Facile Acquisition and Assignment of Oriented Sample NMR Spectra for Bilayer Surface-Associated Proteins[1]

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This communication describes experiments which demonstrate the potential of using magnetically orientable phospholipid mixtures as media in which to carry out oriented sample NMR analyses of bilayer surface-associating peptides and proteins. The use of oriented sample NMR has emerged as a powerful technique for the structural study of membrane proteins.[2] However, most previous oriented sample NMR investigations of membrane proteins have been limited to transmembrane peptides which have been oriented in multilamellar lipid dispersions sandwiched between thin glass plates. This poses a serious obstacle to the study of membrane surface-associating proteins which may not be readily reconstituted into such systems because of problems in protein solubilization, dispersion, and hydration which stem from the multilamellar nature of the sandwiched bilayers. Here, it is demonstrated that these limitations can sometimes be eliminated by the use of recently developed *magnetically* orientable model bilayer media.[3-5] Such media are based upon the formation of micelle-like bilayer fragments in the presence of certain detergents. These lipid assemblies are readily oriented by a strong magnetic field and have been shown to maintain many of the properties of detergent-free bilayers. Furthermore, the detergents used do not generally denature either water-soluble or transmembrane proteins.[3,6]

Figure 1 illustrates ^{13}C NMR spectra of unlabeled leucine enkephalin (LENK, Tyr-Gly-Gly-Phe-Leu) solubilized in mixtures of 1,2-dihexanoyl-*sn*-3-glycerophosphocholine (DHPC) and 1,2-dimyristoyl-*sn*-3-glycerophosphocholine (DMPC). The top spectrum represents a well-oriented sample in which the bilayer fragments are characterized by a whole-assembly order parameter ($S_{bilayer}$) of 0.5 (halfway between the fixed bilayer and isotropic assembly limits). The bottom spectrum represents a sample made isotropic by raising the DHPC:DMPC ratio. Assignments for the isotropic resonances are based upon the work of Khaled et al.[7] Based upon reported association constants[8] and as confirmed in these studies by varying the overall lipid concentrations, LENK is greater than 95% associated with the lipid interface in these experiments. Significant differences in resonance positions are apparent between the two spectra. That CSA accounts for the nature of the shifts between isotropic and oriented spectra was confirmed by examining a series of samples in which $S_{bilayer}$ was systematically varied by changing the DHPC:DMPC ratio.[4,5] A plot of the chemical shifts of the five carbonyl resonances as a function of $S_{bilayer}$ (Figure 2) allows unambiguous spectral assignments and extrapolation of chemical shifts to detergent-free DMPC and reveals an approximately linear relationship between shifts and $S_{bilayer}$, as expected for CSA. Linearity was confirmed for all aromatic resonances (data not shown). The observation of a linear relationship is also significant because it indicates that the structure of the peptide is independent of the DHPC:DMPC ratio, supporting the argument that the oriented assemblies provide a reasonable approximation of the surface of pure phosphatidylcholine bilayers. A number of ^{13}C–^{1}H dipolar couplings were also measured from similar experiments run with the decoupler off or in the presence of MREV-8 ^{1}H–^{1}H homodecoupling. The full data set (not shown) thus produced is suitable for quantitative structural analysis and comprises some 18 CSAs ranging from –3.5 to +5.1 ppm, seven dipolar couplings ranging from –420 to +770 Hz, and upper or lower limits for another 15 dipolar couplings.[9-11]

