CONFORMATIONS OF MODEL PEPTIDES IN MEMBRANE-MIMETIC ENVIRONMENTS

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ABSTRACT The influence of a membrane environment on the conformational energetics of a polypeptide chain has been investigated through studies of model peptides in a variety of membrane-mimetic media. Nuclear magnetic resonance (NMR) and circular dichroism (CD) data have been obtained for the peptides in bulk hydrophobic solvents, normal micelles, and reversed micelles. Several hydrophobic peptides which are sparingly soluble in water have been solubilized in aqueous sodium dodecyl sulfate (SDS) solution. NMR and CD data indicate that the micelle-solubilized peptides experience an environment with the conformational impact of bulk methanol, and have decreased conformational freedom. The site of residence of the peptides interacting with the micelles appears to be near the surfactant head groups, in a region permeated by water, and not in the micelle core. Strongly hydrophilic peptides have been solubilized in nonpolar solvents by reversed micelles. These peptides are located in small water pools in close association with the head groups of the surfactant. NMR and CD data show that there is a conformational impact of this interfacial water region on peptide solubilizates distinct from that of bulk water.

INTRODUCTION

The structure and function of membrane-interactive peptides and proteins are modulated by the heterogeneous environment of the membrane. The thermodynamic forces leading to the adoption of a particular three-dimensional structure by a protein in water are hydrophobic groups clustering and folding to expose a minimal hydrophobic surface area (1). Hydrophilic residues consequently reside at the surface (2). The architecture of an integral membrane protein, which has domains embedded in a region of low dielectric constant, will not follow the same "oil-drop" model. Peripheral membrane proteins must also take up distinct conformations resulting from specific interactions with the polar, interfacial region of a membrane.

We have been exploring the impact of a membrane environment on polypeptide structure through conformational studies of peptides in several membrane-mimetic media. The membrane models have been chosen to be experimentally tractable for analysis by high resolution NMR[1] and by CD. We have invoked micelles as workable models for membrane environments which are free of some of the drawbacks of vesicle systems. NMR in vesicles yields little detail due to the slow tumbling of the vesicle and any tightly associated solubilizate (3–6). By contrast, micelle aggregates behave hydrodynamically like moderately sized proteins (7). An additional heterogeneous system employed in our studies has been reversed micelles (8, 9). These aggregates of surfactants in nonpolar bulk solvents take up water in "pools" contained in the polar interior of the reversed micelle. Hydrophilic or amphiphilic molecules interact with the reversed micelles such that charged or highly polar groups are placed in the water pools. The water inside reversed micelles is strongly interacting with the head groups of the surfactant, and shows unusual properties relative to bulk water (10, 11). It closely resembles the layers of water immediately adjacent to a membrane interface (11), and we have used it to model this region. Results of our micelle studies have been compared to those that we obtain in bulk solvents of varying character. Intramolecular hydrogen bonding schemes which accommodate all N—H's are strongly favored in low dielectric bulk media. Folded structures which utilize proline as the middle residue of a γ-turn seem especially likely. Interactions of the hydrophobic model peptides and detergent micelles suggest that the peptides reside in a region near the surfactant headgroups, rather than in the micelle core, and experience a microenvironment similar in conformational impact to bulk methanol, but also with an immobilizing effect on the peptide. Additionally, we find that there is a distinct

[1] *Abbreviations used in this paper:* NMR, nuclear magnetic resonance; CD, circular dichroism; SDS, sodium dodecyl sulfate; AOT, aerosol OT; TMS, tetramethylsilane; DMSO, dimethyl sulfoxide; *t*-Boc, *tert*-butyloxycarbonyl; OBz, benzyl ester; δ, chemical shift in parts per million downfield from TMS.

impact of the interfacial water region on peptide conformation, and that this region does not stabilize the same conformational distribution as does bulk water.

MATERIALS AND METHODS

Peptides

Lysyl-aspartate dihydrobromide,[2] glycyl-glycyl-tyrosine, and gramicidin S dihydrochloride were obtained from commercial sources (U. S. Biochemicals, Cleveland, OH, for the dipeptide, and Sigma Chemical Co., St. Louis, MO, for the other two). All other peptides were synthesized using procedures which we have described in detail elsewhere (12,13). Amino acid precursors were purchased from Vega Biochemicals (Tucson, AZ), Chemical Dynamics (Plainfield, NJ), or Sigma. The identity and purity of intermediates and products were confirmed by thin layer chromatography, NMR, and infrared spectroscopy. All of the peptides used in the conformational studies are crystalline.

Other Chemicals

Sodium dodecyl sulfate (SDS) was electrophoresis grade (Bio-Rad Laboratories, Richmond, CA) and was used without further purification. Deuterated SDS (97% d_{25}) was custom synthesized by KOR Inc. (Cambridge, MA). Aerosol OT (AOT, sodium diisooctyl sulfosuccinate) was obtained from Fisher Scientific Co. (Fair Lawn, NJ), and was dried *in vacuo* at 40°C for 24 h prior to use. Egg lecithin was prepared by the method of Singleton et al. (14). Deuterated dipalmitoylphosphatidylcholine (perdeuterated, ca. 97%) was custom synthesized by Avanti Polar Lipids (Avanti Biochemicals, Birmingham, AL). Solvents for CD were spectrophotometric grade (Fisher). Deuterated solvents for NMR were purchased from Merck Chemical Division (Rahway, NJ), KOR, Aldrich Chemical Co. (Milwaukee, WI), or Norell Inc. (Landisville, NJ).

Preparation of Micelles and Reversed Micelles

Micelle samples were prepared by addition of dry, solid peptide to solutions of SDS in deionized, distilled water or in D_2O. To facilitate solubilization, samples were sonicated in a bath-type sonicator. Reversed micelles were formed from either of two amphiphiles, AOT or phosphatidylcholine. A dispersion of the amphiphile in the organic solvent was treated with the desired amount of water, followed by the solid peptide. Sonication facilitated solubilization of the peptide. The reversed micelle solutions were quite stable, although generally they were resonicated before any new study of a previously prepared solution was undertaken. Despite reasonable precautions to exclude moisture, samples occasionally exhibited bulk phase separation, presumably due to uptake of additional water from the atmosphere.

NMR Spectroscopy

Fourier transform NMR spectra were obtained on a Bruker WM250 spectrophotometer (Bruker Instruments, Billerica, MA) operating at a frequency of 250.13 MHz for ^1H and 62.90 MHz for ^{13}C. Samples were referenced to internal tetramethylsilane (TMS).

