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Ultraviolet resonance Raman spectroscopy of a β-sheet peptide: a model for membrane protein folding

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The partitioning of a hydrophobic hexapeptide, *N*-acetyl-tryptophan-pentaleucine (AcWL5), into self-associated β-sheets within a vesicle membrane was studied as a model for integral membrane protein folding and insertion *via* vibrational and electronic spectroscopy. Ultraviolet resonance Raman spectroscopy allows selective examination of the structures of amino acid side chains and the peptide backbone and provides information about local environment and molecular conformation. The secondary structure of AcWL5 within a vesicle membrane was investigated using 207.5-nm excitation and found to consist of β-sheets, in agreement with previous studies. The β-sheet peptide shows enhanced Raman scattering cross-sections for all amide modes as well as extensive hydrogen-bonding networks. Tryptophan vibrational structure was probed using 230-nm excitation. Increases in Raman cross-sections of tryptophan modes W1, W3, W7, W10, W16, W17, and W18 of membrane-incorporated AcWL5 are primarily attributed to greater resonance enhancement with the B_b electronic transition. The W17 mode, however, undergoes a much greater enhancement than is expected for a simple resonance effect, and this observation is discussed in terms of hydrogen bonding of the indole ring in a hydrophobic environment. The observed tryptophan mode frequencies and intensities overall support a hydrophobic environment for the indole ring within a vesicle, and these results have implications for the location of tryptophan in membrane protein systems.

Keywords: UV resonance Raman spectroscopy; membrane peptide; protein folding

Introduction

The study of membrane proteins is relevant to many fields, including medicine and drug development. However, these protein systems are difficult to study because of challenging sample requirements and limitations of available methodology for structural characterization.[1] Small peptide models for membrane proteins provide opportunities to gain insight into these large systems.[1] One such model, *N*-acetyl-tryptophan-pentaleucine (AcWL5), has been studied as a model for extended β-sheet formation within lipid bilayers.[2] AcWL5, a highly hydrophobic peptide, has been shown to partition into a synthetic vesicle and self-associate to form antiparallel β-sheets.[2–6] The structure of these β-sheets has been examined using techniques such as circular dichroism[2] and neutron diffraction.[3] Few experimental studies, however, have analyzed the role of tryptophan in the stabilization and localization of AcWL5 in the membrane.

Tryptophan residues in transmembrane proteins are often located at the bilayer-water interface[7] and may serve as molecular anchors to the lipid bilayer due to amphipathic character of the aromatic ring, hydrogen bonding, dipolar interactions, cation–π interactions, or steric structural support.[8] Simulations on the monomer have suggested that hydrogen bonding between the indole nitrogen and membrane head groups stabilizes the AcWL5 peptide–membrane interactions on the 70-ns timescale.[9] A recent neutron diffraction study rules out a totally symmetric structure, and suggests that tryptophan may exist in two different environments due to the heterogeneity of the system.[3] Elucidation of the structure and intermolecular interactions between tryptophan and the lipid bilayer within this membrane-bound β-sheet may provide insight into the mechanism of peptide folding into a vesicle bilayer.

Ultraviolet resonance Raman (UVRR) spectroscopy is a powerful technique that allows structural investigation of biological systems. UVRR spectra provide information about local environment, molecular conformation, and chromophore dynamics. Resonance Raman spectroscopy in the deep ultraviolet (UV) region (<210 nm) selectively probes amide vibrational modes, which are sensitive to backbone conformation.[10] Deep UVRR permits secondary structure determination, and at least two groups have reported basis spectra for the secondary structure components of soluble proteins.[10,11] Longer excitation wavelengths (215–240 nm) probe structure and dynamics of residues.[12,13] Empirical relationships between UVRR wavenumbers/intensities and local environment/structure of aromatic side chains have been reported.[14–17] These relationships have been employed, for example, to explain the role of tyrosine in peptide–membrane interactions involving angiotensin.[18,19] This wealth of information makes UVRR ideally suited for the study of secondary structure and direct characterization of the tryptophan microenvironment of AcWL5 in solution and folded in small unilamellar vesicles (SUVs).

