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VOLTAGE GATING OF A MODEL MEMBRANE SPANNING CHANNEL

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Abstract

Channels in cell membranes are important for intercellular communication and especially for the function of the nervous system in higher vertebrates. These channels consist of proteins made from the 20 common amino acids. Channel proteins are embedded into the lipid bilayer membranes of living cells and function by allowing the specific passage of a positively charged material such as sodium or potassium ions across the membrane in response to an external signal. The external signal is either a chemical signal or a voltage that regulates the opening and closing of channels. In an attempt to understand the voltage-dependent opening of channels (gating), we are investigating model membrane-spanning channels whose properties can be regulated by voltage. Our laboratory has developed the only chemically defined model system for which it is currently possible to investigate the structural basis for the voltage gating response at the molecular level. Our window into the gating process involves deuterium magnetic resonance spectroscopy. We use a small model channel system that we label with deuterium (heavy hydrogen) by specific chemical synthesis and then align in liquid-crystalline arrays of hydrated lipid bilayer membranes. The most novel aspect of this research is the ambitious goal of recording magnetic resonance spectra in the presence of a voltage across a stack of oriented, liquid-crystalline membranes. Experiments toward this goal will be described in our article. Accomplishments to date have prepared the way for the voltage-dependent magnetic resonance experiments. To this end, a series of gated and non-gated (control) channel-forming peptides have been designed, synthesized and incorporated into oriented, hydrated lipid/peptides samples. Spectra that define open and closed channel states have been recorded in the absence of a voltage. An important penultimate step has been the successful replacement of the water of hydration by glycerol in preparation for the voltage-dependent spectroscopy.

Introduction

Biological membranes consisting of proteins and lipids are a necessity for life because they separate the cell from the environment. Within each membrane are numerous channels and pumps, which allow the membrane to behave as a selective permeability barrier. Specific chemicals or physical stimuli can excite the channels in cell membranes. Once excited, the channels allow communication across the membrane. With small changes in the transmembrane voltage, it has been found that ion channels may respond by cycling between closed and open states (1), a process known as voltage gating.

The structural basis for gating is a significant unsolved problem in membrane biology. One source of insight into how ions are transported can be obtained from the study of model systems such as gramicidin A. Naturally, gramicidin is not voltage dependent. However, by changing the amino acid sequence, several gramicidin analogues have been engineered to be voltage-dependent (2). One such gramicidin channel was discovered in 1997 (3) and is the first voltage-dependent homodimeric gramicidin channel (4). In the homo-dimeric channel both gramicidin chains feature the same amino acid sequence. The channels lend simplicity for isotope labeling and magnetic resonance (MR) experiments and offer the first realistic possibility of doing structural experiments with a non-zero voltage across aligned membranes. The work completed to date has involved preliminary studies needed to perform voltage experiments.

Native gramicidin has the following sequence of amino acids: Formyl-VGALAVVVWLWLWLW-ethanolamine. The sequence alternates between D and L chirality where the D-amino acids have been underlined. To achieve voltage gating, the formyl-valine in the first position was dropped, making the peptides fourteen amino acids long, and the second and third positions were modified (Figure 1). The second position, referred to as the trigger position, was kept as formyl-glycine (control) or was dimethylated to formyl-Aib to induce gating (Figure 2). The Aib is abnormal because of its two protruding methyl groups. The third position, which we designate as the sensor position,

was replaced with a deuterium labeled amino acid. Deuterated alanine and valine were chosen because they already appear in the native gA sequence and produce good spectra in oriented samples.

Solid state deuterium MR was used to observe and record the deuterium nuclei using the quadrupole echo sequence (5). Deuterium nuclei produce two signals that are equal in intensity and symmetric about a zero axis. The splitting between the two signals is related to the molecular geometry of the sensor amino acid (6).

Methods

Modified amino acids with either Fmoc or formyl groups were linked together using an Applied Biosystems 431A Peptide Synthesizer. Four analogues of gramicidin A were produced: f-Gly2-L-(Ala3-d4)gA, f-Gly2-L-(Val3-d8)gA, f-Aib2-L-(Ala3-d4)gA, and f-Aib2-L-(Val3-d8)gA. All of the peptides were fourteen amino acids in length with a deuterium labeled amino acid in the third position (Figure 1).