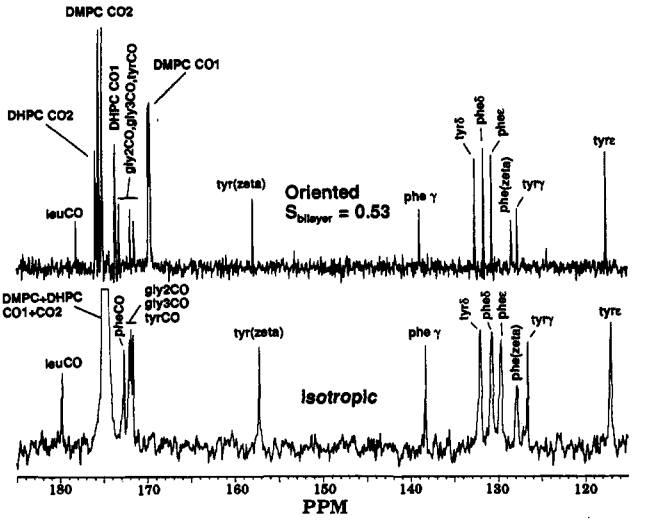

Figure 1. 67.8-MHz ^{13}C (^{1}H-decoupled) NMR spectra of unlabeled leucine enkephalin in DHPC:DMPC mixtures at 40 °C (carbonyl and aromatic regions only). Spectroscopic conditions are described elsewhere.[3] Both samples contained 25% (w/v) total lipid (DHPC + DMPC) and were buffered with 70 mM sodium phosphate, 30 mM KCl in D_2O at pD = 7.0. The DHPC:DMPC mol:mol ratio in the oriented sample was 1:2.7, while the ratio in the isotropic sample was 1:2.0. The DMPC:LENK mol:mol ratio in each case was roughly 10:1. The Phe carbonyl peak in the oriented spectrum is obscured by the DMPC *sn*-1 carbonyl resonance. The specific assignments of the Gly2, Gly3, and Tyr carbonyl resonances can be inferred from the plot in Figure 2 and the isotropic assignments.[7]

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(9) In this paper, CSA refers to the difference between the oriented sample shift and the isotropic shift (see ref 5). The reported CSAs and dipolar couplings were obtained by extrapolating the $S_{bilayer}$–variation plots to $S_{bilayer}$ = 1.0 (detergent-free, globally immobile oriented bilayer). The scalar contribution to coupling was deconvoluted from the dipolar component as described in the following: Sanders, C. R.; Prestegard, J. H. *J. Am. Chem. Soc.* **1991**, *113*, 1987–1996.

(10) The bilayer fragments orient with their normals orthogonal to the magnetic field. As a result, oriented sample spectra are obtained only if the bilayer-associated protein executes rapid axial rotation about the bilayer normal; otherwise, powder patterns would result. The condition of axial rotation is clearly met by both LENK and CytC, although it cannot be ascertained whether this is due to rotation *on* the surface or is *effectively* met by transient dissociation from the surface followed by random reassociation.

(11) The fact that the largest observed ^{13}C CSA and ^{13}C-^{1}H dipolar coupling for both LENK and CytC fall well below maximum rigid-molecule limits (about 50 ppm and 15 000 Hz, respectively in a system with a 90° oriented director) strongly implicates the presence of considerable *orientational* mobility for both molecules. "Orientational mobility" encompasses all motions which would lead to rapid reorientation of CSA and dipolar tensors with respect to the experimental director (the bilayer normal). The deconvolution of the results in terms of contributions from whole-molecule reorientation, conformational mobility, and transient dissociation/reassociation with the oriented surface is beyond the scope of this paper.

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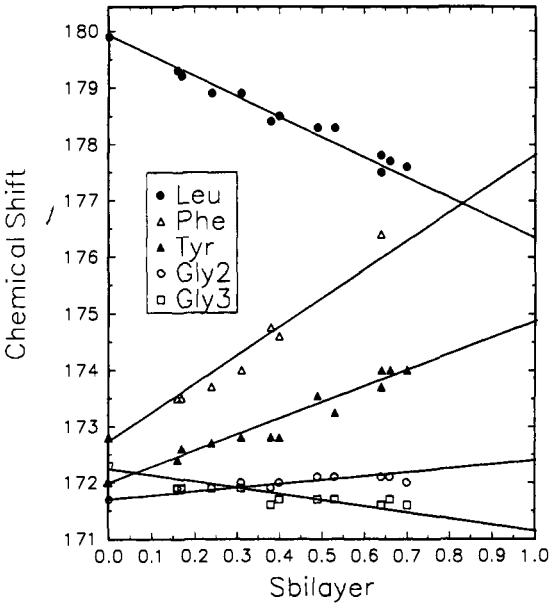

Figure 2. $S_{bilayer}$-dependence of the carbonyl ^{13}C chemical shifts of LENK. $S_{bilayer}$ was systematically varied by changing the DHPC:DMPC ratio (see ref 5) over a total range of about 1:3.5 to 1:1.8 under conditions as described for Figure 1.

The data for LENK demonstrate in prototypical fashion the potential utility of using the magnetically orientable model bilayer systems as media for studying surface-associating proteins. A preliminary but more dramatic demonstration of the potential of this approach is provided by equine ferricytochrome *c* (CytC, 12kDa). CytC does not bind strongly to strictly zwitterionic membrane surfaces,[12] a fact which was confirmed by observing that CytC solubilized in oriented DHPC:DMPC mixtures yields isotropic ^{13}C and ^{1}H NMR spectra (data not shown). However, upon inclusion of a small amount of a negatively charged amphiphile, CytC associates with the DHPC:DMPC assemblies without disturbing the ability of the bilayers to orient as evidenced by dramatic broadening of the CytC resonances in both ^{1}H and ^{13}C (^{1}H-coupled) spectra due to extensive ^{1}H–^{1}H and ^{1}H–^{13}C dipolar coupling (data not shown).

