5-2016

Influence of Cholesterol on Single Arginine-Containing Transmembrane Helical Peptides

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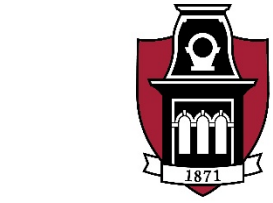
Influence of Cholesterol on Single Arginine-Containing Transmembrane Helical Peptides

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

By

Jordana Kathryn Thibado



UNIVERSITY OF ARKANSAS

Spring 2016

Chemistry & Biochemistry

J. William Fulbright College of Arts and Sciences

The University of Arkansas

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Acknowledgements

First, I would like to thank Dr. Roger E. Koeppe II for including me in his lab and for his invaluable guidance, encouragement, and mentorship over the past 3½ years.

I would also like to thank:

Dr. Denise Greathouse, Ashley Henderson, and the rest of the Koeppe group

Arkansas Department of Higher Education SURF Grant (2013-2014)

University of Arkansas National Science Foundation REU Program (2015)

Honors College Research Grant (2014-2015)

Honors College Travel Grants (2015, 2016)

Abstract

An essential component of animal cells, cholesterol exerts significant influence on the physical properties of the cell membrane and in turn, its constituents. One such category of constituents, the membrane proteins, are responsible for diverse and essential biological functions and often contain polar amino acids. Although sparse within the hydrophobic interior of lipid-bilayer membranes, polar amino acid residues are highly conserved and may play pivotal roles in determining specific structural and functional properties of key proteins. To gain greater understanding of the lipid membrane environment, and more broadly, cellular function, a model peptide framework termed "GWALP23" (acetyl-GGALWLALALAL^{12}AL^{14}ALALWLAGA-amide) can be useful. Designed and created at the University of Arkansas, the peptide is composed of amino acid residues of glycine (G), tryptophan (W), alanine (A), and leucine (L). The central helical core consisting of the W(LA)$_6$LW sequence is critical for defining the transmembrane configuration of this host peptide framework. Furthermore, the limited dynamic averaging of NMR observables such as the deuterium quadrupolar splittings of labeled alanine residues makes GWALP23 favorable for examining the influence of single "guest" residue replacements within the core sequence. Previously, GWALP23 family peptides were characterized with single leucine to arginine (R) mutations at positions 12 and 14 in single-lipid membranes.[1] GWALP23-R14 adopts a defined tilted orientation in DOPC bilayers, whereas GWALP23-R12 displays multi-state behavior.[1] The goal of this research is to further characterize these peptides in cholesterol-containing lipid bilayers. Specific deuterium-labeled alanine residues were incorporated into the R12 and R14 sequences to identify transmembrane peptide orientations by means of solid-

state deuterium NMR spectroscopy. The peptides were incorporated into phospholipid bilayers with varying cholesterol content (0%, 10%, or 20%). Our findings suggest that 10% or 20% cholesterol content has minimal impact on the orientation of the GWALP23-R14 peptide. (Although the NMR signals are broader and weaker in the presence of 20% cholesterol, the deuterium quadrupolar splittings for ^{2}H-Ala residues in GWALP23-R14 change little.) Conversely, cholesterol appears to reduce the multi-state behavior of GWALP23-R12, favoring a single well-defined state for the helix. With 10% or 20% cholesterol content, the spectra exhibit defined quadrupolar splittings, suggesting that GWALP23-R12 adopts a predominant orientation at the membrane surface in the presence of cholesterol. These results convey a conditional sensitivity of a complex multi-state peptide helix to the presence of cholesterol.

Abbreviations

CD, circular dichroism; DLPC, 1,2-dilauroylphosphatidylcholine; DMPC, 1,2-dimyristoylphosphatidylcholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; 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; MtBE, methyl-*t*-butyl ether; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TIPS, triisopropylsilane

Introduction

Upon the discovery and continuing investigations of their vital roles in both disease and basic cell functioning, membrane-bound proteins have become an increasingly important focus of scientific research. Essential to many membrane proteins are polar amino acids, which often determine the structure and function of the protein itself. The particular sequence and length of a largely hydrophobic membrane protein determines its behavior and stability within the cell membrane. Interestingly, the more sparse polar amino acids may be critical for particular functional properties. The examination of polar amino acids is possible through the use of synthetic model peptides such as those of the "GWALP" family. The GWALP peptides were created from an original "WALP" model peptide family by reducing the number of tryptophan (W) residues. WALP contained four aromatic W residues that serve as anchors for the membrane-spanning helix.[2,3,4] Although the WALP peptides have been useful for studying peptide mismatch, their high dynamics made them less than ideal for studying the effects of single residue replacement within the peptide core.[5]

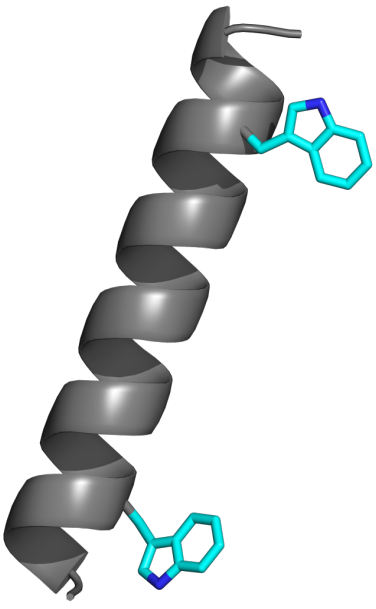

Figure 1. 3D representation of model peptide GWALP23. The indole rings extending from the right side are W^{5} and W^{19} in the 23-residue sequence.

GWALP23 was developed as a control and was found to decrease the high dynamics of WALP (Figure 1). By including only two anchoring tryptophan residues (W) rather than the four contained in WALP, greater stability and

lower dynamics were achieved.[6,7] GWALP23 (GGALW(LA)$_6$LWLAGA) contains a single tryptophan residue at each end (positions 5 and 19) and a repeating leucine-alanine helical core sequence.[8,9] The leucine-alanine helical core prefers the hydrophobic environment of the membrane, while the tryptophan residues prefer a location near the lipid-water interface.[10] GWALP23 and related model peptides fold into helices and adopt defined tilted orientations that can be observed in bilayer membranes by means of solid-state NMR spectroscopy. The solid-state NMR spectra allow for investigations of just one "guest" residue, such as a strongly polar or charged amino acid within the central core of the transmembrane helix. The constrained dynamics of GWALP23 make it more favorable than many other candidate "host" peptides for studies of specific guest residues.

