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Ultraviolet Resonance Raman Spectroscopy of Folded and Unfolded States of an Integral Membrane Protein

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The vibrational structure of native anchoring tryptophan (Trp) and tyrosine residues in an integral membrane protein, bacterial outer membrane protein A (OmpA), have been investigated using UV resonance Raman (UVRR) spectroscopy for the first time. Spectra of native OmpA, a single-Trp mutant, and a Trp-less mutant were recorded in folded and unfolded states, and reveal significant changes in tryptophan structure and local environment. Salient alterations upon folding include loss of hydrogen-bonding character of indole N_1H, evidenced by a shift in W17 frequency from 874 and 878 cm^{-1}, and growth in hydrophobicity of the local tryptophan environment, supported by increase in the ratio I_{1361}/I_{1340}. In addition to these site-specific changes in a single tryptophan residue, modification of the vibrational structure of the remaining native tryptophan and tyrosine amino acids is also evident. Finally, the UVRR data presented here indicate that the structures of OmpA folded in vesicle and folded in detergent may differ, and provide important foundations for ongoing studies of membrane protein folding.

Introduction

Membrane protein folding is an exploding area of research for experimentalists and theorists.[1–6] Despite recent efforts to elucidate the process of protein insertion into lipid bilayers, our knowledge of membrane protein folding remains inferior to our fundamental understanding of soluble protein folding. Experimental studies of the folding dynamics of membrane proteins and peptides are especially challenging because only a limited number of membrane protein systems can realistically be studied, samples are difficult to prepare, and few experimental techniques are capable of providing structural details. In addition, techniques to initiate the folding reaction for membrane proteins have primarily been limited to rapid denaturant dilution methods. Finally, there is added complexity in the study of membrane protein folding due to properties of the bilayer itself. For example, curvature of the bilayer, nature of the phospholipid heads, composition of the lipids, and length of the hydrocarbon chains are important factors that affect the stability and folding mechanisms of membrane proteins.[4,7,8] These and other inherent obstacles have made the problem of membrane protein folding especially challenging.

One of the few membrane protein systems that has been relatively well characterized is the robust bacterial integral membrane protein, outer membrane protein A (OmpA). Here, we present the first UV resonance Raman (UVRR) spectra of OmpA under in vitro folding and unfolding conditions. Folding into small unilamellar vesicles (SUV) and detergent micelles is spontaneous, and we have rigorously characterized the stability and size of the SUVs to ensure reproducible folding conditions (manuscript submitted). Figure 1 displays the structure of OmpA, along with an expanded region of the atoms within 5 Å of the tryptophan (Trp) side chain. The in vitro folding mechanism of this 325-residue, monomeric, β-barrel protein has been studied via electronic spectroscopy, including fluorescence and circular dichroism,[8–11] as well as with nonspecific vibrational techniques.[12,13] In addition, the effects of temperature, bilayer composition, and bilayer curvature have been investigated.[8,10,14] These previous studies have helped establish successful conditions for performing experiments and

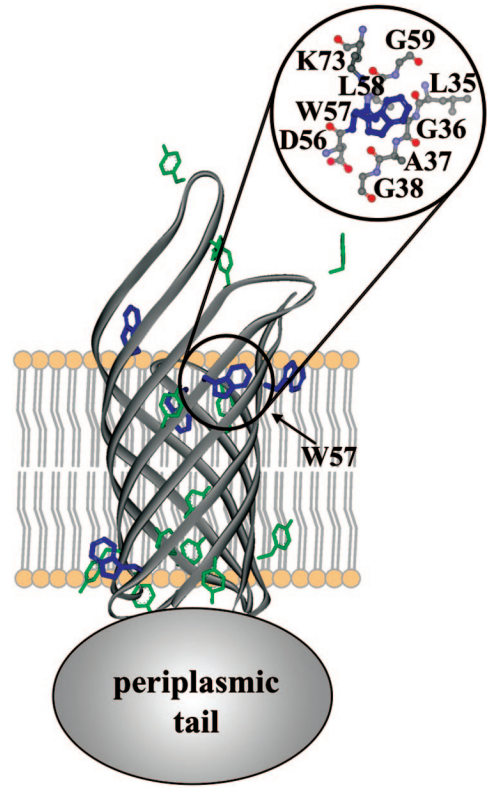

Figure 1. Structure of the OmpA transmembrane domain (residues 1−171; PDB file 1QJP13) highlighting the five native tryptophan residues (blue) and 13 native tyrosine (Tyr) residues (green) in the truncated protein. An expanded region of the local residues within 5 Å of the indole group of W57 is also shown. Cartoon representations of the periplasmic tail and lipid bilayer are included.

