Conformation of a Peptide Solubilizate in a Reversed Micelle Water Pool

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Abstract: Peptide interactions with an aqueous interfacial region have been explored by solubilizing a water-soluble cyclic pentapeptide, *cyclo*(Gly-Pro-Gly-D-Ala-Pro), in Aerosol OT [bis(2-ethylhexyl) sodium sulfosuccinate, AOT] reversed micelles in heptane or octane. The small pools of water within reversed micelles offer an excellent system for spectroscopic studies of water–surfactant and water–peptide interactions, since no large population of bulk water is present to obscure signals from the interfacial water. Nuclear magnetic resonance (NMR), circular dichroism (CD), and infrared (IR) spectroscopies have allowed a definition of the conformational influence of an aqueous interfacial region on a polypeptide chain. Specifically, our results indicate a conformational impact dominated by the counterions of the surfactant. Samples of cyclic peptide in reversed micelles with varying water content and, therefore, varying proportions of interfacial water have been found to display CD spectral shapes and ellipticities, ^{1}H NMR chemical shifts of NH's, and ^{13}C NMR signals for conformationally diagnostic sites in the peptide that correspond to those of a peptide–Na^+ complex. Comparison of the spectral parameters for the peptide in reversed micelles to those of the peptide in NaCl solutions affords an estimate of the effective Na^+ concentration at various H_2O/AOT ratios: in heptane, for example, with H_2O/AOT = 8.3, the Na^+ concentration is ca. 1–2 M by this measure. In addition, our data suggest that the peptide solubilizate does not significantly perturb the population distribution of interfacial and bulk water present in the reversed micelle.

Water close to biological membranes or proteins exhibits behavior markedly different from bulk water.[1-3] Water solubilized in reversed micelles, like this biological interfacial water, has restricted mobility, a depressed freezing point, and characteristic spectroscopic parameters.[4-10] The unusual behavior of this water has been attributed to its strong interaction with the ionic headgroups of the surfactant as well as to an overall disruption of the three-dimensional hydrogen-bonded network usually present in bulk water. Similar reasoning has been invoked in accounting for the behavior of biological interfacial water.[4,11] The sequestered pools of water within reversed micelles offer a unique "isolated" system of interfacial water free from the interference associated with large quantities of bulk water.

We seek to understand the conformational influences of an aqueous interfacial microenvironment on a polypeptide chain. Preliminary studies[12,13] have established that small hydrophilic peptides normally insoluble in apolar solvents can be solubilized in water-containing AOT [bis(2-ethylhexyl) sodium sulfosuccinate] reversed micelles, surrounded by a variety of nonpolar solvents. Spectroscopic data (CD, circular dichroism; NMR, nuclear magnetic resonance; and IR, infrared) have been obtained for these systems yielding information about the conformational changes induced by the interfacial environment.[14] In addition, the water in the reversed micelles appears to be largely unperturbed by the addition of the peptide solubilizate.

In this paper, we report more extensively on the interactions between a solubilized hydrophilic cyclic pentapeptide and the interfacial region. The results of these experiments lead to the suggestion that reversed micelles may be useful in elucidating many biochemical and biophysical phenomena that occur at an interface. In particular, our results emphasize the importance of counterions to charged head groups at an interface; these ions cause significant alterations in the conformational distribution of a molecule in the interfacial region.

Experimental Section

Materials: Bis(2-ethylhexyl) sodium sulfosuccinate (AOT) was obtained from Fisher Scientific and purified by dissolving in methanol, filtration through sintered glass, and solvent evaporation. The resulting white foam was dried in vacuo at 40 °C for 12 h prior to use, yielding a white crumbly solid.

Octane (d_{18}, 99%) was purchased from Cambridge Isotopes for use in ^{1}H and ^{13}C NMR. Spectral grade heptane obtained from Fisher Scientific was used for CD and IR experiments. D_2O (99.8% perdeuterated, Biorad Laboratories) was used for IR experiments.

