JOURNAL OF MAGNETIC RESONANCE, Series B **109,** 60–65 (1995)

The Use of Dodecylphosphocholine Micelles in Solution NMR

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Received December 26, 1994; revised April 24, 1995

Dodecylphosphocholine (DPC) micelles are useful as a model membrane system for solution NMR. Several new observations on dodecylphosphocholine micelles and their interactions with opioid peptides are described. The optimal lipid concentration has been investigated for small peptide NMR studies in DPC micelles for two opioid peptides, a 5-mer and a 17-mer. In contrast to reports in the literature, identical 2D spectra have been observed at low and high lipid concentrations. The chemical shift of resolved peptide proton resonances has been followed as a function of added lipid and indicates that there are changes in the chemical shifts above the critical micelle concentration and up to a ratio of 7:1 (lipid:peptide) for the 17-mer, and 9.6:1 for the 5-mer. These results suggest that conformational changes occur in the peptide significantly above the critical micelle concentration, up to a lipid:peptide ratio which is dependent upon the peptide, here ranging from 7:1 to 9.6:1. To address the stoichiometry more directly, the diffusion coefficients of the lipid alone and the lipid with peptide have been measured using pulsed-field gradient spin-echo NMR experiments. These data have been used to calculate the hydrodynamic radius and the aggregation number of the micelle with and without peptide and show that the aggregation number of the peptide–lipid complex increases at high lipid concentrations without a concomitant change in the peptide conformation. Last, several protonated impurities have been observed in the commercial preparation of DPC which resonate in the amide proton region of the NMR spectrum. These results are significant for researchers using DPC micelles and illustrate that both care in sample preparation and the stoichiometry are important issues with the use of DPC as a model membrane. © 1995 Academic Press, Inc.

Dodecylphosphocholine (DPC) is one of the well-characterized model membrane systems in current use for the study of peptides and proteins which bind to lipids. Its characteristics are such that it forms a stable micelle (*1*) which freely rotates in solution, making it an excellent tool to mimic the anisotropic environment of a lipid membrane, while providing the motional properties desirable for solution NMR. It has electrostatic and hydrophobic components which approximate a cell membrane. It is commercially available in perdeuterated form, making it easily obtainable despite its high cost. Micelles provide a reasonable model of how a peptide or protein may exist in a lipid environment, as evidenced by the numerous examples in the literature of papers by NMR spectroscopists studying micelle-bound peptides and proteins. For example, Arseniev and co-workers have shown that the segments of bacterioopsin in micelles have secondary structural elements which are consistent with the low-resolution electron cryomicroscopy (ECM) structure of bacteriorhodopsin (*2, 3*). Arseniev's results indicate that one may successfully study segments of proteins in a micelle and faithfully reproduce the secondary structure that exists in the holo-protein in the membrane. On the other hand, it has been shown for gramicidin that the orientation of two of the four tryptophans may be different in a bilayer from that in a micelle (*4*). A less subtle example of a difference in a polypeptide structure between a bilayer and a micelle is the specific number of residues involved in a stretch of α-helix in melittin (*5*). It is fair to say that, although some differences may be apparent, they appear to be minor in some cases and significant in others. The overall effects of bilayer versus micelle on peptide or protein secondary structure are thus not yet completely understood. Most assuredly, more comparisons will be forthcoming in the literature.

This laboratory has been using DPC micelles to study the conformation of opioid peptides (*6*) as there is evidence that hormone peptides may bind to the cell membrane as part of the recognition process in interacting with the membrane-bound hormone receptor (*7–10*). Although several key properties of micelle–melittin interactions relevant to the NMR spectroscopist were reported by Wüthrich's laboratory in 1979 (*1*), it was necessary to extend their measurements to the peptides under study in this laboratory. In addition, a recent report in the literature (*11*) suggested a reinvestigation of some issues related to micelle–peptide interactions would be appropriate. Thus, in the course of this laboratory's investigations of peptide–lipid interactions and determination of the optimal lipid concentration for our NMR studies, several phenomena have been observed which are reported here.

