoulos et al., 2010; Epand et al., 2010; Epand et al., 2008b; Arouri et al., 2009;
Polyansky et al., 2010; Epand et al., 2006; Epand et al., 2009; Menger et al., 2003; Epand et al., 2007), or the preferential binding of AMPs to lipid regions that have a lower phase transition temperature (Shaw et al., 2008; Oreopolous et al., 2010; Epand and Epand, 2009b; Joanne et al., 2009; Polyansky et al., 2010), or the preferential binding of peptides to lipids that have a high intrinsic negative curvature (Epand and Epand, 2009a; Schäfer et al., 2011; Gambin et al., 2010).

Regardless of the mechanism by which domains are formed, we can safely assume that the $\tau_D$ distribution of the peptide would be similar or identical to that of the lipid if such domains were composed of tightly packed lipid and peptide molecules so that they diffuse together. The visible ‘tail’ in the $\tau_D$-distributions of mag2 (Figure 4.6) seem to suggest that a small number of mag2 molecules may indeed diffuse together with various lipid domains as their diffusion times are too long to be accounted for as individual peptide species. Mag2 is longer than mpX and contains an additional charged residue as well as two polar residues. In mag2, these charged and polar residues are distributed rather evenly over the entire length of the peptide, whereas in mpX the charged residues are located towards the termini of the peptide. As a result, mag2 exhibits a stronger binding affinity toward anionic lipids. Thus, it is quite plausible that the slow-diffusing peptide species observed in the case of mag2 correspond to a small population of peptides that are tightly bound to and hence diffuse together with lipid domains that are rich in POPG. This notion is consistent with the computer simulation study of Niemelä et al. (Niemelä et al., 2010), which showed that a membrane-bound protein can diffuse as a dynamic complex with the surrounding lipids. In addition, one
might expect that for more potent lipid clustering agents their diffusion could become entirely slaved to that of the clusters thus formed.

However, for both mag2 and mpX our data show that the majority of the peptide diffusion times are comparable to or faster than the mean diffusion time of the lipid in unperturbed membranes (Figures 4.2, 4.3, 4.5 and 4.6), which indicates that the lateral diffusion of most peptides is not hindered by the formation of more slow diffusing lipid domains. In fact, the fast diffusion behavior of the peptides suggests that they are situated in a low-viscosity region of the membrane. For instance, the mean diffusion time of TMR-mpX in the membrane of POPC/POPG (3/1) GUVs is 1.2 ms at 1 µM peptide concentration (Figure 4.3), giving rise to a mean diffusion coefficient of $1.1 \times 10^{-7} \text{cm}^2/\text{s}$, which is significantly larger than that measured for various lipid domains diffusing in the membrane of GUVs (Cicuta et al., 2007). Thus, our results are more consistent with a mechanism wherein the peptides stabilize domains by settling at the interface of the ordered domain and disordered region of the membrane or by partitioning within the more disordered regions of the membrane (Shaw et al., 2008; Epand and Epand, 2009b; Joanne et al., 2009; Polyansky et al., 2010; Almeida et al., 2005; Coibanasu et al., 2010; Hinderliter et al., 2004; Hinderliter et al., 2001). In other words, the peptide molecules in this case behave more like fences or obstacles than nucleation sites for membrane domain formation. It has been observed in simulation studies (Fischer and Vink, 2011; Yethiraj and Weisshaar, 2007), that fixed obstacles in membranes can lead to domain formation by reducing the line tension (Almeida et al., 2005; Schäfer et al., 2011). In light of the current findings, it would be very useful to carry out similar computational studies to further investigate the role of mobile obstacles in membrane domain formations.
Moreover, several studies (Almeida et al., 2005; Saxton and Almeida, 2004; Hammond et al., 2005; McConnell and Vrljic, 2003) have speculated that the size of domains formed due to peptide binding could be too small to detect by conventional optical microscopic methods. Thus, as demonstrated in the current and other studies (Smith-Dupont et al., 2010; Korrbach et al., 1999) FCS provides an easy and alternative method for ‘imaging’ nano- and microdomains in membranes.