Circular Dichroism

CD spectra were obtained using a Jasco-Durrum J10 spectropolarimeter (Jasco, Inc., Easton, MD). Samples were usually 2×10^{-4} M and were run in cylindrical quartz-windowed cells (Precision Cells Inc., Hicksville, NJ) with a pathlength of 1 mm.

[2]All amino acids are of the L configuration unless noted otherwise.

RESULTS

Influence of Membrane-Mimetic Microenvironments on Peptide Conformation:

Normal Micelles. Solubilization of several peptides which are insoluble or only slightly soluble in water has been achieved in aqueous solutions of SDS (0.025 M). ^1H and ^{13}C NMR data and CD spectra of these micelle-solubilized peptides have been obtained. Comparison of these data with those observed for solutions of the peptides in bulk solvents allows an assessment of the overall impact of the micellar environment on the peptide conformation. This can differ from peptide to peptide because the site of residence of the peptide in the micelle may vary. It is

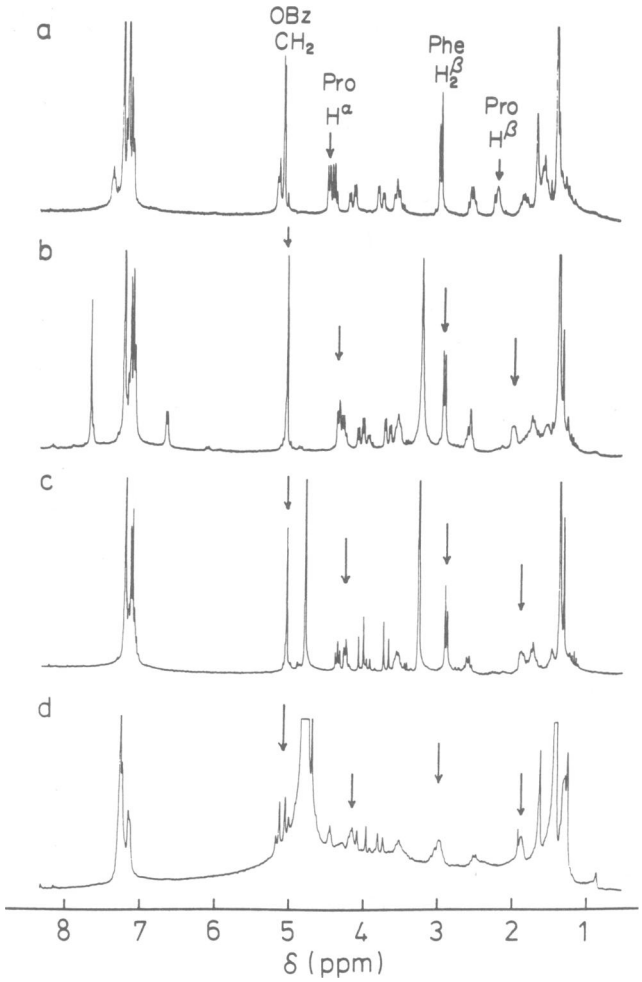

FIGURE 1 ^1H NMR spectra of *t*-Boc-D-Phe-Pro-Gly-OBz in bulk solvents and in aqueous SDS (0.025 M). Peptide concentration 0.02 M in *a*, $CDCl_3$; *b*, 25% DMSO-d_6:75% $CDCl_3$; *c*, CD_3OD; *d*, 0.025 M SDS in D_2O. Note that there is a shift in the Pro H^β resonance to lower field from *a* to *c*, and that its position in *d* corresponds closely to that in *c*. Note further in *d* that the CH_2's of the OBz appear as a quartet and that the Phe CH_2 is distinctly broader than in the bulk solvent spectra.

necessary to establish that the peptide is actually partitioning into the micelle. We have begun preliminary studies of the partitioning using a high pressure liquid chromatographic method (15) but have not as yet obtained useful data. The strongest evidence that the peptides reside in intimate association with the micelles is (*a*) the facilitated solubilization of the peptides in SDS solution relative to water; (*b*) the observation of broadened NMR signals (due to the slower overall tumbling time) for the micelle-associated peptide than for the free peptide; and (*c*) the alterations in conformational parameters in SDS relative to bulk water (in those cases where both environments are observable).

Four peptides have been studied in SDS solutions: two linear tripeptides, *t*-Boc-Gly-Pro-Gly-OBz and *t*-Boc-D-Phe-Pro-Gly-OBz, one linear pentapeptide, *t*-Boc-D-Phe-Pro-Ala-Pro-Gly-OBz, and one cyclic pentapeptide, cyclo(Ala-Pro-Gly-D-Phe-Pro). All have been examined by CD and their spectra compared to those in bulk solvents; two have been studied by NMR as well. The linear peptides chosen take up "folded" γ-turn conformations in chloroform, a low dielectric bulk solvent, and alter their conformational distribution as a function of bulk solvent.[3] The cyclic peptide is in a solvent-dependent equilibrium between an all-*trans* and a one-*cis* form.

The behavior of the peptides on attempted solubilization was not uniform. *t*-Boc-D-Phe-Pro-Gly-OBz was insoluble in water, and *t*-Boc-Gly-Pro-Gly-OBz was of very limited solubility ($< 2 \times 10^{-4}$ M). Both of these tripeptides were solubilized, though slowly, into SDS solutions at peptide concentrations of 2×10^{-4} M. It was not possible to solubilize completely higher concentrations of these peptides in SDS solution. By contrast, the linear and the cyclic pentapeptides were readily soluble in SDS solutions at peptide concentrations of 0.03 M. These latter peptides were more water soluble than the tripeptides; but their solubilities, like those of the tripeptides, were enhanced in the presence of SDS.

CD spectra for the peptides in SDS solution displayed greater ellipticities and narrower bandwidths than those in bulk solvents. These observations may be taken to mean less conformational averaging in SDS solution, but a more detailed conformational interpretation is not possible on the basis of CD alone.