The first observation addresses the effect of the lipid con-

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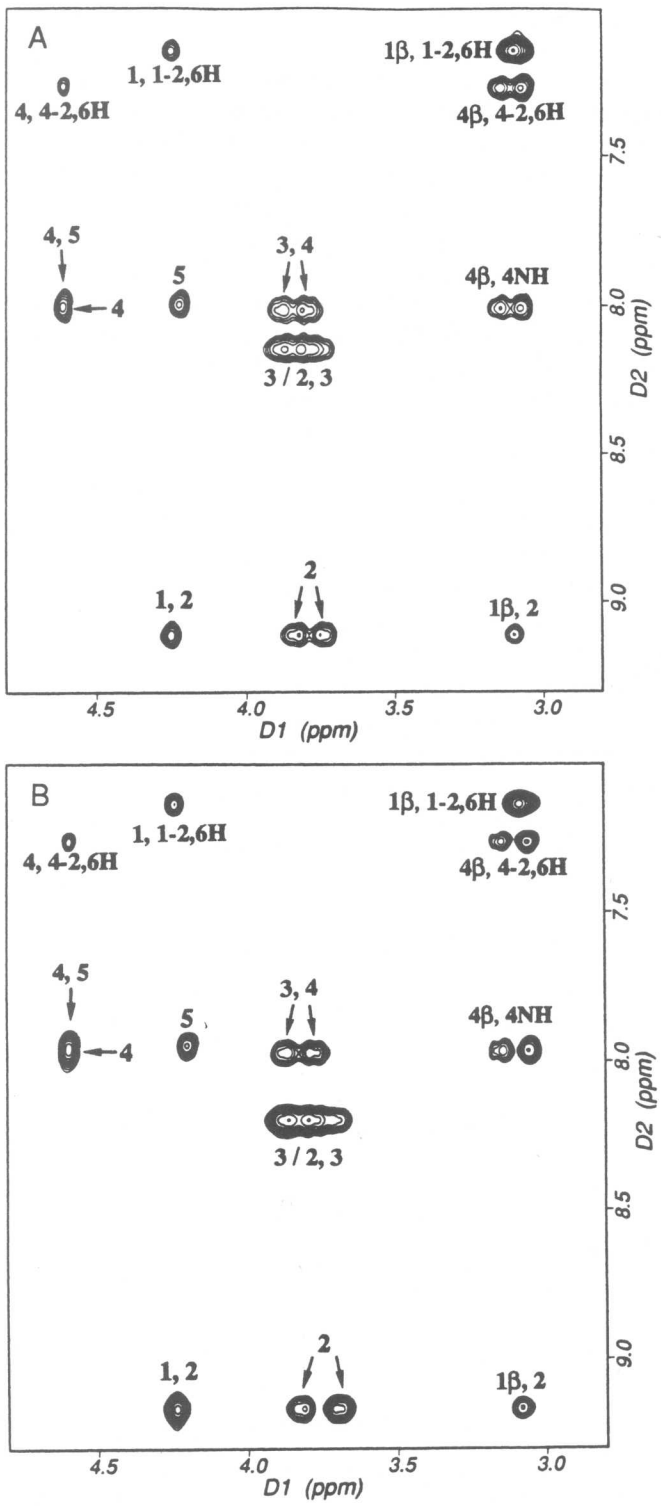

FIG. 1. Expansion of the NH–CαH fingerprint regions of NOESY spectra on LENK in the presence of DPC at two different lipid concentrations: (A) 162 m*M* DPC with 16.7 m*M* LENK [9.71:1.00] and (B) 609 m*M* DPC with 19.6 m*M* LENK [31.0:1.00]. Several cross peaks are labeled. Data were collected on a Bruker AMX-500 spectrometer at pH 3.2, 5°C, 10 m*M* phosphate buffer with 90% H_2O/10% D_2O with all chemical shifts referenced to the TSP internal standard. Spectra were collected with a spectral width of 6578.947 Hz, 2048 complex points, 512 t_1 experiments using TPPI phase cycling to provide quadrature detection in both dimensions, and a mixing time of 200 ms. The H_2O resonance was saturated during the relaxation delay of 1.5 s and mixing period to provide solvent suppression. Data were analyzed using Felix 2.05 (Hare Research), apodizing the t_2 dimension with a skewed sine-bell-squared function over 1024 points, 90° phase shift, and 0.9 skew and the t_1 dimension with a skewed sine-bell-squared function over 512 points, 90° phase shift, and 0.9 skew.

