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Studies of Tryptophans in Membrane- Spanning "WALP" Peptides by Deuterium NMR Spectroscopy

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STUDIES OF TRYPTOPHANS IN MEMBRANE-SPANNING "WALP" PEPTIDES BY DEUTERIUM NMR SPECTROSCOPY

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Abstract.

WALP peptides of sequence acetyl-Gly-Trp-Trp-(Leu-Ala)$_n$-Trp-Trp-Ala-ethanolamine insert into lipid bilayers as membrane-spanning a-helices and modulate the lipid phase behavior as functions of n and the lipid acyl chain length. A key feature of the WALP peptides is the positioning of tryptophan (Trp) indole rings at the membrane/water interface. For the examples WALP19 with n = 6.5 and WALP23 with n = 8.5, we have labeled individual indoles with deuterium and incorporated the labeled peptides in oriented, hydrated bilayers of Dimyristoyl-phosphatidylcholine (DMPC). Deuterium NMR spectra from these samples show sharp resonances when the membrane normal is aligned either parallel (β = 0°) or perpendicular (β = 90°) to the magnetic field. The factor of two reduction in the deuterium quadrupolar splittings when β is changed from 0° to 90° indicates rapid molecular rotation about an axis parallel to the membrane normal. The magnitudes and ring assignments of the quadrupolar splittings vary with the position of an indole ring in a WALP sequence and indicate different average ring orientations for different locations in the sequence.

Nicole Reed and Roger Koeppe

WALP peptides of sequence acetyl-Gly-Trp-Trp-(Leu-Ala)$_n$-Leu-Trp-Trp-Ala-ethanolamine insert into lipid bilayers as membrane-spanning *a*-helices and modulate the lipid phase behavior as functions of *n* and the lipid acyl chain length. A key feature of the WALP peptides is the positioning of tryptophan (Trp) indole rings at the membrane/water interface. For the examples WALP19 with $n = 6$ and WALP23 with $n = 8$, we have labeled individual indoles with deuterium and incorporated the labeled peptides into oriented, hydrated bilayers of Dimyristoylphosphatidylcholine (DMPC). Deuterium magnetic resonance (MR) spectra from WALP19 samples show sharp resonances when the membrane normal is aligned either parallel (♣ = 0°) or perpendicular (ß = 90°) to the magnetic field. The factor of two reduction in the deuterium quadrupolar splittings when ß is changed from 0° to 90° indicates good transmembrane alignment and rapid molecular rotation about an axis parallel to the membrane normal. The magnitudes and ring assignments of the quadrupolar splittings vary with the position of an indole ring in WALP19 and suggest different average ring orientations for different locations in the sequence. With WALP23, a ^{31}P MR spectrum indicates that the DMPC lipids are well aligned (as was also true for WALP19). Nevertheless, the WALP23 deuterium MR spectra are of poorer quality than those observed with WALP19, perhaps because the hydrophobic length of WALP23 is too long for good matching with DMPC. This latter finding only re-emphasizes the high quality of the WALP19/DMPC results.

Introduction

Cell membranes are composed of lipids and numerous proteins that are essential in various biological processes. Membrane proteins catalyze chemical reactions, mediate the flow of nutrients and wastes, and relay information from outside the cell to its interior components Voet, and Pratt)" (Voet, Voet, and Pratt). Interactions between membrane proteins and lipids can affect both the conformation of the protein and the structure of the membrane (Gil, *et al.*; Marsh and Horváth; White and Wimley). Certain amino acids, components of proteins, have particular roles in carrying out protein functions. Interestingly, tryptophans (Figure 1) (and also other aromatic amino acids) have been found to act as 'membrane interface anchors' that hold transmembrane protein segments in a fixed orientation Chang, and Stevens)" (Schiffer, Chang, and Stevens). Although there has been an increasing interest in these anchoring residues, the exact mechanism of anchoring is still unclear. The purpose of this study was to determine the orientation of the tryptophan indole rings at the membrane/water interface in order to gain greater insight into this anchoring mechanism. This information will help to define the boundary between transmembrane segments and exterior domains of membrane proteins, and will contribute to our understanding of membrane protein assembly and topology Planque, (de Planque, *et al.*).