^{1}H NMR spectra of the tripeptide *t*-Boc-D-Phe-Pro-Gly-OBz were examined in several bulk solvents and in SDS micellar media (Fig. 1). It was possible to observe the spectral parameters which have been correlated with the occurrence of a γ-turn (12, 13),[3] and to compare other spectral data for these various samples. Upon addition of DMSO-d_6 to a $CDCl_3$ solution of this tripeptide, the γ-turn indicators (low field H^α and H^β resonances) show a trend towards more usual, nonturn values (see Fig. 1 *b*). The disruption of the γ-turn is more marked in methanol solution. While the values of these parameters in SDS solution are essentially the same as those in methanol, there are notable differences. The spectrum of the tripeptide in SDS solution, while overall somewhat broader than those in bulk solvents, shows fine structure and peak shapes resembling those in methanol except for the methylenes of the benzyl ester and of the phenylalanines. These two groups become markedly more AB in character in the micelle-solubilized peptide (note the AB quartet at 5 ppm, and the Phe $C^\beta H_2$ at 3 ppm in the SDS spectrum, Fig. 1 *d*).

Both ^{1}H and ^{13}C NMR data have been obtained for SDS solubilized cyclo(Ala-Pro-Gly-D-Phe-Pro). As in the case of the linear peptide, the chemical shift data indicate that the impact of the micellar microenvironment is similar to that of the bulk solvents methanol, acetonitrile, or dimethyl sulfoxide (see Fig. 2 and Table I). The spectra in

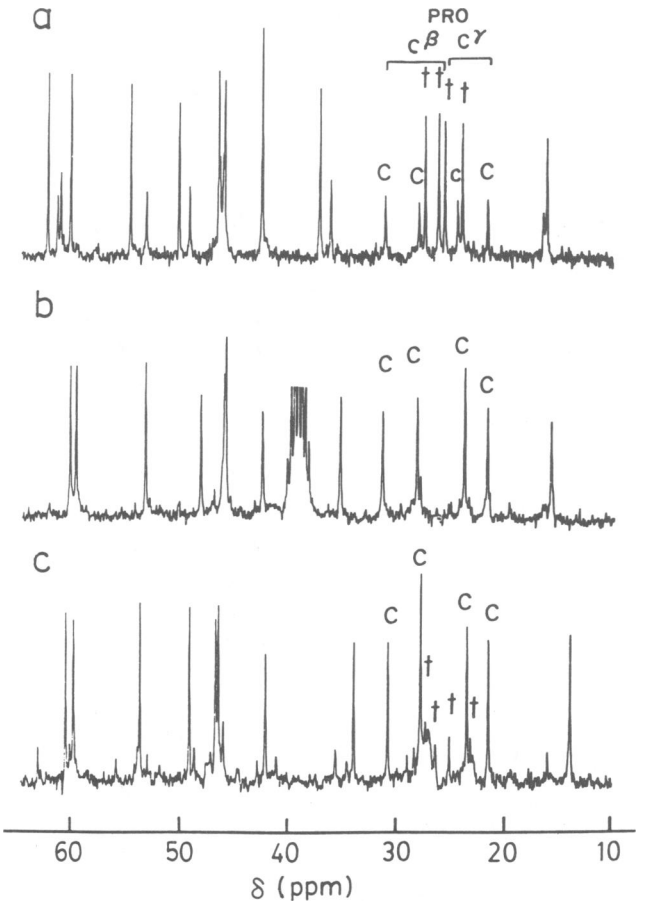

FIGURE 2 Upfield regions of ^{13}C NMR spectra of cyclo(Ala-Pro-Gly-D-Phe-Pro) showing the solvent-dependent equilibrium between the all-*trans* and one-*cis* conformers. Peptide concentration 0.02 M in *a*, $CDCl_3$; *b*, DMSO-d_6; and *c*, 0.025 M SDS in D_2O. Proline C^β and C^γ resonances of the one-*cis* conformer are designated by "c", and those for the all-*trans* by "t". For *cis/trans* ratios and peak positions see Table I.

[3]Gierasch, L. M., P. I. Watnick, J. E. Lacy, and A. L. Rockwell. γ-turns in model peptides: implications for reverse turns in hydrophobic environments. Manuscript in preparation.

TABLE I
^{13}C NMR DATA FOR PROLINES IN CYCLO(ALA-PRO-GLY-D-PHE-PRO): SOLVENT DEPENDENCE OF CONFORMATIONAL DISTRIBUTION*

Solvent	Conformer	%‡	$^{\delta}C\beta$	$^{\delta}C\gamma$	$\Delta\delta\beta\gamma$
$CDCl_3$	all-*trans*	70	28.1	24.5	3.6
			26.8	26.2	0.6
	one-*cis*	30	31.8	22.2	9.6
			28.6	25.0	3.6
CD_3CN	all-*trans*	50	29.0	25.2	3.8
			27.6	26.7	0.9
	one-*cis*	50	32.4	23.0	9.4
			29.7	25.4	4.3
CD_3OD	all-*trans*	35	30.6	25.6	5.0
			28.2	27.3	0.9
	one-*cis*	65	32.9	23.3	9.6
			29.8	25.6	4.2
DMSO-d_6	all-*trans*	0	—	—	—
	one-*cis*	~100	31.6	21.8	9.8
			28.4	23.9	4.5
0.025 M SDS (in D_2O)	all-*trans*	20	29.4	25.2	4.2
			29.1	27.2	1.9
	one-*cis*	80	33.0	23.5	9.5
			29.9	25.5	4.4

*Concentration ~0.05 M. Chemical shift (δ) in ppm.
‡Percent of each conformer was calculated by comparing the heights of well-separated peaks. Several resonances were measured for each case. Estimated error ±5%.

chloroform are markedly different. Furthermore, for this peptide, the solvent-dependent ratio between all-*trans* and one-*cis* conformers provides an additional means of probing the microenvironment. The ^{13}C NMR spectrum of the SDS solution (Fig. 2 *c*) shows a distribution between the two conformations which is close to that in methanol. In DMSO-d_6, the one-*cis* form is favored to the exclusion of the all-*trans* form.

Reversed Micelles. Peptides which would normally not be soluble in nonpolar bulk solvents (e.g. hexane, heptane, chloroform, methylene chloride, or carbon tetrachloride) have been solubilized in solutions containing the surfactant AOT and water. These samples consist of reversed micelles containing small pockets of water which associates strongly with the charged head groups of the surfactant, and thus resembles water in the interfacial region of a membrane (see Fig. 3) (11). Among the peptides studied in this system are Lys-Asp(dihydrobromide), Gly-Gly-Tyr, Gly-Pro-Gly(hydrochloride), gramicidin S(dihydrochloride), and cyclo(Gly-Pro-Gly-D-Ala-Pro). Preliminary ^1H NMR experiments were carried out with the dipeptide to assess the feasibility of this approach. Although resonances of the surfactant obscured large portions of the spectrum, it was possible to see peptide resonances, particularly those of the exchangeable protons at low field. This result demonstrates one practical advantage of this type of sample, that NMR spectra can be obtained in H_2O. Further studies in AOT focused on CD.