In addition, the observation that mpX can effectively induce domain formation in zwitterionic POPC membranes (Smith-Dupont et al., 2010) further suggests that other factors, besides electrostatic interactions, are also important determinants of peptide-induced membrane domain formation. MpX has a large hydrophobic moment, but is too short to fully span the membrane. Nevertheless, mpX can effectively induce membrane thinning, resulting in negative membrane curvature (Gambin et al., 2010; Mouritsen and Sperotto, 1992). Therefore, hydrophobic mismatch between the AMP and membrane thickness may also be a major cause of domain formation (Epand and Epand, 2009a; Salnikov et al., 2009), since it is known that the disordered fluid lipid chains are effectively shorter than the ordered lipid chains (Makowski and Li, 1984) and that each lipid tends to be surrounded by lipids with similar chain lengths in order to protect the hydrophobic core from the surrounding water.

4.5. Conclusions

It is well known that high concentrations of AMPs can cause lipid vesicles to leak or burst. However, at low concentrations the effect of an AMP to the structural integrity of lipid membranes of interest is more difficult to assess. Previously, we demonstrated
that the lateral diffusion of lipids is a sensitive probe of the underlying AMP-membrane interactions, even at very low peptide/lipid ratios, and that AMP-binding leads to formation of a wide variety of lipid species with varying diffusion time. In order to provide a better understanding of the nature of these species, herein we measured the distribution of diffusion times of two membrane-bound AMPs through a well-defined confocal excitation volume using fluorescence correlation spectroscopy. We found that at AMP concentrations of 100 nM and higher the distribution of the characteristic diffusion times of the membrane-bound peptides is much narrower than that of the lipids and also with a faster mean. Thus, these findings indicate that while AMPs induce membrane domain formation, they do not do so by forming tightly bound peptide–lipid clusters. Instead, our results suggest a mechanism of domain formation wherein the AMP molecules (i.e., monomers and/or oligomers) reside within the transition region between domains and behave as mobile fences.
Figure 4.1. $\tau_D$-distributions of fluorescent labeled lipids in POPC GUVs obtained at different bulk mpX concentrations, as indicated (reproduced with permission from Smith-Dupont et al. 2010).
Figure 4.2. $\tau_D$-distributions of TMR-mpX in the membranes of POPC GUVs obtained at different bulk mpX concentrations, as indicated.
Figure 4.3. $\tau_D$-distributions of TMR-mpX in the membranes of POPC/POPG (3/1) GUVs obtained at different bulk mpX concentrations, as indicated.
Figure 4.4. Comparison of the $\tau_D$-distributions of TMR-mpX (red) and TMR-mpX and TR-DHPE (blue) in the membranes of POPC/POPG (3/1) GUVs. In both cases the peptide concentration was 1 $\mu$M.
Figure 4.5. $\tau_D$-distributions of TMR-mag2 in the membranes of POPC GUVs obtained at different bulk mag2 concentrations, as indicated.
Figure 4.6. $\tau_{ij}$-distributions of TMR-mag2 in the membranes of POPC/POPG (3/1) GUVs obtained at different bulk mag2 concentrations, as indicated.
CHAPTER 5

Investigation into the Mechanism of Peptide Translation Across Lipid Bilayers

Cell penetrating peptides (CPPs) play important roles in viral infection and host-pathogen interactions. Although they often carry a high positive charge, they have been observed both computationally and experimentally (in vivo) to translocate across membrane bilayers. The mechanisms of this process are not well understood, partially due to a lack of direct methods to measure peptide diffusion in peptide-membrane systems. Herein, we use a systematic approach to dissect the underlying mechanism of this complex phenomenon using model membrane systems and fluorescence correlation spectroscopy (FCS) as a tool to probe peptide and lipid translocation. Preliminary results of this study suggest that fluorescently labeled CPPs do translocate across model late endosomal membranes and recruit large numbers of lipid molecules in the process.

5.1 Introduction

Cell penetrating peptides (CPPs), like antimicrobial peptides (AMPs), are excellent naturally-occurring systems useful in the study of membrane-peptide interactions. The interactions between CPP primary structure and the chemical composition of lipid membranes lie at the crux of the relationship between structure and function in membrane protein systems. Not only are these excellent examples for studying how membrane proteins interact with the membrane environment, but the translation of these fundamental physical interactions into design of new peptides could
lead to drug discovery and the development of new drug delivery methods. Thus, it is imperative that we dissect these membrane-peptide interactions on a molecular level.