centration on the appearance of the peptide spectra. Shown in Fig. 1 are two NH–CαH NOESY regions for leucine-enkephalin (YGGFL, henceforth called LENK) in DPC micelles. The "low-lipid" spectrum was obtained at 162 m*M* DPC, and the "high-lipid" spectrum was obtained at 609 m*M* DPC. Although the critical micelle concentration for DPC is 1 m*M*, it is surmised from the literature that a much higher concentration of lipid is generally required for NMR studies. It is evident from Fig. 1 that the two spectra are virtually identical. The full spectra are also identical in every respect (data not shown). This is in contrast to the result found for both fd coat protein and Pf1 coat protein, for which it was reported that there is a doubling of peaks at lower lipid concentrations (*11*). Since the coat proteins are larger (fd coat protein has 50 amino acid residues) than the peptides studied here, it is quite possible that the optimal DPC concentration to study proteins is much higher than that required to study peptides. The stoichiometry is difficult to describe unless one knows the number of lipid monomers per micelle in the presence of peptide as discussed below. Another difference between fd coat protein and LENK other than size and charge is that LENK probably lies on the surface of the micelle, while fd coat protein has some of its residues inside the micelle (*12*). For LENK, however, it is clear that a DPC concentration of 162 m*M* yields virtually the same result as a DPC concentration of 609 m*M*.

The effect of lipid concentration on the appearance of the NOESYs for dynorphin A (YGGFLRRIRPKLKWDNQ, henceforth called DYNA) under similar conditions is shown in Fig. 2. An examination of these spectra shows that the low-lipid spectrum and the high-lipid spectrum are very similar, although not identical. There are several peaks that are missing at the higher lipid concentration, but these are NOESY peaks that are consistent with our sequential assignments of the peptide and which are present at a lower temperature (*6, 13*). It is clear, however, that there is no doubling of peaks at the lower lipid concentration. The linewidths at the different lipid concentrations are also comparable. We examined several NOESYs at lipid concentrations which were lower than the 120 m*M* lipid concentration shown here and did not find any peak duplication (data not shown). DYNA does appear to have some of its residues inserted into the micelle (*6, 13*), although none of these resonances are doubled in appearance here. This is in contrast to the coat proteins, which were observed to have some hydrophobic,