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Materials and Methods

Materials

AcWL5 was purchased from GenScript (Piscataway, NJ) and used as received (95% purity). All other chemicals were purchased from Fisher Scientific (Hampton, NH) and used as received. Stock peptide solutions were prepared by adding AcWL5 to deionized water to the level of saturation (~100 μM). Unless otherwise noted, the buffer used was 20-mM potassium phosphate (pH 7.3) with 50-mM sodium perchlorate. Aqueous L-tryptophan solutions were prepared by adding an aliquot of stock 1-mM L-tryptophan in water to buffer. Peptide solutions in buffer were prepared by adding an aliquot of stock peptide solution to buffer. Peptide solutions in lipid were prepared by adding an aliquot of stock peptide solution to a solution of SUVs of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids, Alabaster, AL) in buffer. SUVs with diameters of <50 nm were prepared as described earlier *via* sonication and filtration.[20] All solutions were allowed to equilibrate at 37 °C for >4 h before measurement.

Instrumentation

The Raman instrumentation has been described in detail elsewhere.[20] A tunable Ti : Sapphire laser (Photonics Industries, Bohemia, NY) pumped by a frequency-doubled Nd : YLF laser was used in the current experiments. The 1-kHz, >1-Watt, fundamental output beam was collimated into a lithium triborate (LBO) crystal for second harmonic generation. The resulting beam was then passed through a β-barium borate (BBO) crystal to produce >8 (3) mW of the 230 (207.5) nm UV excitation beam. The UV beam was focused to a spot size of ~230 μm × ~75 μm at the sample. The power at the sample was ~3 (1) mW. Scattered light was collected in a ~135° backscattering geometry and focused onto the entrance slit of a prism-based prefilter. This prefilter has been described elsewhere, and allows for acquisition of UVRR spectra to within 200 cm^{-1} of the Rayleigh line.[21] Raman scattered light was dispersed in a 0.5-m spectrograph (JY Horiba, Spex 500M with 3600 g/mm holographic grating), and imaged onto a charged-coupled device (CCD) detector (Roper Scientific, Pixis 400B). The spectral response was determined by a deuterium lamp, and the bandpass and accuracy (based on ethanol peaks) of the detection system are ~15 and ±1 cm^{-1}, respectively.

Samples were pumped through a vertically mounted, 100-μm i.d. quartz microcapillary at a rate of 0.16 ml/min to ensure fresh sample for each laser pulse, and discarded after a single pass through the laser beam to eliminate artifacts from photolyzed sample. Fifteen-minute UVRR spectra were collected for peptide samples under the following conditions: 25-μM AcWL5 in buffer, 25-μM AcWL5 in vesicles, and 50-μM L-tryptophan in buffer. Spectra of buffer-only solution with and without SUVs were also acquired and subtracted from corresponding AcWL5 and L-tryptophan spectra.

UV-visible absorption spectroscopy was performed using an Agilent UV-visible spectrophotometer (Santa Clara, CA). Absorption spectra were collected in a quartz cuvette with a 1-cm path length. For AcWL5 spectra in SUVs, a spectrum of SUVs alone was subtracted from the raw AcWL5 absorption spectrum to remove signal due to scattering.

Data analysis

Data were analyzed using Igor Pro (WaveMetrics) software. UVRR spectra of buffer and vesicle solutions without AcWL5 were subtracted from sample spectra and residual broad scattering from capillary walls and vesicles was eliminated *via* baseline correction. No resonance Raman peaks were observed in vesicle-only control solutions (see Fig. S2 in Supporting Information); instead, only increased Rayleigh scattering was observed from these control samples. AcWL5 spectra were normalized for intensity with respect to the perchlorate band (938 cm^{-1}) after the perchlorate band intensity was corrected for preresonance effects.[22] Spectral intensities of both AcWL5 and L-tryptophan were corrected for concentration differences. UVRR spectra were smoothed using a binomial smoothing routine. Gaussian decompositions of AcWL5 spectra were accomplished using a least-squares fitting routine. Secondary structure analysis was limited to using peak positions because successful application of established basis spectra[10,11] has not yet been demonstrated for membrane-bound proteins.