Model systems of synthetic peptides and model membranes are often used to study the interactions between membrane proteins and lipids. The peptides used in this experiment are uncharged and a-helical, designed to mimic the membrane-spanning helices often found in membrane proteins (Figure 2). These "WALP" peptides contain two tryptophan anchors at each end, flanking a central hydrophobic region of alternating alanine and leucine residues, for example acetyl-Gly-Trp-Trp-(Leu-Ala)$_5$-Trp-Trp-Ala-ethanolamine. WALP peptides have been found to modulate the phase behavior of membrane lipids (Killian, *et al.*). The model membranes are lipid bilayers of dimyristoyl- phosphatidylcholine (DMPC), a lipid with 14-carbon tails without double bonds. Oriented deuterium MR samples of WALP peptides with deuterium-labeled tryptophans in DMPC were measured to determine the orientations of the indole rings.

Experimental Methods

Deuterium labeled L-tryptophan, with five hydrogens on the indole ring replaced by deuterium (Figure 1), was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). The amino group on tryptophan was protected with 9-Fluorenyl-methylcarbonyl (Fmoc) from N-(9-fluorenyl- methoxy-carbonyloxy)succinimide. The synthesis was tested with an analysis technique called thin layer chromatography. A ninhydrin assay, which tests for free amino groups, confirmed the protection. After recrystallization, product quality was tested by ^{1}H-MR analysis and UV-visible spectroscopy. The deuterium labeled Fmoc-tryptophan was used in solid phase peptide synthesis reactions of four different 19-amino-acid WALP19 peptides and two different 23-amino-acid WALP23 peptides, with one deuterium labeled tryptophan in each peptide (Figure 3). Synthesis was performed on an Applied Biosystems 431A Peptide Synthesizer with an extended coupling time for the deuterium labeled tryptophan (Killian, *et al.*; Greathouse, *et al.*). Peptides were then cleaved with ethanolamine in dichloromethane. Peptide purity was confirmed by high-pressure liquid chromatography and mass spectroscopy. To make the oriented deuterium magnetic resonance samples, each peptide was added to DMPC in a 1:20 peptide to lipid ratio. The solution of peptide and lipid was applied to about 40 small glass plates that were dried and stacked in a sample cuvette, then hydrated with deuterium-depleted water. The cuvette was then sealed to keep the lipids from drying out. The sample was then incubated at 40° C for a week, which allows them to form liquid crystalline lipid bilayers on the glass plates (Figure 4). Phosphorus-31 magnetic resonance (^{31}P-MR) was used to test for proper orientation of the lipid bilayers. Deuterium magnetic resonance spectra were then recorded for each sample with the normal of the plates at both 08° and 90° with respect to the magnetic field.

Results and Discussion

The ^{31}P magnetic resonance spectra indicate the extent of alignment of the lipid head groups. The peak at 30 ppm for WALP23 and 27 ppm for WALP19 (Figure 5) is the signal from the lipid head groups that are properly aligned in the lipid bilayer. Lipids in alternative alignments give the signal for the peaks at –15 and –13.9 ppm. The quality of lipid bilayer alignment is determined by the ratio of the peak sizes. These ^{31}P-MR results show that both the WALP19 peptide and WALP23 peptide samples contain well-oriented lipid bilayers.

In deuterium magnetic resonance, characteristics of deuterium nuclei are measured by subjecting the nuclei to a magnetic field and recording the signal at which radio frequency (RF) waves are absorbed. Two peaks are observed separated by a splitting ($\Delta\nu$) that is affected by the orientation and motion of the carbon-deuterium bond. For peptides that rotate rapidly about the helix axis, the deuterium magnetic resonance splittings are related to the orientation of the labeled indole ring by the following equation Taylor, and Koeppe)" (Killian, Taylor, and Koeppe):

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

(Equation 1). Here, *theta* is the angle between the carbon-deuterium bond and the helix axis, and ß is the angle between the magnetic field and the membrane normal, either 0° or 90° (Figure 6). The static coupling constant (e^2qQ/h) is approximately 180 kHz for an aromatic carbon-deuterium bond.

For the WALP19 peptides, reasonably sharp peaks obtained at ß= 0°and ß= 90°indicate that the peptides are well oriented in the lipid bilayer (Figure 7). The tilt of the helix axis (which contributes to the angle b) and the structure of the peptide (which could affect the angle *theta*) must be consistent to give a clear spectrum. Furthermore, the sharp peaks when ß = 90°indicate rapid rotational motion about the helix axis that allows the signals at various rotational positions to be averaged to a single signal, called motional averaging.