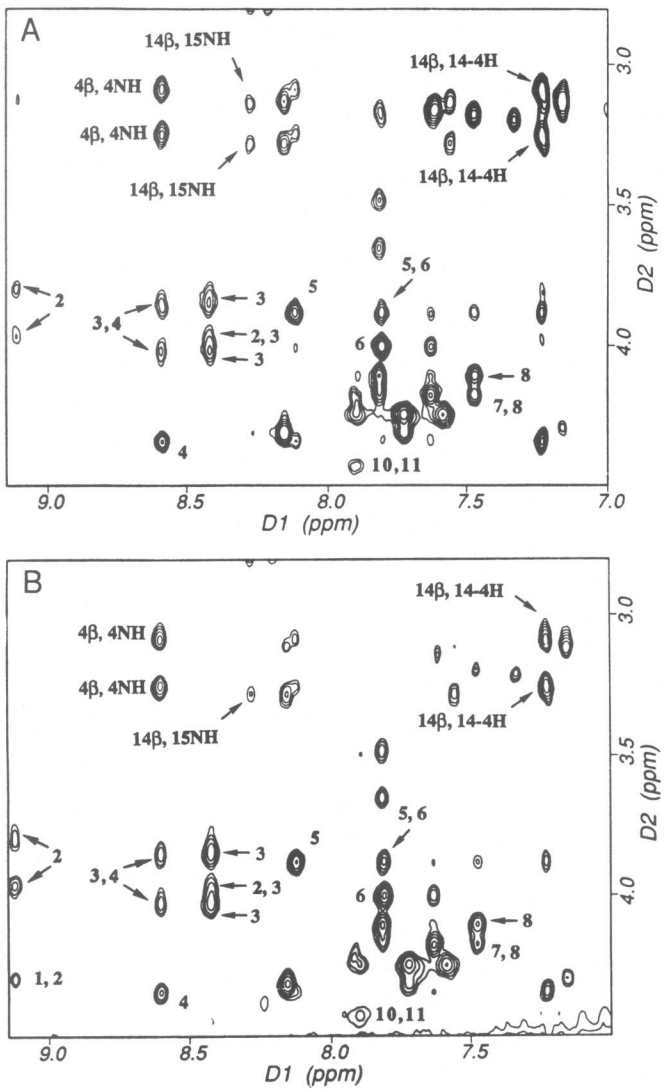

FIG. 2. Expansion of the NH–CαH fingerprint regions of NOESY spectra of DYNA in the presence of DPC at two different lipid concentrations: (A) 120 m*M* DPC with 4.88 m*M* DYNA [24.60:1.00] and (B) 400 m*M* DPC with 4.88 m*M* DYNA [81.96:1.00]. Several cross peaks are labeled as reported elsewhere (*6*). Data were collected on a Bruker AMX-500 spectrometer at pH 3.5, 37°C, 10 m*M* phosphate buffer with 90% H_2O/10% D_2O referencing all chemical shifts to the TSP internal standard. Spectra were collected with a spectral width of 6578.947 Hz, 2048 complex points, 512 t_1 experiments using TPPI phase cycling to provide quadrature detection in both dimensions, and a mixing time of 200 ms. The H_2O resonance was saturated during the relaxation delay of 1.5 s and mixing period to provide solvent suppression. Data were analyzed using Felix 2.05 (Hare Research), apodizing the t_2 dimension with a skewed sine-bell-squared function over 1024 points, 90° phase shift, and 0.9 skew and the t_1 dimension with a skewed sine-bell-squared function over 512 points, 90° phase shift, and 0.9 skew.

membrane-spanning residues doubled in appearance at the lower lipid concentrations (*12*).

DPC was titrated into both LENK and DYNA to further determine the effect of the DPC concentration on the state of the peptide as determined by proton chemical shift as shown in Figs. 3 and 4. Similar trends were observed with the enkephalins and lysophosphatidylcholine (*14*) and with melittin, a 26-residue peptide, and dodecyldimethylamine oxide micelles (*1*). As shown in the DPC-LENK titration in Fig. 3, there are significant changes that occur in chemical shift from the beginning of the titration to about 150 m*M* lipid (a mole ratio of 9.6:1), which is well above the critical micelle concentration. Note that the G2 moves fairly steadily downfield, while the G3 amide proton moves upfield, then downfield, the F4 amide proton moves upfield, and, finally, the L5 amide proton moves slightly downfield before moving upfield. These changes then level off as noted. A similar shifting and leveling off of chemical shift was observed when the lipid was titrated into DYNA (Fig. 4). Figure 4 indicates that the leveling off occurs at a lipid:peptide ratio of about 7:1. Other key similarities between the LENK and the DYNA titrations are that both are in fast chemical exchange and both G2 amide protons move downfield with increasing lipid concentration. The DYNA W14 indole proton also moves downfield, and it is noted in this regard that fluorescence data indicate it is associated with the hydrophobic portion of the DPC (*13*). In each titration, the peptide resonances cease to change at a particular lipid:peptide ratio which is about 9.6:1 for LENK and about 7:1 for DYNA. We interpret these data as follows. At the low lipid concentrations, the peptide is not yet saturated with lipid, so even