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have expanded our general understanding of β-barrel membrane protein folding; however, critical molecular details remain missing.

UVRR spectroscopy is a powerful technique capable of providing highly specific and selective molecular detail in complex systems. UVRR has been applied to a variety of topics in biophysics, including folding of soluble proteins,[15–18] allosteric transitions,[19–21] activation of G-protein coupled receptors,[22,23] and ultrafast protein dynamics.[23–25] Variation of the excitation wavelength from ~195 to 240 nm allows for selective enhancement of Raman signal from peptide backbone or aromatic amino acids.[16,26,27] Extensive analysis of UVRR spectra of peptide backbone[28,29] and aromatic amino acids[30–33] have been reported. Off-resonance Raman studies of aromatic residues have also played a critical role in the interpretation of UVRR spectra.[34–36] These and other Raman studies have established important empirical relationships between Raman frequencies/intensities and molecular detail, such as secondary structure, hydrogen bonding strength, microenvironment, and static structure. In particular, systematic UVRR studies of tryptophan reveal spectral signatures that report upon local hydrophobicity, strength of indole N,H hydrogen bond, and torsional angle, χ2,1 about the C-2-C-3-Cβ-Cα linkage. The wealth of vibrational information makes UVRR studies of tryptophan an ideal probe in our studies of protein folding in a bilayer environment.

Tryptophan and other aromatic residues in membrane proteins are preferentially located at the bilayer−water interface[37–40] and can serve as membrane anchors. Therefore, it is likely that folding of a membrane protein causes significant modification in tryptophan vibrational structure and microenvironment. Comparison of UVRR spectra of wild-type OmpA (five native tryptophan residues), a single-Trp mutant (W57), and the Trp-less mutant (W0) enables systematic identification of vibrational changes from a single tryptophan residue in folded and unfolded protein states. In addition, structural differences of OmpA folded in detergent and folded in vesicles may be elucidated via UVRR. The current work provides the first set of residue-specific details associated with protein insertion into a synthetic bilayer and establishes a foundation for ongoing experimental studies of membrane protein folding.

Experimental Methods

Preparation of OmpA Mutants. The expression, isolation, and purification of OmpA were adapted from previously published procedures with some revisions[9–11,41] and presented briefly here. Site-directed mutagenesis was performed on a pet1102 plasmid for OmpA to produce the single-tryptophan mutant at position 57 (W57); the other four native Trp residues (positions 7, 15, 102, and 143) were mutated to phenylalanine (Phe) residues. A Trp-free mutant of OmpA (W0) was also generated, in which all five Trp residues were replaced with Phe residues. Wild-type OmpA, containing five native tryptophan residues, was obtained from *E. coli* strain JF701 (Genetic Stock Center, Yale University). JF701 cells were grown overnight in 50 mL sterile media (1% bactotryptone, 0.5% yeast extract, and 25 μg/mL streptomycin) at 37 °C and then transferred to a 6 L batch for continued growth. W57 and W0 OmpA mutants were overexpressed in *E. coli* strain JF733 (Genetic Stock Center, Yale University). JF733 cells were grown overnight in 300 mL of sterile LB media (0.5% glucose, 50 μg/mL ampicillin) at 37 °C and then transferred to a 6 L batch where OmpA overexpression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Harvested wild-type and mutant OmpA cells were lysed, and the membrane fragments were collected. Peripheral membrane proteins were removed with a 3.5 M urea solution, followed by extraction of OmpA using a 1:1 8 M urea/isopropanol solution. Crude OmpA was purified by anion exchange chromatography (Q Sepharose Fast Flow column, GE Healthcare). Fractions containing purified protein were combined, washed, and concentrated via ultrafiltration (Amicon, PM-10). Stock samples were stored as unfolded protein in an 8 M urea, potassium phosphate (KP_i) buffer solution at −80 °C.