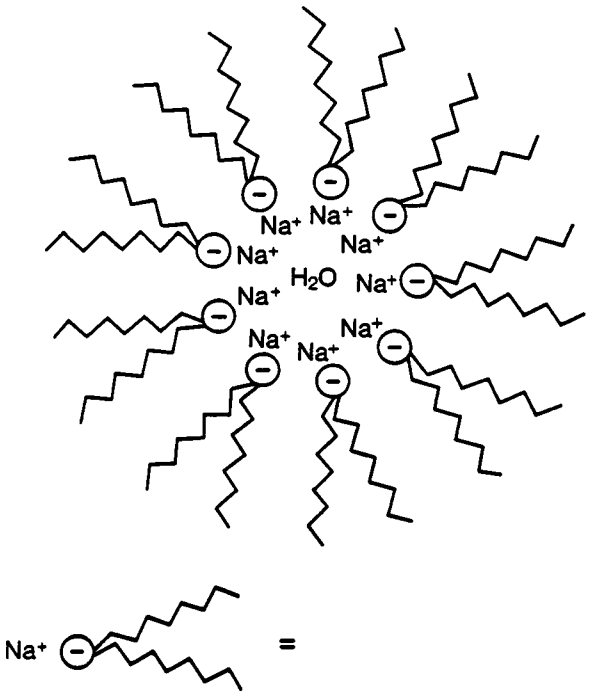

FIGURE 3 Diagram of an AOT reversed micelle, showing the "nonpolar out-polar in" structure and the included water pool. (Adapted from reference 11).

The CD spectra for peptides solubilized in reversed micelles were generally distinct from those for bulk water solutions (see Figs. 4 and 5). Addition of increasing amounts of water throughout a range which does not lead to destabilization of the reversed micelle (up to 6%) caused the spectra to approach those in bulk water. Only in the case of Gly-Pro-Gly was the CD spectrum observed to be the same for peptide solubilized in reversed micelles and dissolved in bulk water.

The spectral changes seen for cyclo(Gly-Pro-Gly-D-Ala-Pro) are particularly interesting. The spectrum in AOT reversed micelles resembles the reported CD for this peptide upon interaction with cations (12). Interpretation of this qualitative observation requires that further studies be done, since several conformational adjustments accompany the ion binding. Not only does the major all-*trans* conformer rearrange itself to present a binding face to the cation, but also an energetically competitive one-*cis* isomer, which also binds cations, increases in concentration as salt is added (12). In the present case, it is possible that binding interactions account for the altered CD, or that

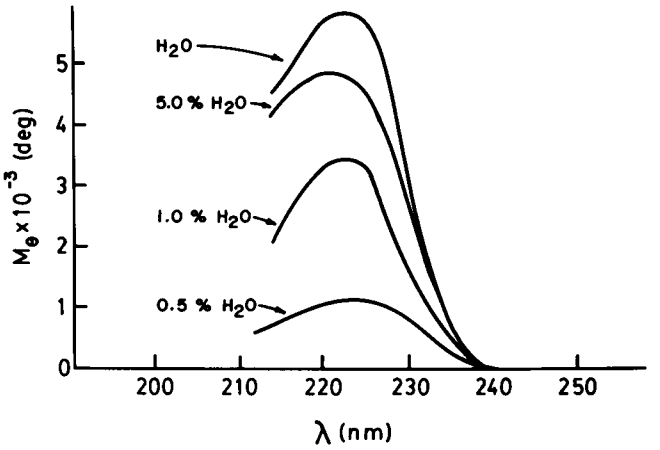

FIGURE 4 CD spectra of Gly-Gly-Tyr (1.1×10^{-3} M) in bulk water and in AOT reversed micelles (3% AOT wt/vol in heptane). The amount of water present is indicated in the Figure. Note that the peptide CD in reversed micelles is distinct from that in bulk water, and that it approaches the bulk water spectrum as the water content of the micellar system increases.

more one-*cis* conformer occurs in the micellar water pool than in bulk water. Both of these intriguing possibilities are consistent with the observed return to a bulk water spectrum as the water content of the sample is increased.

The naturally-occurring cyclic decapeptide gramicidin S, postulated to be membrane-interactive, contains both

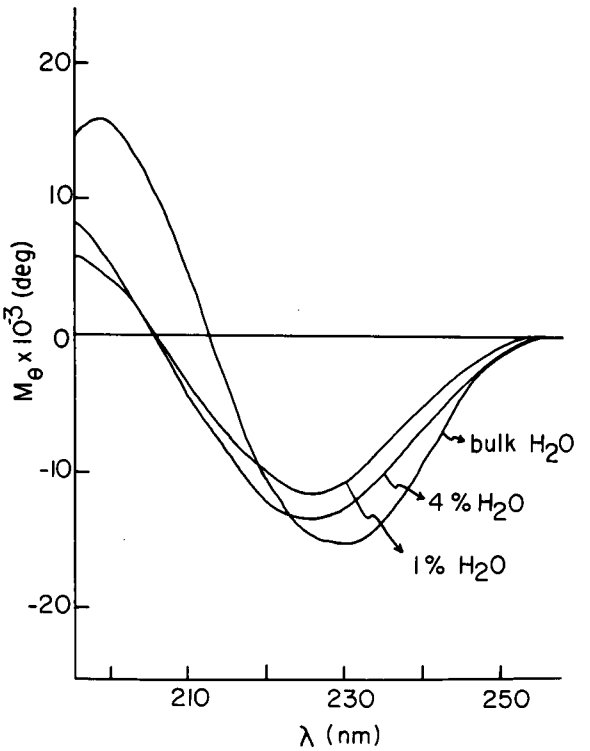

FIGURE 5 CD spectra of cyclo(Gly-Pro-Gly-D-Ala-Pro) (0.89×10^{-3} M) in bulk water and in AOT reversed micelles (3% AOT wt/vol in heptane). Increasing water content leads to a more bulk water-like spectrum in the reversed micelle sample.