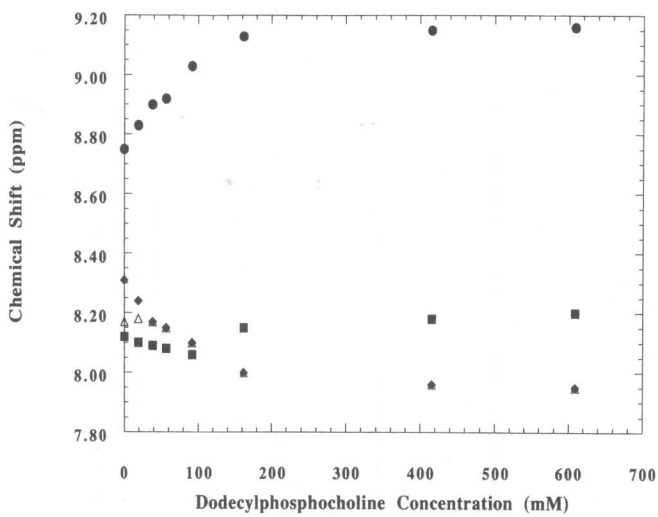

FIG. 3. Results of the titration of LENK with DPC performed on a Bruker AMX-500 spectrometer at pH 3.2, 5°C, 10 m*M* phosphate buffer with 90% H_2O/10% D_2O referencing all chemical shifts to the TSP internal standard. The following amide peaks were clearly identifiable throughout the titration and shown on the graph: glycine 2 (●), glycine 3 (■), phenylalanine 4 (◆), and leucine 5 (△). The concentration of peptide and the corresponding lipid-to-peptide ratio [DPC:LENK] of the experimental data points are as follows: 19.6 m*M* [0.00:1.00], 19.3 m*M* [1.01:1.00], 18.9 m*M* [2.04:1.00], 18.6 m*M* [3.06:1.00], 17.9 m*M* [5.14:1.00], 16.7 m*M* [9.71:1.00], 16.7 m*M* [24.9:1.00], and 19.6 m*M* [31.0:1.00].

TABLE 1
Summary of NMR Diffusion Measurements and Resultant Calculations at 300 MHz, 310 K on DPC and DPC–DYNA

DPC concentration[a] (m*M*)	Dynorphin A concentration (m*M*)	Diffusion constant, average (cm^2/s)	Hydrodynamic radius[b] (Å)	Aggregation number[c]
228	—	9.17×10^{-7}	18.65 ± 0.3	44 ± 5
55	4.88	9.23×10^{-7}	21.36 ± 0.7	50 ± 5
400	4.88	6.53×10^{-7}	23.13 ± 0.3	83 ± 5

[a] d_{38} DPC with 10 m*M* phosphate, pH 7.
[b] With excluded volume effect and radius of hydration sphere corrections.
[c] From $N\# = \{\frac{4}{3}\pi r^3(N)\rho\}/M_{avg}$; Nis Avogadro's number; M_{avg} is average molecular weight; ρ is from Lauterwein *et al.* (*1*).

though micelles have formed, not all the peptide is bound. The lipid concentration at which the peptide conformation ceases to change represents the lipid concentration at which all the peptide is bound to lipid. In light of our observation of fast chemical exchange for both DYNA and LENK in the presence of DPC, it seems possible that the doubling of peaks observed for the coat proteins (*11*) may be due to the bound and unbound protein being in slow chemical exchange on the chemical-shift time scale at the lower lipid concentrations which were employed.

A further understanding of the behavior and stoichiometry of the peptide–lipid complexes was sought by using NMR to measure diffusion coefficients. Lauterwein and co-workers made diffusion measurements on DPC and melittin/DPC complexes at 20 m*M* DPC using analytical centrifugation and quasi-electric-light-scattering techniques (*1*). Another method which allows measurement of the diffusion constant, and thereby the calculation of the hydrodynamic radius via the Stokes–Einstein equation, is the Fourier-transform pulsed-field-gradient spin-echo (PGSE) experiment (*15*). Our measurements were performed on both DPC alone and on two different DYNA–DPC samples: one at high lipid concentration and one at relatively low lipid concentration. Although perdeuterated lipid was used, the proton signals of the undeuterated fraction of lipid are clearly visible at these higher lipid concentrations. The measurements were made on a Nicolet 300 MHz spectrometer equipped with a proton diffusion probe (Cryomagnetic Systems Inc., Indianapolis, Indiana). The sample temperature was the same as that used for the collection of the 2D data (310 K), and the magnetic field gradient pulses were generated by a home-built electronic apparatus. The results are summarized in Table 1. These data show that the numbers of lipid monomers with and without peptide at DPC concentrations at the lower end of that used in the literature are consistent with the earlier measurements made on DPC and melittin/DPC (*1*). Since DYNA has eight less residues than melittin, it would be expected that the hydrodynamic radius be smaller but the aggregation number be about the same. Our measurements are consistent within experimental error with this expectation. Lauterwein *et al.* (*1*) presumed that when the peptide complexes with the lipid, the lipid monomers are replaced by peptides, resulting in a complex with a greater mass but a reduced aggregation number of the lipid. Our low-lipid-concentration results are consistent with this interpretation, but our results at the higher lipid concentration indicate that the hydrodynamic radius increases with a resultant increase in the aggregation number of the complex to about 83. Our results are meaningful for several reasons. They show that even though the aggregation number increases, the conformation of the peptide is the same. Perhaps this suggests a "core" of less fluidity in the micelle where peptide is bound as compared to the other regions of the micelle, which would be expected to be fluid and dynamic.