Each WALP19 peptide gives a unique spectrum, indicating that the indole rings of the four tryptophans in the peptide have four different orientations. The distance between symmetrical peaks in these spectra, or splittings ($\Delta\nu$), are compiled in Table 1. Four symmetrical peaks are expected from the five deuterons on each labeled tryptophan, because positions 4 and 7 are geometrically equivalent (Figure 1). The expected result is obtained for WALP19 with tryptophan labeled at position 17. WALP19 with tryptophan labeled at position 2 shows four peaks at ß = 90° but only three peaks are detected at ß = 0°. For WALP19 peptides labeled at positions 3 or 18, only three peaks can be discerned. The deuterium MR splittings are reduced by a factor of two when the sample is measured at ß = 90°, compared to their values at ß = 0°(Table 1). This result is predicted by equation 1, and further indicates that the WALP19 peptides rotate rapidly about their helix axis and display motional averaging (Killian, Taylor, and Koeppe).

From the deuterium MR splittings, it may be possible to calculate possible orientations for the indole rings on the labeled tryptophans. This approach has been successfully applied to the membrane channel gramicidin A (Koeppe, Killian, and Greathouse). However, assignment of each signal in these spectra to a particular deuterium label on the ring (Figure 1) must be accomplished first. Future experiments will address this issue by using tryptophan indoles labeled with deuterium at only one position, which requires a more difficult synthesis. Results from those experiments should facilitate the assigning of peaks to the correct positions on the indole ring. The deuterium MR spectra for the WALP23 peptide did not contain sharp peaks (Figure 8). Because the ^{31}P-MR spectrum for this sample indicates that the lipid bilayer is oriented properly, the poor quality of the deuterium MR spectra suggests that the WALP23 peptides may be too long to be stable in a DMPC bilayer. (This behavior of WALP23 contrasts with the very nice deuterium MR spectra obtained using WALP19 (Figure 7) or the shorter WALP16 der Wel, (van der Wel, *et al.*)). The stability of peptide/bilayer systems depends on matching between the hydrophobic acyl chain length of the lipids and the length of the hydrophobic transmembrane domain of the peptide. If the peptide's transmembrane domain is longer than the bilayer thickness, there is an energetically unfavorable exposure of hydrophobic surfaces to the aqueous environment outside of the bilayer. This mismatch can force peptides that are too long to be excluded from the lipid bilayer der Wel, (van der Wel, *et al.*), which may be the case with the WALP23 sample. Further deuterium MR studies on WALP23 samples may require lipids with longer acyl chains. However, bilayers with longer lipids will not maintain their fluidity unless the lipid acyl chains contain double bonds. A possible model membrane system for WALP23 would be dioleoylphosphatidylcholine (DOPC), a lipid with 18-carbon tails with one double bond.

This project has shown that labeled WALP19 peptides orient well in DMPC bilayers and can be measured by deuterium magnetic resonance. The two-fold reduction in splittings between ß= 0°and ß = 90°demonstrates that the peptides are rotating rapidly about the helix axis. Different tryptophans in the peptides yield different spectra, indicating that their indole rings have different orientations. Future experiments using this approach can yield useful information about the particular orientations of the labeled tryptophan indole rings at the membrane/water interface.

Abbreviations

DMPC	dimyristoylphosphatidylcholine
DOPC	dioleoylphosphatidylcholine
Leu (L)	leucine
Ala (A)	alanine
Trp (W)	tryptophan
Gly (G)	glycine
ea	ethanolamine
MR	magnetic resonance
UV	ultraviolet

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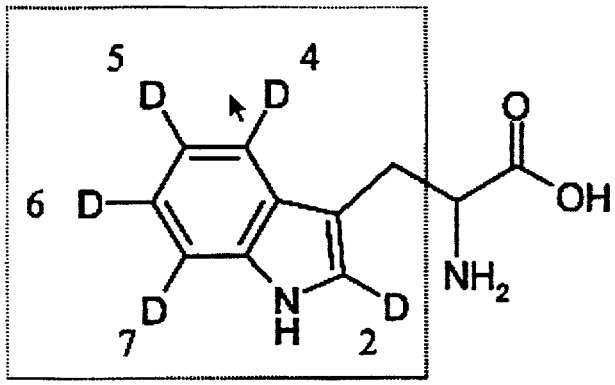


Figure 1. Tryptophan amino acid (the indole ring is boxed). Here deuterium has replaced hydrogen at positions 2, 4, 5, 6, and 7.