Preparation of SUVs. SUVs were prepared using a modification of a previously published procedure.[10] Briefly, a 20 mg/mL lipid solution of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids) in $CHCl_3$ was dried for 1 h under a stream of argon. The dried lipids were dissolved in a 20 mM, pH 7.3 KP_i buffer to a final lipid concentration of 5 mg/mL. This solution was sonicated using an ultrasonicator microtip for 30 min at 50% duty cycle in a water bath and filtered by passing the SUV solution through a 0.22 μm membrane. The stock vesicle solution was further diluted to a final lipid concentration of 1 mg/mL in 20 mM KP_i buffer. All SUV solutions were equilibrated overnight at 35 °C and used the following day. Vesicle size (~50 nm diameter) and stability (>12 h) were determined via dynamic light scattering measurements.

Refolding and Unfolding of OmpA. OmpA samples were refolded into detergent micelles of *n*-octyl-β-D-glucopyranoside (OG) or SUVs. The final concentration of OmpA was ~20 μM based on UV−visible (UV−vis) absorption measurements and the following calculated extinction coefficients: 54 390, 32 330, and 26 810 M^{-1} cm^{-1} for OmpA wild type, W57, and W0, respectively. For folding in detergent, stock OmpA was added to a 10 mg/mL solution of OG (20 mM KP_i, pH 7.3), and allowed to equilibrate for 30 min prior to spectroscopic experiments. For folding in SUVs, stock OmpA was added to a 1 mg/mL solution of sonicated DMPC (20 mM KP_i, pH 7.3) and equilibrated for ~6 h before spectroscopic measurements were made. For unfolded OmpA, the stock solution was diluted to <1 M urea in KP_i buffer solution. Protein conformational states were confirmed by the differential gel electrophoretic mobility of folded (30 kDa) and unfolded (35 kDa) OmpA and fluorescence spectroscopy;[10,11] Trp emission in folded OmpA peaks near 330 nm, while, in unfolded protein, OmpA emits maximally at ~350 nm.[11]

UVRR Spectroscopy. A tunable Ti:Sapphire laser (Photonics Industries) pumped by a frequency-doubled Nd:YLF laser was used in the current experiments. The 1 kHz, >1 W, 920 nm fundamental output beam was collimated into a lithium triborate (LBO) crystal for second harmonic generation; the resulting 460 nm beam was then passed through a β-barium borate (BBO) crystal to produce >10 mW of the 230 nm excitation beam. The UV beam was isolated via a Pellin-Broca prism, and focused by two cylindrical lenses (200 mm and 75 mm focal lengths) to a spot size of ~230 μm × ~75 μm at the sample. The power at the sample was ~3 mW. Scattered light was collected in a 135° backscattering geometry by a UV compound lens 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.[42] Raman scattered light was dispersed in a 0.5 m spectrograph (JY Horiba, Spex 500 M with 3600 gr/mm holographic grating), and imaged onto a CCD detector (Roper Scientific, Pixis 400B). The spectral response was determined by a deuterium lamp, and the bandpass and accuracy (based on

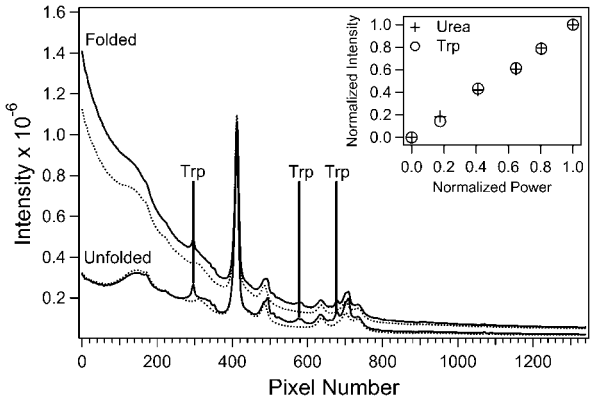

Figure 2. Raw UVRR spectra of WT OmpA folded in vesicle and unfolded in KP_i buffer. The solid curves are spectra of protein solution; dotted curves are spectra of corresponding buffer solution. Unique protein peaks from tryptophan are indicated; the large peak near pixel 400 is due to urea. Inset: Normalized urea and trp peak intensities as a function of normalized power.

ethanol peaks) of the detection system are <14 cm^{-1} and ± 1 cm^{-1}, respectively.