Alanine residues within the GWALP23 core are labeled with deuterium in order to study the dynamics and orientation of the peptide by means of solid-state deuterium (^{2}H) NMR spectroscopy. For convenience and experimental efficiency, alanine residues are labeled in pairs in core positions as follows: 7 and 9, 11 and 13, or 15 and 17. Within the alanine pairs that are labeled, the alanine closest to the N terminus is labeled with 50% deuterium. The other alanine is labeled with 100% deuterium. This is so that quadrupolar splittings in NMR spectra may be distinguished and assigned, as shown in Figure 2.

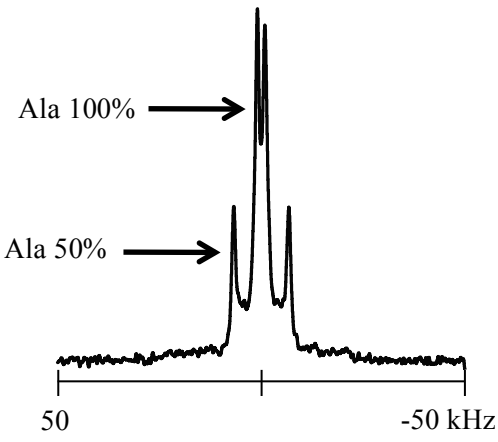

Figure 2. Deuterated alanine residues are incorporated into the core of the peptide with 50% or 100% relative abundance to distinguish the quadrupolar splittings by solid-state deuterium NMR spectroscopy.

The first focus of this project is to study the effects of replacing a single hydrophobic leucine residue (L) with the very polar, and positively charged, arginine (R) at position 14 of the GWALP23 peptide. Previous experiments have shown that R14 causes a 10° change in helix tilt, which is invariant over a pH range of 4 to 9, where the arginine is charged.[1] The equivalent peptide with lysine at position 14 (GWALP23-K14) shows an interesting pH-dependent tilt that indicates a pK_a of 6.5 for the lysine at 37°C in membranes of DOPC.[11] Based on the established titration of the lysine, my project has focused on extending the NMR observations for GWALP23-R14 to higher pH, by using bilayers composed of the more stable ether-linked lipids. The ether-linked DOPC bilayers are used as a means of preventing the base-catalyzed hydrolysis of ester lipids. Deuterium labels are incorporated in alanine residues 15 and 17 in GWALP23-R14 and the magnitude and orientation of the helical tilt was assessed using solid-state deuterium NMR spectroscopy at pH 9.5 and above.

This research project has also been directed to studying GWALP23-R14 and GWALP23-R12 in cholesterol-containing lipid bilayer membranes. Calculations based on questionable approximations for the lipid-bilayer environment have suggested that cholesterol may have a direct effect on the pK_a of buried arginine residues; perhaps shifting the pK_a of arginine from 12.5 to 3.8 in the presence of cholesterol.[12] Using a combination of DOPC phospholipids and cholesterol, I conducted experiments on GWALP23-R14 and GWALP23-R12 using 0%, 10%, and 20% cholesterol content (relative to the major lipid component DOPC) in the lipid bilayer.

The results of this research are important for understanding the effect of a changing chemical environment on the ionization behavior of arginine and, more generally, the

basic properties of proteins in cell membranes. Arginine plays an important role in cell-to-cell communication. If the functioning of arginine is better understood in a realistic cell environment, this will serve as an invaluable resource for computational scientists modeling arginine-containing systems, as well as for researchers working to advance drug design and delivery mechanisms. The sequences of model peptides used in this research are shown in Table 1 and cartoon representations of peptides GWALP23-R12 and GWALP23-R14 are displayed in Figure 3.

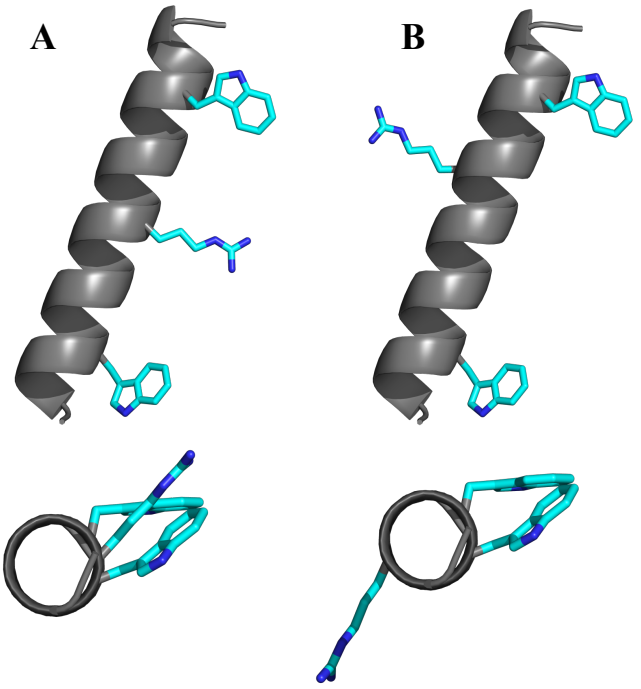

Figure 3. Side view and top-down representations of peptides (A) GWALP23-R12 and (B) GWALP23-R14.

Table 1. Model Peptide Sequences: Table 1 displays the sequence of model peptide GWALP23 as well as the sequences of model peptides I have synthesized (GWALP23-R12 and GWALP23-R14). Deuterated alanine residues are denoted as **A**$^{\#}$.