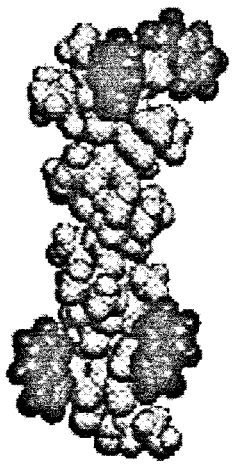


Figure 2. Molecular model of Walp19 peptide obtained using Insight II v. 908.0 (Molecular Simulations Inc., San diego, CA). Tryptophan indole rings are dark gray and NH groups are black

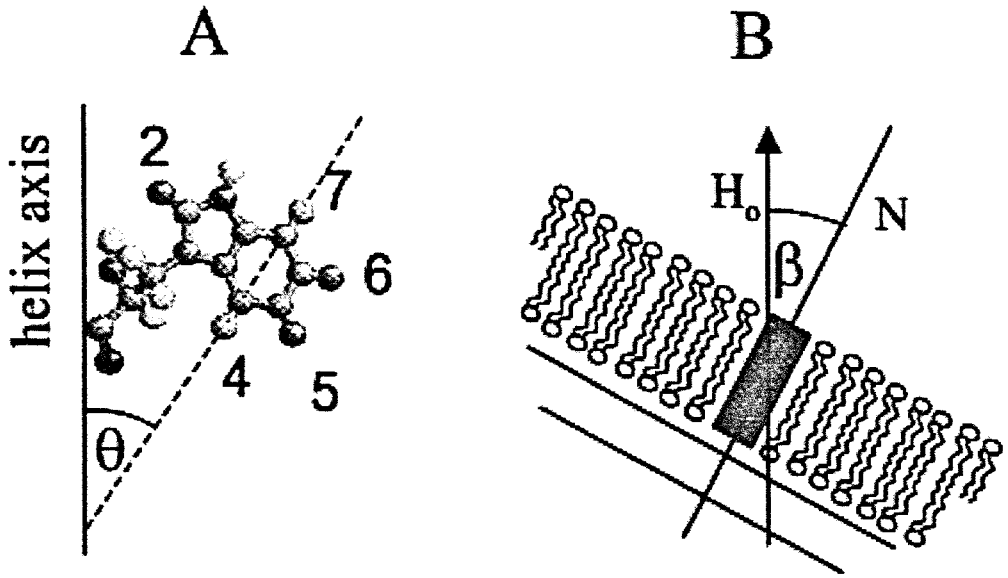


Figure 6. Illustrations of the variables in equation 1. The angle theta is between the axis of the WALP peptide helix and the carbon-deuterium bond (A). The angle ß is between the membrane normal N and the magnetic field direction H_0(B).

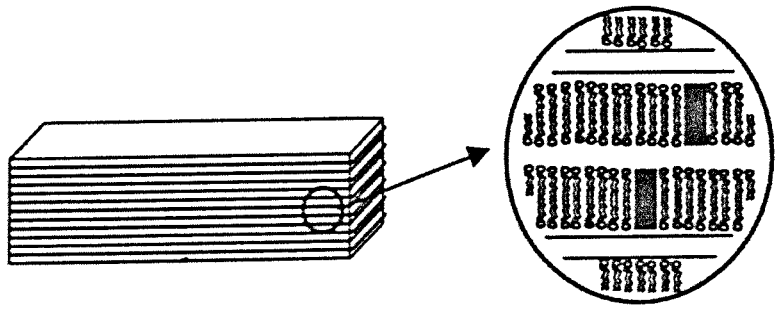
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Ac-G	W*	W	L	A	L	A	L	A	L	A	L	A	L	A	L	W	W	A-ea				
Ac-G	W	W*	L	A	L	A	L	A	L	A	L	A	L	A	L	W	W	A-ea				
Ac-G	W	W	L	A	L	A	L	A	L	A	L	A	L	A	L	W*	W	A-ea				
Ac-G	W	W	L	A	L	A	L	A	L	A	L	A	L	A	L	W	W*	A-ea				
Ac-G	W*	W	L	A	L	A	L	A	L	A	L	A	L	A	L	A	L	A	L	W	W	A-ea
Ac-G	W	W	L	A	L	A	L	A	L	A	L	A	L	A	L	A	L	A	L	W	W*	A-ea

