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Influence of a Potentially Destabilizing Central Tryptophan on Transmembrane Helix Domains

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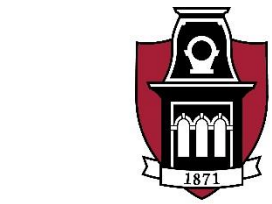
Influence of a Potentially Destabilizing Central Tryptophan on Transmembrane Helix Domains

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies in Chemistry & Biochemistry

By Vasupradha Suresh Kumar



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Abstract

The vital role that membrane proteins play in cell mechanisms and the inner workings of disease in our bodies has been universally accepted. Thus, the study of protein-membrane and transmembrane interactions has become increasingly significant in understanding these membrane proteins. Synthetic model peptides, such as GWALP23 (acetyl-GGALW^{5}LALALALALALALW^{19}LAGA-amide)—designed at the University of Arkansas and composed of the amino acid residues of glycine (G), alanine (A), leucine (L) and tryptophan (W) —provide a favorable "host" framework for investigations of the influence of chosen "guest" amino acids. For example, it is of interest to know the consequences of having a third, centrally located, tryptophan (Trp) within the hydrophobic core of a well characterized, anchored, transmembrane helix. It is crucial to note that the orientation and rotation of GWALP23 are sensitive to single-residue replacements, in part because the membrane-spanning helix exhibits only limited dynamic averaging of solid-state NMR observables such as the deuterium (^{2}H) quadrupolar splitting. A single Trp residue was introduced in the 12th or 13th position of GWALP23, and specific deuterated alanine labels (^{2}H-Ala) were included as probes within the core helical sequence. The ^{2}H quadrupolar splittings from solid-state NMR spectra of GWALP23-W12 and GWALP23-W13 show that the peptide remains helical and retains a dominant preferred tilted transmembrane orientation (similar to GWALP23) in lipid bilayer membranes of DLPC, DMPC, and DOPC. Modified Gaussian and semi-static treatments of the peptide dynamics yield similar conclusions. While a central Trp at position 12 or 13 does not alter the characteristics of bilayer-spanning GWALP23, incorporation of the peptide helix into the bilayer membrane becomes more difficult. The properties of Trp^{4,5} GWALP 23 are also being investigated for comparison with the

highly dynamic $Tyr^{4,5}$ and the less dynamic $Phe^{4,5}$ peptides. Preliminary results through solid-state ^{2}H NMR reveal $Trp^{4,5}$ to be more dynamic than $Phe^{4,5}$ and less dynamic than $Tyr^{4,5}$. Deuterium labels at Ala^{3} and Ala^{21} reveal possible fraying of the ends of selected helices in bilayers comprised of lipids of acyl chains of differing lengths. The partial helix unwinding could help to stabilize the peptide with a central Trp residue.

ABBREVIATIONS

CD, circular dichroism; DLPC, 1,2-dilauroylphosphatidylcholine; DMPC, 1,2-dimyristoylphosphatidylcholine; DOPC, 1,2-dioleoylphosphatidylcholine; Fmoc, Fluorenylmethoxycarbonyl; GALA, Geometric analysis of labeled alanines; GWALP23, acetyl-GGALW$(LA)_6$LWLAGA-[ethanol]amide; HPLC, high-performance liquid chromatography; kHz, kilohertz; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MRE, mean residue ellipticity; MtBE, methyl-*t*-butyl ether; NMR, nuclear magnetic resonance; RMSD, root mean squared deviation; RPM, revolutions per minute; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TIPS, triisopropylsilane

Introduction

The study of proteins and transmembrane interactions is in the forefront of scientific research because of the intricate role membrane proteins play in many cellular processes like cell signaling and the opening and closing of voltage-gated channels (1). In our lab, we use synthetic model peptides ("GWALP" families; see Table 1) to analyze the intricate relationship between peptides and lipid bilayers. The GWALP peptides arose from an original WALP model peptide which contained four aromatic residues that served as anchors for a membrane-spanning helix (3)(8)(14). Additionally, the length of the hydrophobic core of the WALP peptide was altered and analyzed with various lengths of phosphatidylcholine lipids to study hydrophobic mismatch in WALP peptides and lipid interactions. Though useful for studying peptide mismatch, the WALP peptides were highly dynamic and were not good model peptides to analyze the effects of altering a single residue on the peptide (17).

The GWALP23 model peptide, a cognate of the WALP peptide, was developed to reduce the high dynamics that was seen in the WALP model peptide. The GWALP23 model peptide consists of two anchoring tryptophan (Trp/W) residues instead of the initial four, which (remarkably) allows for greater stability and lower dynamics of the transmembrane helix (15)(16). GWALP23 (GGALW$(LA)_6$LWLAGA) contains one tryptophan residue on each end (at positions 5 and 19), and a repeating leucine-alanine helical core sequence (5) (10). The repeating leucine-alanine helical core prefers the hydrophobic environment of the membrane, whereas the Trp residues prefer a location near the lipid-water interface (11). The leucine-alanine core accentuates the peptide's response to the thickness of the membrane (that is a result of hydrophobic mismatch).

This hydrophobic mismatch is from the difference between the thickness of the lipid bilayer's hydrophobic portion and the length of the peptide's hydrophobic core. The mismatch can affect both the orientation of the peptide in the lipid bilayer and the alignment of the lipid themselves (8). This sensitivity of the GWALP23 peptide makes it the preferred model for studying the influence of single and multiple residue replacements on peptide orientation and dynamics.

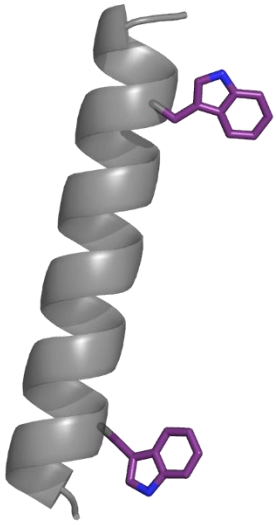

Figure 1: 3D representation of the favored GWALP23 model peptide is shown along with the indole rings of W^5 and W^{19} in the 23-residue sequence

The orientation and dynamics of GWALP23 and a tyrosine derivative (containing Y^5 instead of W^5) are well understood (5). In this project, the GWALP23 peptide was modified by adding a central tryptophan in the 12^{th} or 13^{th} position in the hydrophobic core of the GWALP23 peptide. Alanine residues in the helical core were labeled with deuterium in order to study the dynamics and orientation of the peptide in lipid bilayers by solid-state deuterium NMR. Depending on the intent of the study, alanine can be labeled either in the core positions of 7 and 9, 11 and 13, 15 and 17, or in the end positions of 3 and 21. In the alanine pairs that are labeled, the alanine closer to the N-

terminal is labeled with 50% deuterated alanine and the other alanine is labeled with 100% deuterated alanine.