Peptide Name	Sequence
GWALP23	acetyl-GGALWLALALALALALALWLAGA-amide
GWALP23-R12	acetyl-GGALWL**A**^{7}L**A**^{9}LA**R**ALALALWLAGA-amide
GWALP23-R12	acetyl-GGALWLALALA**R**AL**A**^{15}L**A**^{17}LWLAGA-amide
GWALP23-R14	acetyl-GGALWLALAL**A**^{11}L**A**^{13}**R**ALALWLAGA-amide
GWALP23-R14	acetyl-GGALWLALALALA**R****A**^{15}L**A**^{17}LWLAGA-amide

Materials and Methods

Solid Phase Peptide Synthesis

Peptides containing deuterium-labeled alanine residues were synthesized using an Applied Biosystems 433A Peptide Synthesizer.[13] Fmoc-ala-d_4 was created and recrystallized in the lab, using a process which takes several weeks.[8] To create peptides by solid-phase peptide synthesis, 0.57 mmole of each amino acid in the peptide sequence was obtained (weighed with a balance) and placed in an individual cartridge (one per amino acid). The two alanine residues in the peptide sequence labeled with deuterium were labeled in different proportions. One alanine was labeled with 50% deuterium (0.285 mmoles of Fmoc-ala-d_4 and 0.285 mmoles of alanine) and the other was labeled with 100% deuterium (0.57 mmoles Fmoc-ala-d_4). Alanine residues were labeled in pairs at positions 7 and 9, 11 and 13, and 15 and 17. The amino acid cartridges are placed in order from C to N-terminal in the delivery system of the peptide synthesizer. Thus, peptides are synthesized from the carboxyl terminus back to the amino terminus. "FastMoc" chemistry method was used to remove blocking groups, couple the amino acids in the proper order, and thereby perform the peptide synthesis.

Peptide Cleavage

Following completion of peptide synthesis, the peptide is cleaved using a trifluoroacetic acid (TFA) cleavage cocktail, which is composed of TFA:TIPS:H_2O:phenol in a ratio of 85:5:5:5 (v/v, or w/v for phenol).[14] To begin the cleavage, approximately 100 mg of resin with peptide attached is weighed and placed in a glass scintillation vial. Next, the TFA cleavage cocktail is added to the resin. The vial is wrapped in foil and placed on a shaker for 3 hours at room temperature. Afterward, the

resin is transferred with a plastic pipette to short glass Pasteur pipette containing glass wool. The glass wool is used as a filter to separate peptide from the resin. The filter is rinsed with 2 mL of TFA and purged using a plastic pipette. Afterwards, the peptide solution is dried under nitrogen gas flow until approximately ¼ of the volume remains. The peptide product is then transferred to a 50 mL plastic centrifuge vial and is precipitated with 25 mL of methyl-t-butyl ether:hexane solution on ice for 30 minutes. The solution is centrifuged at 4°C for 10 minutes at approximately 1500 RPM. The supernatant is discarded and the pellet is re-suspended in 25 mL methyl-t-butyl ether:hexane. This procedure is repeated twice more. After the final centrifugation, the supernatant is poured off and the pellet is dried under a low flow stream of nitrogen gas. The final product is lyophilized overnight to a white powder from 2 mL of acetonitrile/water.

Crude Peptide Quantitation and Purification

After the peptide has been cleaved from the resin, the amount of recovered peptide is determined. First, 0.5 mL of TFE is added to the centrifuge tube containing the dried peptide to dissolve the peptide and begin the quantitation process. A UV-Vis sample is prepared from mixing 2 μL of the peptide/TFE mixture with 1.5 mL of methanol. The UV-Vis is first run with a blank of methanol and is set to read at 280 nm. After a sample is run, the spectra and optical density at 280 nm allows for calculation of total milligrams of peptide present. Next, an analytical HPLC sample is prepared from a 1 μL aliquot of the peptide solution diluted to 1 mg/mL with TFE. This is injected into the analytical HPLC and is called a “test injection.” The analytical HPLC reveals a spectrum that indicates peptide impurities. This is useful for determining a method for collection of

pure peptide. Another sample is prepared from the dried peptide/TFE mixture for mass spectrometry. This is created from a 50 μL aliquot and 450 μL of additional TFE. This sample vial is sent to the Arkansas Mass Spectrometry facility for analysis. The MALDI-TOF mass spectrometry profile confirms the peptide molecular mass and deuteration pattern. Finally, the remaining peptide solution is ready for purification by semi-preparative HPLC. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) is run with the Hitachi L1700 system and a Zorbax RX-C8 column (9.4 mm x 250 mm) from Agilent Technology. The HPLC must initially be purged with methanol and a desired gradient set to 92-96% for GWALP23. A test sample of 10 μL is injected into the HPLC for determination of the peak positions on the spectrum. The time the peak occurs corresponds to the time the peptide will be collected from the HPLC. After the test injection, the range of the HPLC detector is changed to 10 and a 300 μL aliquot of peptide is injected. The pure peptide product is collected approximately 15 minutes after the injection. Once collected, the aqueous product is dried under nitrogen until only a white film remains. Quantitation is performed again to determine the amount of pure peptide collected. For this process, the peptide film first is dissolved in 1 mL of TFE. A UV-Vis sample is created with 10 μL of dissolved peptide and 990 μL of methanol. The optical density at 280 nm is used again to calculate the total mass of pure peptide in milligrams. A sample of the peptide is sent once again to the Arkansas Mass Spectrometry facility for analysis by MALDI-TOF mass spectrometry. This sample is prepared using a 50 μL aliquot of peptide and 450 μL of TFE. Finally, the peptide is aliquoted for initial sample preparation for solid-state ^{2}H NMR and circular dichroism spectroscopy. Solid-state ^{2}H NMR samples require 1.3 μmol of peptide. The CD samples

need 0.0625 μmol of peptide. The remaining pure peptide and the aqueous samples are all dried under nitrogen until only a film remains. Lastly, any remaining pure peptide not aliquoted for samples is lyophilized. To lyophilize, 0.5 mL of acetonitrile and 0.5 mL of water is added to the peptide film and the solution is immersed in liquid nitrogen. The frozen centrifuge tube is then left under vacuum overnight.