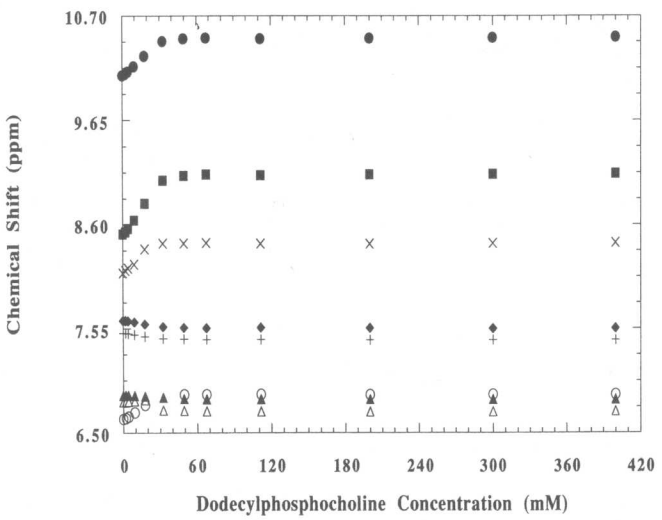

FIG. 4. Results of the DYNA titration with DPC. The titration was performed on a Bruker AMX-500 spectrometer at pH 3.4, 37°C, 10 m*M* phosphate buffer with 90% H_2O/10% D_2O referencing all chemical shifts to the TSP standard. The concentration of DYNA was 4.88 m*M* throughout the titration. Assignments for the peaks in the graph are as follows: W14 indole (●), G2 amide (■), G3 amide (×), W14 4H (◆), W14 7H (+), Y1 3,5H (▲), R9 εNH (△), and side-chain amino (○).

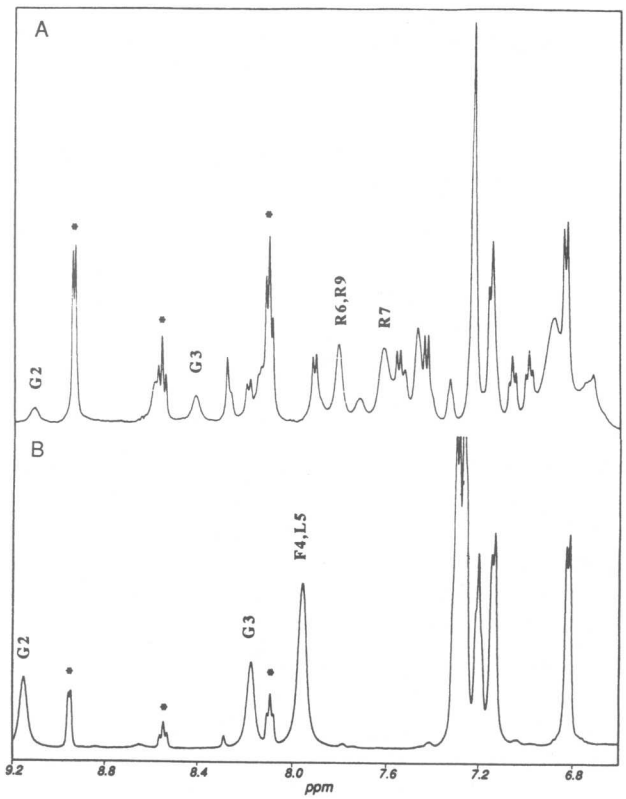

FIG. 5. Expansion of the downfield regions of (A) dynorphin A [1–17] YGGFLRRIRPKLKWDNQ, tryptophan indole proton at 10.49 ppm not shown, and (B) leucine-enkephalin YGGFL, both in the presence of dodecylphosphocholine-d_{38} (DPC). Impurities are marked by asterisk (*) and several peptide NH resonances are also labeled. Spectra were collected at 500 MHz similarly to those in previous figures. Sample conditions were as follows: (A) 4.88 m*M* dynorphin A, 400 m*M* DPC, 10 m*M* phosphate buffer, pH 3.5 with 90% H_2O/10% D_2O, 37°C; and (B) 16.7 m*M* leucine-enkephalin, 400 m*M* DPC, 10 m*M* phosphate buffer, pH 3.2 with 90% H_2O/10% D_2O, 5°C.