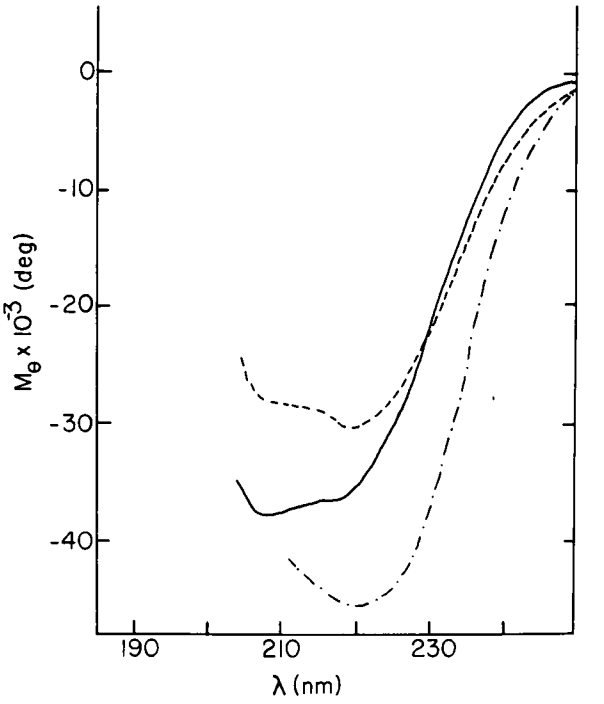

FIGURE 6 CD spectra of gramicidin S, a cyclic decapeptide antibiotic of sequence $(Val\text{-}Orn\text{-}Leu\text{-}D\text{-}Phe\text{-}Pro)_2$, in bulk water (solid line), and in micellar media; —·—·, in 0.025 M aqueous SDS solution; and ---, in 3% AOT, 5% H_2O, in heptane.

hydrophobic and hydrophilic residues. This molecule was examined in a reversed micelle environment. CD data were obtained in AOT-reversed micelles in hexane, in SDS micelles, and in bulk solvents (see Fig. 6). The various environments can be seen to have distinct impacts on the peptide. Preliminary ^1H NMR spectra of this peptide have been obtained using reversed micelles formed from perdeuterated dipalmitoyl phosphatidylcholine in carbon tetrachloride (see Fig. 7). The exchangeable proton resonances are visible (and disappear in a sample prepared from D_2O), but detailed analysis has not as yet been accomplished.

DISCUSSION

Influence of a Membrane-mimetic Microenvironment on Peptide Conformation

Normal Micelles. The peptides which we examined in SDS solution are hydrophobic and sparingly soluble in water; all are clearly solubilized through interaction with the micellar phase. The types of interactions that may be operative in this system are the same ones that are important in protein-lipid associations in membranes, namely: surface interactions due to head groups or counterions; interfacial absorption at the micelle-water boundary; partitioning into the hydrophobic core; or co-micelli-

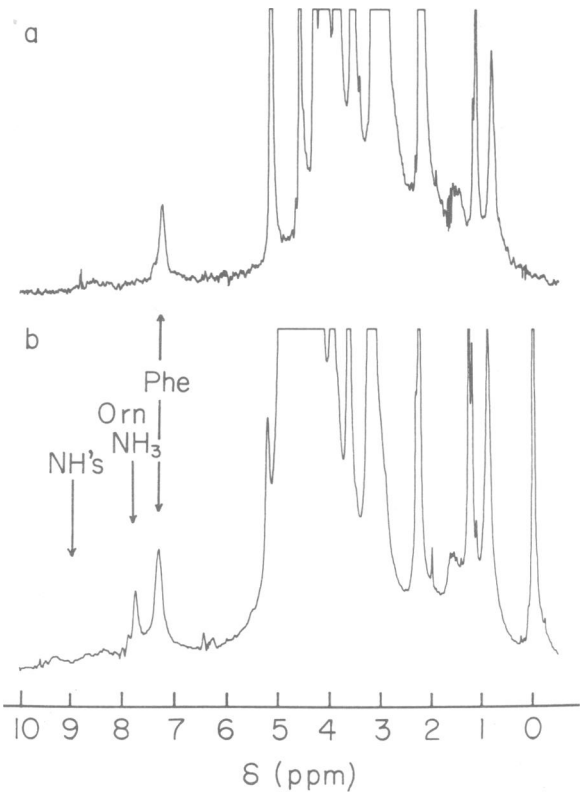

FIGURE 7 ^1H NMR spectra of gramicidin S (0.026 M) in dipalmitoyl phosphatidylcholine reversed micelles (50 mg phosphatidylcholine in 0.5 ml CCl_4 + 1% water). *a*, with D_2O; *b*, with H_2O. Note the loss of intensity in the downfield region due to exchange of NH's and Orn NH_3^+ protons in the D_2O sample. The spectrum in *b* is the result of 6970 accumulations with a block size of 32 K. A low receiver gain was used to minimize spurious folded peaks from the strong solvent resonance. (Note that there are still some glitches visible, e.g., at 6.2, 8.0, and 9.5 ppm.) Spectrum *a* is the result of 2,000 16 K acquisitions.

zation due to both hydrophobic and hydrophilic interactions.[4] Comparisons with bulk solvent data indicate that the predominant impact of the micellar environment on peptide conformation is effectively like that of methanol. Furthermore, the enhanced CD extrema and the example of decreased aromatic ring mobility (*t*-Boc-D-Phe-Pro-Gly-OBz) indicate that the micelle-solubilized peptide has decreased conformational freedom relative to the peptide in bulk methanol. Enhanced CD's have been observed for several membrane-interactive proteins in detergent solution relative to their CD's in bulk solvent (16–18); our finding of reduced conformational freedom, especially of aromatic rings, provides a plausible molecular interpretation.