Synthesis of the cyclic pentapeptide *cyclo*(glycyl-L-prolylglycyl-D-alanyl-L-prolyl), *cyclo*(Gly^1-Pro^2-Gly^3-D-Ala^4-Pro^5), has been reported elsewhere.[15] For some of the experiments, a sample of the cyclic peptide enriched 95% with ^{15}N at Gly^1 and D-Ala was used to facilitate assignments. The ^{15}N amino acids were purchased from Cambridge Isotopes.

Preparation of Reversed Micelle Samples. All samples were prepared by addition of measured volumes of appropriate solvent to dry, preweighed quantities of AOT (weight of AOT/volume of solvent) under nitrogen. For NMR and CD experiments, the desired quantities of H_2O were then added (v/v) and the samples shaken until clear. The quantity of water present in these reversed micelle samples was determined by quantitative integration of the H_2O ^{1}H NMR signal. It was found that very little residual water remained in the dried AOT (<0.5 H_2O/AOT), and no correction was applied in reported H_2O/AOT ratios. For IR experiments, desired quantities of 11 M HOD (10% H_2O in D_2O) were added to the sample with analogous quantities of D_2O added to the reference sample. For samples with peptide solubilizate, dry peptide was added to the reversed micelle sample which was then shaken or sonicated until the peptide was solubilized.

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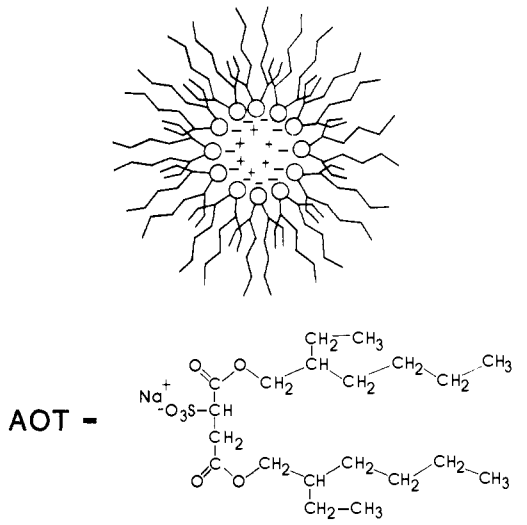

Figure 1. Schematic diagram of the accepted structure of an AOT reversed micelle. Water is located in a pool in the center of the structure adjacent to charged surfactant headgroups (designated by circles). Also shown is the structure of the surfactant used in these studies: AOT, bis(2-ethylhexyl) sodium sulfosuccinate.

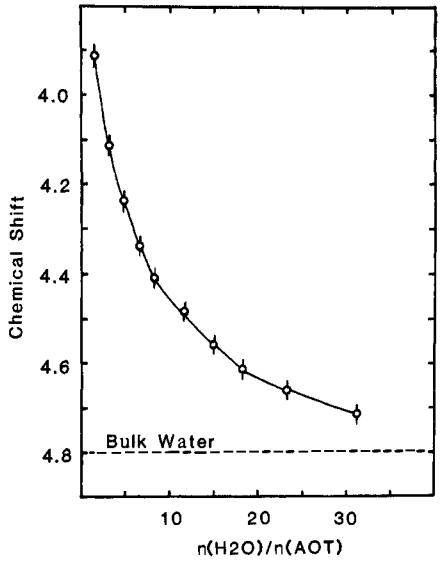

Figure 2. ^{1}H NMR (250 MHz) chemical shift of H_2O resonance as a function of water content in AOT reversed micelles. Water content is expressed as the molar ratio of water to surfactant (3% AOT w/v, bulk solvent octane-d_{18}).

Nuclear Magnetic Resonance. All spectra were obtained in the Fourier transform mode on a Bruker WM250 Spectrometer operating at 250.13 MHz for ^{1}H and 62.9 MHz for ^{13}C. Chemical shifts are referenced to internal tetramethylsilane (Me_4Si) or dioxane (for ^{13}C NMR in water).

Circular Dichroism. All spectra were obtained on a Jasco J10 spectropolarimeter using cylindrical quartz cells with a 0.5- or 1.0-mm path length (Precision Cells).

Infrared. Infrared spectra were obtained on a Cary 17D spectrophotometer using quartz IR cells with a 1-cm path length. Samples were referenced to a reversed micelle sample containing an equivalent quantity of D_2O.