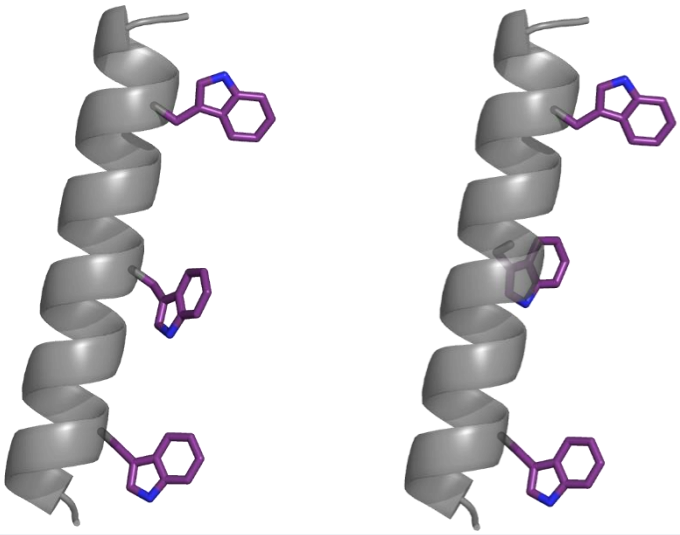

Figure 2: The GWALP23-W12 is shown on the left and GWALP23-W13 is shown on the right

The orientations of GWALP23-W12 and GWALP23-W13 were investigated in bilayers of DLPC (12:0), DMPC (14:0), and DOPC (18:1). It can be hypothesized that the indole ring of all tryptophans, including the central W12 or W13, will prefer the membrane-water interface. Results for GWALP23-W12 and GWALP23-W13 will be compared to those of GWALP23 to observe the changes that have occurred in peptide orientation (specifically the helix tilt and rotation).

In addition to studying the effects of a central tryptophan, the effects of altering the aromatic anchors on a GWALP23 are also studied. After a single anchor peptide ,Y^{5}GWALP23, was initially synthesized and found to have similar dynamics to that of GWALP23, another model peptide with two tyrosine residues, $Y^{4,5}$GWALP23, was created to study the influence of an 'extra' aromatic residue in peptide/lipid interactions (5). Unlike Y^{5}GWALP23, $Y^{4,5}$GWALP23 displayed a large difference in its

dynamics in comparison to GWALP23. The $Y^{4,5}$GWALP23 model peptide seemed to have a much higher dynamic averaging, perhaps a result of the multiple aromatic tyrosine anchors, than that of GWALP23. A third model peptide, $F^{4,5}$GWALP23, was later analyzed and displayed a lowered dynamic averaging in comparison to GWALP23 and especially $Y^{4,5}$GWALP23 (12) . To better study the effect of altering the type of 'extra' aromatic residue anchor on peptide behavior and orientation, $W^{4,5}$GWALP23 was investigated and compared to both $Y^{4,5}$GWALP23 and $F^{4,5}$GWALP23.

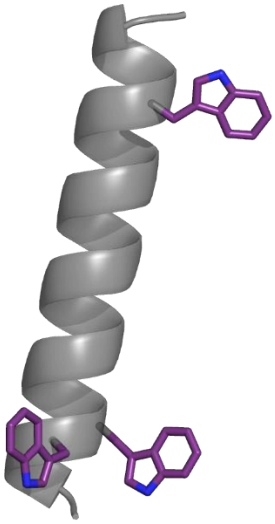

Figure 3: Model for $W^{4,5}$GWALP23

$W^{4,5}$GWALP23 , like GWALP23-W12 and GWALP23-W13 will be studied in DLPC, DMPC, and DOPC lipid bilayers. Additionally, Nuclear Magnetic Resonance (NMR) can be used to study the orientation of the peptide in the different lipids and can also be used for tilt analysis (performed through either a semi-static or Gaussian calculation method) (9). The tilt analysis will contribute to the understanding of the behavior of peptides with multiple aromatic anchoring residues.

To study the partial unwinding of the ends of the transmembrane helix, alanines on the ends of the peptide (at positions 3 and 21) are labeled with ^{2}H-Ala. Positions 3 and

21 are located 18 residues apart in the sequence of the peptide. Because an alpha helix geometry requires 3.6 amino acids per turn of the helix, this places Ala 3 and Ala 21 in the same radial position on the helix, separated by exactly five helical turns. This study provides an opportunity to monitor the unwinding of the alpha helix at the N and C termini. If both residues are part of the helix, they should give identical quadrupolar splittings in ^{2}H NMR. If the ends are indeed unwound, the quadrupolar splittings for Ala^{3} and Ala^{21} will differ and will not lie on the quadrupolar wave from the orientation analysis of the labeled Ala residues within the core of the peptide. The labeled ends will allow for the thorough analysis of the influence of an extra tryptophan, specifically a central tryptophan, on the unwinding of the transmembrane helix ends.

The focus of this work is to study the effects of an extra tryptophan residue. GWALP23-W12 and GWALP-W13 probe the influence of a central tryptophan in comparison to GWALP23. $W^{4,5}$GWALP23 allows for the comparison between the dynamic averaging of the various 'extra' aromatic residues (W, Y or F) at the 4^{th} and 5^{th} positions. Finally, ^{2}H-labels are used to label the alanine at position 3 and 21 to specifically study the effect of a central tryptophan on the possibility of unwinding of the ends of a transmembrane helix domain. The various peptides analyzed as a part of the project are listed in Table 1 below.

Table 1. Model Peptide Sequence: The table below displays the sequence of GWALP23 and the peptides I have synthesized as a part of my project

Name	Sequence
GWALP23	**Ac-GGA^{3}LWL$\underline{A}^{7}$LA^{9}LA^{11}LA^{13}LA^{15}LA^{17}LWLA^{21}GA-EtNH2**
GWALP23-W12	**Ac-GGA^{3}LWL$\underline{A}^{7}$L$\underline{A}^{9}$LA^{11}WA^{13}LA^{15}LA^{17}LWLA^{21}GA-EtNH2**
GWALP23-W12	**Ac-GGA^{3}LWLA^{7}LA^{9}LA^{11}WA^{13}L$\underline{A}^{15}$L$\underline{A}^{17}$LWLA^{21}GA-EtNH2**
GWALP23-W12	**Ac-GG$\underline{A}^{3}$LWLA^{7}LA^{9}LA^{11}WA^{13}LA^{15}LA^{17}LWL$\underline{A}^{21}$GA-EtNH2**
GWALP23-W13	**Ac-GGA^{3}LWL$\underline{A}^{7}$L$\underline{A}^{9}$LA^{11}LWLA^{15}LA^{17}LWLA^{21}GA-EtNH2**
GWALP23-W13	**Ac-GGA^{3}LWLA^{7}LA^{9}LA^{11}LWL$\underline{A}^{15}$L$\underline{A}^{17}$LWLA^{21}GA-EtNH2**
W^{4,5}-GWALP23	**Ac-GGA^{3}WWL$\underline{A}^{7}$L$\underline{A}^{9}$LA^{11}LA^{13}LA^{15}LA^{17}LWLA^{21}GA-EtNH2**
W^{4,5}-GWALP23	**Ac-GGA^{3}WWLA^{7}LA^{9}L$\underline{A}^{11}$L$\underline{A}^{13}$LA^{15}LA^{17}LWLA^{21}GA-EtNH2**
W^{4,5}-GWALP23	**Ac-GGA^{3}WWLA^{7}LA^{9}LA^{11}LA^{13}L$\underline{A}^{15}$L$\underline{A}^{17}$LWLA^{21}GA-EtNH2**