Results and Discussion

Secondary structure determination of AcWL5

The secondary structure of AcWL5 was probed using 207.5-nm excitation UVRR spectroscopy (Fig. 1). Overlapping amide bands (1200–1700 cm^{-1}) were decomposed into Gaussian peaks to determine mode energies; these peak positions were then correlated to secondary structure elements.[10,11] The UVRR spectrum of AcWL5 in a lipid bilayer is dominated by peptide backbone peaks with wavenumbers typical of β-sheets.[11] The amide I and II positions support this secondary structure. The amide III vibrations, which arise from in-phase combinations of C–N stretching and N–H bending, are particularly sensitive to conformation. The low-wavenumber peak ranges from ~1235 to ~1275 cm^{-1} and is highly dependent on hydration; with minimal hydrogen bonding to water,[11] the mode is shifted to lower energies. This amide III position of AcWL5 in vesicles is 1238 cm^{-1},

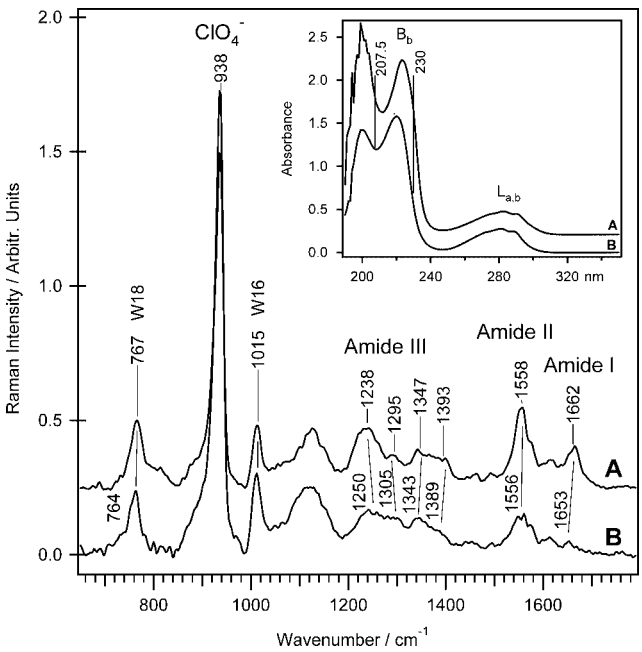

Figure 1. UVRR spectra and absorption spectra (*inset*) of AcWL5 (207.5 nm excitation) in vesicles (A) and 20-mM phosphate buffer (pH 7.3) (B). Spectra have been offset for clarity. The perchlorate peak of the internal standard is indicated.

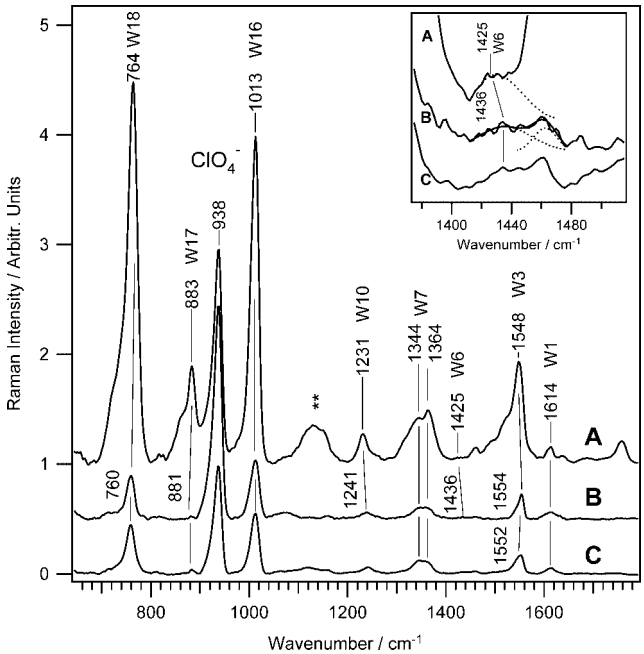

Figure 2. UVRR spectra (230-nm excitation) of AcWL5 in vesicles (A) and 20-mM phosphate buffer (pH 7.3) (B), and L-tryptophan in 20-mM phosphate buffer (pH 7.3) (C). Residual scattering from quartz capillary is indicated with **. *Inset*: Expanded region of W6 bands with Gaussian decompositions for peak positions indicated by dotted lines. Decomposition of the W5 band is also shown for (*B*).

