Reconstitution of Membrane Proteins into Lipid-Rich Bilayered Mixed Micelles for NMR Studies†

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ABSTRACT: This paper describes a study undertaken to assess the possibility and practical consequences of reconstituting integral and peripheral membrane proteins into bilayered discoidal mixed micelles ("bicelles") composed of dimyristoylphosphatidylcholine and smaller amounts of either CHAPSO or short-chain phosphatidylcholine. The amphiphilic assemblies in these mixtures are uniquely suited for use in NMR structural studies because they can be magnetically oriented with experimentally-tunable system order. The first step of this study was to test about 15 membrane-associating polypeptides and proteins for their ability to interfere with magnetic orientation of the bicellar assemblies. A variety of results were obtained ranging from no perturbation to a complete disruption of orientation. Second, the suitability of bicelles as mimics of natural bilayers was tested by reconstituting diacylglycerol kinase, an integral membrane enzyme. The kinase was observed to be functional and completely stable for at least 24 h when incubated at 38 °C in bicelles. Third, the NMR spectra from a number of bicelle-reconstituted proteins were examined. In some cases, ^{13}C NMR resonances from reconstituted proteins were extremely broad and asymmetric. In other cases, resonances from reconstituted proteins were moderately broad, but much less so than resonances from proteins reconstituted into multilayers oriented by mechanical methods. In the cases of two surface-associating proteins (cytochrome *c* and leucine enkephalin), oriented sample ^{13}C NMR spectra of extremely high resolution were obtained. For these proteins it was also demonstrated that the experimentally variable order of the bicellar assemblies could be exploited to provide a means of screening for detergent-specific structural perturbations, for making spectral assignments, and for measuring chemical shift anisotropies and dipolar couplings. Taken as a whole, these results indicate that bicelles may be uniquely and effectively employed as model membranes to facilitate NMR structural studies of many, but not all, membrane proteins.

The use of NMR as a tool for the structural study of membrane proteins remains in a relatively early state of development, partly due to limitations imposed by the model membrane systems generally employed in such studies. Most *solid state* NMR investigations of membrane proteins have relied upon the use of mechanically oriented or randomly dispersed multilamellar vesicles (for review see Cross & Opella, 1994), a medium which sometimes does not readily lend itself to studies of peripheral membrane proteins or to integral membrane proteins with large extramembrane domains. Many *solution* NMR studies have relied upon the use of detergent micelles as model membranes (see review in McDonnell & Opella, 1993). Among the problems which may be encountered using micelles are (i) that it may be difficult to reconstitute some transmembrane proteins which require a *bilayer* to adopt a native-like conformational state and (ii) that it is difficult to experimentally assess the degree to which a given micellar system may suitably mimic the environment of an actual membrane bilayer for a given membrane protein. This paper probes the possibility that the above model membrane-specific problems may sometimes be overcome by employing a class of model systems which lies at the interface between vesicles and classical micellar systems.

It is known that mixtures of dimyristoylphosphatidylcholine (DMPC) with certain detergents form lipid-rich bilayer fragments which are edge-stabilized by the detergent component. For the sake of convenience, the lipid−detergent aggregates in these systems will be referred to as *bicelles*.[1] The detergents usually employed are either dihexanoylphosphatidylcholine (DHPC; Sanders & Schwonek, 1992) or a zwitterionic bile salt derivative, CHAPSO (Sanders & Prestegard, 1990). The lipid:detergent ratios present in the bicellar systems are relatively high (ca. 4:1 to 1.5:1) compared to most other micellar systems which have previously been employed as model membranes in biochemical or structural studies. Furthermore, the bicellar systems are unusual in that bicellar assemblies can be magnetically oriented at relatively high lipid:detergent ratios to yield oriented "solid state" NMR spectra of very high quality. Titration of additional CHAPSO or DHPC into these samples results in the systematic reduction of the orientational order of the bicelles as the bilayer fragments are transformed into smaller and smaller units. At sufficiently high detergent:

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[1] For **bi**nary, **bi**layered, mixed mic**elles** bearing a resemblance to the classical model for **bi**le salt−phosphatidylcholine aggregates (see Muller, 1981).