Deuterated Alanines are underlined as $\underline{\mathbf{A}}$ in the sequence

Ac=acetyl, EtNH2=ethanolamine

Materials and Methods

Solid Phase Peptide Synthesis with ^{2}H Labels

Peptides with deuterium labeled alanines were synthesized using an applied Biosystems 433A Peptide Synthesizer (7). The Fmoc-ala-d_4 was made by members in the lab as described in (5). For the amino acids that comprise the peptide, 0.57 mmoles of the amino acid was weighed out and placed in separate cartridges. The peptide was labeled with two deuterium labeled alanines (one 50% labeled and one 100% labeled) at the core of the peptide at positions 7 and 9, 11 and 13, 15 and 17, or the ends of the peptide at position 3 and 21. For the labeled alanines, 0.28 mmoles of Fmoc-Ala-d_4 with 0.28 mmoles of Alanine were placed in a cartridge (for the 50% deuterium labeled Alanine). 0.57 mmoles of only fmoc-Ala-d_4 was used for the 100% deuterium labeled Alanine. The peptide was synthesized from the carboxy end of a Wang resin (company), achieved by ordering the cartridges from the C to N-terminal. During the synthesis, the "FastMoc" method was used on the synthesizer and a series of deprotecting, coupling, and rinsing was performed (6).

Cleavage of the Peptide:

After the completion of the peptide synthesis, the peptide is cleaved from the resin with Ethanolamine. The resin with the peptide is placed in a glass vial and 11 mL of Dichloromethane and 4 mL of Ethanolamine is added to the resin. The glass vial is wrapped in foil and placed on a shaker for 48 hours at 480 min-1. After the 48 hours, the resin slurry is filtered with a 10mL glass filter. The resin solution is rinsed with 5mL of Dichloromethane four times and then 5 mL of TFE four times. After the rinse, the rotovap is set up and used to minimize the volume of the filtered liquid. After rotovaping, the resulting peptide substance is equally poured into two centrifuge tubes and filled with DI H20 (about 80% of the total volume of the centrifuge tube should be DI water). The

tubes are placed in a refrigerator and allowed to precipiate in DI water overnight. The next day, the JA-20 rotor is used to centrifuge the peptide mixture at 14,000 RPM for two hours. The resulting supernatant should be disposed and the pellet should be dried on the vacuum line overnight.

Crude GWALP Quantitation and Purification:

After the peptide has been cleaved off of the resin, the amount of peptide that has been recovered needs to be determined. This process is called peptide quantitation. To quantitate the peptide, 0.5 mL of TFE is added into the centrifuge tubes with the dried peptide. A UV-Vis sample is prepared by mixing 2 μL of the dried peptide with 1.5 mL of Methanol. The UV-Vis is blanked with methanol and the sample is run at 280 nm. The resulting spectra and the optical density (at 280nm), allows for the calculation of the total milligrams of peptide that is present. To test the peptide, analytical HPLC is run with a 1μL aliquot of the peptide solution (diluted to 1mg/ml with TFE). The sample is then prepped for mass spectrometry with 50 μL of the peptide is diluted with 450 μL of TFE. After the peptide cleavage, the peptide is ready to purify using the Semi-prep HPLC. Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) with the Hitachi L1700 system and the Zorbax RX-C8 column, 9.4 mm x 250mm (Agilent Technology). The HPLC machine is first purged and a desired gradient is set at 95-99% for the GWALP23 with an extra tryptophan. A test injection of 10 μL is initially injected to determine the approximate peak position (time the peptide will come off the machine). After the test injection, the range of the RP-HPLC is set to 10 and a 500μL aliquot of the peptide is injected. The purified product is usually collected after 23-25 minutes and then dried. Finally, the purified peptide is quantitated to determine how much pure peptide (in

milligrams) is collected. For this, the peptide is redissolved in 1 mL of TFE and a UV-Vis sample is made with 10 μL of the peptide and 990μL of MeOH. Again, from the Optical Density at 28nm, the weight of the total peptide (in mg) can be calculated. After rechecking the peptide wih MALDI-TOF mass spectrometry with a 50 μL aliquot o the peptide and 450 μL of TFE), the peptide is aliquoted for solid-state ^{2}H NMR and circular dichroism (CD) and tryptophan emission fluorescence spectroscopy. For the solid-state ^{2}H NMR , 1.3 μmols of the peptide is aliquoted . For the CD and Trp fluorescence, 0.0625 μmols is measured out. The solvent is evaporated under N_2 for an hour. The samples are then lyophilized by adding 0.5 mL of acetonitrile and 0.5 mL of H_20 to the dried peptide. The solution is frozen in liquid N_2 and left under vacuum overnight.

Mass Spectrometry

The mass of the mass spectrometry spectra, allows for the verification of our intended peptide by comparing expected and actual mass of peptide. The molecular mass of the peptide was confirmed by MALDI- TOF mass spectrometry (MS) MALDI run by the University of Arkansas-Arkansas Statewide Mass spectrometry. The satellite peaks that are observed represent the different ionization states of the peptide. As described, peptides can also be purified using the semi-preparative HPLC to eliminate miscellaneous substances.

Circular Dichroism Spectroscopy

When working with peptides, it is important to know the structural conformation of the peptide. The secondary structure can be ascertained by running a circular dichroism (CD) sample. For CD, our peptides (with a central tryptophan or a tryptophan at the 4th position) are incorporated into small unilamellar lipid vesicles (SUVs) in DLPC,

DMPC, DOPC lipids. For CD, the samples were prepared by using 0.0625 μmols of peptide from the stock solution with a 1:40 peptide to lipid ratio. In making the CD sample, 50:50 acetonitrile: water is added to the 0.0625 μmols of peptide. Then, 2.5 μmols of lipid (DLPC, DMPC, or DOPC) is added to the peptide and acentonitrile:water solution. The mixture of peptide and lipid is dried under N_2 gas and put under vacuum for 48 hours to remove the solvent.

Tryptophan Emission Fluorescence Spectroscopy:

Tryptophan fluorescence spectroscopy is used to observe the membrane burial depth of tryptophan (both the central tryptophan and the tryptophan in position 4) in SUVs made of DLPC, DMPC, and DOPC lipid bilayers. Samples for tryptophan fluorescence were prepared by performing a 1:10 dilution of the circular dichroism samples (with a final concentration around 10 μM) . The machine used for tryptophan fluorescence was the ISS Photon Counting Spectrofluorometer with the VINCI software (ver. 1.6. SP7). While samples were running, they were excited at 280 nm (the excitation wavelength for Trp) and the emission was recorded at 300 to 450nm. The emission measurement is scanned at a rate of 60nm/min with 5mm excitation and emission slit widths. The excitation pathlength was 10nm and the emission path length was 1nm.

Oriented Sample Preparation:

Oriented Samples made for NMR spectroscopy were prepared in a 1:40 peptide to lipid ratio using DLPC, DMPC, and DOPC lipid membranes. 1.33 μmols of peptide and 90μmols of a lipid with 45% (w/w-water/peptide-lipid) hydration were made. To prepare the oriented samples, 40 glass plates were placed in two separate petri dishes (20 glass plates per each petri dish). The 1.3 μmols of peptide was dissolved in1 0.5mL of TFE.

