Effect of pH and lipid composition on membrane-spanning helices with glutamic acid examined by solid-state NMR

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Effect of pH and lipid composition on membrane-spanning helices with glutamic acid examined by solid-state NMR

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

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Acknowledgements

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This semester didn’t end like anyone expected it to, but I am very thankful for the past three and half years as an undergraduate at the University of Arkansas.
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Abstract

Transmembrane proteins constitute about 30% of the proteins in a mammalian cell and are involved in major biological processes [1]. The dynamic properties of membrane proteins and the ionization states of particular side chains are important for biological function [2]. The biophysical properties of membrane proteins nevertheless can be difficult to decode, particularly for glutamic acid in the lipid environment of cell membranes. To study the ionization of glutamic acid in transmembrane peptides, guest glutamic acid residues were substituted into the well-defined model helix of GWALP23 (acetyl-GGAL^4WLALALALALAL^16ALWLAGA-amide). These guest residues were placed at position L16 or L4 and specific $^2$H-labeled alanine residues were incorporated in the central helix to enable detection by solid-state NMR. These chemically synthesized GWALP23 derivatives were placed in lipid membranes with 90% zwitterionic and 10% negatively or positively charged lipids, DMPG or DMTAP, buffered at pH 3.0, 6.1 or 8.0 in deuterium-depleted water. Lipid bilayer alignment was confirmed with $^{31}$P NMR. The core alanines of the GWALP23 E16 helix give distinguishable $^2$H quadrupolar splittings at pH 6.1 with both positive and negatively charged lipids. Samples containing DMTAP had identical splittings from pH 3-8, indicating a lack of titration or lack of helix response. However, the samples with DMPG did not maintain distinguishable quadrupolar splittings above or below pH 6. The findings with DMPG are puzzling yet may reveal changes in helix aggregation or dynamics. Ongoing investigations of GWALP23 with E4 are focused on better understanding of the pH dependence of helix properties. The present results and anticipated future results are important for understanding membrane protein function.
Abbreviations

A: alanine
CD: circular dichroism
DLPC: 1,2-dilauroyl-glycero-3-phosphocholine
DMPC: 1,2-dimyristoylphosphatidylcholine
DMPG: 1,2-dimyristoylphosphatidylglycerol
DMTAP: 1,2-dimyristoyl-3-trimethylammonium-propane
DOPC: 1,2-dioleoylglycerol-3-phosphocholine
E: glutamic acid
Fmoc: fluorenyl methoxycarbonyl
G: glycine
GALA: geometric analysis of labeled alanine
GWALP23: acetyl-GGA\textsuperscript{L4}WLALALALALAL\textsuperscript{L16}ALWLAGA-amide
HEPES: 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid
HPLC: high-performance liquid chromatography
kHz: kilohertz
L: leucine
MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight
MeOH: methanol
MtBE: methyl-t-butyl ether
NMR: nuclear magnetic resonance
Q: glutamine
TFA: trifluoroacetic acid
TFE: 2,2,2-trifluoroethanol
TIPS: triisopropylsilane
UV-Vis: Ultraviolet-visible spectroscopy
W: tryptophan
Introduction

Membrane proteins are essential to the cell and its processes, namely in cell signaling and transport of ions or small molecules across the membrane. The cell membrane is comprised of closely packed amphiphilic phospholipids and membrane proteins, sprinkled with various hydrophobic compounds such as cholesterol. [3] With the inside of the bilayer being hydrophobic due to the long carbon lipid chains and the outside as hydrophilic from the phosphate head groups, the membrane proteins that cross the bilayer also follow similar composition. The portion of the protein that crosses the membrane are typically made of hydrophobic amino acids in conformations such as $\alpha$-helices or $\beta$-barrels due to the favorable hydrophobic free energy. [4]

The polar and ionizable amino acids within these primarily hydrophobic transmembrane proteins are often essential to the protein’s structure and function. Many of the antibiotic resistant bacteria use transmembrane proteins with key ionizable amino acid residues to efflux drugs. [5] While the ionization of glutamic acid and other titratable amino acids have been studied in solution, their ionization has been relatively little studied in a lipid bilayer environment necessary for transmembrane proteins.