Mass Spectrometry

A MALDI-TOF (matrix-assisted laser desorption ionization, time of flight) mass spectrum reveals the peptide mass and deuteration pattern. If correct, this pattern will confirm the identity of the peptide created by solid-phase peptide synthesis. The mass spectrometry is conducted by the Arkansas Statewide Mass Spectrometry Facility at the University of Arkansas. The satellite peaks in the spectrum correspond to ionization states of the peptide. As described in the "Crude Quantitation and Purification" section, mass spectrometry samples can be prepared both before and after peptide purification by semi-preparative HPLC.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy for samples in the lipid bilayer environment may be used to confirm the secondary structure of the peptides created by solid-state peptide synthesis. To create CD samples, peptide is incorporated into small unilamellar lipid vesicles (SUVs) in DLPC, DMPC or DOPC lipids. These samples require 0.0625 μmol of pure peptide, and peptide is added in a 1:40 peptide to lipid ratio. 1 mL of acetonitrile:water (50:50) solution is added to the peptide. Next, 2.5 μmols of lipid is added to the peptide and acetonitrile:water solution. This mixture is then dried

under nitrogen gas until a film remains. Finally, the samples are put under vacuum for ~48 hours to remove solvent.

Oriented Sample Preparation

Samples are created for analysis by NMR spectroscopy through preparation in a 1:40 peptide:lipid ratio. Samples can be prepared using DLPC, DMPC, and DOPC lipid membranes and cholesterol. However, the focus of this project has used DOPC phospholipids and cholesterol only. The samples are comprised of 1.3 μmols of peptide and 90 μmols of lipid with 45% hydration (w/w-water/peptide-lipid). After the lipids are added to dried peptide, the sample is dried under nitrogen gas. Once dry, 1 mL (95% methanol and 5% water) is added to the dried peptide/lipid combination. To create samples, 40 glass slides are dispersed in two petri dishes (20 glass slides per petri dish). A 50 μL syringe is used to add 25 μL peptide/lipid solution to each glass slide. Once the peptide/lipid solution is evenly dispersed on all slides, the open petri dishes are covered with glass lids and dried under vacuum in a dessicator for 48 hours. The second step of oriented sample preparation is hydrating and stacking the glass slides. Approximately 1.2 μL of deuterium-depleted water or buffer is used to hydrate the glass slides. The water must be free of deuterium so that the only deuterium present in the system is the deuterium-labeled alanine residues in the peptide core. The hydrating liquid is added as approximately 3 drops to each slide. Once a slide has been hydrated with water or buffer, it is stacked on another slide and carefully pressed down with tweezers. This pressure helps to mechanically form a lipid bilayer stack between the slides. The final result is a single stack of glass slides that is placed in a cuvette and sealed with a glass top using epoxy glue. The cuvette is sealed twice to ensure a complete seal and is then placed in a

dark heating plate at 40°C for several days to achieve orientation of liquid-crystalline lipid bilayers. The sample is removed when it appears clear. The oriented sample preparation process is illustrated in Figure 4.

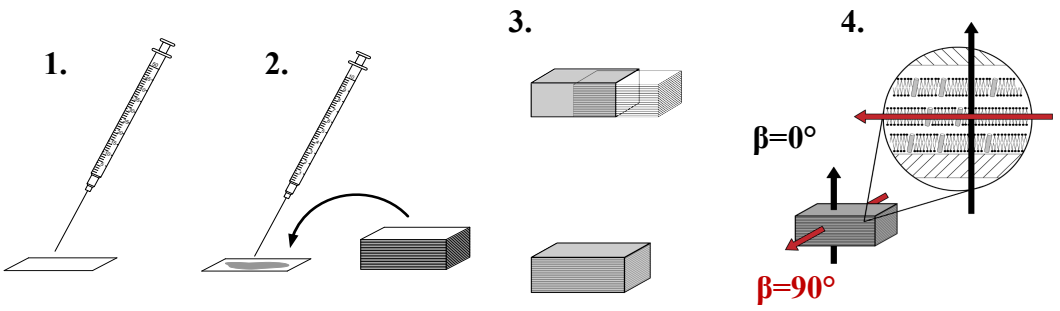

Figure 4. Visual representation of oriented NMR sample preparation. (1) First, the peptide/lipid mixture is applied to glass slides. (2) Next, the glass slides are hydrated with deuterium-depleted water or buffer and stacked. (3) Third, the glass slides are placed in a cuvette and sealed. (4) Finally, the oriented sample is placed in the NMR probe at 90° or 0° with respect to the magnetic field.

^{31}P NMR Spectroscopy

^{31}P (phosphorous) NMR spectroscopy verifies bilayer alignment of oriented samples by monitoring the lipid head groups. Samples are run at 50 °C on a Bruker Avance 300 spectrometer at both β=0° and β=90° orientation. At β=0°, the bilayer normal is parallel to the applied magnetic field. At β=90°, the bilayer normal is perpendicular to the magnetic field.

Solid-State ^{2}H NMR Spectroscopy

Solid-state ^{2}H NMR spectroscopy is conducted similarly to ^{31}P NMR spectroscopy. Samples are run at 50 °C on a Bruker Avance 300 spectrometer at both β=0° and β=90° orientations. Resulting spectra display peaks that can be used to measure the respective ^{2}H quadrupolar splittings. These measurements are important in determining information about the peptide tilt and rotation in various lipid environments.

Results

After GWALP23-R12 and GWALP23-R14 peptides were synthesized and purified, MALDI-TOF mass spectrometry was used to confirm the molecular mass and deuteration pattern. The expected molecular mass of 2304 + ~8 deuterons was confirmed for the two peptides, as shown in Figure 5. (The individual peaks within the "4D" (four deuteron) and "8D" envelopes in Figure 5 represent integral differences in the content of ^{13}C, which has 1.1% natural abundance.)