Results and Discussion

I. Characterization of Interfacial Water. Nuclear Magnetic Resonance. Various spectroscopic parameters of water in reversed micelles have indicated that micellar solubilized water is perturbed by the highly charged interior surface of the micelle.[4] Figure 1 depicts schematically the accepted structure of an AOT reversed micelle; note that the waters enclosed in the micelle are immediately adjacent to the surfactant head groups. As shown previously,[4] ^{1}H NMR data indicate that the chemical shift of H_2O in reversed micelles is a function of water present. The ^{1}H NMR chemical shift observed for H_2O in reversed micelles is a weighted average of the shifts for the various environments sampled by the water. Initially at high field ($\simeq$3.8 ppm) with low water content ($\simeq$1.5 H_2O/AOT), the water resonance moves downfield with increased water content approaching the value for bulk water (4.8 ppm) (Figure 2), consistent with the hypothesis that the first waters added, i.e., those closest to the micellar surface, are perturbed the most by that surface. As previously described,[4,7,11] once ca. 8 waters per AOT are present, the water inside the reversed micelle behaves increasingly like bulk water, suggesting that the perturbations on water several layers away from the headgroups are less pronounced.

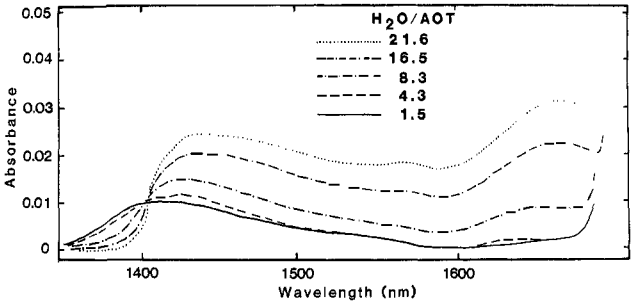

Figure 3. Near-IR spectra of water in AOT reversed micelles as a function of water content (3% AOT w/v, bulk solvent *n*-heptane, water phase 11 M HOD). Note the appearance of a second band at 1670 nm and the shift from ca. 1400 to ca. 1420 nm in the shorter wavelength band with increasing water content.

Infrared. Infrared data were obtained in the overtone (2ν) region for OH stretching vibration. Analysis was simplified by using HOD instead of H_2O, so that the OH stretching modes within one molecule would be uncoupled.[16] Others have reported that "perturbed" (disrupted from the usual hydrogen-bonded structure generally observed in liquid water) and bulk water can be distinguished by their characteristic vibrational frequencies in this region.[17-19] Since IR experiments involve a measurement with a much faster time scale than NMR, distinct bands can be assigned to populations of water with various degrees of perturbation. Water not involved in a tetrahedral array of hydrogen bonds exhibits an OH stretching band at shorter wavelengths in the near IR region (ca. 1400 nm); conversely, bulk-like water produces an absorption band at longer wavelengths (ca. 1670 nm).[20,21] As shown in Figure 3, when fewer than 8 H_2O/AOT are solubilized in reversed micelles, a short wavelength (ca. 1400 nm) band predominates. This band is attributable to the most strongly perturbed water. The water titration with reversed micelles shows a second band at longer wavelengths (1670 nm), increasing in intensity with increasing water content. Hence, as the size of the internal pool of water increases, we observe an increased absorbance at the longer wavelength band and a slight red shift in the maximum absorbance at shorter wavelength. Zana has suggested a mechanism for headgroup hydration that may explain the red shift in the short wavelength band.[22] Initially, the water hydrates an ion pair complex; with addition of larger amounts of water, a water molecule is inserted between the cation and anion, inducing a shift in the absorption band for the most strongly perturbed water species.