Protein solution was 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 was discarded after a single-pass through the laser beam to eliminate artifacts from photolyzed protein. Ten-minute UVRR spectra were collected for wild-type and mutant OmpA solutions under the following conditions: folded in SUV, folded in detergent, and unfolded in KP_i buffer. Spectra of each of the three buffer solutions without OmpA were also acquired and subtracted from the corresponding OmpA spectra. Fluorescence and absorption spectra were measured before and after photolysis. Data were analyzed using Igor Pro (WaveMetrics) software.

Results and Discussion

Typical raw spectra of folded and unfolded $\sim$20 μM WT OmpA along with corresponding buffer spectra are shown in Figure 2. To our knowledge, these data are the first UVRR spectra of membrane proteins in highly scattering synthetic vesicles. Despite intense Rayleigh scattering from SUVs (left-side of Figure 2), resonance Raman bands from OmpA are apparent in these 10 min spectra. Raman signal from vibrational modes coupled to tryptophan $L_{a\text{-}b}$, and B_b electronic transitions are enhanced with 230 nm excitation.[27,30,31] Signal from residual urea ($\sim$0.8 M), fused silica, and water are also evident. Linear dependence of Raman intensities for the W18 mode and 1005 cm^{-1} urea mode as a function of laser power up to 6.5 mW is shown as an inset.

Figure 3 shows the 230 nm UVRR spectra of folded and unfolded WT, W57, and W0 OmpA after subtraction of appropriate buffer spectra and removal of residual scatter. For example, the top spectrum in Figure 3 was determined via the following analysis: [(raw OmpA in OG buffer $-$ raw OG buffer) $-$ residual scattering background]. The residual scattering background was determined by interpolation from the remaining background after removal of buffer contribution. The feature near 1000 cm^{-1} is due to urea and, if relevant, overlapping W16 peak at $\sim$1013 cm^{-1}; upon dilution of the stock solution of OmpA in 8 M urea/KP_i into appropriate folding or unfolding buffers, residual ($\sim$0.8M) urea remained. Despite our attempt to replicate the residual urea concentrations in analogous, protein-free buffer solutions, it was difficult to quantitatively eliminate signal from urea without simultaneously over- or under-subtracting other features, such as the water bend peak at $\sim$1640 cm^{-1}.

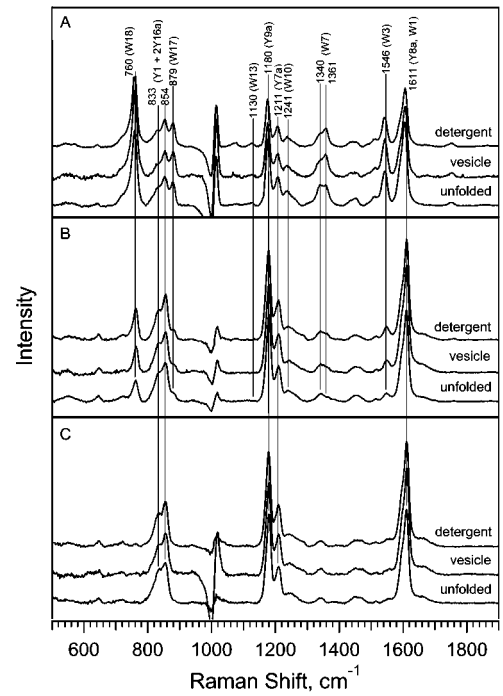

Figure 3. Corrected UVRR spectra of WT OmpA (A), W57 OmpA (B), and W0 OmpA (C) folded in detergent, folded in vesicle, and unfolded in KP_i buffer. Trp and Tyr peaks are indicated. The feature near 1000 cm^{-1} is due to incomplete subtraction of the large urea peak.