Shown in Figure 3 are ^{13}C NMR spectra of CytC in oriented and isotropic samples acquired in the presence of proton decoupling. The samples represented differ only in their DHPC:DMPC ratios. The oriented sample spectrum exhibits high resolution while at the same time exhibiting a number of differences from the isotropic case. The differences in resonance positions between isotropic and oriented samples can again be ascribed to CSA as confirmed by observing a linear dependence upon $S_{bilayer}$ of resonance shifts for peaks 1–8 in Figure 3 (titration data not shown). Unlike the case of LENK, no attempt was made to assign these peaks based upon available isotropic resonance assignments.

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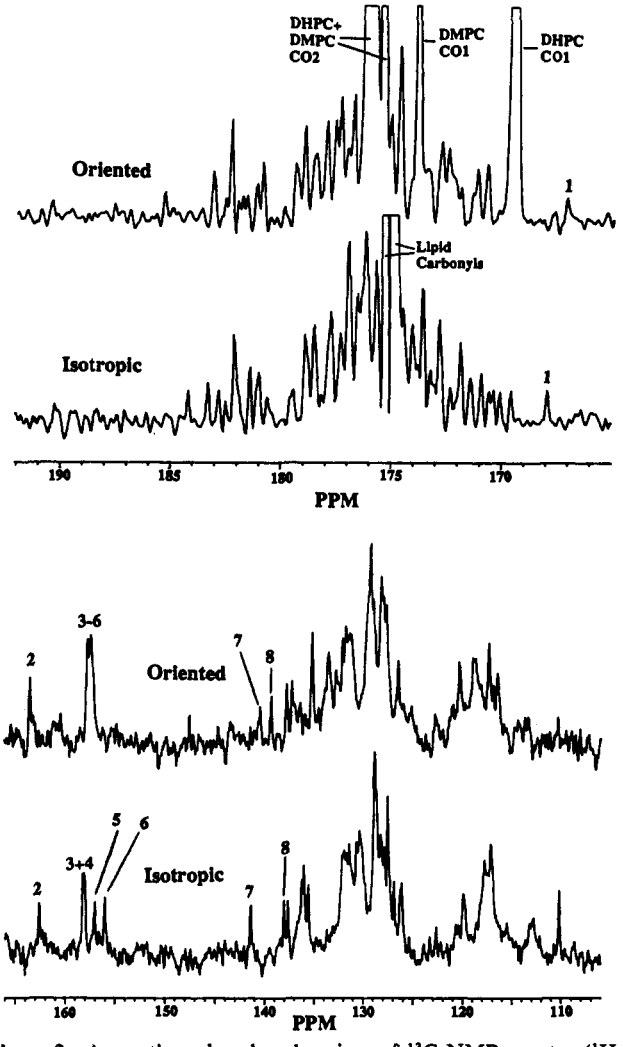

Figure 3. Aromatic and carbonyl regions of ^{13}C NMR spectra (^{1}H-decoupled) from CytC in DHPC:DMPC mixtures at 38. The aromatic spectra were produced following 8 Hz of exponential line broadening, while the carbonyl spectra are resolution-enhanced. The samples contained a fixed molar ratio of DMPC to lauryl sulfate (12:1) at a total amphiphile concentration of 20%. The oriented sample spectrum represents an $S_{bilayer}$ of 0.62 and a DHPC:DMPC ratio of 1:2.5. The ratio for the isotropic sample was 1:1.6. The CytC concentration was ca. 6 mM in 50 mM sodium phosphate, 50 mM Cl^-, pD = 6.3. Sodium is the preferred counterion in such studies because K^+ can induce precipitation of lauryl sulfate.

While the CytC results are very preliminary, they are intriguing because they suggest that once isotropic ^{13}C resonance assignments are made it may be possible to obtain a large body of CSA and dipolar coupling data from $S_{bilayer}$-variation experiments. Such data could, in principle, be used for total structural analysis of surface-associated CytC. Perhaps more importantly, the results for both LENK and CytC suggest the possibility of extending the basic methodology outlined herein to studies of other surface-associating proteins.