The aggregation numbers of many types of micelles have been determined using NMR and other methods, while peptide–lipid aggregation numbers have been rarely investigated. Although diffusion measurements have been made for zwitterionic micelles (e.g., *16*) they have not been made at this higher concentration with a peptide bound. Herein evidence is provided for an increase in aggregation number at higher concentrations for DPC in the presence of a 17-residue peptide.

The foregoing observations lead to the conclusions that the lipid-to-peptide ratio which is optimal for NMR measurements is well above the CMC and at least as high as the concentration where the peptide resonances cease to change, but much higher is not necessary for the peptides studied here. A generalization to the stoichiometry of other systems is not yet possible. It likely depends not only on the size of the peptide or protein, but also on the electrostatic and hydrophobic interactions between the polypeptide and the zwitterionic DPC. The micelle concentration where the peptide resonances cease to change represents the lower limit at which all the peptide is bound to micelle.

The last observation reported here concerns impurities from the lipid synthesis. At high concentrations of perdeuterated lipid, several peaks which may be mistaken for amide protons are observed in the downfield region of the spectrum. Shown in Fig. 5A is a 1D NMR spectrum of DYNA in DPC micelles and in 5B a 1D NMR spectrum of LENK in DPC micelles. Both spectra in Fig. 5 display (in addition to the amino acid resonances) three peaks at 8.92, 8.56, and 8.11 ppm, although the two upfield peaks are obscured by peptide resonances in Fig. 5A. These three protonated impurities do not resonate in a region that would be expected for DPC impurities. There are of course the usual proton signals at aliphatic chemical shifts due to the fraction of undeuterated lipid remaining (data not shown). These downfield resonating pyridinium salt impurities have been found in three different lots of perdeuterated DPC from two different manufacturers (data not shown). The use of a lipid concentration of 100 m*M* will mean that only a 1% impurity will be on the order of the intensity of the peptide peaks, and at the 400–600 m*M* concentrations recommended for proteins (*11*), the impurities will be much more intense. To our knowledge, the impurities at the amide proton chemical shift have not been previously reported.

Our results on the effect of lipid concentration on the conformation of peptides are significant for several reasons. The changes in chemical shift at a lower-than-optimal lipid concentration imply that erroneous conclusions may be reached in structure determination for lipid-bound peptides. In this, we concur with the literature. We have demonstrated that a lipid:peptide ratio of about 7–10:1 is the optimal lower limit for the peptides studied here, which is a lower ratio than that recommended in the literature, albeit for larger polypeptides (*11*). We have shown that the hydrodynamic radius of the DYNA–lipid complex at a 12:1 lipid-to-peptide ratio is consistent with a complex in which about four peptides per 45 lipid monomers forms. At the 82:1 lipid-to-peptide ratio, it is likely that one peptide per 82 lipid monomers compose a larger micelle. As the aggregation number increases, the peptide remains in the same observed conformation, a conclusion which is supported by both the 1D titration and the 2D data. These results represent significant and illuminating caveats for the NMR spectroscopist studying lipid-bound peptides, as the behavior of the lipid in such complexes has been rarely investigated. One must clearly use DPC carefully in the structural determination of peptides which interact with model membranes.

ACKNOWLEDGMENTS

The authors gratefully acknowledge support by the Petroleum Research Fund of the American Chemical Society (to D.A.K.), the Samuel Melendy

Graduate Fellowship of the College of Pharmacy at the University of Minnesota (to M.R.T.), and the Molecular Biophysics Training Program NIH 5T32 GM08277-07 (to C.R.W.). The D.A.K. group thanks Dr. Vikram Roongta and the Biomedical Engineering/Biochemistry NMR Center at the University of Minnesota for continuing support, and Professor Timothy Wiedmann for helpful discussions.

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