The peptides then need to be oriented and prepared for NMR. Each peptide was mixed with the lipid DMPC in a molar ratio of 4:80 moles. The peptide/lipid mixture was dissolved in a 1mL solution of 95% methanol and 5% deuterium depleted water. The mixture should be applied evenly over 40 glass plates (4.8 x 23 x 0.07 mm) and allowed to dry for 48 hours. When dried, the glass plates are hydrated with deuterium depleted water, stacked together, and sealed in a cuvette. Incubation at 40 C for 72 hours in a heating block allows the peptide molecules to orientate within the lipid bilayer. Once oriented, the samples are ready to be analyzed by solid state deuterium (^2H) NMR. Phosphorous (^{31}P) NMR also is used to monitor the alignment of the lipids.

Results and Discussion

The NMR data and spectra will be grouped and discussed according to whether the peptides contained deuterated alanine or deuterated valine. The first peptides to be discussed are those labeled with deuterated alanine.

The control peptide for these experiments was [f-Gly-L-Ala-d4]gA. The NMR spectra in Figure 3 are shown for two sample orientations, with the peptide long axis either parallel ($\theta=0^\circ$) to the magnetic field or perpendicular ($\theta=90^\circ$). Figure 3a illustrates the signal produced by alanine's backbone deuterium (A). It has a splitting of 208 kHz, and the signal produced by the methyl group (B) has a splitting of 33 kHz. Comparing this to Figure 3b, we find that the same peaks are present with half the splitting, characteristic of solid state NMR. The central peaks are due to randomly oriented material and water.

The other peptide labeled with alanine was [f-Aib-L-Ala-d4]gA. Its two spectra are shown as Figure 4a and 4b. Much like the f-Gly spectra, Figure 4a and 4b both illustrate the backbone

deuterium (A) and with almost the same splitting. The difference is in the methyl peaks (B). Figure 4a shows two methyl peaks with a difference of 6 kHz. If these two peaks are both signals from the methyl group, then they should also appear on Figure 4b but with half the peak separation, and they do. The methyl peaks (B) on Figure 4b have a separation of 3 kHz, half of that in Figure 4a. This implies that the bulky methyl groups on the Aib are causing the gramicidin to acquire two different conformations.

The two peptides that are labeled with deuterated valine are [f-Gly-L-Val-d8]gA and [f-Aib-L-Val-d8]gA. Figure 5a and 5b are the [f-Gly-L-Val-d8]gA spectra. Spectra for deuterated valine differ from alanine in that valine has individual deuterons attached to the beta carbon (B) as well as to the alpha carbon (A). There are also two protruding methyl groups (C), whose signals overlap and are compact in the center of the spectra. For Figure 5a and 5b, the individual deuterons, (A) and (B), are present in both; and characteristically, the splittings in Figure 5b are half of those in Figure 5a. The methyl groups (C) are compact in the center and are not resolved into distinct splittings.

Figure 6a and 6b illustrate the other valine labeled peptide, [f-Aib-L-Val-d8]gA. The single deuterons, (A) and (B), in Figure 6a and 6b are present and have almost the same splittings as those in Figure 5. The difference is in the methyl groups. The methyl peaks (C) in both Figure 6a and 6b are more distinct than those in Figure 5 and have a lot more separation. This reinforces what was said earlier. The f-Aib is definitely acting differently than the f-Gly, and it may be causing a second conformation for the gramicidin channel.

For a voltage experiment to be feasible, the water of hydration needs to be replaced with glycerol. Water has a high conductance and the excess heat will deteriorate the lipid and peptide when a voltage is applied (7). Samples with glycerol should not have this effect. A comparison between a water-hydrated and a glycerol-hydrated sample is shown in Figure 7 using the peptide [f-Aib-L-Ala-d4]gA. The two ^2H NMR spectra are similar. Each spectrum has multiple peaks with similar quadrupolar splittings. Phosphorous (^{31}P) NMR provides information about the lipid orientation. It is evident from Figure 8b that hydration with glycerol does not disrupt the lipid's orientation. In fact, the unoriented peak is much smaller in the glycerol sample. Glycerol is therefore a feasible alternative for hydrating the samples.

This project has completed all of the preliminary work needed prior to the actual voltage experiments. The information gathered has provided valuable insight and a firm foundation from which to continue. The spectra serve as references for comparison when the voltage experiments are initiated and provide information about the relationship between the labeled deuterium's orientation and the corresponding peak position. The NMR spectra also provide data for preliminary interpretations.