We characterized the interior water pools at different ratios of water to surfactant by two spectroscopic methods. Then, the conditions appropriate for exploring the influence of the interfacial (nonbulk-like) water on a peptide solubilizate were chosen. Both

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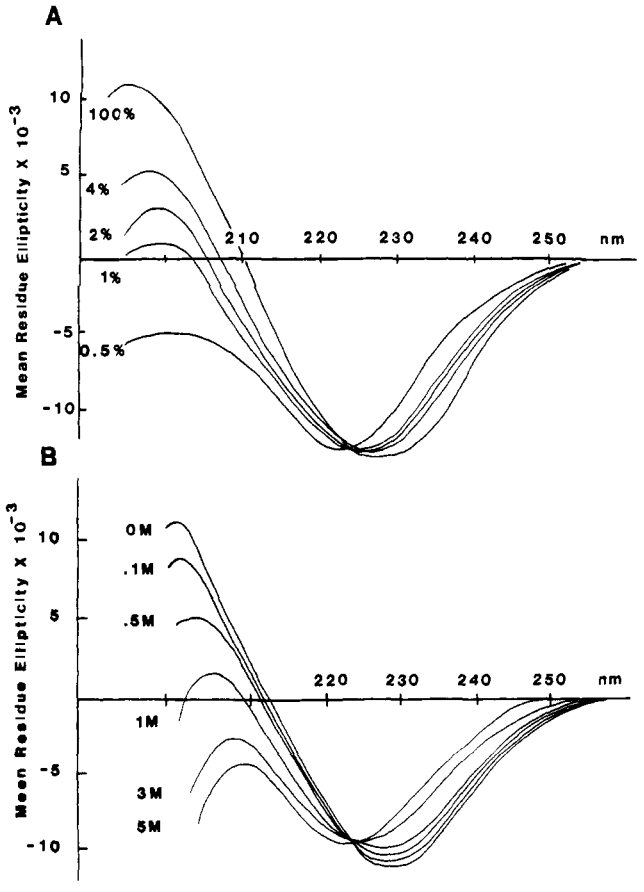

Figure 4. Circular dichroism spectra of *cyclo*(Gly-Pro-Gly-D-Ala-Pro): (A) in AOT reversed micelles as a function of water content (peptide 0.67 M in water phase, AOT 3% w/v, bulk solvent *n*-heptane, water concentrations given as % v/v; note that 1% H_2O = 8.3 H_2O/AOT); (B) in aqueous solution as a function of NaCl concentration (peptide 0.67 M).

the NMR and the IR data indicate that at water contents of 1–10 H_2O/AOT much of the water is not hydrogen bonded in the same manner as bulk water; a significant proportion is strongly perturbed by the charged surface of the micelle. Upon addition of larger amounts of water we observe that the water displays more bulk water characteristics; the 1670-nm band appears in the IR, and the NMR shift approaches that of bulk water.

II. Interactions of Model Peptide in the Interfacial Region. Dimensional Considerations. The peptide solubilizate studied is a neutral cyclic pentapeptide, with a compact, spheroidal shape of largest dimension ca. 10 Å. Samples have been prepared with a constant concentration of peptide in the water pseudophase (0.67 M). At the lowest water content examined (0.5%, 4.1 H_2O/AOT), assuming no effect of the peptide on the reported aggregation behavior of AOT,[9,23] on the average one peptide molecule would be solubilized per micelle water pool (diameter ca. 24 Å). Reversed micelle water pools increase in size as the H_2O/AOT ratio increases,[9,23] and the same assumption as above (no perturbation of AOT aggregation number and water pool size by peptide) would imply multiple peptide molecules per water pool at higher water contents (1%, 8.3 H_2O/AOT, and above). Since peptide–peptide interactions have not been observed in aqueous solutions of this peptide,[24] such interactions are not likely to be important sources of conformational perturbation in the micellar water pools. The samples thus provide a means of exploring the influence of a range of water environments (bulk/interfacial), determined by varying H_2O/AOT ratios, on the conformation of the peptide solubilizate.

Circular Dichroism. The conformational impact of an aqueous interfacial environment on the peptide solubilizate has been examined using several physical techniques. CD spectra (Figure 4A) of *cyclo*(Gly-Pro-Gly-D-Ala-Pro) in AOT/heptane reversed micelles monitored as a function of H_2O content clearly indicate a conformational change. At low water concentrations, the CD observed is distinctly different from that observed in bulk water. At higher water concentrations, the CD approaches that observed for bulk water. In particular, there is a similarity between the CD obtained for this peptide in reversed micelles with a small ratio of H_2O to AOT and previously reported CD spectra of a cation complex of this peptide (e.g., the decreased ellipticity at 200 nm).[15] A clear isosbestic point at 225 nm is observed in the reversed micelle samples. As shown in Figure 4B, a family of curves was generated by titrating this peptide in H_2O with NaCl. A strong resemblance to the curves seen in reversed micelle samples on variation of H_2O concentration (Figure 4A) can be noted. Again an isosbestic point is observed at ca. 224 nm with a decreased ellipticity at 200 nm as NaCl concentration increases. These results suggest that the model peptide undergoes a conformational transition inside the reversed micelle due to a high effective cation concentration in the micellar water pool.