There are many naturally-occurring CPPs and a growing number of designed CPP mimics (Smith et al., 2008; Marks et al., 2011; Ziegler and Seelig, 2011) available for study. A well-known CPP is the human immunodeficiency virus transactivator of transcription (HIV1-TAT) peptide (Frankel and Pabo, 1988; Green and Loewenstein, 1988; Ruben et al., 1989). The HIV1-TAT peptide comprises the C-terminal region of the TAT protein, which is rich in arginine and lysine, and carries a charge of +8 at neutral pH. A recent NMR study by Su et al. (2010) revealed that HIV1-TAT (48-60) exhibits a random coil structure in the interfacial region of DMPC/DMPG bilayers. The peptide assists in nuclear transport of TAT in latent and actively replicating HIV-infected host cells by enhancing the processivity of RNA polymerase II transcription (Frankel and Young, 1998). The arginine side chains mediate interactions between the peptide and RNA (Puglisi et al., 1992). In light of the function of HIV1-TAT, the question arises: How does HIV1-TAT gain access to the cytosol and nucleus of host cells?

The current paradigm for internalization of HIV1-TAT and other CPPs is supported by observations in vivo and consists of a multistep, energy-dependent, receptor-independent process that includes macropinocytosis of CPPs from the extracellular space, followed by endosomal escape (Wadia et al., 2004; Futaki et al., 2007; Smith et al., 2008; Richard et al., 2003; Gump and Dowdy, 2007). Each of these steps is dependent on a complex set of interactions between the peptide and host cell. Specifically, macropinocytosis is mediated by electrostatic interactions between the arginine guanidine groups and proteoglycans on the plasma membrane, as well as
interactions between heparin sulfate and guanidine groups that increase the hydrophobicity of the peptide (Futaki et al., 2007; Kaplan et al., 2005). Endosomal escape is mediated by pH, membrane composition, and membrane potential (Wadia et al., 2004; Rothbard et al., 2004; Gump and Dowdy, 2007).

It is important to take into account the effects of membrane composition and dynamics on peptide activity and function. This is especially intriguing and important for understanding the phenomenon of “endosomal escape”. Late endosomes are unique structures in the cell in terms of their membrane composition (Kobayashi et al., 2002; Kobayashi et al., 2001; Record et al., 2011). Specifically, bis(monoacylglycerol)-phosphate (BMP) comprises less than 1% of total phospholipid in the cell, but can account for 10% of the late endosomal membrane (Kobayashi et al., 2002; Record et al., 2011).

We hypothesized that the energy-independent translocation of HIV1-TAT molecules would be dependent on both pH and membrane composition. To address this, we used fluorescence correlation spectroscopy (FCS) to monitor diffusion into the interior of giant unilamellar vesicles (GUVs) composed of a late endosomal model membrane as a result of incubation with fluorescently labeled HIV1-TAT. We also tested for the possible translocation of lipid species from the membrane into the GUV interior. Our preliminary results suggest that there is some translocation of labeled lipid and peptide molecules, and that the population of these species is heterogeneous in size.
5.2 Experimental Methods

5.2.1 Materials

All materials were used as received. Fmoc-protected amino acids were purchased from Advanced Chem Tech (Louisville, KY). Human immunodeficiency virus (HIV) transactivator of transcription (HIV1-TAT) (sequence: YGRKKRRQRRR-NH$_2$) was synthesized using standard fluoren-9-ulmethoxycarbonyl (Fmoc)-based solid-phase method on a PS3 automated peptide synthesizer (Protein Technologies, MA). The deprotected synthesis product was either cleaved using a trifluoroacetic acid/diisopropylsaline/water (95/2.5/2.5) cleavage cocktail, or conjugated at the N-terminus with 5(6)-carboxytetramethylrhodamine dye (Anaspec, CA). Conjugation to the TAMRA dye was performed according to the procedure described in Bilgiçer and Kumar (2004). The resulting cleaved HIV1-TAT and TAMRA-HIV1TAT were then purified by reverse-phase chromatography, and the identity of each peptide was verified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3[phospho-rac(1-glycerol)] (sodium salt) (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), L-α-phosphatidylinositol (Soy) (sodium salt) (Soy PI), and sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-1’-(3’-oleoyl-2’-hydroxy)-glycerol (ammonium salt) (BMP) were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red® 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (referred to hereafter as TR-DHPE) was purchased from Molecular Probe (Eugene, OR). Fatty-acid-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO).
5.2.2 Preparation of Giant Unilamellar Vesicles