While these transmembrane proteins and their secondary structures are of great interest in both biochemical and pharmaceutical research, it is very difficult to study these systems in vivo due to factors such as lipid bilayer thickness, component mixing, water-lipid interactions at the bilayer interface, and varying cholesterol levels. Therefore, the use of model protein systems has become important for research to understand the biophysical properties of membrane proteins and lipids. The model peptide system of GWALP23 (acetyl-GGAL$^4$WLALALALALAL$^{16}$ALWLAGA-amide) is notable for its well-defined orientation in
lipid bilayers. The alanine-leucine core maintains a stable \(\alpha\)-helix while the two flanking tryptophans at positions 5 and 19 anchor the peptide with respect to the lipid head groups. With this stability, it is possible to analyze the effects of a single point mutations in the peptide sequence.

This project has been focused on the insertion of a guest glutamic acid (E) or glutamine (Q) residue into the GWALP23 peptide sequence at position L16 and studying the effect of pH on these residues in varying anionic or cationic lipid bilayers. A schematic of the GWALP23 \(\alpha\)-helix and the GWALP23 derivative sequences are seen below in Figure 1 and Table 1, respectively:

![Figure 1: GWALP23 host framework (left), GWALP23 Q16 (right), and GWALP23 E16 (middle). Tryptophan residues are shown in pink while the glutamate is blue and glutamine orange.](image-url)
Table 1: Sequences of GWALP derivatives with Glutamic Acid and Varying Controls

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>GWALP23</td>
<td>acetyl-GGALWLALALALALALALWLAGA-amide</td>
</tr>
<tr>
<td>GWALP23 Q16</td>
<td>acetyl-GGALWLALALALALQALWLAGA-amide</td>
</tr>
<tr>
<td>GWALP23 E16</td>
<td>acetyl-GGALWLALALALALEALWLAGA-amide</td>
</tr>
</tbody>
</table>

These chemically synthesized GWALP23 derivatives written out in Table 1 will be subjected to varying lipid environments containing both positive and negatively charged lipid head groups along with the control zwitterionic lipids, identified below in Table 2.

Table 2: Lipids of Interest

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-dimyristoylphosphatidylcholine (DMPC) – zwitterionic</td>
<td><img src="image" alt="DMPC" /></td>
</tr>
<tr>
<td>1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP) – positively charged</td>
<td><img src="image" alt="DMTAP" /></td>
</tr>
<tr>
<td>1,2-dimyristoyl phosphatidylglycerol (DMPG) – negatively charged</td>
<td><img src="image" alt="DMPG" /></td>
</tr>
</tbody>
</table>

Inserting GWALP23 E16 and Q16 into lipid-bilayer membranes composed of different molar ratios of DMPC with DMTAP or DMPG will be done to approach our key goal of obtaining NMR spectra which will reveal the transmembrane helix properties. Previous work in using GWALP23 E16 focused solely on the titration of the glutamic acid in zwitterionic neutral lipids. It was reported that the pKa of the glutamic acid in this system was likely to be above 12.5, significantly higher than the 4.1 reported for glutamic acid in solution. [6, 7]. Nevertheless, there were lingering uncertainties about the limited lipid environment and the poor spectral
resolution involving small spectral changes with pH. I have investigated the prospects for improving the quality of the $^2$H NMR spectra by optimizing the membrane lipid composition. While most of my efforts have been focused on glutamic acid residue E16, I have also obtained new and recent promising results with residue E4 which will be mentioned in the discussion.
Materials and Methods

The derivatives of GWALP23 were synthesized using solid phase Fastmoc™ chemistry with an Applied Biosystems 433A peptide synthesizer from Life Technologies ®. All amino acids used, with the exception of the deuterated alanine, were purchased from NovaBiochem® with Fmoc protective side chains. In addition, glutamic acid contained a t-butyl ester protecting group and tryptophan had a t-butoxy carbonyl protecting group.[6] Fmoc-Ala-d₄ was synthesized in lab for detection in ²H NMR Spectroscopy. The terminal N and C charges were blocked with acetyl and amide groups, respectively. With terminal charges blocked, the glutamate or glutamic acid guest residues determines the ionization state of the peptide as a whole. Specific deuterated alanine residues at 50% and 100% abundances were incorporated into each peptide at position A15 and A17, respectively.

Each peptide was cleaved from the rink amide resin through an 85:5:5:5 (v/v) TFA:TIPS:water:phenol solution. Once the cleavage is complete after shaking for three hours, the newly freed peptide was filtered from the resin and precipitated using 25 mL of a 1:1 MtBE:hexane solution. Peptides were collected by centrifugation with the precipitate lyophilized twice from a 1:1 (v/v) acetonitrile: water solution on the vacuum line.