The 14 μL of peptide was mixed with 1.5 mL of MeOH. The solution was then placed in UV-Vis spectrometry and an absorbance reading was taken at 280 nm. From the absorbance, the amount of lipid required for the NMR samples was calculated. The amount of lipid was then added to the peptide solution and dried under nitrogen flow and then under vacuum overnight. To the dried peptide/lipid film, 1 mL (95% methanol and 5% H20) was added. Using a 50 μL syringe, the peptide/lipid solution was added evenly to the 40 glass slides on the petri dish. The glass plates were dried under vacuum in a desiccator for 48 hours. The final step of the oriented NMR sample preparation was hydration and stacking the glass plates. The dried plates were hydrated using about 1.2 μL of deuterium depleted water. The water (about 3-4 drops per plate) was used as the 'glue' to stack the 40 glass plates. Once the plates were stacked, they were inserted into a cuvette. The, empty glass plates were then added to the outside of the stack to fill the cuvette. Finally, the cuvette was sealed with a glass square cover using epoxy glue. Once the cuvette was sealed twice (with one hour between the first and second seal), the plates was placed inside a dark, heating plate at 40 C for several days (until sample looks clear).

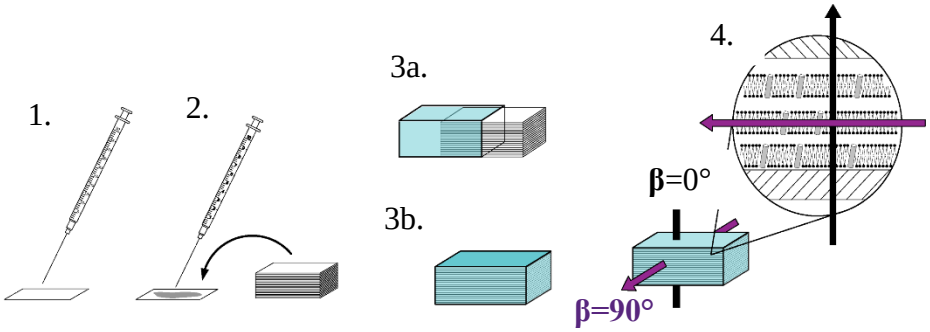

Figure 4: A visual representation of preparing an oriented NMR sample is shown below

^{31}P NMR:

A type of NMR called, ^{31}P NMR spectroscopy is used to verify bilayer alignment. The sample was run at 50 ºC on a Bruker Avance 300 spectrometer (Billerica, MA) in both β=0° orientation (where the bilayer is parallel to the applied magnetic field) and β=90º orientation (where the bilayer is perpendicular to the applied magnetic field).

^{2}H NMR:

Similar to ^{31}P NMR, solid-state ^{2}H NMR at 50 °C on a Bruker Avance 300 spectrometer. The samples were run at β=0° and β=90°. The resulting spectrum gives information about quadrupolar splitting that will serve useful in creating a tilt analysis to study the behavior of the peptides in various lipids.

Tilt Analysis:

From the ^{2}H quadrupolar splittings ($\Delta\nu_q$) that was collected in various lipids (DLPC, DMPC, DOPC) with different set of alanines labels, the orientation of the peptide in the lipid bilayers was determined. Two methods of calculations, GALA fit and modified Gaussian were used to perform a semistatic model with variable parameters. The parameters for the calculations are the tilt of helix τ, helix rotation ρ, and $S_{zz,}$ a principal order parameter. This principal order parameter gives more information about the overall motion of the peptide relative to the orientation of the peptide.

Results

After the target peptides were synthesized and purified, their molecular masses were confirmed by MALDI-TOF mass spectrometry. From the resulting mass spectrum, the expected mass of (2378 + 8 for the deuterons + 23 for sodium) can be compared with the actual mass of 2408.4 as shown on the spectrum.

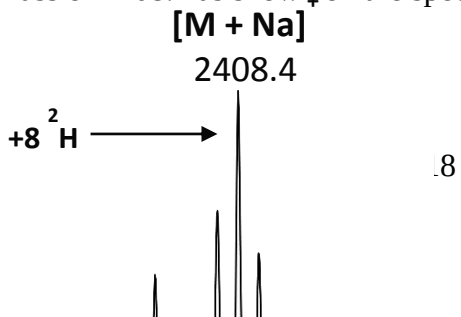

Figure 5: The figure below is the mass spectrum for $W^{4,5}$-GWALP23

From the displayed isotope distributions for $W^{4,5}$ GWALP23, it can be seen that the expected mass of 2377.9 Da for $W^{4,5}$ GWALP23 matches the actual mass of $W^{4,5}$ GWALP23 (which includes the mass of Na^{+}). (Successive peaks in each of the (+4) and (+8) "envelopes" indicate different content of ^{13}C (1.1% natural abundance) versus ^{12}C in a peptide having about 100 carbon atoms.)

After verification that the desired peptide had been synthesized, an estimate of the secondary structure of the peptide was assessed using circular dichroism (CD) spectroscopy. The repeating Leu-Ala sequence in the core of the GWALP23 peptides prefers an alpha–helical secondary structure that is in the hydrophobic region of the lipid bilayer.

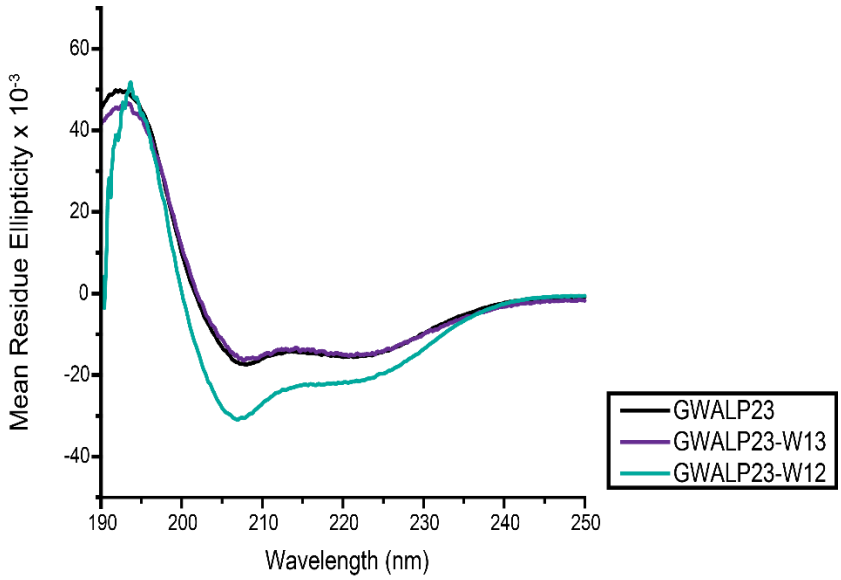

Figure 6: CD spectra for GWALP23, GWALP23-W12, and GWALP23-W13 in DMPC vesicles

Each CD spectrum in Figure 6 is characteristic of an alpha-helical secondary structure, with the expected characteristic minima observed at 208 nm and 222 nm. Minor differences among the three spectra could be due to complex interactions of the polarized light with the multiple Trp residues.