Giant unilamellar vesicles (GUVs) were prepared by the standard methods of electroswelling (Mathivet, 1996) and as described previously (Guo et al., 2011). The resulting GUVs were then further treated to label the outer leaflet of the GUVs, or used directly in FCS experiments. LEM GUVs were prepared from a stock solution of 1 mM of POPC/POPG/SoyPI/BMP (5/2/1/2) in chloroform. TR-DHPE was conjugated to BSA separately. BSA was dissolved to a concentration of 50 mg/mL in 50 mM PBS (pH 7.4). TR-DHPE was diluted in ethanol and added in a 1/750 mol/mol ratio with BSA. The solution was then dialyzed in a 10K MWCO dialysis cassette against 200x volume of 50 mM PBS (pH 7.4) at room temperature for a total of six hours, with three total solution changes. The dialysis chamber was kept away from light to reduce bleaching to the TR dye. The TR-DHPE-BSA solution was then removed and centrifuged at 100k x g 4°C at for 30 minutes to pellet any insoluble aggregates. The resulting concentration of TR-DHPE-BSA was then measured from the absorption of 591 nm light, and stored for up to one week in the dark at 4°C. Lipid concentration of GUV stock samples was verified to approximately 500 µM using a phosphate assay (Bartlett, 1959).

5.2.3 FCS Sample Preparation

Open coverslip chambers were manually prepared for each sample. For samples of unlabeled GUVs, 5 µL of 500 µM stock GUV solution in 100 mM sucrose was placed on the surface of the coverslip and then diluted with pH 5 or pH 7.4 50 mM PBS to a final concentration of 55.6 µM. A coverglass containing wetted lens paper was used to protect the sample and prevent evaporation. The GUVs were allowed to settle on the surface of the coverslip for approximately 15 minutes before measurements were taken.
Outerlabeling of the GUVs was also prepared on the coverslip. TR-DHPE-conjugated BSA solution was diluted to approximately 5 µM, and 0.5 µL was added to the GUV stock solution on the coverslip, to a final TR-DHPE-conjugated BSA of 0.1%. For unlabeled GUVs, the TAMRA-HIV1-TAT peptide was added. For labeled GUVs, the unlabeled HIV1-TAT peptide was added. Outer-leaflet labeled GUV samples were used within 6 hours of labeling. Stock solutions of 2 µM peptide in pH 5 and pH 7.4 50 mM PBS were used. In the case of TAMRA-HIV1-TAT stock solutions, the labeled peptide consisted of only 10% of the total peptide in solution. The final concentration of peptide was approximately 100 nM.

5.2.4 FCS Setup and Data Analysis

The FCS set up has been previously described by Chowdhury et al. (2007). The geometry of the confocal volume was calibrated by measurement of the diffusion time of rhodamine-6G at a concentration of 1 nM in PBS. Fluorescence intensity fluctuations were acquired for 40 s. All collected correlation curves were individually assessed and fit to the following multi-component model for three-dimensional diffusion as described in Chapter 1 (Equation 1.25). Correlation curves obscured by noise, shifts from the baseline due to significant changes in the overall fluorescence intensity, or exhibiting diffusion times of less than 10 µs were noted as “uncounted”.

5.3 Results

5.3.1 TMR-HIV Diffusion in Solution

We first assessed the diffusion dynamics of soluble TAMRA-HIV1-TAT (hereafter referred to TMR-HIV) at pH 7.4 and pH 5 in 50 mM PBS. The fluctuation of fluorescence correlation was fit to a three-dimensional model for diffusion containing one
or two components. A two-component fit was utilized only if a single component did not successfully fit the data. The measured single component diffusion times for 20 nM TMR-HIV in 50 mM PBS at pH 7.4 and pH 5 were 77 ± 9 µs and 61 ± 8 µs, respectively. Only one of six measurements in pH 5 PBS required the use of a second component with a diffusion times of 57 µs and 402 µs, making up 93% and 7% of the relative amplitude of the signal, respectively. Out of a total of 32 measurements in pH 7.4 PBS, 27 of these measurements required the use of a second fitting component. The two-component fit for diffusion of TMR-HIV in pH 7.4 PBS resulted in two amplitudes and two diffusion times: 57 ± 15 µs (85 ± 13%) and 3634 ± 5352 µs (15 ± 13%). At both pH 7.4 and pH 5, the majority of the signal arises from the faster of the two diffusion times, and this diffusion time is within the margin of error for the single component fit for diffusion under each condition.