The crude peptide, now in a white powder, was purified by reversed-phase HPLC on an octyl-silica column (Zorbax Rx-C8, 9.4x250 mm. 5 μm particle size) from Agilent Technologies with Hitachi L1700 UV-Vis system. A gradient of 88-92% MeOH was used over 13 minutes and purified peptide was collected and dried down under nitrogen flow. Dried peptide was lyophilized in the same procedure as after cleavage.[8] Purified peptide was quantified by UV-Vis spectroscopy based on the UV absorbance of W at 280 nm. To confirm the correct molecular
mass and level of deuteration, the peptide was analyzed through MALDI-TOF Mass Spectrometry at the Arkansas Statewide Mass Spectrometry Facility.

CD and fluorescence spectra were collected on a Jasco J710 CD Spectropolarimeter using a 1 mm cell path length, 1.0 nm bandwidth, 0.1 nm slit. The CD samples were made of small lipid vesicles at a 1:60 peptide:lipid ratio. An average of 10 CD scans were taken with a scan speed of 20 nm/min. Steady-state fluorescence experiments were performed after diluting CD samples with water (1/20). The samples were excited at 280 nm and excitation slit width of 5 nm. Emission spectra were recorded with a 5 nm emission slit width between 300-450 nm, with an averaging of 5 scans. [9]

Aliquots of purified peptides was combined with either 100% DMPC, or varying lipid ratios of DMPC with DMTAP or DMPG in a 1:60 peptide to lipid ratio. Lipids were purchased from Avanti Polar Lipids. The peptide: lipid sample was distributed evenly onto 34 glass slides and dried under vacuum for 48 hours to remove solvent. The slides were then hydrated to 45% w/w using either citrate or HEPES buffer in deuterium-depleted water from Cambridge Isotopes Laboratory. The hydrated slides were stacked to mechanically create lipid bilayers and placed in a glass cuvette. The sample is then sealed and incubated at 40°C in order for the peptide: lipid sample to orient and come to equilibrium.

Figure 2: Schematic of Mechanically Oriented Lipid Bilayers Sample Preparation

Bilayer alignment at both $\beta = 90^\circ$ and $\beta = 0^\circ$ sample orientations was confirmed with $^{31}$P NMR spectroscopy on a Bruker Avance 300 MHz spectrometer at $^1$H broadband decoupling and
128 scans. Solid-state $^2$H NMR experiments were performed with a quadrupolar echo pulse sequence using a Bruker Avance 300 MHz spectrometer at both $\beta = 90^\circ$ and $\beta = 0^\circ$ orientations. $\beta = 0^\circ$ is oriented with the mechanically made bilayer parallel to the NMR’s magnetic field where as $\beta = 90^\circ$ is perpendicular to the magnetic field. The pulse sequence included a pulse time of 3.2 $\mu$s, an echo delay of 115 $\mu$s, and a recycle delay of 90 ms. Each $^2$H NMR experiment acquired approximately 1.5 million scans. Fourier transformation was applied using an exponential weighting function with 150 Hz line broadening. [8]
Results and Discussion

MALDI-TOF mass spectrometry was run on the purified peptide GWALP23 E16 to determine the molecular mass and level of deuteration of alanines at the Arkansas Statewide Mass Spectrometry Facility. The peak located at 2302.3 m/z corresponds to the +4D level of A15 with 50% deuterated alanine while the peak at 2306.3 m/z corresponds to the +8D level of A17 with 100% deuterated alanine. The same analysis was run on synthesized GWALP23 Q16 with A15 at 100% deuterated alanine and 50% at A17 with peaks in Figure 3 at 2304.3 and 2301.3, respectively.

Figure 3: MALDI-TOF Chromatograph of E16 (left) & Q16 (right)

CD and fluorescence spectroscopy were used to determine the alpha-helical properties of both the synthesized GWALP23 E16 and Q16 samples. The peptide-containing lipid vesicles were made corresponding to the samples made for NMR spectroscopy (seen in Figure 6 and Figure 7) with 100% DMPC, 90:10 DMPC: DMTPAP, or 90:10 DMPC: DMPG.
The maximum absorbance in the fluorescence spectra for all Q16 samples was 335 nm whereas the E16 maximum absorbances differed for each lipid system with DMPC only at 333 nm, 10% DMPG at 335 nm, and 10% DMTAP at 338 nm. As seen in Table 3, a higher $\varepsilon_{222}/\varepsilon_{208}$ ratio is observed in GWALP23E16 in bilayers composed of only DMPC. The $\varepsilon_{222}/\varepsilon_{208}$ ratio is similarly higher for GWALP23Q16 in 10% DMTAP bilayers. Ultimately, the
circular dichroism spectra show that the peptide is primarily alpha-helical while the fluorescence shows that tryptophans are anchored within the varying lipid bilayers for both Q16 and E16.