Splittings (Δν) for Deuterium-Labeled WALP19 Peptides		
Labeled Residue #	β = 0°	β = 90°
2	81 61 21	75 41 28 10
3	155 85 77	78 41 26
17	77 62 44 11	38 28 23 6
18	63 22 11	32 10 7

Figure 3 (above). Sequence of WALP peptides. Abbreviations: Ac-Gly = acetyl-glycine, W = tryptophan, W denotes deuterium-labeled tryptophan, L = leucine, A = alanine, and ea = ethanolamine.*

Table 1 (left). Deuterium MR Splittings for WALP19 peptides. Note the factor of two reduction between splitting at ß = 0° and those at ß = 90°

Figure 4 (below). Liquid crystalline lipid bilayers form on the stacked glass plates with the WALP peptides (represented by shaded rectangles) included.



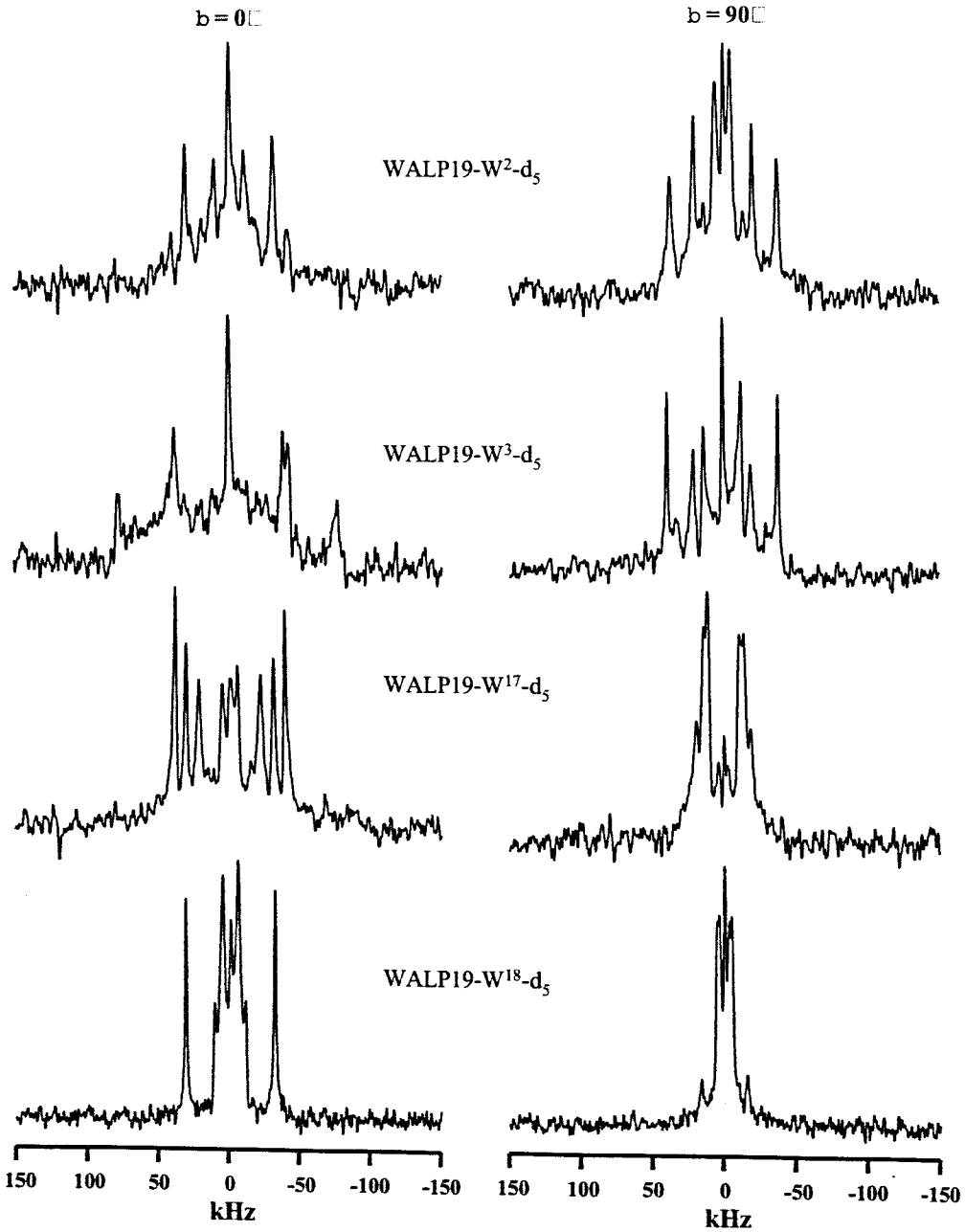


Figure 7. 2H-NMR spectra for tryptophans in WALP23 at ß = 0° (left) and ß = 90°.