GWALP peptides could potentially produce nonlamellar phases like the inverted hexagonal and cubic phases of the phosphatidycholine lipids (DLPC, DMPC, DOPC) when there is negative mismatch (at a high peptide concentration) (4). The proper alignment of the lipid bilayers can be verified by ^{31}P NMR. The peptide/lipid oriented samples in the three lipids (DLPC, DMPC, DOPC) were all verified to have aligned bilayers. The spectra display a single peak at expected place for the ^{31}P resonance in both $\beta = 0°$ (membrane normal parallel to the applied magnetic field) and $\beta = 90°$ (perpendicular to the applied magnetic field).

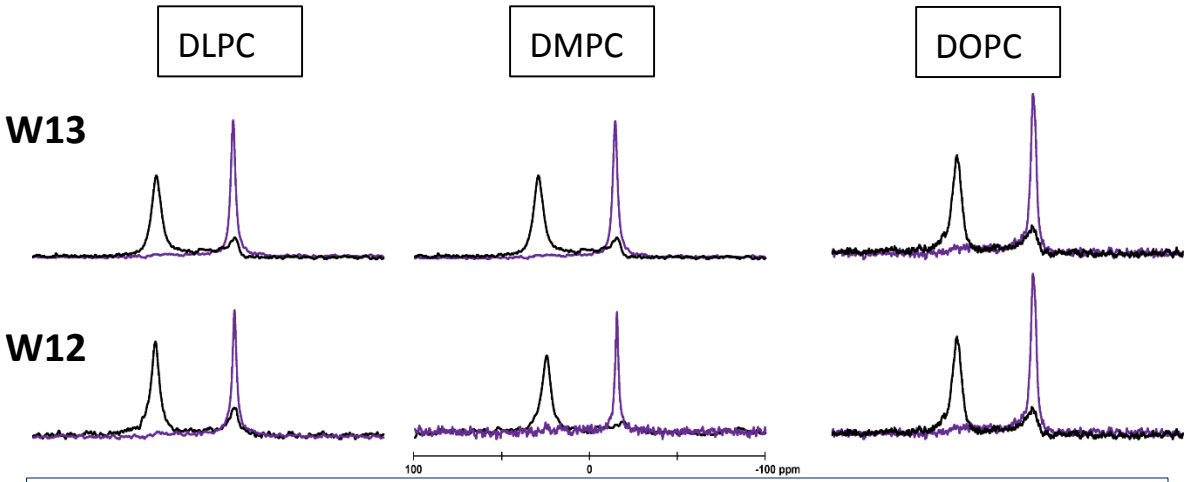

Figure 7: ^{31}P NMR spectra at both β = 0° and β = 90° sample orientations (overlaid) in DLPC, DMPC and DOPC, showing signals characteristic of aligned lipid bilayers

The ^{31}P NMR spectra display peaks at around +28 ppm β= 0° and -14ppm for β = 90°. The peak shapes are indicative of relatively well-aligned lipid bilayers. Some unaligned lipids are evident from the smaller peaks at -14 ppm in the β = 0° spectra, which are more sensitive to the lipid alignment.

An effective method to study peptide incorporation and approximate location in various lipid membranes is through tryptophan fluorescence emission spectroscopy. Specifically for this project, there are three tryotphans. The emission spectra will represent an average of the depths of the three tryptophan residues of the peptide in the membrane. The λ_{max} that is determined through tryptophan fluorescence varies depending upon the polarity of the environment around the indole ring. A λ_{max} of 320-325 nm indicates a deeply buried tryotphan within the membrane, a value of 335-345 nm indicates a tryptophan at the water/membrane interface, and a value of 350-360 nm is indicative of tryptophan in water solution (2)(9).

Table 3. Tryptophan Fluorescence Emission Results: The table below displays the fluorescence emission λ_{max} values for the various GWALP23 and the derivative peptides excited at 295nm

λ_{max} (nm)			
Peptide	Lipid		
	DLPC	DMPC	DOPC
GWALP23	331	330	331
W12	331	332	329
W13	332	329	327
$W^{4,5}$	330	329	---
$F^{4,5}$	331	331	332
$Y^{4,5}$	331	330	---

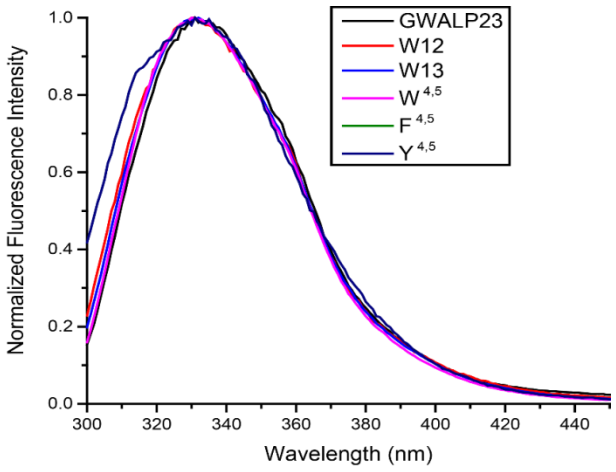

Figure 8: Tryptophan fluorescence emissions spectra for GWALP23 and selected derivative peptides having one, two or three Trp residues

As seen in the figure above, the λ_{max} for all the peptides is mostly between 325-330nm, suggesting the tryptophans on all of the peptides are somewhat buried within the membrane.

Finally, peptide behavior and orientation in various lipid bilayers can be studied by performing solid-state ^{2}H NMR with the prepared oriented samples. As described, the outer tryptophans (one at each end of the peptide) are expected to aid in the stabilization of the peptide at the membrane-water interface. Another important factor is the extent of hydrophobic mismatch between the length of the peptide and the thickness of the lipids. In the core of the peptides, I incorporated two deuterated alanines in positions 7/9, 11/13, or 15/17. These ^{2}H-labeled alanines allow for the analysis of peptide interaction and orientation in the lipids through the use of solid-state ^{2}H NMR spectroscopy. In deuterium (^{2}H), the nucleus is a quadrupole, giving two sets of resonance peaks for the CD_3 group. The distance between the pair of peaks for each alanine methyl (CD_3) group

(the ^{2}H quadrupolar splittings) was then measured. The quadrupolar splittings, $\Delta\nu_q$, that are observed in the solid-state ^{2}H NMR spectra, vary based on the orientation of each CD_3 groups with respect to the external magnetic field.

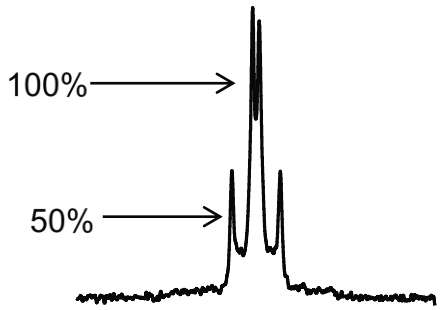

Figure 9: This is an example spectra from the ^{2}H NMR analysis from an aligned lipid-bilayer sample of $W^{4,5}$-GWALP23 containing a GWALP peptide with two deuterated alanines at positions 15 (50% deuerated alanine) and 17 (100% deuterated alanine)

By measuring the distance between the respective peaks and finding the quadrupolar splittings ($\Delta\nu_q$), the peptide orientation in the lipid membrane can later be understood through GALA (Geometric Analysis of Labeled Alanines) and modified Gaussian calculation methods (5)(12)(14). Values for $\sigma\tau$ were fixed for calculation (15º for DLPC and 9º for DOPC) in the modified Gaussian analysis (12).