$^{31}$P NMR was implemented to determine the bilayer alignment within samples. Two sets of spectra were made: one set with E16 incorporated in the lipid bilayer and one set with only lipids.

**Figure 6:** $^{31}$P NMR of lipid bilayers containing E16 (left) and without peptide (right) at increasing amounts of either DMPG or DMTAP

The sharp peak shapes show the well-oriented bilayer alignment with both the control DMPC alone and also containing E16. Lipid bilayers with varying anionic and cationic lipid
ratios (5%-10%) have also been confirmed with and without E16. Samples made with higher levels of charged lipids didn’t give the same patterned peaks as in Figure 6 and suggested a large amount of unaligned lipids in the sample.

$^2$H NMR was utilized to analyze the E16 peptide behavior and orientation in the different cationic and anionic lipid bilayers based on the alanine methyl-group quadrupolar splittings. Samples held at pH 6 were made with increasing ratios of the charged lipids, starting with 0% (DMPC only) going up to 5% and then 10%. Samples with 20% charged lipid ratios were made; however, the splittings were not resolved. Alanines 15 and 17 were deuterated with 50% and 100%, respectively.

![2H NMR Spectra](image)

**Figure 7:** $^2$H NMR Spectra of GWALP23 E16 in 100% DMPC, 95% DMPC with 5% DMTAP (right) or 5% DMPG (left), and 90% DMPC with 10% DMTAP (right) or 5% DMPG (left)
The quadrupolar splittings for the increasing amounts of DMTAP and DMPG along with the control DMPC were found to be the same with A15 at 21 kHz and A17 at 10 kHz.

An experiment was conducted to determine if there were changes in peptide dynamics with changing pH to indicate titration of the glutamic acid within the bilayer. While spectra seen in Figure 8 is for samples at pH 3 & 8 for DMTAP samples, there were no distinguishable spectra seen for DMPG above or below pH 6.

![Figure 8: $^2$H NMR Spectra of GWALP23 E16 in pH 3 & 8 in 9:1 DMPC:DMTAP bilayers](image)

The quadrupolar splittings in samples of 9:1 DMPC: DMTAP remained relatively consistent with the sample across pH 3 and 8 with splittings of A15 at 20 kHz and A17 at 8.0 kHz. Ultimately, the $^2$H quadrupolar splittings of GWALP23 E16 do not vary between different lipid environments.
After the creation of these charged lipid bilayers, experiments with GWALP23 E4 were done in lipid bilayers of various lengths: DLPC with saturated 12-carbon chains, DMPC with saturated 14-carbon chain, and DOPC with unsaturated 18-carbon chains. Previous work in the Koeppe lab by Matthew McKay that focused on the titration of glutamic acid within the GWALP system and placed a guest glutamic acid residue at L4 and deuterium labeled alanine at A3. The titration of GWALP23 E4 from pH 3 to 13 in different lipid bilayers was carried out in solid-state NMR experiments. It was found that the titration curve of glutamic acid in thinner lipids (DLPC and DMPC), reported by residue A3, showed lower pKa values than the titration of glutamic acid in thicker lipids (DOPC) and is seen in Figure 9 below [8].

Figure 9: Titration curves of GWALP23E4 in DLPC (black), DMPC (blue), and DOPC (Red). Dotted lines are the pH midpoints of 4.8 (DLPC), 6.3 (DMPC), and 11.0 (DOPC) [8]

The data points represent the measured $^2$H quadrupolar splittings for alanine A3

However, these titration experiments with alanine labeled at position 3 at the lipid head groups do not reflect the influence of glutamic acid ionization in the core helix. By deuterium labeling of the core alanine residues within the helix of GWALP23 E4, it is feasible to
characterize the peptide’s position during protonation/deprotonation at the lipid head groups. By having a full analysis at both the lipid head groups and alpha helix of GWALP23 E4, we can uncover the full effects of protonation/deprotonation on the structure of transmembrane peptides.