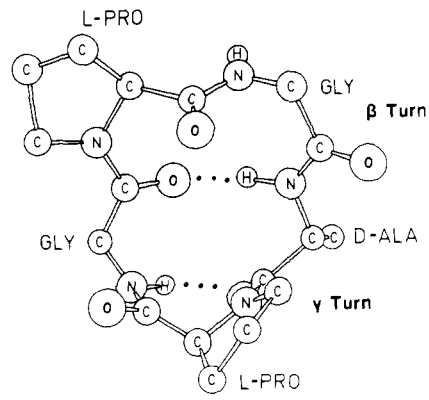

Figure 5. Diagram showing the model cyclic peptide, *cyclo*(Gly-Pro-Gly-D-Ala-Pro). The conformation illustrated contains two intramolecular hydrogen bonds (indicated by dots) and is the preferred conformer of the free peptide in bulk solvents. Upon formation of a cation complex, the hydrogen bonds are disrupted, as three carbonyl oxygens orient to form a binding site.

The conformation of the model peptide *cyclo*(Gly-Pro-Gly-D-Ala-Pro), chosen as a probe to determine the conformational impact of the environment presented to a polypeptide in an interfacial water region, has been well characterized in a variety of solvents. In particular, it has been reported that the conformation of this cyclic pentapeptide is remarkably insensitive to solvent polarity.[15] The peptide is conformationally very rigid, adopting an all-trans conformation containing both a β and a γ turn (Figure 5) in a wide variety of solvents.[25] The conformation of the peptide is altered by complexation with cations. Pease and Watson have reported ion binding constants for several divalent cations and for Li^+ with this peptide in acetonitrile but saw no evidence for Na^+ binding by the peptide in this solvent at the concentrations studied.[15] It was suggested that upon ion binding, a conformational adjustment occurs whereby three carbonyls orient to form an ion binding site, causing the rupture of both internal hydrogen bonds. The present titration of *cyclo*(Gly-Pro-Gly-D-Ala-Pro) in water indicates that a complexation of Na^+ does occur with a concomitant conformational change. A control experiment wherein the peptide was titrated with $NH_4^+Cl^-$ showed no alterations in the CD, indicating that the observed CD changes were due to the Na^+ and not the Cl^-, and that ionic strength changes were not causing the changes in the conformational distribution.

Since we can mimic the CD curves for *cyclo*(Gly-Pro-Gly-D-Ala-Pro) in reversed micelles as a function of water content by titrating the peptide in bulk water with NaCl, we propose that the peptide is binding Na^+ ions in the reversed micelles. When the water to surfactant ratio is about 4.1, the effective Na^+

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(25) A small proportion of a one-cis X-Pro bond-containing conformer occurs in H_2O.

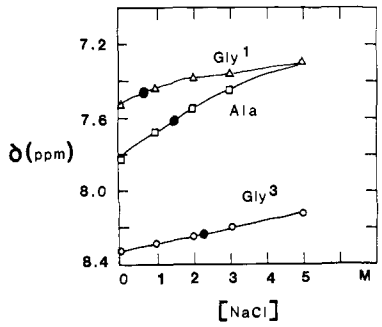

Figure 6. Chemical shift of the N-H resonances of Gly[1] (Δ), Ala (□), and Gly[3] (○) of *cyclo*(Gly[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]) in aqueous solution (peptide 0.67 M) as a function of NaCl concentration. N-H resonance positions of the peptide in reversed micelles (3% AOT w/v, 8.3 H_2O/AOT, bulk solvent octane-d_{18}) are indicated by filled dots. Spectra were obtained by using a solvent suppression routine to reduce dynamic range problems.