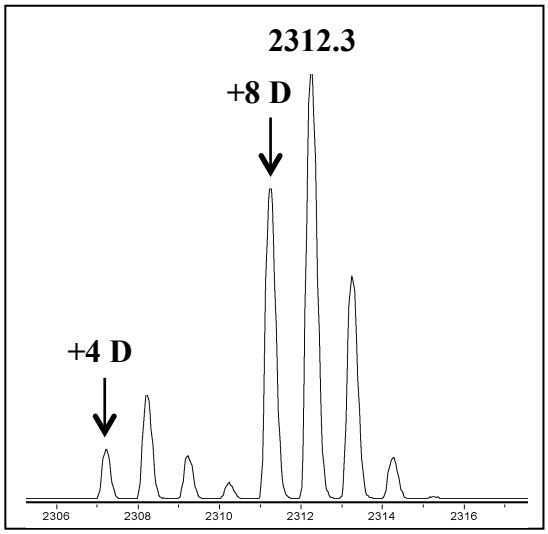

Figure 5. MALDI-TOF mass spectrum for GWALP23-R12. GWALP23-R14 gives the same spectrum because its molecular weight and composition is identical.

Next, the peptides were analyzed using circular dichroism (CD) spectroscopy to confirm peptide secondary structure. The repeating Leu-Ala sequence of the GWALP23 peptide core forms an alpha-helical secondary structure in the hydrophobic region of the lipid bilayer. The CD spectra for both the R12 and R14 peptides shown in Figure 6 are characteristically alpha-

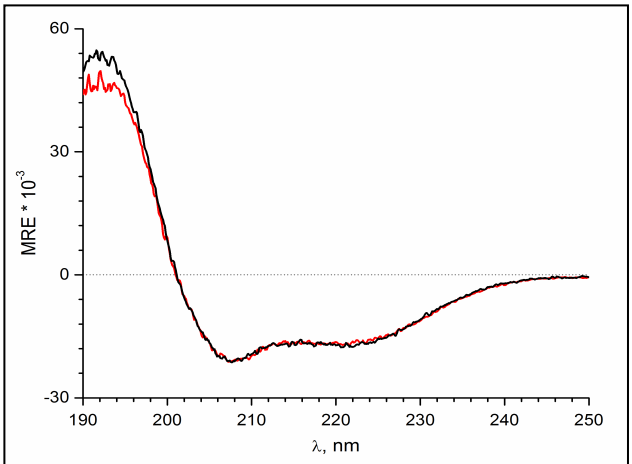

Figure 6. Circular dichorism spectra for peptides in DLPC vesicles. GWALP23-R14 is shown in black and GWALP23-R12 is shown in red. Both spectra are representative for peptides that are alpha-helical.

helical. The helicity is indicated by the presence of minima at approximately 208 nm and 222 nm.

Phosphorous NMR (^{31}P NMR) is used to confirm lipid bilayer alignment in oriented samples. ^{31}P NMR spectra display a single well-defined peak at +28 ppm for β = 0° (membrane normal parallel to the applied magnetic field) or -14 ppm for β = 90° (membrane normal perpendicular to the applied magnetic field) for samples with proper bilayer alignment. Smaller secondary peaks can occur in the β = 0° spectra, which indicates some lipid unalignment. This occurs because the β = 0° orientation is more sensitive to lipid alignment. Representative ^{31}P spectra for three distinct peptide-lipid conditions are displayed in Figure 7.

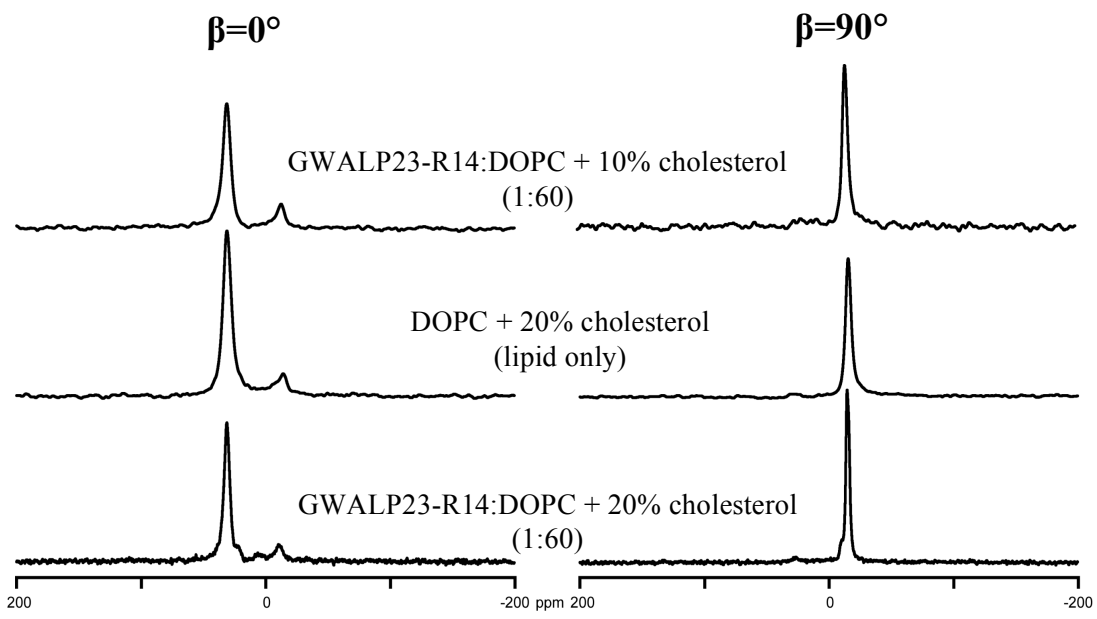

Figure 7. From top spectrum: ^{31}P NMR spectra of GWALP23-R14 with 90% DOPC and 10% cholesterol content; DOPC with 20% cholesterol content (no peptide); GWALP23-R14 with 80% DOPC and 20% cholesterol. Samples containing peptide and lipid are composed in a 1:60 peptide:lipid molar ratio. Well-defined single peaks in β = 90° and β = 0° spectra indicate that the lipid bilayer is aligned.

Peptide behavior and orientation is assessed in various bilayers by studying oriented samples with solid-state ^{2}H NMR spectroscopy to monitor the side-chain methyl groups of the labeled alanine residues. Prior to the addition of cholesterol, GWALP23-R14 was investigated over an extensive pH range of 2-13. This was to establish the limits for the pK_a of arginine in a lipid bilayer. The results of these experiments are displayed in Figure 8 and indicate that the pK_a of arginine is above 13.