Table 4. Quadrupolar splittings for GWALP23-W12: Quadrupolar splittings (($\Delta\nu_q$) in kHz) for labeled alanines in GWALP23-W12. β=0° sample orientation. Temperature (º C)

Ala-d_4	DLPC	DMPC	DOPC
7	24.0	20.2	16.0
9	19.0	8.3	0.5
15	26.0	16.0	14.4
17	5.0	2.6	1.9

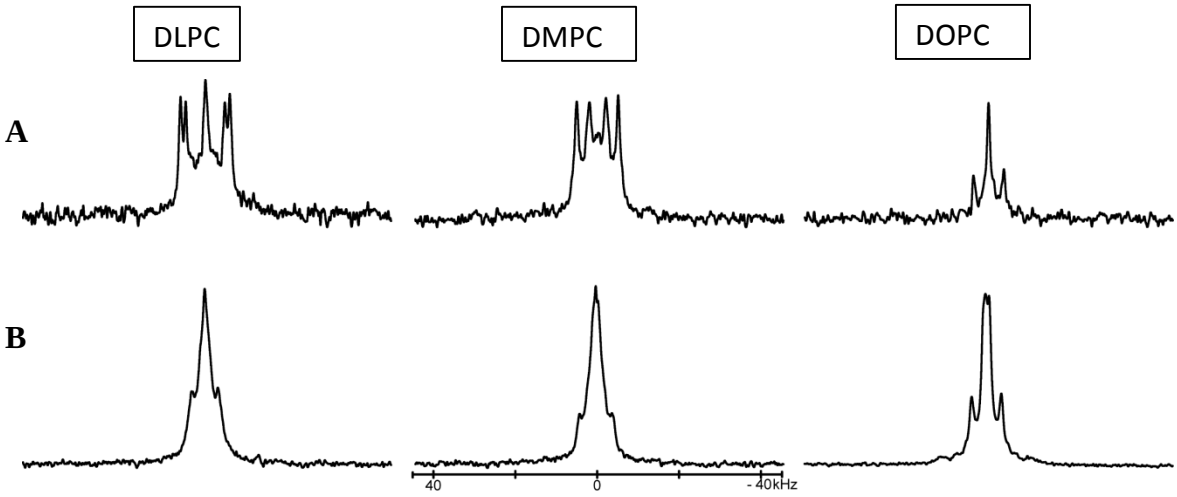

Figure 10: The figure above displays the solid-state ^{2}H NMR for GWALP23-W12 in the β=90º orientation. The spectra on row **A** displays GWALP23-W12 peptides with deuterated alanines in position 7 $^{(100\%)}$ and 9 $^{(100\%)}$. The spectra on row **B** display the ^{2}H NMR results for GWALP23-W12 with alanines labeled at position 15 $^{(50\%)}$ and 17 $^{(100\%)}$

Table 5. Quadrupolar splittings for GWALP23-W13: Quadrupolar splittings ($\Delta\nu_q$) for labeled alanines (β=0°) in GWALP23-W13

Ala-d_4	DLPC	DMPC	DOPC
7	23.0	19.0	---
9	22.0	9.0	---
15	14.0	12.0	15.0
17	9.9	5.2	0.4

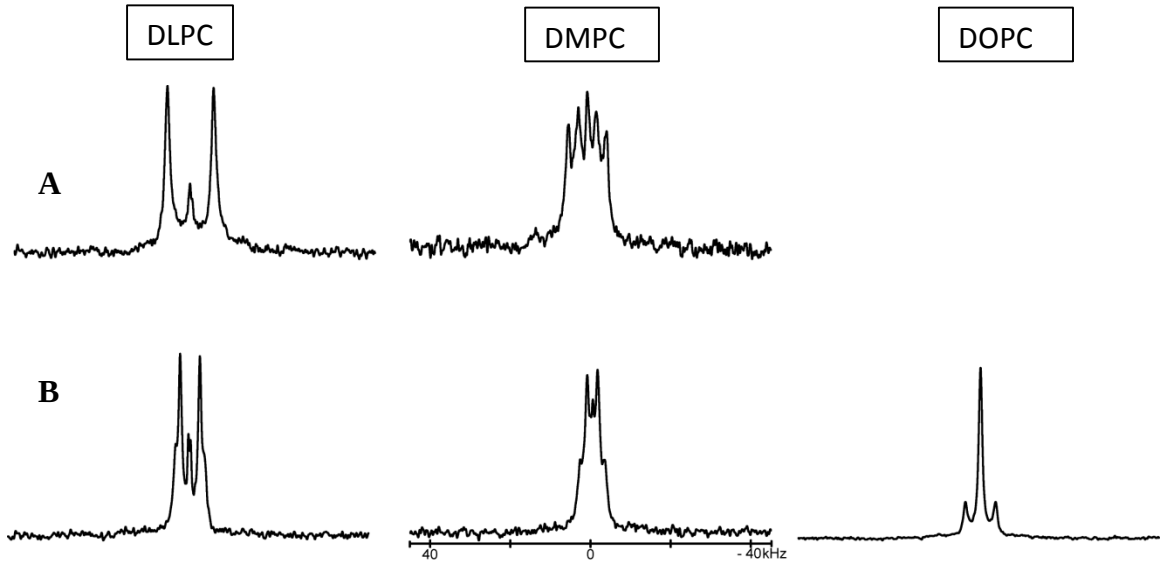

Figure 11: The figure above displays the solid-state ^{2}H NMR for GWALP23-W13 in the β=90º orientation. The spectra on row **A** displays GWALP23-W12 peptides with deuterated alanines in position 7 $^{(50\%)}$ and 9 $^{(100\%)}$. The spectra on row **B** display the ^{2}H NMR results for GWALP23-W13 with alanines labeled at position 15 $^{(50\%)}$ and 17 $^{(100\%)}$

Table 6. Quadrupolar splittings for $W^{4,5}$-GWALP23: Quadrupolar splittings ($\Delta\nu_q$) (kHz) for labeled alanines (β=0°) in $W^{4,5}$GWALP23 and related peptides

	DLPC			DMPC			DOPC		
Ala-d_4	$W^{4,5}$	$F^{4,5}$	$W^{5,19}$	$W^{4,5}$	$F^{4,5}$	$W^{5,19}$	$W^{4,5}$	$F^{4,5}$	$W^{5,19}$
15	17.4	23.4	20.7	16.2	20.2	17.6	14.9	18.6	15.4
17	3.3	1.8	3.4	1.5	3.8	2.9	1.0	1.9	2.6