concentration in the reversed micelle appears to exceed 5 M NaCl. With water content at 8.3 H_2O/AOT (1% H_2O), we observed that the peptide is experiencing the impact of 1–2 M NaCl. These effective Na^+ concentrations allow an estimate to be made of the extent of dissociation of Na^+–AOT in reversed micelle water pools. By correlating the stoichiometric concentration of Na^+–AOT head groups in the water pools (assuming no Na^+–AOT in the nonpolar phase) with the effective Na^+ concentration experienced by the model peptide probe, we can calculate that the Na^+ ions are approximately 30% dissociated from the micellar surface at 8.3 H_2O/AOT. This value agrees well with that previously reported by Wong, Thomas, and Nowak[4] from ^{23}Na line-width measurements.

Nuclear Magnetic Resonance. We have also observed the conformational impact of the micellar aqueous core by monitoring the N-H chemical shift of the peptide in reversed micelles and in bulk water by ^1H NMR. ^1H NMR data correlate well with CD data in terms of a high effective Na^+ concentration experienced by the peptide in the reversed micelle. The N-H chemical shifts of the peptide in AOT/octane reversed micelles at 8.3 H_2O/AOT are $\delta_{Gly^1\text{-}NH}$ 7.46, $\delta_{D\text{-}Ala\text{-}NH}$ 7.62, $\delta_{Gly^3\text{-}NH}$ 8.24. These are significantly different from their values in bulk water: $\delta_{Gly^1\text{-}NH}$ 7.53, $\delta_{D\text{-}Ala\text{-}NH}$ 7.83, $\delta_{Gly^3\text{-}NH}$ 8.33. Titration of the peptide in water with NaCl caused shifts analogous to those observed for the peptide in reversed micelles (Figure 6). By correlation of NH resonance positions in the reversed micelle with those observed in the NaCl titration in bulk water, we conclude that the effective Na^+ concentration in the AOT/octane reversed micelle at 8.3 H_2O/AOT is 1–2 M.

^{13}C NMR parameters have been well characterized for this peptide in a wide variety of solvents.[15] In the absence of salt, *cyclo*(Gly-Pro-Gly-D-Ala-Pro) adopts both a β and a γ turn (see above and Figure 5). Several parameters have previously been shown to be diagnostic for the presence of a γ turn; ^{13}C NMR chemical shifts for prolines in γ turns are particularly informative.[15] Specifically, an upfield proline C^β resonance position is usually indicative of a proline in a γ turn. The resonance of the proline C^β involved in the γ turn in this peptide is shifted upfield by 5 ppm from the usual position for a proline C^β in an X-Pro trans peptide bond. When salt is added to *cyclo*(Gly-Pro-Gly-D-Ala-Pro), the carbonyls from Pro[2], Gly[3], and Pro[5] come together