In order to calculate the radius of a diffusing object in a three-dimensional volume, we used the Stokes-Einstein relation (1905)

\[ D_T = \frac{k_B T}{6 \mu \pi R} \]  

(5.1)

in which the diffusion coefficient \(D_T\) is inversely related to the radius of a spherical diffusing object \(R\), where \(\mu\) is solvent viscosity, \(T\) is temperature, and \(k_B\) is Boltzmann’s constant. Here, we accounted for the geometry of the confocal volume based on calibration of the instrument using rhodamine-6G, which has a reported diffusion coefficient of approximately 2.8E-6 cm²/s (Magde et al., 1974). The TMR-HIV molecule, if unfolded in solution, has an approximate radius of 1.7 nm. Using the above stokes Einstein equation, we calculated that the single component diffusion times and the main component of the two-component diffusion times correspond to diffusing objects
with hydrodynamic radii between 1.1 and 1.4 nm. In addition, these diffusion times are consistent with the diffusion time of $82 \pm 12 \mu s$ measured for a 23-residue peptide, mag2, labeled at the N-terminus with TAMRA (Guo et al., 2011).

The larger diffusion times associated with diffusion in pH 7.4 and pH 5 PBS correspond to diffusing objects with hydrodynamic radii of 67.4 and 7.4 nm, respectively. It is possible that these diffusing objects are large aggregated species in solution. A concentration titration experiment would be useful for addressing this hypothesis. If these are indeed aggregated species in solution, then the fractional component of the species should increase if the concentration of bulk peptide is increased.

5.3.2 Translocation of TMR-HIV Molecules Across Unlabeled LEM

The simplest experiment to assess whether HIV1-TAT peptide translocation across GUV membranes occurs is to monitor diffusion within the center of unlabeled GUVs after exposure to fluorescently labeled HIV peptides (TMR-HIV). We expected to observe correlation of our fluorescent signal fluctuations only under conditions where fluorescent molecules diffused through our confocal volume. Figure 5.1.A (unshaded bars) indicates that approximately 40% of the total events recorded showed some measureable correlation of fluorescence fluctuation. Approximately 60% of the total measured events were too noisy, contained poor baselines, or reported diffusion times that were indistinguishable from possible triplet state lifetime measurements (i.e., $<10 \mu s$) and were left uncounted. More than 30% of the total number of measured events showed calculated correlation times between 0 and 1.0 ms. Under acidic conditions (Figure 5.1.B, unshaded bars), approximately 42% of the total number of events showed calculated correlation times between 0 and 1.0 ms, with 40% of the total number of
events uncounted. Under acidic conditions, approximately 10% of the total events observed showed measurable diffusion times above 1.0 ms. GUVs were prepared without any fluorescent labeling; therefore, we did not expect to see any correlation curves. It is possible that during the GUV electroformation process, small lipid aggregates, micelles, and small vesicles, may form within a larger GUV. Currently, we can attribute these measured diffusion times to be caused by scattered light by these small aggregate species in the center of the GUVs. These effects are observed throughout all of the diffusion time histograms (Figure 5.1).