Different conformations and behaviors are already visible between the gramicidin analogues. The peptides containing Aib, for example, are showing evidence of at least two conformations. By following the fate of the deuterium signals from these conformations in the presence of transmembrane voltages of increasing magnitude, future researchers will begin to learn the structural basis for voltage gating.

Abbreviations

NMR	Nuclear Magnetic Resonance
Val (V)	Valine
Gly (G)	Glycine
Ala (A)	Alanine
Leu (L)	Leucine
Trp (W)	Tryptophan
Aib	Aminoisobutyric acid
gA	gramicidin A
HPLC	High Performance Liquid Chromatography
DMPC	dimyristoylphosphatidylcholine (14:0)
DOPC	dioleoylphosphatidylcholine (18:1)

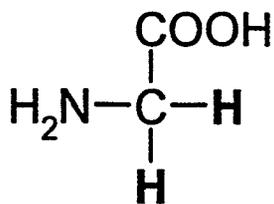
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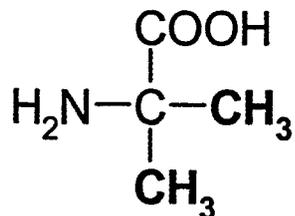
Figure 1: Amino acid sequence for the four synthesized peptides and native gramicin A.

		Sequence															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
#1	a		f-Aib	A	<u>L</u>	A	<u>V</u>	V	<u>V</u>	W	<u>L</u>	W	<u>L</u>	W	<u>L</u>	W	e
	b		f-G	A	<u>L</u>	A	<u>V</u>	V	<u>V</u>	W	<u>L</u>	W	<u>L</u>	W	<u>L</u>	W	e
#2	a		f-Aib	V	<u>L</u>	A	<u>V</u>	V	<u>V</u>	W	<u>L</u>	W	<u>L</u>	W	<u>L</u>	W	e
	b		f-G	V	<u>L</u>	A	<u>V</u>	V	<u>V</u>	W	<u>L</u>	W	<u>L</u>	W	<u>L</u>	W	e
Native gA		f-V	G	A	<u>L</u>	A	<u>V</u>	V	<u>V</u>	W	<u>L</u>	W	<u>L</u>	W	<u>L</u>	W	e

Figure 2

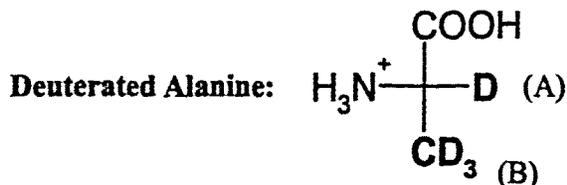
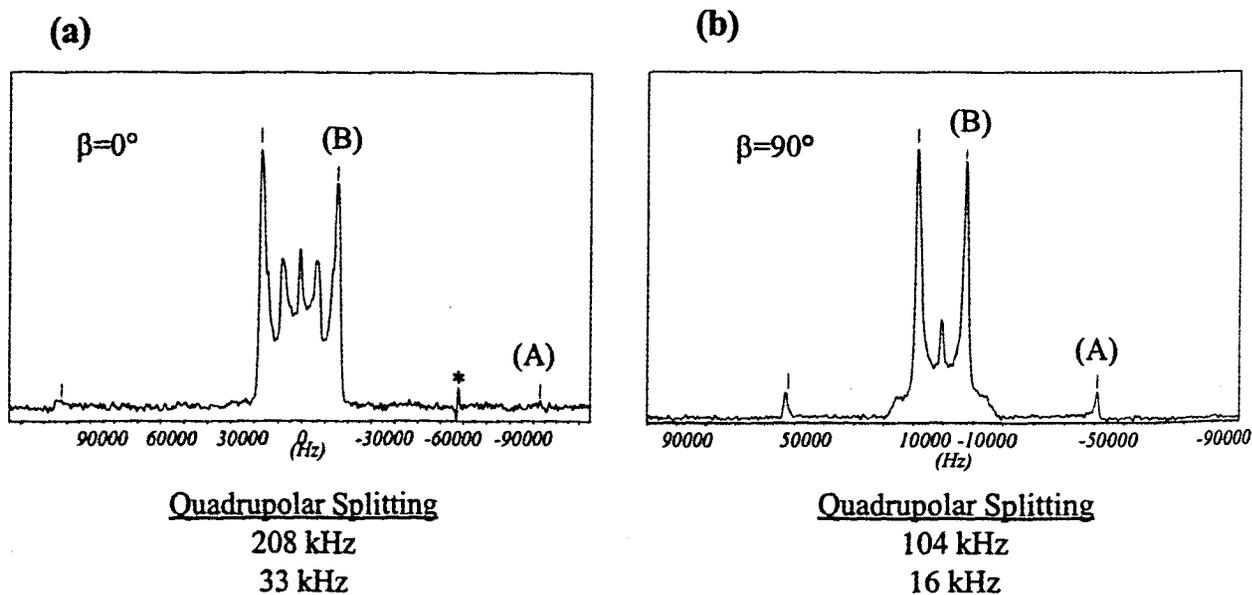