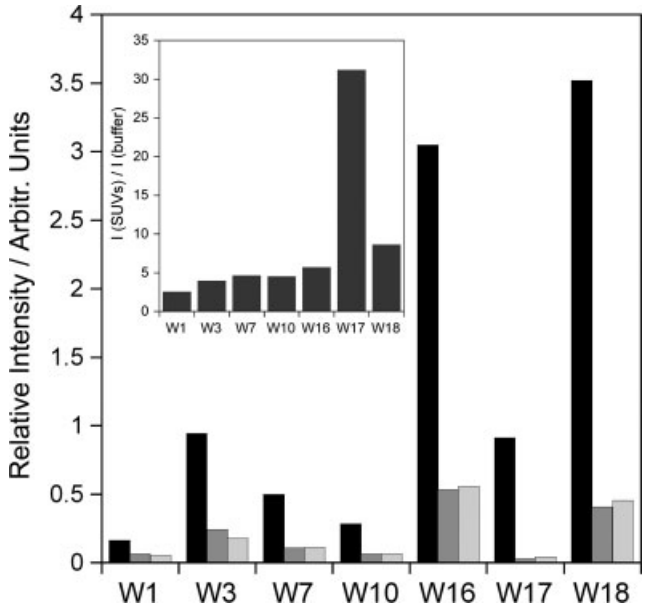

Figure 3. Intensities of Trp normal modes (230-nm excitation) with respect to ClO_4^- peak intensity; abscissa represents Trp mode. (■) Intensity of AcWL5 in vesicles; (■) intensity of AcWL5 in 20-mM phosphate buffer (pH 7.3); (■) intensity of L-tryptophan in 20-mM phosphate buffer (pH 7.3). *Inset*: Intensity ratio of AcWL5 in vesicles to AcWL5 in 20-mM phosphate buffer (pH 7.3).

suggesting weak hydrogen bonding to solvent and enhanced interpeptide hydrogen bonding.[23]

In the absence of vesicles, AcWL5 lacks significant secondary structure; the amide mode energies are characteristic of unordered polypeptide chains.[10] Furthermore, Raman cross-sections for all AcWL5 modes were lower in aqueous solution relative to a lipid bilayer. This finding is consistent with the observed increase in absorption coefficient and redshift of the C=O $\pi \rightarrow \pi^*$ electronic transition for membrane-inserted AcWL5 (Fig. 1 inset) and has been observed in previous studies.[24] The backbone frequencies of AcWL5 in a lipid bilayer provide strong support to previous claims that AcWL5, while unordered in aqueous solution, spontaneously forms an oligomeric β-sheet network in vesicle membranes.[2]

Tryptophan microenvironment

While excitation in the deep UV region probes vibrations coupled to the peptide backbone, longer wavelengths are used to probe structure and environment of specific amino acid side chains. Excitation at 230 nm enhances scattering from tryptophan residues. Specifically, vibrational modes coupled to both B_b and $L_{a,b}$ electronic states will display strong resonance Raman signal,[17] shown in Fig. 2. The energy of the transitions to the B_b and $L_{a,b}$ states are known to shift depending on solvent and environment hydrogen bond strength and polarizability[25–27]; within proteins, a redshift correlates to a reduction in solvent-exposed surface area for the tryptophan indole ring.[28] The stabilization of the electronic transitions of tryptophan in AcWL5 upon membrane insertion (Fig. 1 inset) support an increase in hydrophobic environment and exclusion of water and lead to differential enhancement of tryptophan modes of vesicle-inserted AcWL5 (Fig. 3). Tryptophan modes W1, W3, W7, W16, W17, and W18 are known to be coupled to the B_b transition and show an increase in intensity upon insertion into a membrane.[17,29,30] The intensity of the W10 mode also increases upon insertion into a bilayer, which suggests coupling to the B_b electronic transition. The average increase in Raman cross-section for all peaks with the exception of W17 was found to be ~5-fold (Fig. 3 inset), which is consistent with A-term resonance Raman enhancement with a red-shifted B_b band.[12]