When we incubate HIV1-TAT in a 1:500 (peptide: bulk lipid) ratio with the LEM GUVs, we observe small changes in the diffusion time distributions at both neutral and acidic pH (Figure 5.1.A and B, shaded bars). At neutral pH (Figure 5.1.A, shaded bars), 44% of the total events observed are uncounted, 25% of the observed events exhibit diffusion times between 0 and 1.0 ms, and the remaining 31% of events exhibit diffusion times above 1.0 ms. The highest number of events counted exhibit diffusion times between 9.0 and 10.0 ms and 1.0 and 6.0 ms. According to Equation 5.1, these correspond to radii of approximately 160 nm and between 37 and 110 nm, respectively. Under acidic conditions, the measured diffusion times above 1.0 ms count for approximately 44% of the total number of events observed. These diffusion times are distributed from 1.0 ms to more than 20.0 ms. The radii of these objects are similar in size to large unilamellar vesicles (LUVs), which are approximately 10,000 times smaller than GUVs. It is important to note that the GUV membranes remain intact throughout the experiment (data not shown). It is possible that these LUVs could be released from the GUV membrane without destruction of the GUV, as suggested in the literature.
(Piantavigna et al., 2011; Säälik et al., 2011). However, this does not rule out fluctuations in membrane structure or increased leakage of BMP-enriched vesicles, which may facilitate translocation, as observed by Yang et al. (2010). Taken together, these data suggest that the addition of TMR-HIV to LEM GUVs causes an increase in observable diffusion of molecules through the confocal volume located in the center of the GUV. These events are rare, but increase in frequency under acidic conditions and in the presence of TMR-HIV.

5.3.3 Translocation of HIV molecules across labeled LEM

The underlying mechanism of translocation of highly charged molecules across lipid membranes cannot be fully realized without a full understanding of how the peptides interact with lipid molecules during and after translocation across the membrane. Because of possible pre-existing lipid aggregates and vesicles in the GUV interior, we could not discriminate between TMR-HIV molecules that attached to lipid molecules post-translocation and TMR-HIV molecules that recruited lipid molecules during translocation from the GUV membrane. The next set of experiments was designed to answer the question: does HIV1-TAT recruit lipids during translocation and exit from the membrane? We incorporated a bovine serum albumin-Texas Red-DHPE (BSA-TRDHPE) lipid conjugate into LEM GUVs in order to label the outer leaflet of formed GUV structures. As shown in Figure 5.1.C and 5.1.D (unshaded bars), over 80% of the total events observed did not exhibit measurable diffusion times, or exhibit diffusion times below 1.0 ms. In the presence of unlabeled HIV1-TAT (~100 nM), the distribution (shaded bars) of diffusion times widens under both acidic and neutral conditions.
5.4 Discussion

Our aim in this work is to provide an *in vitro* systematic assessment of the determining factors required for translocation of a CPP, HIV1-TAT, across model membranes. Once completed, this body of information may provide essential clues for dissecting the molecular level mechanisms that drive peptide translocation. Our results are consistent with computational and experimental studies on the effects of CPPs on model membranes (Yang et al., 2010; Herce and Garcia, 2007), specifically that the fluorescently labeled peptide and lipid molecules are translocated into the vesicle interior. However, the experimental design and data analysis need to be critically assessed due to the inherent heterogeneity and background noise present in the interior of GUVs.

Even in the presence of background noise, the presence peptide-lipid species in the GUV interior is clear. We observe a wide range of diffusion times within the GUV interior, corresponding to a range of radii calculated using the Stokes-Einstein equation for diffusion. These radii range from 37 nm to more than 160 nm, indicating that micelles and even large vesicles could be formed as a result of peptide translocation. This is consistent with molecular dynamics simulations, in which peptide translocation from one leaflet to the next is coupled with lipid translocation (Herce and Garcia, 2007). By selectively labeling the outer leaflet of the GUVs, we were able to visualize the recruitment of these lipids into the vesicle interior by HIV1-TAT.

5.5 Conclusions

In summary, we demonstrated the use of FCS to monitor translocation of HIV1-TAT across GUV model membrane systems. The preliminary results obtained in this study suggest that labeled peptide as well as labeled lipid species can be translocated into
the GUV interior at both neutral and acidic pH. Future work will include sampling a series of GUV model membrane compositions, to pinpoint the role of specific lipid molecules, like BMP, in translocation. In addition, a systematic investigation of a series of peptide sequences may reveal the role of specific amino acids and chemical characteristics (i.e., charge distribution, hydrophobic moment) on translocation.
Figure 5.1. Diffusion time distributions from FCS experiments, in which the confocal volume was positioned in the center of the GUV. All GUVs used were late endosomal membrane model compositions as described in the experimental methods. The bulk lipid concentration was approximately 25-50 µM. Unlabeled LEM GUV interiors (unshaded bars) and unlabeled LEM GUV interiors in the presence of approximately 100 nM TMR-HIV (shaded bars) monitored at pH 7.4 (A) and pH 5 (B). Outer leaflet labeled LEM GUV interiors (unshaded bars) and outer leaflet labeled LEM GUV interiors in the presence of approximately 100 nM HIV1-TAT (shaded bars) monitored at pH 7.4 (C) and pH 5 (D).
CHAPTER 6