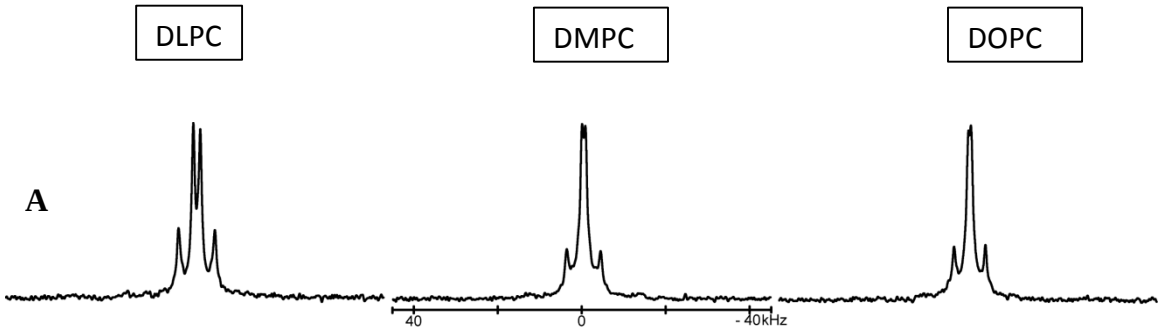

Figure 11: The figure above displays the solid-state ^{2}H NMR for $W^{4,5}$-GWALP23 in the β=90º orientation. The spectra on row **A** display the ^{2}H NMR results for $W^{4,5}$-GWALP23 with alanines labeled at position 15 (50%) and 17 (100%)

In DLPC, the shortest lipid, the quadrupolar splittings for $W^{4,5}$ GWALP23 range from 3.3 to 17.4 kHz, $F^{4,5}$ GWALP23 ranges from 1.8 to 20.7 kHz. From the rather similar results seen in Table 5, it can be concluded that $F^{4,5}$ GWALP23 nevertheless seems to tilt to a slightly greater degree or tends to be less dynamic than $W^{4,5}$ GWALP23. The reason for the small difference is unknown but could relate to the differing properties of the different Phe and Trp aromatic rings.

In addition, from Tables 3, 4, and 5, it is evident that the labeled alanines in each lipid have different kHz quadrupolar splitting values, indicating that each helix is tilted. If the helix were perfectly straight in the membrane, the different labeled alanines would have the same quadrupolar splitting values.

With the quadrupolar splittings, further calculations to determine the tilt and rotation are made by performing tilt analysis using the GALA and modified Gaussian methods (12) (14). Both forms of calculations use the following equation (1) in their calculations:

$$\Delta\nu_q = \frac{3}{4}\frac{e^2qQ}{h}\left(\frac{1}{2}\left[3\cos^2\theta - 1\right]\right)\left(\frac{1}{2}\left[3\cos^2\beta - 1\right]\right)S_{zz}$$

In the above equation, β represents the angle of the peptide in relation to the applied magnetic field. $\frac{e^2qQ}{h}$ in the equation is a known quadrupolar coupling constant (168 kHz). The equation displays the relationship between the ^{2}H quadrupolar splittings value ($\Delta\nu_q$) and the orientation of the peptide (the angle θ that is between the alanines of C_α and C_β) with the external magnetic field (either 0º or 90º) . Equation (1) can be fitted to any set of $\Delta\nu_q$ values. In GALA Fit, this θ value can also be described through equation (2):

$$\theta = \varepsilon_{//}\left[\cos\tau - \sin\tau\cdot\cos\left(\rho + \varepsilon_{\perp} + \phi\right)\cdot\tan\varepsilon_{//}\right]$$

In equation (2), the $\varepsilon_{//}$ is the angle between C_α-C_β and the peptide axis. This coupling constant accounts for the C-D with no dynamic averaging. In the calculation, the S_{zz},τ, and ρ values are determined. The S_{zz} is the principal order parameter which describes overall motion of the peptide such as the wobbling of the molecule or vibrations (13). The τ is the tilt of the peptide in the bilayer, and ρ is the rotation of the helix based on the glycine at position one.

Table 7. GALA Fit DLPC Calculations: Calculations of tilt (τ), S_{zz}, and rotation (ρ) through the GALA fit method comparing GWALP, GWALP23-W12, and GWALP23-W13 in DLPC (12 carbons)

DLPC	GALA Fit Results			
	S_{zz}	τ	ρ	RMSD (kHz)
GWALP23	0.71	20.7°	305°	0.66
W12	0.81	17.0°	326°	0.50
W13	0.70	18.7°	291°	1.64

Table 8. Modified Gaussian Calculations DLPC Results: Calculations of tilt (τ), S_{zz}, and rotation (ρ) through the modified Gaussian method comparing GWALP, GWALP23-W12, and GWALP23-W13 in DLPC (12 carbons)

DLPC	Modified Gaussian				
	τ	ρ	σρ	*στ	RMSD (kHz)
GWALP23	23°	304°	33°	15°	0.7
W12	19°	326°	21°	15°	1.0

In tables 6 and7, the tilt (τ) and rotation (ρ) can be compared between the peptides with a central tryptophan and the regular GWALP23 peptide. The GALA Fit method shows the tilt for GWALP23 to be 20.7°, the tilt for GWALP23-W12 to be 17.0° and the tilt for GWALP23-W13 to be 18.7°. All the values for tilt, regardless of the type of peptide, seem to have tilt values very similar to each other (between 17-21°). Similarly, in the modified Gaussian method, the rotation for GWALP23 is calculated to be 304° and the rotation for GWALP23-W12 is 326°. The rotation, like the tilt, does not differ much between peptides in DLPC lipid bilayers.

Table 9. GALA Fit DMPC Calculations: The table below shows the calculations of tilt (τ), S_{zz}, and rotation (ρ) through the GALA fit method comparing GWALP, GWALP23-W12, and GWALP23-W13 in DMPC (14 Carbons)

DMPC	GALA Fit Results			
	S_{zz}	τ	ρ	RMSD (kHz)
GWALP23	0.89	9.0°	311°	1.06
W12	0.79	10.0°	312°	0.58
W13	0.79	9.3°	300°	1.33

Table 10.Modified Gaussian Calculations DMPC Results: The table below shows the calculations of tilt (τ), S_{zz}, and rotation (ρ) through the modified Gaussian comparing GWALP, GWALP23-W12, and GWALP23-W13 in DMPC (14 Carbons)

DMPC	Modified Gaussian				
	τ	ρ	σρ	*στ	RMSD (kHz)
GWALP23	13°	308°	42°	10°	1.2
W12	16°	310°	56°	10°	0.2

GALA fit and modified Gaussian calculations for GWALP, GWALP23-W12 and GWALP23-W13 in DMPC lipid bilayer give tilt values of 9.0°, 10.0°, and 9.3° respectively in the GALA Fit model. Similarly, the modified Gaussian gives tilt values of 13° and 16° for GWALP23 and GWALP23-W12 respectively (in DMPC). Like in DLPC, both calculation methods demonstrate that the tilt does not change significantly whether there is a central tryptophan or not. The GALA Fit calculations show the rotation (p) to be 311° for GWALP23, 312 GWALP23-W12, and 300° for GWALP23-W13. It

can thus be concluded that both the tilt and rotation do not vary as a result of altering the GWALP23 sequence by adding a central tryptophan.