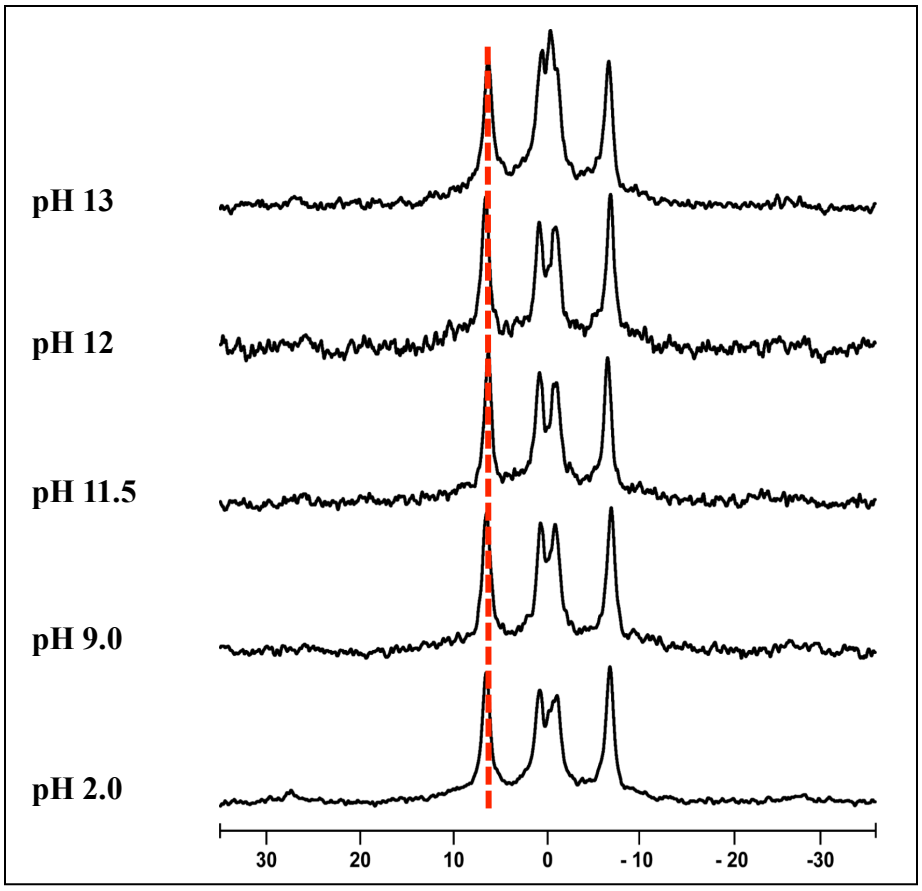

Figure 8. ^{2}H NMR spectra of GWALP23-R14 in DOPC ester or ether linked bilayers, over a pH range of 2-13. Samples above pH 8.5 required ether-linked lipids. Alanines 15 and 17 are deuterated at 50% and 100%, respectively. No significant changes in the quadrupolar splittings are observed between spectra.

Typically, ester-linked DOPC phospholipids are the lipid of choice for creating a model mammalian cell environment because they are natural phosphatidylcholine (PC) lipids. However, for the results displayed in Figure 8, the extreme pH conditions would lead to degradation of the ester-linked lipids at high or low pH, by means of alkaline or acid hydrolysis to release the acyl chains. Ether-linked DOPC phospholipids were discovered to be suitable for replacement as they are resistant to acid and base-catalyzed degradation while providing a highly similar lipid environment. Small changes of 0-2 kHz are observed in NMR spectra obtained from samples with DOPC bilayers formed with ether lipids rather than ester lipids, as shown in Figure 9.

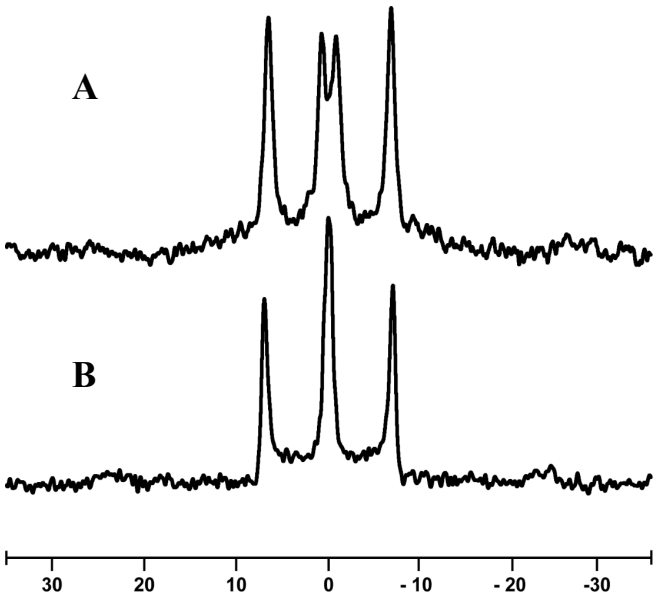

Figure 9. (A) ^{2}H NMR spectra of GWALP23-R14 with ether-linked DOPC phospholipids and with (B) ester-linked DOPC phospholipids.

^{2}H NMR experiments were then conducted at pH 5.5 to investigate the influence of 10% cholesterol content on the GWALP23-R14 peptide. The results of this experiment are displayed in Figure 10. No significant differences in the alanine methyl group quadrupolar splittings were observed between GWALP23-R14 in an environment of 100% DOPC phospholipids and that of 90% DOPC and 10% cholesterol content,

suggesting that 10% cholesterol content does not affect the charge of arginine or the orientation and dynamic behavior of GWALP23-R14 in the bilayer membranes.