Previous studies with nonpeptide micellar solubilizates reveal that preferred sites of residence are often within a few carbons of the head groups (7, 19–21), where water molecules are thought to penetrate readily (21). Several micelle solubilizates have been reported to experience an effective solvent environment similar to, but more ordered than, bulk methanol (20, 22). Although we cannot yet specify the site of residence of the peptides, the interfacial region seems likely. Further work with varying concentrations of surfactant and peptide, with other surfactants and counterions, and with spin-labeled surfactants or aqueous probes, will shed light on the details of the peptide-micelle interaction. Our results are consistent with current pictures of micelle structure which describe very dynamic aggregates, with penetration of water between the head groups of the amphiphiles, or "fjords" (21), and with rapid monomer-micelle exchange rates (monomer residence times of $\sim 10^{-5}$ s [7]) (see Fig. 8). The observation of a single set of somewhat broadened peptide resonances (see Fig. 1) in SDS solutions indicates that peptide molecules undergo fast exchange (on the NMR time scale) between micelle-"resident" and free states, as do SDS monomers. By contrast, residence times of lipids in bilayers (vesicles) are much longer (several hours [23]). The assembly of amphiphiles in a bilayer appears to be significantly more static, and the surface is most likely not so irregular and accessible to water as in a micelle. These differences are consistent with previous studies of proteins in detergents, where both reactivity and conformational parameters were different from vesicle-protein preparations (24). However, extension of the conformational impact of these two ordered supramolecular assemblies to biological membranes is not straightforward. Recent suggestions of lipid polymorphism in biological membranes (25) point out the necessity to understand the nature of interactions between peptides or proteins and amphiphiles in a variety of ordered systems.

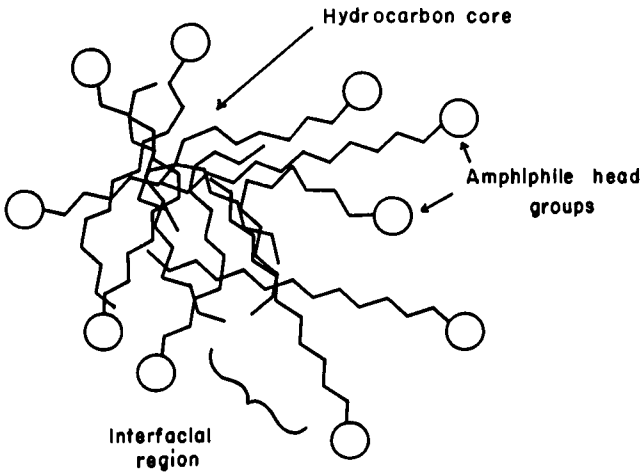

FIGURE 8 Dynamic model for the SDS micelle characterized by an irregular surface contour and significant penetration of the hydrocarbon region by water. This model is consistent with the conclusions reached in this study about the conformational impact of the micelle environment on a peptide solubilizate. (Figure adapted from reference 19).

[4]McIntire, G. L., D. M. Chiappardi, R. L. Casselberry, and H. N. Blount. Electrochemistry in ordered systems. II. Electrochemical and spectroscopic examinations of the interactions between nitrobenzene and anionic, cationic, and nonionic micelles. Manuscript in preparation.

Reversed Micelles. The use of reversed micelles has facilitated examination of an environment resembling the water-membrane interface (11). Previous work had established that the water pools inside reversed micelles differ markedly from bulk water (10, 11). A portion of this water is strongly associated with the surfactant head groups (6 H_2O per head group in AOT, corresponding to 1% vol/vol H_2O in 3% AOT wt/vol [11], or 12 H_2O per head group in lecithin reversed micelles [26]), and, like the Stern layer and Guoy-Chapman electrical double layer of normal micelles, this water pool is enriched in the counterions of the head groups (11). Our results indicate a conformational impact of this highly ordered, interactive water, which is mollified as the pool size increases. Possible origins of this conformational impact include peptide-surfactant interactions (including of counterions), peptide-peptide interactions as a consequence of the high effective peptide concentration (0.1–2.5 M), hindered mobility of the peptides due to the small size of the water pool (20–80 Å [11]), and peptide-water interactions involving the unusual interfacial water. The resemblance of the CD for the cyclic pentapeptide in reversed micelles to that observed when cation is bound by the peptide (12) favors peptide-surfactant interactions. However, additional data are needed to reach a more definitive conclusion.

CONCLUSIONS

We have carried out preliminary NMR and CD studies of peptides solubilized in solutions of normal micelles and reversed micelles. These systems provide tractable samples for spectral analysis, and illustrate many of the interactions which are critical to the assembly of proteins and lipids in membranes. Our results show that the interfacial region is a favorable one for a small, hydrophobic peptide, and that in this region such a molecule experiences an effective solvation similar to bulk methanol, but with reduced conformational freedom. Reversed micelle studies demonstrate that the water near the interface has a distinct conformational impact on a peptide, and may influence the peptide as a result of locally high concentrations of surfactant counterions. Each of these necessarily qualitative conclusions suggests many further experiments, and directs attention to novel aspects of peptide-amphiphile interactions in ordered systems.

We thank Professor H. N. Blount for valuable discussions.

This research was supported by grant GM 27616 from the U.S. Public Health Service and a grant from the University of Delaware Research Foundation. Acknowledgment is also made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this work.

Received for publication 25 April 1981.

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DISCUSSION

Session Chairman: Thomas E. Thompson *Scribe*: Donald M. Laird

DEBER: How far can you take this approach? Is it really possible to get specific or molecular-level information about the actual conformational structure of the micelle-peptide complex? For example, can you say how much of the peptide is actually "bound" to the micelle rather than "solubilized?" Can you tell which specific groups on the peptide may be interacting with the micelle and can you tell if it is buried or exposed?

GIERASCH: We are presently working on, and are going to be doing, experiments that will resolve some of these questions. Larry Brown has been doing some work to define the site of residence of a molecule in a micelle by using paramagnetic probes at different depths in the micelle. I think that this is the most promising experiment for determining where in the micelle the peptide actually resides. One can also monitor the perturbation of the surfactant by the peptide, using relaxation times and chemical shift changes. To study binding and how much peptide is actually resident in the micelle, one can determine the tumbling time of the peptide, i.e., whether it is tumbling with the micelle or not. The line broadening obtained indicates that some of the peptide population is tumbling with the micelle at least some of the time. Furthermore, an experiment that can be done to determine the partition coefficient of something in and out of SDS is high-pressure liquid chromatography (HPLC) with an SDS solution as the mobile phase. In a reverse-phase system, as one increases the concentration of SDS and hence the concentration of micelles in the mobile phase, one tends to cause shorter retention times for molecules that interact with the micelle. The mobile phase becomes more and more attractive to it. From this, one can calculate the partition coefficient.