I have been successful with creation of GWALP23 E4 with deuterated alanine labels 7/9, 11/13, and 15/17. The E4 peptide has been placed in lipid bilayers of DMPC and DOPC at pH 3 and 8, spectra seen in Figure 10. The quadrupolar splittings (Δvq) are recorded in Table 4 below.

![Figure 10: 2H spectra of GWALP23 E4 with deuterated A750% and A9100% in DMPC and DOPC lipid bilayers at pH 3 and 8](image)

<table>
<thead>
<tr>
<th>Table 4: Quadrupolar Splitting Magnitudes (Δvq) in kHz at β=0°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
</tr>
<tr>
<td>DMPC</td>
</tr>
<tr>
<td>DOPC</td>
</tr>
</tbody>
</table>

The quadrupolar splittings of 10 and 3.5 kHz are maintained in the DMPC sample at both pH 3 and 8. This indicates that there is little to no core helix movement during the titration of
glutamic acid at the estimated midpoint of pH 6.3. The E4 DOPC quadrupolar splittings are also the same; however, this is expected since the samples have not been buffered around the high midpoint of pH 11 in this lipid. This peptide in the DLPC lipid has been measured at the same pH values; however, these spectra are inaccessible due to the COVID-19 emergency. These same experiments have been replicated with peptides labeled at A11 and A13. GALA analysis of these spectra unfortunately was halted due to the abrupt laboratory shut down.
Conclusions

The synthesis and purification process of the GWALP23 E16 and Q16 with labels A15 and A17 were successful, as determined by the MALDI-TOF spectrometry. The peptides were added in bilayers originally made with 80:20 DMPC:DMPG or 80:20 DMPC:DMTAP at pH 6. However, the $^3$P and $^2$H NMR spectra were unresolved, likely indicating that the bilayers were unaligned, or the peptide was not fully integrated into the bilayer. By lowering the percentage of cationic or anionic lipid in the system to 10% or below, a successful bilayer was created as seen in Figures 6 and 7. The CD and Fluorescence experiments were therefore done under the same lipid bilayer in the liposomes, presented in Figures 4 and 5. The GWALP23 E16 and Q16 maintained the desired alpha-helical secondary structure as seen in the shape of the CD spectra. The fluorescence spectra of the two peptides indicates the anchoring of the peptides’ tryptophans near the lipid head groups. It can therefore be concluded that the GWALP23 E16 and Q16 peptides are maintaining ideal secondary structure and bilayer integrity that validates the $^2$H NMR spectra for comparison to the parent GWALP23 characterization.

After the lipid optimization, $^2$H NMR spectra for the 90:10 DMPC:DMPG and 90:10 DMPC:DMTAP were obtained at pH 6 and compared to the 100% DMPC peptide-lipid bilayers of the GWALP23 E16. The quadrupolar splittings of the deuterated E16 peptide in all three types of bilayers (DMPC only, 10% DMTAP, and 10% DMPG) were identical with the A15 at 50% deuteration measuring 21 kHz and A17 at 100% deuteration showing a splitting of 10 kHz (Figure 7). Titration experiments were performed with the same peptide-lipid compositions at pH 3 and 8. Notably, the DMTAP lipid samples produced spectra with reduced noise and better resolution compared to those in DMPG. As seen in Figure 8, the DMTAP samples at pH 3 and 8 maintained the same $^2$H quadrupolar splittings as the DMTAP samples at pH 6. While there are
not clear conclusions as to why there are minimal changes to the spectra, the results suggest a lack of titration or lack of helix response since there are no significant changes in the quadrupolar splittings observed between spectra seen in Figure 7 and Figure 8. Even with the charged lipid environment, the lack of helix response at lower pH supports a lack of titration and points to the experiments measuring a pKa of about 12.5 in the GWALP23 E16 system, even when a positively charged lipid is present.

Due to the successful titration by Matthew McKay of glutamic acid in the GWALP23 system at position 4 in different thickness of lipid bilayers, I attempted to characterize the helical tilt and rotation of the secondary structure during titration. Deuterated alanines were labeled at positions A7/9, A11/13, and A15/17 and placed in DLPC, DOPC, and DMPC at varying pH. The labels for A7/9 in DOPC and DMPC were measured and reported in Table 4. These results are encouraging because they seem to indicate that the core helix maintains a stable orientation when residue E4 titrates. However, the experiments to fulfill a full GALA analysis of the E4 in the three lipid bilayers were halted because of the COVID-19 pandemic.
References