Glycine



Aib (Aminoisobutyric Acid)

Figure 3: [Formyl-Gly-L-Ala-d₄] gA



* Artifact from NMR

Figure 4: [Formyl-Aib-L-Ala-d₄] gA

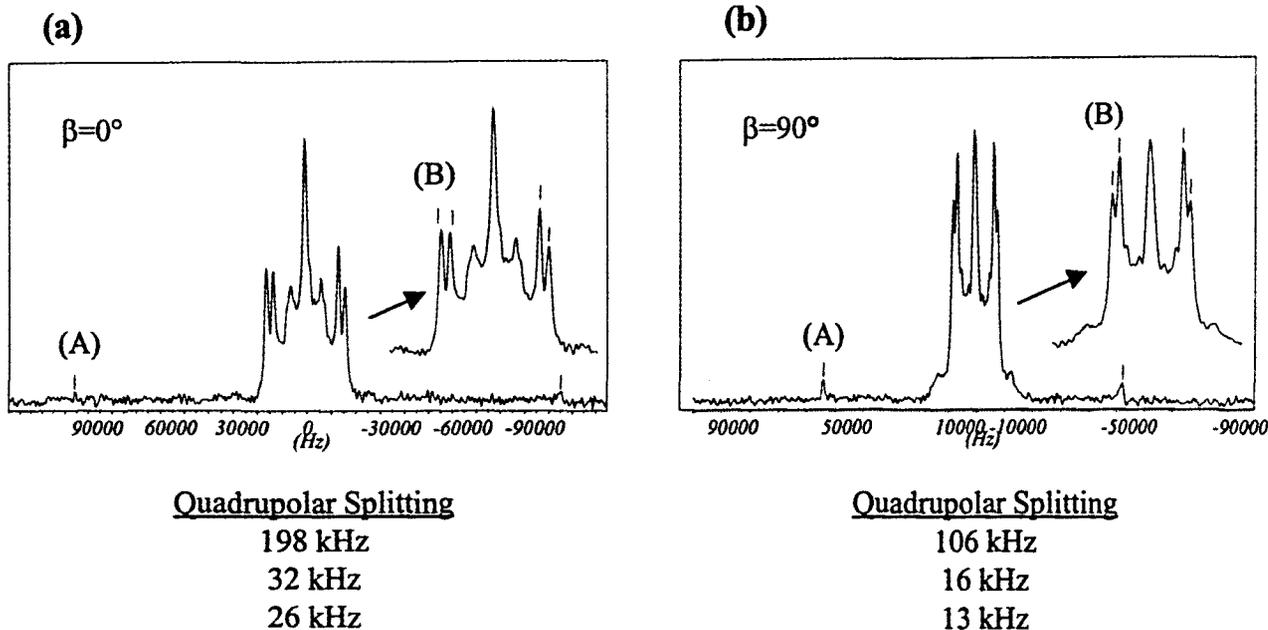