Table 11. GALA Fit DOPC Calculations: The table below shows the calculations of tilt (τ), S_{zz}, and rotation (ρ) through the GALA fit method comparing GWALP, GWALP23-W12, and GWALP23-W13 in DOPC (18 Carbons)

DOPC	GALA Fit Results			
	S_{zz}	τ	ρ	RMSD (kHz)
GWALP23	0.87	6.0°	323°	0.61
W12	0.78	6.7°	325°	0.36

Table 12. Modified Gaussian Calculations DOPC Results: The table below shows the calculations of tilt (τ), S_{zz}, and rotation (ρ) through the modified Gaussian method comparing GWALP, GWALP23-W12, and GWALP23-W13 in DOPC (18 Carbons)

DOPC	Modified Gaussian				
	τ	ρ	$\sigma\rho$	$*\sigma\tau$	RMSD (kHz)
GWALP23	9°	321°	48°	9°	0.7
W12	6°	330°	11°	9°	1.0

In DOPC, the GALA fit method calculates the tilt to be 6° for GWALP23 and 6.7° for GWALP23-W12. The modified Gaussian determines the rotation to be 321° for GWALP23 and 330° for GWALP23-W12. Both the tilt and rotation of the peptide stay pretty consistent in DOPC regardless of the presence of a central tryptophan.

It can be concluded from GALA fit and modified Gaussian tilt analysis in DLPC, DMPC, and DOPC that the orientation of the peptide is largely unaffected by a central tryptophan. Nevertheless, I have observed that the samples are more difficult to make

and the NMR signal-to-noise ratio is less when a central tryptophan is present in the peptide helix.

In addition to studying the orientation and behavior of peptides with a central tryptophan, it is also a point of interest to study the possibility of 'ends-fraying' of peptide helices with a central tryptophan. The possible fraying near the ends of helices of GWALP23-W12 or GWALP23-W13 could lead to stabilization of the helix in the membrane. To investigate the ends, deuterium labels were incorporated in residues A^3 and A^{21} of selected peptides. If there is helix unwinding, the $\Delta\nu_q$ values will differ between these alanines, and either A^3 or A^{21}, or both of them, will not be part of the helix

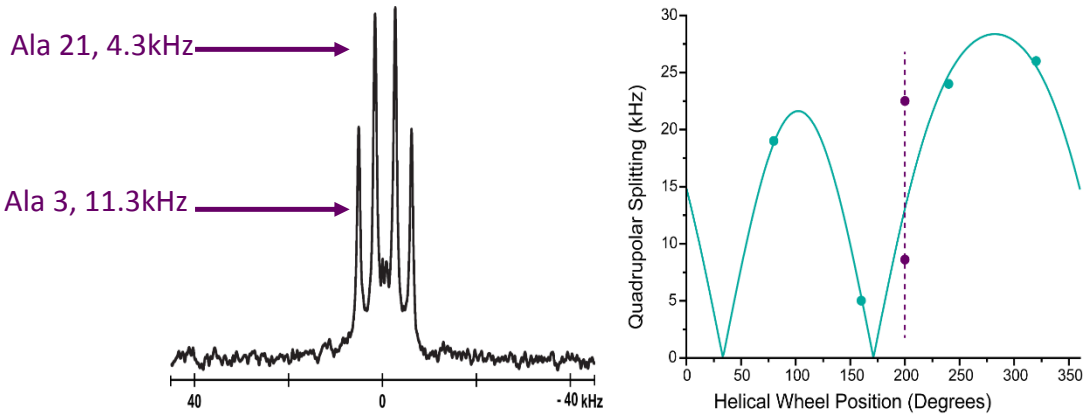

Figure 10: A. Solid-state ^{2}H NMR spectra for GWALP23-W12 with Ala^3 and Ala^{21} labeled. **B.** Helical wheel position graph from GALA analysis that shows that the Ala^3 and Ala^{21} $\Delta\nu_q$ values do not fit the core helical wheel

With the quadrupolar splitting data, the helical wheel position graph is made to display a visualization of where Ala^3 and Ala^{21} are in relation to the helical wheel defined by the core leucine-alanine residues of the peptide. It is evident that Ala^3 and Ala^{21} are both far from the helical wheel, indicating fraying of both ends. Additionally, the

quadrupolar splitting values (kHz) for Ala^3 and Ala^{21} are far from being identical; instead, they are 11.3 and 4.3 kHz respectively. With different kHz values for the two deuterium labeled alanines, separated by an exact helical repeat distance of 18 residues, it is determined that there is unwinding at the ends of the GWALP23-W12 peptide.

Discussion

Through this project, the following principles can be understood: (1) Although there is a centrally located tryptophan (in position 12 or 13) incorporated into the GWALP23 model peptide, it exerts little influence on the orientation or dynamics of the GWALP23 helix in DLPC, DMPC, or DOPC. (2) Though a central tryptophan (W12 or W13) does not affect the tilt or rotation of a GWALP23 model peptide, it is nevertheless more difficult to incorporate the peptide into some lipid bilayers, especially those of DOPC. There is a perceived difficulty in burying the central Trp residue, especially in GWALP23-W13. (3) The ^2H quadrupolar splittings observed to date for $W^{4,5}$GWALP23 are somewhat smaller than those for GWALP23 or $F^{4,5}$GWALP23, perhaps indicating increased motional averaging when W^4 and W^5 are present together. The dynamic averaging with W^4/W^5 appears to be intermediate between the previously studied cases of F^4/F^5 (low dynamics) and Y^4/Y^5 (high dynamics). (4) The analysis of solid-state ^2H NMR of GWALP23-W12 with deuterated alanines 3 and 21 shows that the ends of the transmembrane helix fray or unwind.

A central tryptophan in position 12 or 13 in a GWALP23 model peptide would be expected to favor the membrane/water interface because of the amphipathic indole group on the tryptophan. As seen by quadrupolar splitting ($\Delta\nu_{q)}$ magnitudes and the

corresponding ^{2}H NMR spectra, it can be concluded that GWALP23-W12 and GWALP23-W13 exhibit little change in their orientation compared to the regular GWALP23 model peptide. Though solid-state ^{2}H NMR indicates that the principal orientation of GWAPL23 is not affected by incorporating a centrally located tryptophan, there is indication that the central tryptophan poses some difficulty for peptide incorporation into lipid bilayers (especially DOPC). It is especially difficult to bury the central tryptophan in GWALP23-W13 in DOPC lipid bilayers.

In addition to the labeling the alanines in the core helix, the labeling of Ala^{3} and Ala^{21} in GWALP23-W12 lends insight into the unwinding of the ends of the transmembrane helix. It can be concluded from the quadrupolar splitting ($\Delta\nu_q$) magnitudes that the fraying of the helix ends is evident. The unwinding of the ends of the helix is significant as it could be important for stabilizing the peptide in a tilted orientation and minimizing the motion of the transmembrane helix.

The extra tryptophan added in the 4^{th} position of $W^{4,5}$-GWALP23 is used to study the dynamics induced by an additional tryptophan on the GWALP23 peptide in comparison to an additional phenylalanine or tyrosine. The results show that the helix dynamic averaging decreases as the polarity of the paired aromatic residues decreases in the series YY > WW > FF. These results could be important for understanding of membrane protein dynamics generally. In this context, extensions of the studies reported here to cholesterol-containing membranes would be of interest.

GWALP23 peptides serve as an effective model for understanding the role of transmembrane helices in biological functions. Results from specific alterations, such as

those presented in this thesis, of the basic GWALP23 model sequence (like the addition of a central tryptophan) allow us to learn principles that underlie the behavior of membrane proteins as a whole.

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