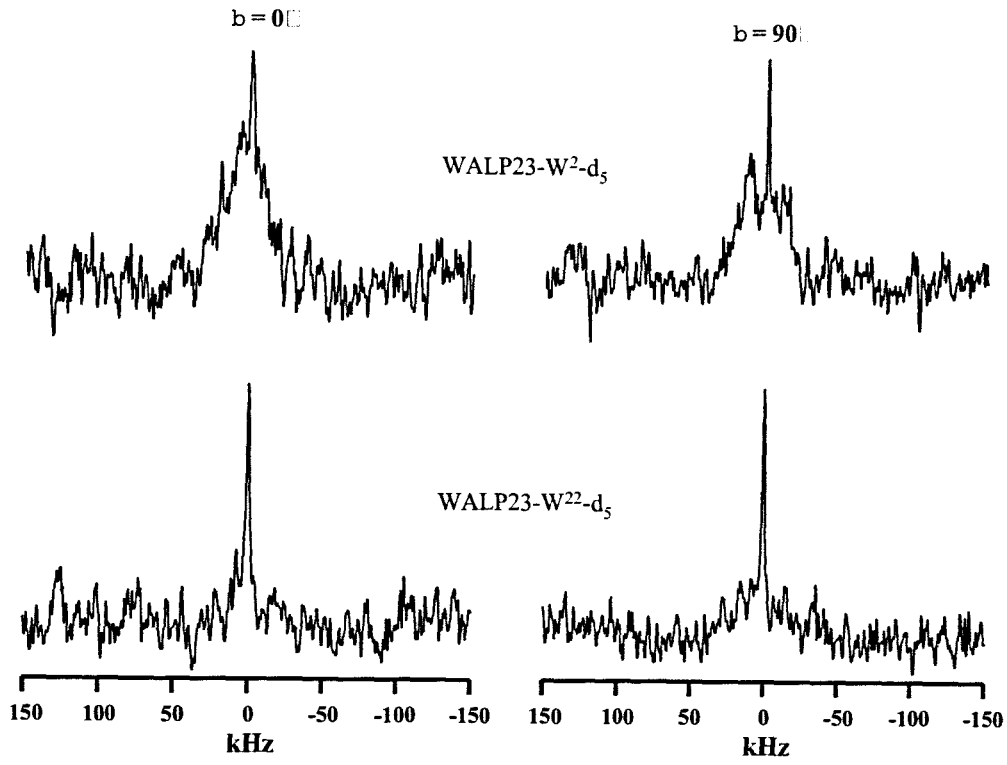


Figure 8. ^{2}H-NMR spectra for selected tryptophans in WALP23 at ß = 0° and ß = 90°.

Faculty Comments:

University Professor **Roger E. Koeppe II**, Ms. Reed's mentor, described her work as follows:

> Nicole is an outstanding student who has done a superb job on her project. She has used an innovative approach to apply the methods or solid-phase peptide synthesis and solid-state deuterium magnetic resonance spectroscopy to investigate the interactions between proteins and lipids in biological membranes. The approach and Nicole's results have important implications for our understanding of the chemistry of cell membranes and intercellular trafficking.
>
> Nicole is a student leader with a remarkable breadth of interests. She has served for two years as President of the local student affiliate section of the American Chemical Society. Under her leadership, this group has been active in numerous public service projects, including training events for Boy Scouts, and for elementary school students at Springfest each April and at the Northwest Arkansas Mall during National Chemistry Week each November. Nicole has been awarded and currently holds the prestigious national Morris Udall Fellowship for studies in environmental policy. For her career plans, she will apply her diverse expertise and leadership abilities to combine the fields of chemistry and law into a coherent whole. Approaches to complex problems such as public policy require such interdisciplinary efforts.
>
> The quality of Nicole's research project was evident when she presented her results at the national meeting of the Biophysical Society in New Orleans in February of this year. At this meeting, she defended her research alongside graduate students and postdoctoral fellows who are doing similar or competing experiments to hers in esteemed laboratories around the world. In this setting and as a "mere" undergraduate student, she held her ground well and was a marvelous representative of the University of Arkansas! One version of her abstract has been published in the

Biophysical Journal (volume 78, page 413A), and we will submit a final comprehensive account of her research to a peer-reviewed national journal such as *Biochemistry* (published by the American Chemical Society).