Raman cross-sections of tryptophan modes may also increase due to environmental hydrophobicity.[15] Specifically, large enhancements of W16, W17, and W18 resonance Raman intensities have been reported to correlate with environment hydrophobicity and hydrogen bonding in hydrophobic solvents.[17] Our results indicate that mode W16 shows a ~6-fold-increase upon AcWL5 insertion into a vesicle whereas the W18 band intensity demonstrates a ~9-fold-increase. The W17 band intensity undergoes a ~30-fold increase, which is much greater than that can be expected from simple resonance enhancement. These unexpectedly large scattering cross-sections suggest that in addition to the expected ~5-fold enhancement due to resonance conditions, an additional effect must contribute to the observed intensities of AcWL5 in vesicles. A previous UV resonance Raman study concluded that the addition of a strong hydrogen-bond acceptor in a hexane solution of skatole increased the W17 signal by ~5-fold.[17] Therefore, we attribute the observed ~30-fold increase in W17 intensity to a combination of resonance enhancement (~5-fold) and hydrogen-bonding in a hydrophobic environment (~5-fold). This scenario is not unprecedented; a previous study reported a significant intensity increase of W17 and attributed this change to tryptophan side chains buried within the interior of a protein.[31] The significant change in W17 band intensity for AcWL5 observed here indicates that the indole ring may have van der Waals contact with nearby aliphatic groups, either from peptide–peptide or peptide–lipid interactions.

Additional evidence of a local hydrophobic tryptophan environment is found in the intensity ratio of the Fermi-resonance doublet

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(W7) between 1340 and 1365 cm^{-1}. This intensity ratio, I_{1360}/I_{1340}, is sensitive to the environment, has been well characterized for a number of different solvents, and ranges from 0.65 to 0.92 in hydrophilic solvents such as methanol, 1.02–1.11 in benzene, and 1.23–1.32 in saturated hydrocarbons under nonresonant conditions.[29] While a systematic study of this ratio in different solvents has not yet been reported using UVRR, similar trends and sensitivity to local environment have been observed for ~229-nm excitation.[17,20,32] For example, the extremely hydrophobic tryptophan environment in the copper protein azurin[33] exhibits a Fermi-resonance doublet intensity ratio of 1.3 at 230-nm excitation (data not shown). For AcWL5, this ratio is 1.2 when folded in a vesicle and 1.0 when unfolded in buffer; aqueous L-tryptophan shows a ratio of 0.9. Again, these results support a picture in which the indole ring is buried in a hydrophobic environment.

Both intensity and energy of tryptophan modes are indicators of structure and local environment. There are several tryptophan modes sensitive to indole hydrogen bonding; W6 wavenumber increases with hydrogen-bonding strength, whereas W17 shows the opposite trend.[14,34] The energy of the W6 mode for AcWL5 decreases from 1436 to 1425 cm^{-1} on folding into a hydrophobic vesicle, reflecting loss in hydrogen bonding to solvent (Fig. 2 inset). Likewise, the W17 frequency increases from 881 to 883 cm^{-1} (Fig. 2). Aqueous L-tryptophan exhibits W6 and W17 frequency similar to those of aqueous, unfolded AcWL5. Finally, a correlation has been established between frequency of the W3 tryptophan mode and torsion angle, $\chi_{2,1}$, of the indole ring with respect to the $C_2-C_3-C_\beta-C_\alpha$ linkage.[14] In buffer, $\chi_{2,1}$ for AcWL5 is calculated to be 102°, which is similar to that of aqueous L-tryptophan (97°). In the lipid bilayer, $\chi_{2,1}$ of the indole ring is calculated to be ~88°. This angle is very close to the reported $\chi_{2,1}$ value for a buried tryptophan residue in the membrane-inserted β-barrel protein OmpA,[20] suggesting that the tryptophan residue in β-sheet and β-barrel proteins may have similar conformations.