DEBER: You state in your paper that the water in the reversed micelles is characterized by a rather high cation concentration due to the counterions on the inside headgroups and also that a cation-binding cyclic peptide changes its conformation and becomes in effect the cation complex when it is in this interfacial water region. What is the relationship between the peptide and the membrane in this situation? Could you merely have trapped the peptide in a rather high sodium chloride concentration medium and therefore have a small amount of the peptide-cation complex that happens to be located inside of a reversed micelle, or is there a more specific relationship between the peptide and micelle?

GIERASCH: The layer of water adjacent to the headgroups is a special characteristic of a charged interface. It does not have to possess a net charge. Any charged interface including a zwitterionic head group on a lipid would give you the same type of Gouy-Chapman layer which has unusual electrical properties. Any processes which take place at the interface would involve the same sorts of conformational impact that we are seeing with this peptide. Many things go on within a 10-Å layer adjacent to the interface in any membrane system. Many membrane proteins have their hydrophilic components extended into this layer. Therefore it is important to understand what is going on in this region. The character of the water is also changed in that it is highly-immobilized water that is not water-water hydrogen-bonded. I think that this can also have an impact on the functional aspects of processes taking place in that region.

DEBER: You say that when you add more water to the system that the properties approach that of bulk water. Are the reverse micelles expanding to accommodate more water inside of them?

GIERASCH: Yes, exactly. The size is defined by the ratio of water to AOT present. It takes ~0.8% water to hydrate fully the headgroups in the AOT system at 3% surfactant concentration. As you get beyond this you are building up a pool of water which is "normal." That is to say, it is involved in water-water hydrogen bonding and it behaves as expected for bulk water. What we are seeing with this peptide is an occupation of the bulk pool as well, so there is an exchange. However, if you tie something into that region, it doesn't have a choice and will be experiencing the interfacial character all of the time.

WALLACE: What is the partition coefficient of the peptide into the reversed micelles? That is, what proportion of the molecules you are viewing are actually experiencing the water phase vs. those experiencing the hydrophobic lipid phase? Secondly, how are you going to probe whether the peptides are looking at the interfacial region of the micelle vs. the interior of the micelle? Have you examined the use of paramagnetic probes to determine the location of the peptides perpendicular to the vesicle surface? Finally and possibly most important, what evidence is there that the structures you are looking at have anything to do with the structure in actual bilayer membranes?

GIERASCH: The peptides in the normal micelle system that we have been working with are not water-soluble or only sparingly soluble so that one can anticipate a favored partitioning into the hydrophobic region. We will be checking this with bulk dodecanol two-phase partitioning experiments and also with the HPLC experiment which I have described. In the reversed micelles the situation is even more extreme since we are dealing with a heptane bulk phase and initially with charged peptides. We can be sure that they are not in the bulk phase. They might be interfacially-resident if they have some hydrophobic groups on them, but these peptides are small enough that I don't think that any hydrophobicity of the side chain would dominate their behavior. The cyclic pentapeptide that we have worked with is also not soluble in heptane, so it is definitely in the reversed micelle.

I have already addressed the question concerning probes, and believe that your work on gramicidin A in vesicle systems using paramagnetic probes and line broadening exemplifies the way to probe the depth of residence of a molecule in a bilayer.

The relationship of the conformations observed in micelles to those expected in vesicles or multilayers is something which needs to be checked. I think that one can use CD as a comparison, although care must be taken to avoid light-scattering effects. Another possibility, which we are working on, is solid-state NMR experiments with multilayers. We can synthesize these peptides enriched, and the solid-state NMR technology with magic-angle spinning allows us to work with samples that are not rapidly averaging the chemical shift anisotropy and the dipole-dipole interactions between spins. It is also possible to vary the character of the micelle by varying the components that are present, i.e., addition of a third component to a surfactant-water mixture in order to tighten up the

interface. This is the so-called "swollen-micelle" concept, where short-chain alcohols are involved as a third component and serve to plug up the holes. I think that one of the big differences between a micelle and a vesicle, in addition to differing dynamics, is the degree of water penetration into the hydrocarbon region. This is greater in the micelle than it is in a vesicle. We will be looking to see whether swollen micelles cause any different conformational changes from what we've seen in unplugged micelles.

LOVRIEN: Table I and the recent work by Brandts (L-n. Lin and J. F. Brandts. 1980. *Biochemistry.* 19:3055–3059) show that the presence of a proline in a peptide oftentimes imposes really major time-dependencies on the ability of the peptide to switch conformations, which in turn depends on which amino acids are near that proline in addition to its environment. In your ability to switch environments around this cyclic peptide, do you see any time dependencies which would reflect proline control over rate processes like this?

GIERASCH: We are precluded from doing that because of the length of the time involved in obtaining the NMR spectra. What we sample is the equilibrium that is established after a certain period of time.

LOVRIEN: What if you remain at a single wavelength in the CD spectra?

GIERASCH: We could do that, and although CD is not the most diagnostic technique for the *cis-trans* equilibrium, we might expect to see a difference.

DEGRADO: Can you comment on the conformational impact on peptides of the reversed micelle-water interface compared to your normal micelle-water interfaces?

GIERASCH: For the moment we have asked two different questions since we have focused on the interfacial water in the reversed micelle system. Clearly, you have an interface in the micelle as well. For a hydrophilic peptide you would see the same types of phenomena that we see in the reversed micelle. The advantage of the reversed micelle is that you are looking at a very small percentage of the water—only that which is affected by the interface. A comparison between the two systems could be made with amphiphilic molecules that tend to penetrate into the surfactant hydrocarbon region. Gramicidin S is the only case which was examined in both systems. The preliminary data demonstrate a difference in the circular dichroism spectrum, but we are not able to make a conformational statement without supporting NMR data.

DEGRADO: On your reverse-phase HPLC separation, can you rule out the possibility that what you are seeing is the SDS binding to the reverse phase column (with increasing concentrations) and thus affecting your separation?

GIERASCH: It does that, and it actually coats the column. The column is affected by the SDS, but surprisingly you get very reproducible results even with increasing concentrations. It is actually possible to force this situation and get a stationary phase that has been intentionally coated with a surfactant or lipid. This may be a possible system for looking at the partitioning in and out of the lipid phase.

MOORE: What would the actual ionic concentration be, in terms of molarity, in the interior of the reversed micelle? Have you any data concerning the effective dielectric constant of the water in the reversed micelle?