The OmpA UVRR spectra in Figure 3 show strong signal from the 17 native tyrosine (Tyr) residues in WT, W57, and W0 OmpA. Strong enhancements are also observed for the native tryptophan residues in WT and W57 OmpA.[27] Comparison of the WT and W57 spectra to the Trp-less W0 mutant shows strong signal from tryptophan modes W18 ($\sim$760 cm^{-1}), W17 ($\sim$879 cm^{-1}), W13 ($\sim$1130 cm^{-1}), W10 ($\sim$1241 cm^{-1}), W7 Fermi doublet ($\sim$1340 and $\sim$1361 cm^{-1}), and W3 ($\sim$1546 cm^{-1}). Unique tyrosine peaks are also evident, such as the Y1 + 2Y16a Fermi doublet ($\sim$833 and $\sim$854 cm^{-1}), Y9a ($\sim$1180 cm^{-1}), and Y7a ($\sim$1211 cm^{-1}). Strong signal from overlapping Trp and Tyr peaks appears near 1611 cm^{-1} (Y8a and W1). Because W57 OmpA has a single tryptophan residue at position 57, our W57 spectra unambiguously illustrate vibrational spectra of this single residue under the current folding and unfolding conditions. The frequency of the indole ring vibration (W3) quantitatively reflects the torsional angle, χ2,1 about the C-2-C-3-Cβ-Cα linkage.[35] The calculation of the angle from our UVRR spectra yields a χ2,1 value of $\sim$91° for W57 folded in vesicle and detergent, which is similar to the value of 93° from the crystal structure of detergent-solubilized OmpA.[43,44]

In addition to structure, peak positions are sensitive to microenvironment. The W17 band at $\sim$879 cm^{-1} is sensitive to hydrogen bonding by the N_1H group of the indole.[34] In W57 OmpA, decomposition of the peaks near W17 into three Gaussian bands centered at $\sim$833 and $\sim$854 cm^{-1} (Y1+Y16a Fermi double), and $\sim$879 cm^{-1} yield W17 frequencies of 874 and 878 cm^{-1} in unfolded and folded states, respectively. The 4 cm^{-1} increase in W17 frequency illustrates a loss of hydrogen-bonding character of the indole N_1H upon folding into detergent micelles.[34] Our result coincides with the crystal structure that shows no hydrogen-bonding partners within 4 Å of the indole N_1H. Presumably, this loss in hydrogen-bonding structure is due

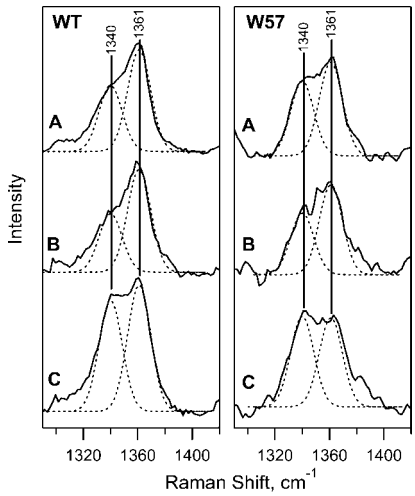

Figure 4. Expanded region of W7 Fermi doublet for WT (left) and W57 (right) OmpA folded in detergent (A), folded in vesicle (B), and unfolded in KP_i buffer (A). Tyrosine and protein signals observed in the W0 spectra were removed. Gaussian decompositions are indicated by the dotted curves.

to the expulsion of hydrogen-bonding water partners upon transfer of OmpA from aqueous solution, where it remains unfolded, to detergent micelles, where it forms β-barrel structure. The removal of water upon folding in detergent is consistent with the observed increase in hydrophobicity as measured via steady-state fluorescence experiments.[11]

An expanded region of the Fermi resonance doublet of Trp near 1340−1360 cm^{-1} is illustrated in Figure 4 for WT and W57 OmpA. Only features from Trp contribute to these spectra because a W0 spectrum was subtracted from the corresponding WT or W57 spectrum. The doublet has been decomposed into two Gaussian peaks centered at 1340 and 1361 cm^{-1} (20 cm^{-1} breadth for each peak). The relative intensity, R, of this doublet resonance (I_{1361}/I_{1340}) is sensitive to local environment; excitation with 488 nm results in a value of R that ranges from 0.65−0.92 in hydrophilic solvents such as methanol, 1.02−1.11 in benzene, and 1.23−1.32 in saturated hydrocarbons.[36] This ratio is wavelength-dependent, but remains a sensitive indicator of local environment with UV excitation. For WT OmpA, the ratios, R, when OmpA is folded in detergent, folded in SUV, and unfolded in KP_i, are 1.6, 1.8, and 1.1, respectively. These large values for R averaged over all five native Trp residues in folded OmpA are consistent with burial of the Trp residues in a highly hydrophobic region, such as in a local hydrophobic pocket or in the hydrocarbon core of the vesicle. For W57 OmpA, R is 1.2 (folded in detergent), 1.3 (folded in SUV), and 0.9 (unfolded in KP_i). These data support a picture in which the single Trp residue at position 57 remains in a relatively hydrophilic region when unfolded, and transfers to a hydrophobic microenvironment upon folding into vesicles or detergent, consistent with burial of the Trp residue upon folding.