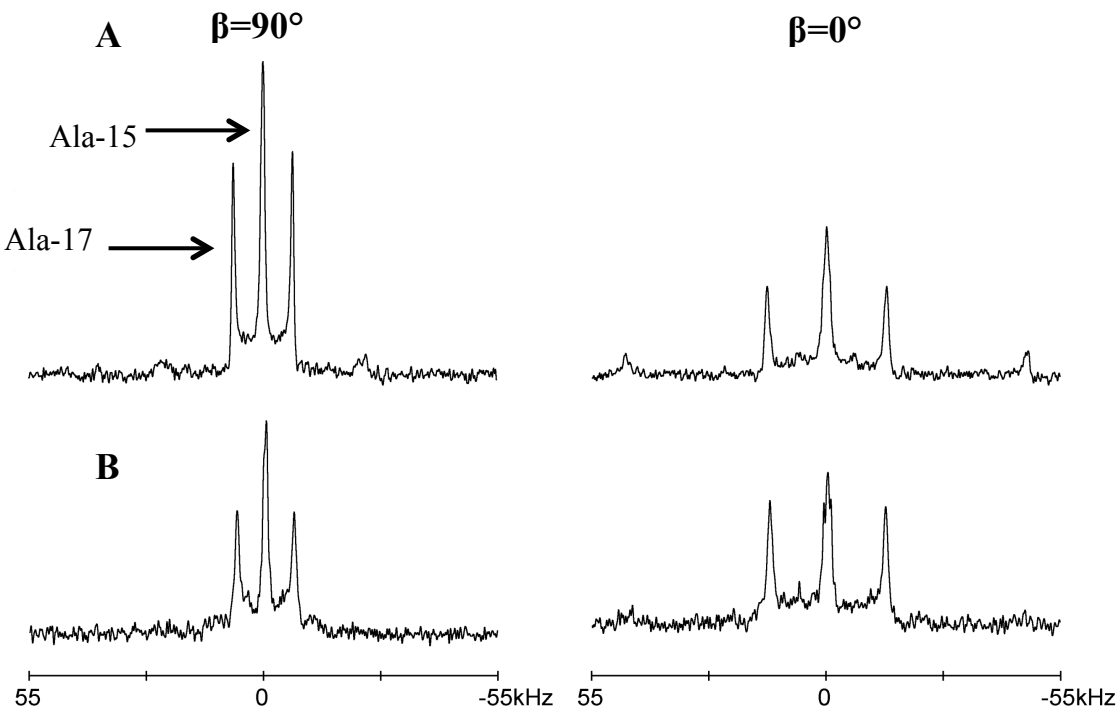

Figure 10. ^{2}H NMR spectra of (A) GWALP23-R14 with 100% DOPC and (B) GWALP23-R14 with 90% DOPC and 10% cholesterol content (B). Deuterated alanine residues in this experiment are Ala-15 and Ala-17. There are no significant differences in the quadrupolar splittings between spectra contained in (A) and (B).

An experiment was also conducted to investigate the influence of 20% cholesterol on GWALP23-R14 at pH 5.5. The spectra obtained from this experiment are shown in Figure 11. Similar to the results displayed in Fig. 10, no significant differences in quadrupolar splittings were observed for GWALP23-R14 in an environment of 100% DOPC phospholipids and that of 80% DOPC and 20% cholesterol content. This suggests that 20% cholesterol content does not affect the charge of arginine or the behavior and dynamics of GWALP23-R14.

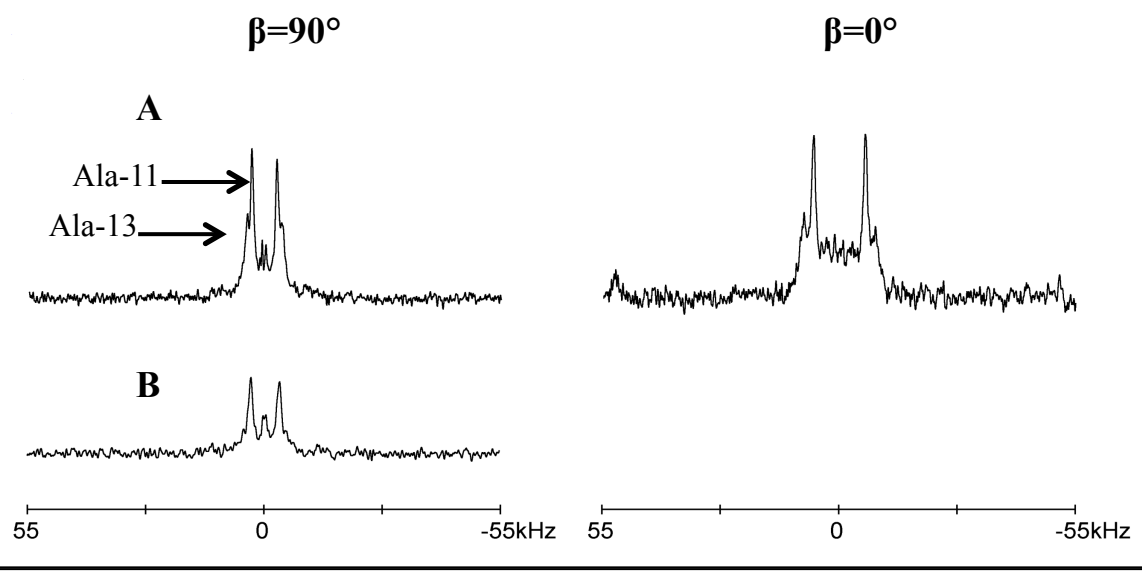

Figure 11. ^{2}H NMR spectra of (A) GWALP23-R14 with 100% DOPC and (B) GWALP23-R14 with 80% DOPC and 20% cholesterol content (B). Deuterated alanine residues in this experiment are Ala-11 and Ala-13. There are no significant differences in the quadrupolar splittings between spectra contained in (A) and (B).

Further ^{2}H NMR experiments were conducted to investigate the influence of cholesterol on GWALP23-R12 at pH 5.5. GWALP23-R12 has been characterized previously in DOPC bilayers and was shown to display multi-state behavior.[1] This means that the peptide molecules exist in a variety of different orientations within the lipid bilayer, including one populated state that moves to the membrane surface, rather than existing in primarily one single (tilted) transmembrane orientation. Multi-state ^{2}H NMR spectra are characterized by low signal strength of peaks. Spectra obtained for the GWALP23-R12 peptide in 0%, 10%, and 20% cholesterol conditions with DOPC phospholipids are displayed in Figure 12. In the presence of 10% and 20% cholesterol, the GWALP23-R12 helix transitions from multiple states in DOPC bilayers to instead occupy one major state (see Discussion).