GIERASCH: The overall concentration of sodium AOT in the bulk phase is 0.067 M in the system we have worked with. However, if you deal with the minimum water size that we have used, you have on the order of 6–12 M. In this case you have nothing but interface present, with all the water serving to hydrate the head groups, and as you increase the water concentration, you get up to 1–3 M concentrations corresponding to what you are dealing with in the high-percentage water systems. Using CD as a monitor of what the peptide experiences, we pointed out the similarity in a 0.5–1% water sample with bulk 5 M sodium chloride. This is slightly lower than the maximum sodium concentration that we ever see. The effective dielectric constant of the bound water has been quoted to be ~75–77 (Wong et al. 1977. *J. Amer. Chem. Soc.* 99:4730–4735), which is not that much different from bulk water. The water is unique in its behavior, but it is difficult to use a bulk property to describe it.

PARSEGIAN: This is to the point of water activity near the lipid-water interface. Peter Rand and I, with several co-workers, have completed a series of papers on the activity of water near several phospholipid/water interfaces (LeNeveu et al. 1977. *Biophys. J.* 18:209–230; Cowley et al. 1978. *Biochemistry.* 17:3163; Parsegian et al. 1978. *Proc. Natl. Acad. Sci. U.S.A.* 76:2750–2754; Lis et al. 1981. *Biochemistry.* 20:1761–1770; Rand et al. 1980. *Canad. J. Bioch.* 58:959–968; Rand. 1981. *Annu. Revs. Biophys. Bioeng.* 10:277–314; Lis et al. 1982. *Biophys. J.*. 37. In press.). We find that the one or two water molecules nearest the zwitterionic polar groups of phosphatidylcholine are bound with much the tenacity one expects of water next to a charged group, that is to say activities of the order of kilocalories/mole different from bulk water. But there is an exponential decay in this perturbation with a decay distance about the size of a water molecule, extending 20 or 30 Å from the interface. What we are able to do is to measure molecule-by-molecule the work of removing water from that interface.

The converse of that removal measurement is to ask, "what kind of a solvent is that boundary water?". That is to say, if a solute is to come into the boundary region, and to displace this water, which kind of behavior do we see? There are two qualitatively different kinds of behavior observed. Small sugars are excluded by 12–15 of the water molecules/phospholipid. But there is no evidence at all that ions are unable to enter any place in the aqueous boundary region. If you are a charged molecule, that water acts like a very nice solvent.

Another class of experiments I am surprised people have not mentioned is the diffusion experiments that have been done in the aqueous clefts in the lamellar lattices formed by phospholipids. There again the magic number seems to be ~12–15 mol of water/phosphatidylcholine show funny diffusion behavior. This is either the thermal diffusivity of water itself (Chan and Pershan. 1978. *Biophys. J.* 23:427–449) or the movement of solutes through that region. (Benyoucef et al. 1978. *Biochim. Biophys. Acta.* 507:219–299). The remaining water acts as bulk water in these probes.

GIERASCH: That is very interesting. It is interesting that peptides get in there more than the sugars from what you said. It is also true that, by relaxation-time criteria, water is immobilized, so a very clean-cut property, consistent with the solubility and diffusion, results.

LITMAN: In Fig. 5 you show CD spectra that show increasing red-shift with increasing water content; under these conditions your micelles are increasing in size. I would like to know how you have been able to rule out scattering effects in your CD spectra?

GIERASCH:The reversed micelles that we have dealt with, between 0.5–3% water and 3% AOT, are not very large particles. They have radii from ~20 to ~70 Å (Wong et al. 1977). In addition, the correspondence of that series of spectra with the perturbation by salt is so striking that for the moment we are reassured that the changes are not a result of the increase in size.

WOLBER: The suggestion has been put forward that molecules which change the structure of water, in particular a lot of molecules used as energetic uncouplers or those used to measure *trans*-membrane poten-

tials, change the structure of the interfacial region. Have you done any work with these types of molecules or do you have any plans to?

GIERASCH: We have not, but there has been work done with things like *p*-nitrophenol and other species, which are not very different from DNP and other uncouplers that are interfacially resident. You can vary the pKa of the molecule by putting a hydrophobic anchor on it and causing it to sit at the interface or by letting it sample the interior of the reversed micelle. Striking changes in its properties (focusing on the probe itself, not on the water) are observed.

WOLBER: I was wondering what would happen to your NMR signal from the interfacially bound water in the systems that are not very hydrated depending on whether you were able to get enough of some uncoupler in there.

L. BROWN: In order to make the kinds of conclusions that you have reached it is imperative to know exactly what you have in your system. For instance, I wonder whether it makes sense to talk about a peptide bound to an interface when you have a 1:1 ratio of peptide to detergent. Secondly, if you are going to interpret line width as being due to the peptide bound to the micelle or reversed micelle you must know how much of it is bound so that you know that it isn't an exchange between bound and free. And you must know whether you have a unique conformation on the micelle.

GIERASCH: I do agree with your point. In the system with a 1:1 ratio we are seeing a population that is unbound and a population that is interacting with the micelle. These are dynamically averaging so that the changes in our spectral parameters indicate shifts between the two populations. This allows us to get an idea of the impact of the environment. In the complementary CD experiments, where we do have the limited population criteria (<1 peptide/micelle) met for the peptide and micelle, we can interpret more directly what we are seeing.

MOORE: I would like to return to the subject concerning the state of the water in the reversed micelle. From the previously discussed material I had the impression that the dielectric constant and molarity seemed relatively normal; however you mentioned that the NMR data unequivocally showed that the water was not freely mobile. I was wondering if you could briefly recapitulate the NMR basis for that important conclusion, which would suggest a dielectric constant lower than that of bulk water.

GIERASCH: Those are T_1 and T_2 measurements from ^{17}O, ^{2}H, and proton NMR studies obtained by looking at the change in those properties and the correlation time of the water as a function of the amount of water present in the reversed micelle. What you see is an extremely broad line for the low-water-percentage sample and a decline in the line width regardless of which nucleus you monitor. This parameter then follows the extent of hydration which you know numerically would be required for the head groups. In other words, when you get to ~1% water you have started to create a pool of water that is mobile. The immobile water tumbles with the AOT micelle. Actually it has a fourfold difference in its tumbling time (it is fourfold more mobile than AOT-reversed micelles in its most immobile state) and then goes to the regular correlation time of bulk water as you increase the amount of water. So it is a correlation time measurement by NMR.