Summary and Future Directions

Much of what we know about membrane protein folding can be summarized in a multi-component thermodynamic cycle (Popot and Engelman, 1990; Popot and Engelman, 2000; Wimley and White, 1999; Almeida et al., 2012). The thermodynamics of these processes, while useful for understanding the principles of membrane protein folding, must be complimented by kinetic measurements of membrane-protein association, folding, and oligomerization. In this thesis, we explore the application of time-resolved and single molecule fluorescence spectroscopic techniques to elucidate the kinetics of membrane protein folding and the molecular details of membrane-peptide interactions. Our aim is to provide a molecular level, quantitative understanding of the kinetic mechanisms of folding of specific protein structure motifs and peptide-membrane interaction dynamics. While our results provide useful evidence for understanding membrane protein folding, many questions still remain.

In chapter 2, we explore the folding kinetics of a helical hairpin peptide, HAFp. Our results provide a self consistent model for folding and membrane association, as well as information on how the protein responds to changes in pH, which has implications for its function as a membrane fusion peptide. What is most exciting, from a protein folding perspective, is that this work highlights the importance of the membrane in folding. The membrane provides an environment which favors intra- and inter-peptide hydrogen bond formation, and that is supported by our FRET measurements. Much like intrinsically disordered proteins, membrane proteins are often unstructured in solution, and fold upon
exposure to the membrane interfacial region. While our study is not the first to use stopped-flow fluorescence spectroscopy in conjunction with a novel FRET pair (Tang and Gai, 2008; Tucker et al. 2006b) to investigate membrane protein folding rates, it shows that these methods can be used for proteins that contain secondary and tertiary structural elements. Future mutational analysis of HAfp would be useful for dissecting the role of individual amino acids in folding and membrane binding. In addition, future multi-step FRET experiments (Rogers et al., 2010) will provide a wider range of probes for dissecting events involved in secondary and tertiary structure formation in proteins like HAfp.

In chapters 3 and 4, we demonstrate that fluorescence correlation spectroscopy (FCS) is a useful tool for measuring diffusion as a probe for membrane structural changes in response to peptide association. The application of this single-molecule fluorescence spectroscopic method to the complex process of antimicrobial peptide activity is useful for directly measuring changes in lipid and peptide diffusion under conditions that are not observable using conventional ensemble fluorescence methods. Our results show that the antimicrobial peptide-membrane interactions are very sensitive to peptide primary structure and membrane composition, which is important for AMP function (Matsuzaki, 2009). By directly measuring diffusion of both peptide and lipid molecules, we find that the AMP binding induces heterogeneous domain formation in a concentration dependent manner, which is consistent with the results of both computational and experimental work. Future directions for this project would entail a systematic investigation of the activity of additional AMPs with non-alpha-helical motifs, like Thanatin, which folds into a beta-hairpin when bound to membranes, and indolicidin, which does not exhibit
distinct secondary structural elements when bound to membranes. There are hundreds of AMPs with varying chemical characteristics, which could be studied systematically in order to elucidate the role of the primary sequence of AMPs on their function. In addition, systematic investigation of the effects of membrane composition and structure would be useful for better understanding AMP selectivity for bacterial membranes over eukaryotic membranes. Applying eukaryotic and bacterial cell extracts in these experiments is a clear next step for understanding the molecular determinants of AMP selectivity. Further, in vivo FCS techniques could be used to study the effects of these peptides on the complex membranes of live cells. FCS could also be applied to test designed AMP activity for development of new drug delivery systems and antibiotics.

In chapter 5 we expand the application of FCS to study other types of peptide-membrane interactions, namely, the activity of cell penetrating peptides (CPPs). Preliminary results from a FCS study of HIV1-TAT in late endosomal model membranes reveals that the HIV1-TAT peptide indeed translocates across these membranes. Future systematic investigations into the pH dependence of this process, as well as the influence of peptide primary structure and role of membrane composition would help elucidate the mechanism of translocation.
REFERENCES


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