Implications for the molecular interactions between tryptophan and the lipid bilayer

Tryptophan residues in transmembrane peptides have been demonstrated to reside in a number of locations throughout the lipid bilayer: the tryptophan in the WALP peptides is strongly hydrogen-bonded on the surface of the lipid to the phosphate head group or solvent molecules[35]; studies on PMP1 place tryptophan below the polar head groups[36]; and synthetic peptides have been engineered to place the tryptophan in the center of the lipid bilayer.[37] The tryptophan indole ring and the lipid molecules may interact in a variety of ways, including indole hydrogen bonding to phosphate head groups or solvent,[35] indole hydrogen bonding to lipid acyl carbonyl groups,[38] cation–π interactions,[39] or by providing structural support.[8] The tryptophan in AcWL5 may be involved in any of these interactions, but the resonance Raman data suggest that the dominant interaction involves hydrogen bonding of the indole groups in a hydrophobic environment. Such an interaction is consistent with tryptophan residues localized *below* the phospholipid head groups and hydrogen bonded to the lipid acyl carbonyls. A previous report suggested that the tryptophan residue is located at the lipid–water interface based on the observed fluorescence emission maximum of ~339 nm.[2] While emission maxima may indicate general trends, tryptophan emission blue shifts do not strictly correlate to exclusion of water.[40] For example, in the case of the well-studied transmembrane peptide gramicidin, the tryptophan fluorescence maximum redshifts from 333 to 335 nm even though the average distance between the tryptophans and the center of the lipid bilayer decreases from 11 to 7 Å.[41] In the case of AcWL5, we hypothesize that the tryptophan residues are hydrogen bonded to the lipid acyl groups in a hydrophobic environment. Our data do not preclude the existence of alternate tryptophan populations, and this picture complements the recent result from a neutron diffraction study that suggested a staggered conformation of antiparallel β-sheets that would give rise to a heterogeneous population of tryptophan residues.[3]

To draw further analogy between AcWL5 and gramicidin, the tryptophan residues at positions 11 and 13 of gramicidin have torsion angle $\chi_{2,1}$ values that are similar to the calculated value for AcWL5[42,43] and are believed to strongly hydrogen bond to the carbonyl groups in the membrane.[39,44,45] A tryptophan residue that is involved in hydrogen bonding to the lipid acyl carbonyl groups would exhibit deeper penetration into the membrane with an increase in environmental hydrophobicity but may not result in a dramatic fluorescence blue shift; this is the model that we propose for the location of the tryptophan in membrane-inserted AcWL5 based on the observed UVRR wavenumbers and intensities and is not unprecedented for membrane-bound peptides.[39,41]

Conclusion

Membrane protein folding mechanisms pose significant challenges, and an increasing number of experimental techniques are being applied to understand these complicated systems. UVRR spectroscopy provides comprehensive structural and environmental information about specific amino acid residues and the peptide backbone that is not easily accessible with other techniques. This article presents the first study aimed at elucidating the location of the tryptophan residue of membrane-incorporated AcWL5 within the lipid bilayer and identifying specific molecular interactions guiding that localization. Resonance Raman peak positions and intensities provide structural information at a much higher level of detail than can be obtained from fluorescence bands.[46] The positions and intensities of the W18, W17, W16, W7, and W3 Raman bands indicate that the tryptophan in AcWL5 is found in a hydrophobic, hydrogen-bonded environment, which contrasts with previous reports that the tryptophan side chain exclusively forms hydrogen bonds with the solvent and the lipid phosphate head groups.[7,9] Instead, the indole ring may play a role in localizing the peptide within a hydrophobic region of the membrane through hydrogen bonding to lipid acyl carbonyls. This characterization of AcWL5 as a model for hydrophobic β-sheet assembly in a vesicle may help advance our understanding of integral membrane protein systems.

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