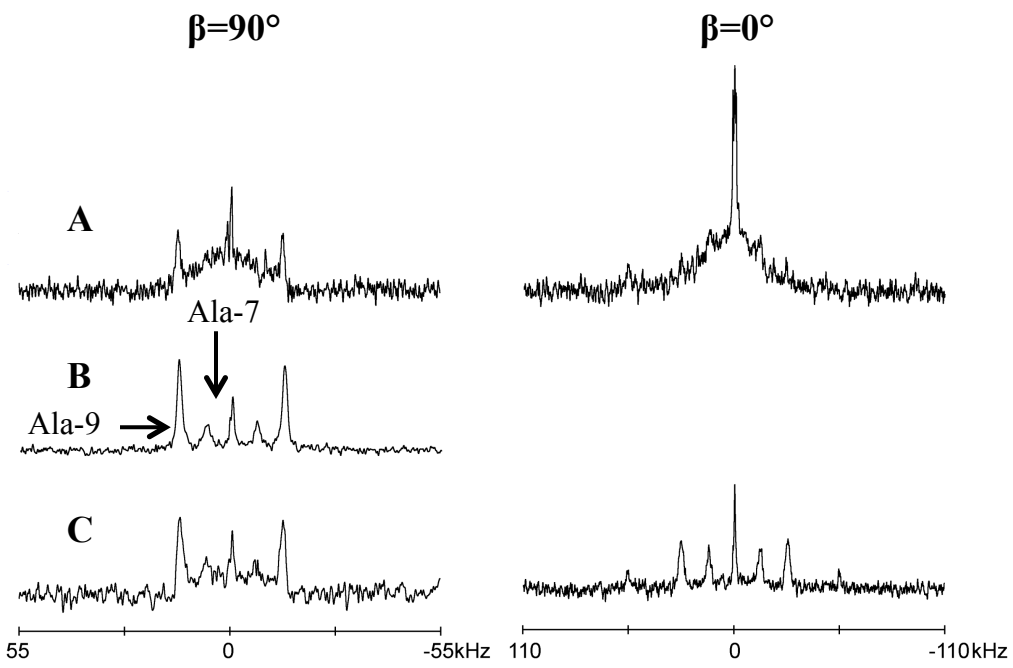

Figure 12. ^{2}H NMR spectra of (A) GWALP23-R12 with 100% DOPC (0% cholesterol), (B) GWALP23-R12 with 90% DOPC and 10% cholesterol content, and (C) GWALP23-R12 with 80% DOPC and 20% cholesterol content. Deuterated alanine residues in this experiment are Ala-7 and Ala-9. Spectra become more resolved in the presence of cholesterol.

Discussion

This research project has resulted in several significant discoveries. Most notably, the pK_a of arginine in a lipid bilayer was found to be above 13. Previously, the pK_a of the arginine guanidinium group in aqueous solution has been considered to be ~12.5. This value dates from the 1920s, is cited in biochemistry textbooks and frequently is used in electrostatics calculations. Within the hydrophobic lipid bilayer interior, one might expect the arginine pK_a to be lower, since the pK_a for lysine in a DOPC bilayer is about 4 pH units lower than in aqueous solution.[11] Nevertheless, my results indicate that the arginine pK_a remains high in the lipid bilayer environment, perhaps because other mechanisms such as membrane deformation and helix translocation tend to ensure that hydration of the guanidinium group is maintained.[1] This finding was corroborated by a striking recent publication which reported that the longstanding pK_a of arginine in aqueous solution should be revised to 13.8.[15] The high pK_a values for both aqueous and membrane-incorporated arginine are particularly significant because a difference of a single pH unit corresponds to a 10-fold change in hydrogen ion concentration.

Secondly, it was discovered that ether-linked DOPC lipids can be substituted for ester-linked DOPC lipids in experiments that require extreme pH conditions. Spectra obtained from samples containing ether-linked lipids are nearly indistinguishable from spectra of samples containing ester-linked lipids. The ether-linked lipids display small changes of 0-2 kHz in the deuterium quadrupolar splittings, but they are a suitable replacement for ester-linked lipids due to their resistance to acid and base-catalyzed degradation.

Thirdly, it was found that the pK_a of arginine remains high, even in the presence of 10% and 20% cholesterol content in DOPC bilayers. The quadrupolar splittings of ^2H-labeled alanine side chains of GWALP23-R14 in a cholesterol-containing environment were not significantly different from those in an environment without cholesterol. This finding was particularly surprising because it is in direct opposition to the computational findings from Cui et al., who suggested through molecular dynamics simulations that the pK_a of arginine may drop to 3.8 in the presence of cholesterol.[12]

Although the pK_a of arginine was shown to remain unchanged in the presence of cholesterol, in experiments with GWALP23-R12, the peptide itself was affected. GWALP23-R12 did not exhibit its characteristic multi-state behavior in DOPC membranes in the presence of cholesterol. With the addition of 10% and 20% cholesterol content, ^2H NMR spectra became resolved, indicating that the peptide has a single primary orientation near the surface of the DOPC bilayers when cholesterol is present. The cholesterol effectively seems to drive GWALP23-R12 out of the bilayer interior and to the membrane surface.

GWALP23 peptides serve as an invaluable model for studying the role of polar residues and transmembrane helices in a biologically relevant environment. The findings of this project are important for advancing computational modeling of arginine and arginine-containing proteins. For example, arginine is critical for the voltage-gating domains of potassium channels. These channels propagate electrical signals in the nervous system, which contribute to cellular communication. If both the pK_a of arginine in a lipid bilayer and the role of cholesterol are better understood, more accurate computational models can be produced. This advancement is also important for drug

development. Many neurodegenerative diseases, such as Alzheimer's and Parkinson's, are related to disruptions in cellular communication. If cellular function and the role of arginine in these systems is better understood, drug development for such diseases may be advanced.

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