Figure 5: [Formyl-Gly-L-Val-d₃] gA

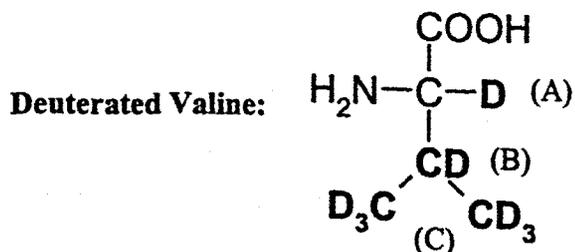
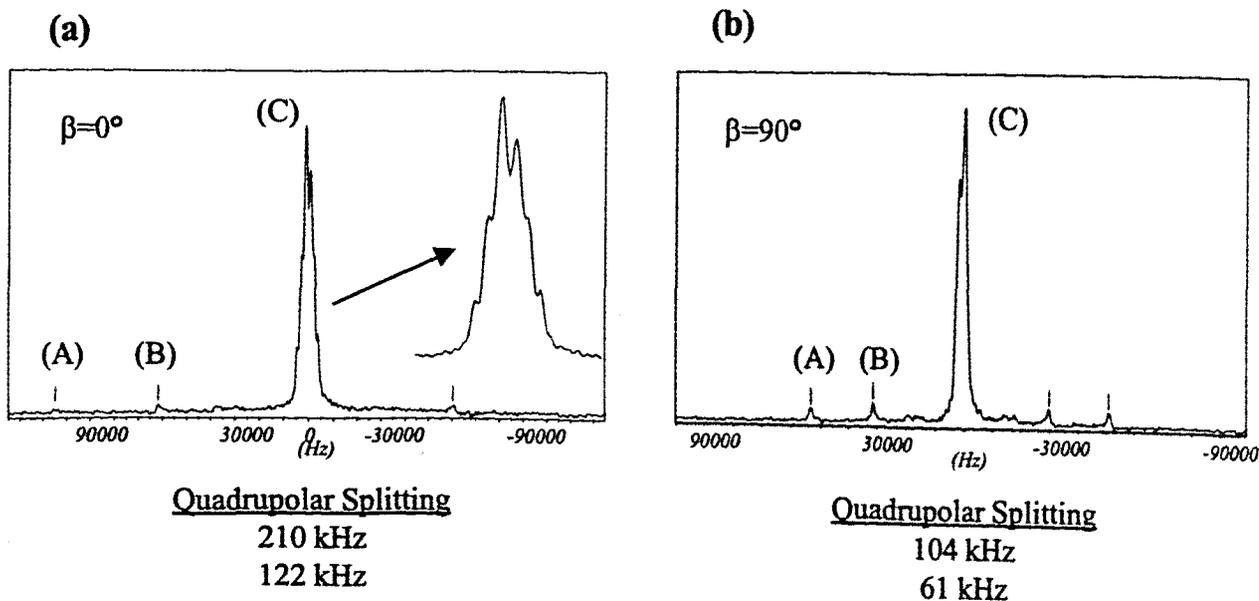
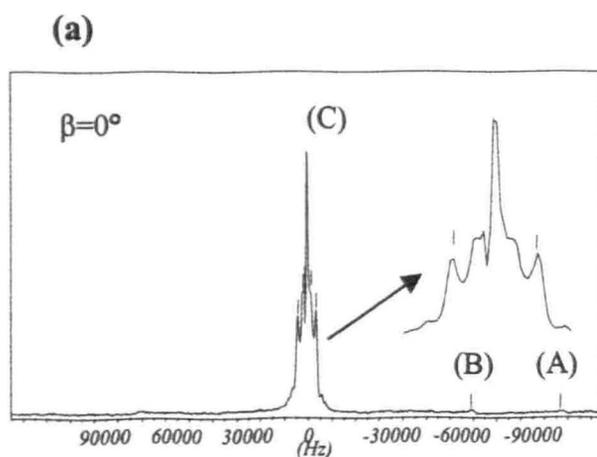
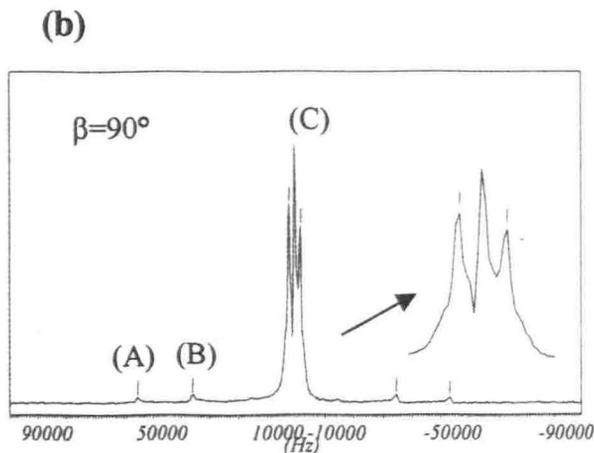