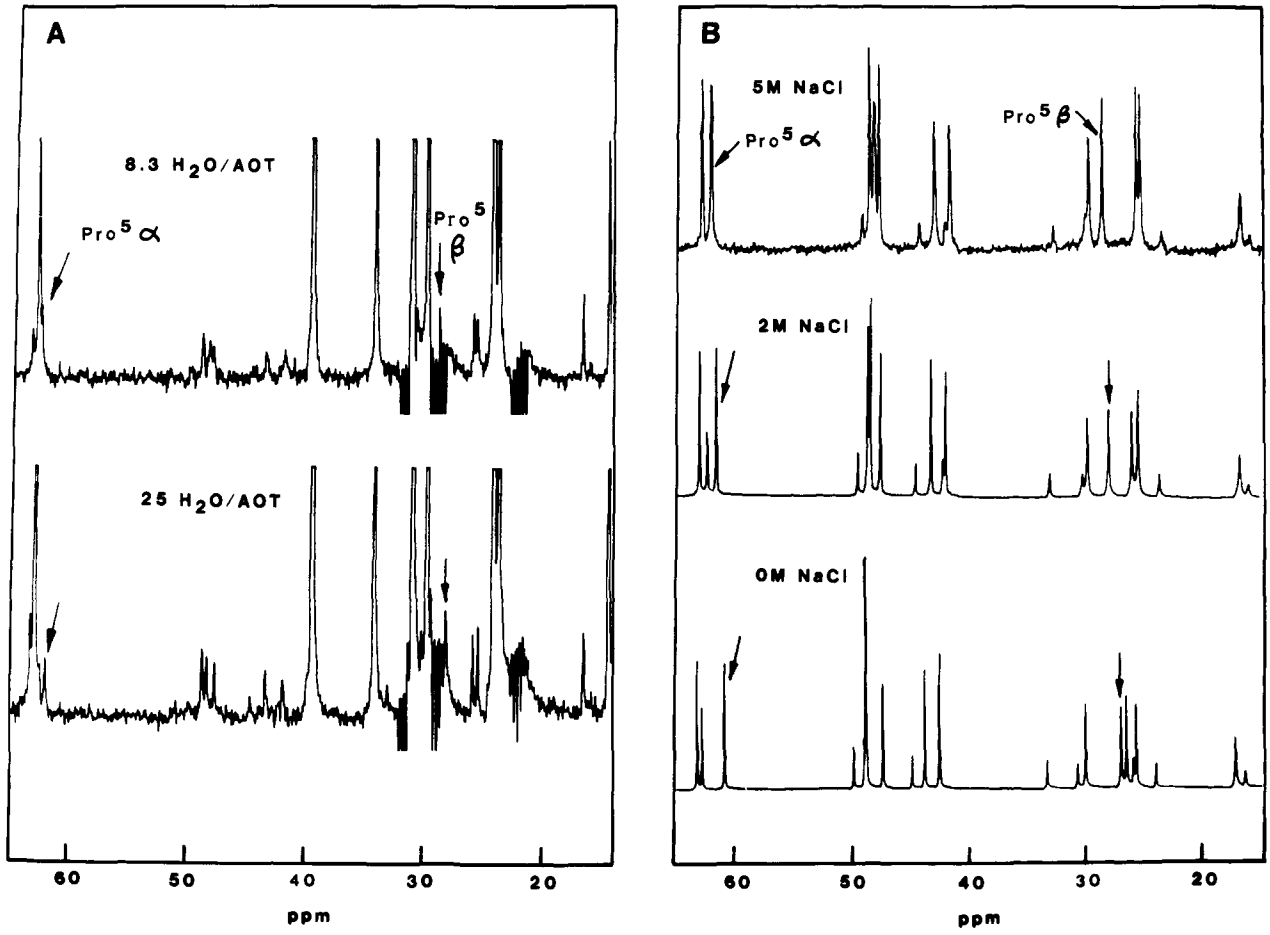

Figure 7. ^{13}C NMR (62.9 MHz) spectra of *cyclo*(Gly[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]): (A) in AOT reversed micelles as a function of water content (AOT 3% w/v, bulk solvent *n*-octane-d_{18}, peptide 0.67 M in water phase); (B) in aqueous solution as a function of NaCl concentration (peptide 0.67 M). Note particularly the changes in the Pro[5] C^α and C^β resonances. In reversed micelles at 8.3 H_2O/AOT, $\delta_{Pro^5C^\alpha}$ 62.21, $\delta_{Pro^5C^\beta}$ 28.70; at 25 H_2O/AOT, $\delta_{Pro^5C^\alpha}$ 61.72, $\delta_{Pro^5C^\beta}$ 28.10. In aqueous solution at O M NaCl, $\delta_{Pro^5C^\alpha}$ 60.16, $\delta_{Pro^5C^\beta}$ 26.20; at 2 M NaCl, $\delta_{Pro^5C^\alpha}$ 61.29, $\delta_{Pro^5C^\beta}$ 27.65; and at 5M NaCl, $\delta_{Pro^5C^\alpha}$ 61.97, $\delta_{Pro^5C^\beta}$ 28.45. The spectra for the peptide in reversed micelles were obtained by using an inversion recovery pulse sequence (180°–τ–90°) with τ = 1.0 s and a delay between pulse trains of 2.0 s, to minimize interference from solvent resonances.