D. Mack Ivey, Associate Professor in the Department of Biological Sciences, commented:

> I have had Nicole in class, and I serve on her Honors thesis committee. Her academic skills are top-notch, and her thesis work is excellent. She is investigating the determinants of transmembrane helix formation and stability in membrane proteins. This is a worthy endeavor. Membrane protein structure is a very active field of research, but the traditional methods used to study protein structure are difficult and often unproductive when applied to extremely hydrophobic proteins. Nicole's approach involves the use of deuterium NMR, synthetic peptides, and artificial membrane bilayers in a spectroscopic analysis of structure. This is a highly original and creative approach, which is already producing interesting and readily interpretable results. I predict that this research will have a great impact, and I strongly recommend it for inclusion in the undergraduate research journal.

Suzanne McCray, Director of the Office of Post-Graduate Fellowships and Associate Director of Honors Studies, describes Ms. Reed and her research project in glowing terms:

> Recommending Nicole Reed for this recognition is a pleasure. Nicole Reed is a Sturgis Fellow who has greatly exceeded our very high original expectations. She is a very motivated student, who commits completely to each project. She has served as an Honors Council Student Representative and as President of the Association of Honors Students. She is committed to undergraduate research and created a student organization with the goal of interdisciplinary student research. The group has its own web page. They meet monthly with faculty. It has been a wonderful experience for everyone concerned–faculty and students alike. I count Nicole Reed among the top five students I have known in my ten years of working with high ability students at the University of Arkansas
>
> Last year, Ms. Reed received the nationally competitive ***Morris Udall Scholarship,*** recognizing outstanding achievement in environmental studies. This fall she was awarded a *Science Information Liaison Office Undergraduate Research Fellowship* for the project currently under consideration. When I asked her to explain the project to me in a way that a non-science oriented person could understand, she basically said she wanted to learn more about the interactions of proteins embedded in cell membranes with the lipids that are the main building blocks of those membranes: "Proteins that are embedded in the cell membrane carry out many functions essential for life," she wrote in an enthusiastic e-mail describing her project. "For example, the reactions that generate the main energy source for all organisms are carried out in membrane proteins. Scientists are trying to learn more about how proteins fold into the membrane, how they interact with the membrane, how they move in the membrane, etc. My projects looks at the edges of transmembrane (membrane-spanning) peptides to find out how they are 'anchored' in the membrane -- what mechanism is responsible for keeping these proteins stretched out across the membrane rather than '"lobbed' up inside the membrane or coming out of the membrane altogether? It has been discovered that tryptophan (amino acid) residues anchor pep/ides/protein segments in the membrane, but the mechanism that allows them to act as membrane anchors is not known. In this project, I am trying to find the orientation of the tryptophan side chains (indole rings), which will help answer the question of how they work as membrane anchors." As I understand it, knowing how the membrane anchors will impact on other studies in the future including those in biomedical research.

Ms. Reed has been the President of the campus American Chemical Society for two years. She was elected in part due to her research interests and because of her ability to make these interests appealing to a wide audience. In celebration of National Chemistry Week last fall, she coordinated a mall event for youngsters, which required several months of planning. The event involved demonstrations and hands-on chemistry reactions geared toward children, but was also popular with their parents. She wanted to attract these children to science and to spark their interest in learning more about their world and its connection to chemistry. The event was an enormous success. Ms. Reed did an excellent job of describing the events and its impact on those involved when she was interviewed at length by KUAF. The national American Chemical Society included a report and a photo of the event in their national publication. She is our undergraduate Stephen J. Gould, bringing science to the masses.

As you can tell from the description of her project, Ms. Reed is majoring in biochemistry, but she will attend **Stanford Law School.** Her focus will be environmental law. Five thousand undergraduates applied to the Stanford's law program; 180 were accepted. Nicole was admitted early, having scored a 171 on the LSAT (a score of 180 is perfect; 163 was the highest mark I had previously seen). Stanford awarded her a full, renewable fellowship to cover her expenses. She will no doubt thrive in law school both academically and personally.