Figure 6: [Formyl-Aib-L-Val-d₃] gA



Quadrupolar Splitting

214 kHz
138 kHz
8 kHz



Quadrupolar Splitting

108 kHz
70 kHz
4 kHz

Faculty comments

Mr. Miller's mentor, Roger Koeppel, gives him extraordinary credit for contributing significantly to the department's research efforts through his research. He says:

While I have advised many talented undergraduate research students through the years, Eric's project is the most innovative that I have seen by an undergraduate student during my career.

He was awarded a nationally competitive Pfizer Fellowship for this work. Eric has presented his exciting results both at Pfizer headquarters in Groton, Connecticut, in October, 2000, and at the annual national meeting of the Biophysical Society in Boston, Massachusetts, in February, 2001. The foundation that Eric has established has given my laboratory the best possible chance to date for understanding the conformational transitions (i.e., structural rearrangements) that accompany voltage-dependent opening ("gating") of a membrane-spanning channel of known structure at the molecular level. (Since we will not insist on keeping him here following his graduation in May, Eric's fine work will be extended by graduate student Sigrid Schmutzer as part of her Ph.D. thesis.)

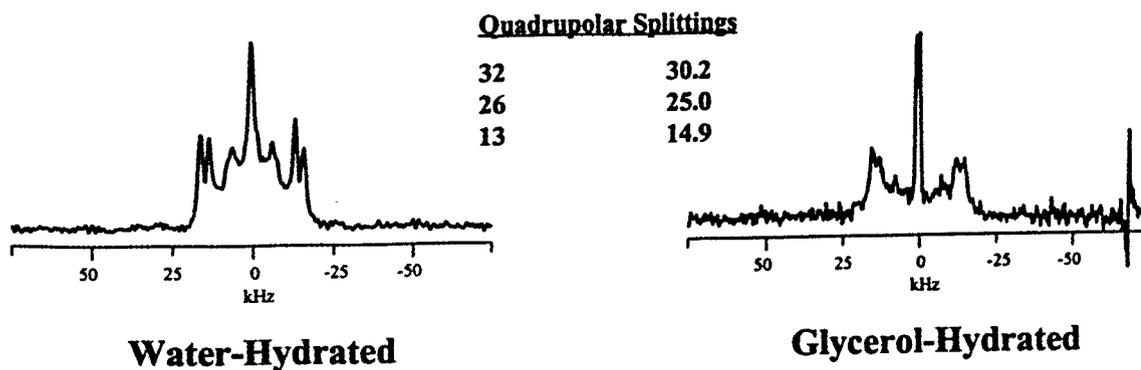
Eric is a delightful, motivated, hard-working and imaginative student. His research project is highly challenging, in both concept and execution. Admittedly, some of the background for Eric's project was in place even before his arrival in our laboratory.



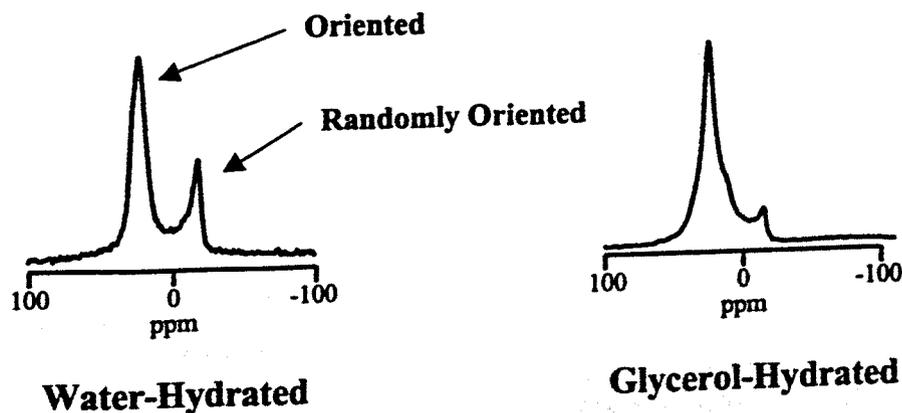
Eric Miller

Figure 7: Comparison of Water-Hydrated versus Glycerol-Hydrated [formyl-Aib-L-Ala-d₄]gA samples using (a) ²H NMR and (b) ³¹P NMR