In addition to the single Trp residue in W57, the remaining four native tryptophan residues also undergo structural changes upon folding; these shifts can be determined from further comparison of WT, W57, and W0 OmpA UVRR spectra. In W57, the combined Y8a and W1 peak near 1611 cm^{-1} appears in the same spectral region as the isolated Y8a peak in W0 spectra, suggesting that any change in W1 frequency caused by protein folding is masked by the large signal from tyrosine. In WT OmpA, however, frequency shifts of the W1 tryptophan band are evident in this spectral region; the large peak appears at 1614, 1609, and 1612 cm^{-1} for protein folded in vesicle, protein folded in detergent, and unfolded protein, respectively. This W1 mode has contributions from the phenyl ring and pyrrole N_1C_8 stretch,[27] and the frequency shift from 1614 to 1609 cm^{-1} of protein folded in vesicle and detergent, respectively, suggests different folded structures in these two media. Additional support for distinguishable folded structures is evident in the W3 peak of WT OmpA. Unlike the fixed W3 peak of W57 OmpA at 1548 cm^{-1}, the W3 peak of WT OmpA shifts from 1548 (folded in vesicle) to 1544 (folded in detergent) cm^{-1}. This shift may reflect a change in the average torsional angle, χ2,1, from ∼91° to ∼79° for the remaining four trp residues in OmpA folded in vesicle and detergent, respectively. Alternatively, the shift of W3 frequency in WT OmpA could be attributed to a large change in χ2,1 of a single Trp residue for the protein folded in vesicle versus detergent.

Inspection of the W0 OmpA UVRR spectra indicates that tyrosine residues also undergo changes upon folding. The large peak near 1611 cm^{-1} is dominated by the 17 native tyrosine residues; in W0, the isolated tyrosine peak, Y8a, appears at 1612 (folded in detergent), 1612 (folded in SUV), and 1609 (unfolded) cm^{-1}. The Y8a ring-stretching mode is sensitive to hydrogen bonding, and has been shown to undergo a systematic decrease in frequency with increasing hydrogen bond strength.[19] The observed shift in Y8a frequency suggests that, on average, the tyrosine residues have weaker hydrogen bonds in folded OmpA than in unfolded OmpA. This loss of tyrosine hydrogen bonding is consistent with our conclusion, based on W17 analysis above, that Trp-57 experiences decreased hydrogen bonding upon folding. Comparison of the Y8a frequency in unfolded OmpA to the model compound (Y8a for L-Tyr in 0.5 M urea/KP_i appears at ∼1609 cm^{-1}) shows similar hydrogen bonding in the unfolded protein relative to the fully solvent-exposed model compound. Collectively, these data support a picture in which solvent exposure to aromatic amino acids is decreased in the folded state because of water expulsion upon folding, and are consistent with previous UVRR studies of peptide folding in micelles.[45,46]

Summary

UVRR spectroscopy is a powerful technique that is capable of elucidating site-specific, molecular changes associated with protein folding. Here, we report 230 nm excitation UVRR spectra of an integral membrane protein folded in a synthetic bilayer vesicle for the first time. Comparison of these UVRR spectra to those of protein folded in detergent micelles and protein unfolded in buffer reveals structural changes in these different states, including modifications in tryptophan hydrogen-bonding strength and microenvironment. Our results coincide with previous studies based on other techniques. However, UVRR spectroscopy is unique because the spectra yield *residue-specific*, *structural* details of a protein in the native environment of a bilayer membrane. These and other ongoing studies, including UVRR spectroscopy of additional OmpA mutants in our own laboratory, will contribute to the growing understanding of membrane protein folding.

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Supporting Information Available: Regions near the W1 and W3 peaks of wild-type OmpA and the Y8a peak of W0 OmpA have been expanded. This material is available free of charge via the Internet at http://pubs.acs.org.

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