to form an ion binding site. Both the β and the γ turns are lost. Consequently, the resonance of the proline C^{β} involved in the γ turn moves downfield to the more usual position. These shifts are clearly visible in spectra of this peptide in water with varying NaCl concentration (Figure 7B). In AOT reversed micelles at 8.3 H_2O/AOT, we have observed that the proline C^{β} resonance is downfield of the position characteristic of a γ turn (Figure 7a). The resonance position reflects the proportion of the cyclic peptide that exists as the cation complex. Addition of more water, raising the H_2O/AOT ratio to ca. 25, shifts the proline C^{β} resonance upfield by ca. 0.6 ppm, indicating that a greater proportion of the peptide is in the free conformation when larger pools of water are present than when smaller amounts of water are solubilized in reversed micelles. This parameter, like the CD spectral shape and the NH chemical shift, can be correlated with an effective Na^+ concentration in the reversed micelle. At 8.3 H_2O/AOT, the ^{13}C NMR shift for the Pro^5 C^{β} suggests an effective Na^+ concentration higher than that deduced from ^1H NMR and CD data: greater than 5 M NaCl.

Although the predominant influence of the water pools on the conformational distribution of the peptide is the high effective counterion concentration, some of the observations we have made indicate that there are additional conformational influences in the aqueous interfacial region in this system: (1) While the NH chemical shifts of peptide in reversed micelles show the same trends relative to free peptide in bulk water as those of the aqueous peptide titrated with NaCl, the observed shifts of the three NH resonances are not exactly matched by any one NaCl concentration. (2) Similarly, even though the shape and magnitude of the CD for peptide in reversed micelles is very close to that of peptide in NaCl solution, the observed curves are not superimposable on those at particular NaCl concentrations. (3) The ^{13}C NMR data for peptide in reversed micelles suggest a conformational perturbation consistent with a higher NaCl concentration than indicated by the other methods. These differences may be due to the presence of other conformational influences not yet defined. Possibilities include electric field effects or anionic head group interaction.

III. Influence of Peptide on Interfacial Water. No significant changes in the population distribution of interfacial and bulk water in AOT reversed micelles have been observed either by IR or by NMR techniques upon solubilization of peptide. Addition of the peptide solubilizate did not alter the position of the H_2O ^1H NMR resonance to within ± 0.005 ppm. IR absorption bands remained unaltered also.

Conclusions

cyclo(Gly-Pro-Gly-D-Ala-Pro), a hydrophilic peptide, has been solubilized in nonpolar solvents by reversed micelles. The conformation of the peptide was monitored as a function of water content in AOT reversed micelles by spectroscopic methods. All data indicated that the conformation of the peptide was perturbed predominantly by the high effective cation concentration in the aqueous core of the reversed micelle. The sodium counterions of the surfactant effectively increase the cation concentration in the water pool so that the peptide undergoes a conformational transition to an ion binding conformation. The decreased ellipticity at long wavelengths in CD spectra indicated the loss of the preferred γ turn conformation of the peptide in bulk water. In addition, ^1H and ^{13}C NMR data demonstrated that the peptide was in the ion binding conformation in reversed micelles. Conformational changes have been closely mimicked by NaCl titrations of the peptide in bulk water by CD and NMR techniques. At very low water to surfactant ratios, the effective Na^+ concentration was as high as 5 M. As the water pool became larger, the apparent Na^+ concentration decreased until at 8.3 H_2O/AOT the effective Na^+ concentration was 1–2 M in heptane or octane.

The water present in reversed micelles has been studied by IR. Our data support a simplistic treatment of the water pool as a population distribution between interfacial and bulk water. Our observations agree with previous reports[4,7,11,23] that a pool of more bulk-like water develops in the reversed micelle as hydration of surfactant becomes complete. Addition of peptide solubilizate did not significantly alter the population distribution of interfacial and bulk water in reversed micelles as monitored by IR and ^1H NMR.

Acknowledgment. We thank Jeffrey Lacy, Arlene Rockwell, and Arnold Hagler for helpful discussions and Lois Weyer for assistance in obtaining near-IR spectra. This work was supported by grants from the NIH (GM 27616), the American Cancer Society (Institutional funds), and Merck Sharp & Dohme Research Laboratories.

Registry No. *cyclo*(Gly-Pro-Gly-D-Ala-Pro), 66143-40-6.