(a) ²H NMR Spectra



(b) ³¹P NMR Spectra



We had discovered the first gramicidin peptides that gated as homodimers instead of heterodimers, and the observation raised the possibility of characterization by magnetic resonance. But it was Eric who seized this opportunity, developed and synthesized appropriate deuterium-labeled peptides, performed the deuterium magnetic resonance measurements, and turned a phenomenon into an experimental reality. With Eric's very significant progress on sample development and methods development, we are now near the very difficult goal of "voltage-dependent" magnetic resonance experiments (in which an alternating electric field must be established across oriented membranes inside a superconducting magnet).

Our laboratory now occupies a fortunate position. Because of our unique opportunity for characterizing for the first time the molecular details of voltage gating, our laboratory is envied as well as respected within the international biophysics community. I cannot say enough about Eric's contributions to this favorable situation: Without his undergraduate thesis work, we would not be now poised to solve this molecular gating problem. Only Eric's impending graduation (and subsequent graduate study in another prestigious chemistry department) will preclude his seeing the forthcoming voltage-dependent magnetic resonance experiments to their ultimate conclusion. Others will enjoy the benefits of the groundwork that he has prepared and will have the privilege of being coauthors with Eric at the time of publication.

In summary, Eric Miller is imaginative in experimental design and productive in performing experiments in the laboratory. His contributions to our research efforts on voltage-dependent gating of channels have been outstanding.

A member of Mr. Miller's honors committee from the Department of Biological Sciences, C. L. Sagers, is also very complimentary. She says:

I met Eric nearly two years ago in a course I was teaching in evolutionary biology and I now serve as a member of his honor's committee. Eric's honors work focuses on understanding voltage dependence of gramicidin A channels. I now find this project especially engaging (a credit to Eric's teaching skills) even though it is far afield of my research specialty. From my view, Eric is addressing in his undergraduate research a series of weighty questions. The Koeppel lab has found that gramicidin channels may be engineered to be voltage-dependent, and that these may be used as a tool to understand channel gating in non-zero voltage. Eric has made remarkable progress in characterizing these phenomena and has presented a draft of the results to his committee. Through this project, Eric has honed his laboratory and analytical skills, and has proven to be talented as a writer. His successes in his undergraduate research suggest that

Eric has the potential to become an exceptional young scientist.

Beyond his academic accomplishments, Eric is one of the more mature and poised students that I have met on this campus. Interacting with him is always a pleasure and whether the discussion centers on interpreting NMR spectra or the behaviors of Labrador Retrievers, I usually learn a little something.

Donald Bobbitt, chemistry professor and Associate Dean of Fulbright College, also worked with Mr. Miller and shares the views they expressed. He says:

I am writing to offer my strongest recommendation for Mr. Eric Miller in support of his manuscript under consideration for publication in *Inquiry*. I am familiar with Eric's credentials and capabilities through the classroom, and from having served as the Director of the Department of Chemistry and Biochemistry's NSF-supported, 2000 Research Experience for Undergraduates site project. Eric was a participant in that program but raised his own funds through his successful competition for a prestigious Pfizer Undergraduate Research Fellowship.

Eric is a very gifted student; his intellectual abilities clearly place him in the top one percent of students I have observed over the past fifteen years. What truly makes Eric special is that he combines these substantial intellectual capabilities with an exceptional work ethic. He is quick to accept challenges and will work hard to meet them. Further, Eric has a caring and easygoing personality; he is a pleasure to know and work with. I am positive he will be extremely successful at whatever he chooses to do after his graduate work.

As mentioned, during the summer of 2000 Eric participated in the Department's summer research program. Eric, working with Professor Koeppel, accepted a very significant and challenging research problem. At the conclusion of the summer, each student had to write up their results in a journal format, and present their findings in a departmental seminar. Eric's performance on both of these tasks was exceptional. He demonstrated a profound understanding of the problem, and was able to produce meaningful results in only eight weeks. His presentation was clear and focused. I would rate his performance as being similar to that expected of a second year graduate student rather than that of an undergraduate researcher. His research has progressed exceptionally well since that point and I am positive his work will be an important and original contribution to the journal.