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DMPC ratios the assemblies lose significant orientational preference in a magnetic field, at which point spectroscopic conditions revert to the solution NMR regime. The characterization and prior utilization of the CHAPSO-DMPC and DHPC-DMPC systems have recently been reviewed (Sanders et al., 1994).

The possibility of conducting structural studies of membrane proteins solubilized in bicelles is worthy of investigation for several reasons.[2] Unlike multilamellar systems, the bicellar assemblies are monodisperse and fully hydrated (see review and Hare et al., in press). Unlike classical micelles, the dominant morphology of the orientable systems is a lipid bilayer. Furthermore, because it is possible to systematically vary the CHAPSO or DHPC to DMPC ratio without changing aggregate morphology, it is possible to screen for detergent-specific artifacts in structural studies by making NMR measurements at a series of detergent:DMPC ratios and then extrapolating data to 0 detergent content. Finally, the ability to conduct both solution *and* solid state NMR studies on samples which are identical except for modest differences in lipid:detergent ratios potentially offers a uniform approach to bringing *both* regimes of NMR to bear upon structural problems.

Previous structural studies employing the CHAPSO-DMPC and DHPC-DMPC systems have focused upon bicelle-solubilized lipids or small molecule solutes (Sanders et al., 1994; Sanders, 1994; Henderson et. al. 1994). In this contribution, extension to membrane proteins is investigated by probing the following questions. (i) Do reconstituted proteins disrupt the morphology or magnetic orientational properties of the bicelles? (ii) What are the consequences of bicellar reconstitution upon the functional and spectroscopic properties of the proteins? (iii) What is the potential for exploiting the unique properties of the bicellar systems in NMR structural studies of membrane proteins?

MATERIALS AND METHODS

Lipids and Polypeptides. DMPC was purchased from Princeton Lipids (Princeton, NJ). Dimyristoylphosphatidic acid (sodium salt) and DHPC were purchased from Avanti Polar Lipids (Birmingham, AL). 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPSO), lauryl sulfate (sodium salt, SDS), gramicidin D, gramicidin S, bacteriorhodopsin (BR), alamethicin, unlabeled leucine enkephalin (LENK), carbobenzoxy-D-Phe-L-Phe-Gly (CBz-fFG), and bovine heart cytochrome *c* were purchased from Sigma (St. Louis, MO) and used without further purification. Myelin basic protein was purchased from Gibco BRL (Gaithersburg, MD) and was observed to yield a single ca. 19 kDa band by SDS−PAGE (15% gel). (1-^{13}C-Ile$_{20}$)-Melittin was a gift from Dr. Frances Separovic (CSIRO, Australia) while unlabeled P16 ($K_2GL_{16}K_2A$) and P24 ($K_2GL_{24}K_2A$) were gifts from Drs. Robert Hodges (University of Alberta) and Myer Bloom (University of British Columbia). Phe-Phe-Gly-OBz (FFG-Bz) was a gift from Dr. Richard Epand (McMaster University, Hamilton, ON). The β-amyloid (1−39) peptide labeled with C13 at the α-carbons of Gly-9, -25, and -33 and on the methyl of Met-35 was a gift from Dr. Michael Zagorski of Case Western Reserve University.

Acetyl-KWSFGYP-(2-^{13}C)G-(^{15}N)AIIFMILAG-(1-^{13}C)L-(^{15}N)APYLYPKR-amide ("Cor3") represents the third transmembrane segment of the microbial Mg^{2+} transporter CorA (Smith et. al, 1993). This peptide was contract-synthesized by Dr. James Elliot of the Keck Biotechnology Laboratory at Yale University and purified at CWRU by reversed phase HPLC (RPHPLC) according to a modification of a method suggested to us by Dr. John Hunt (University of Texas, SW Medical Center). Before each injection onto a Vydac C18 preparative column, 50−100 mg of crude peptide was dissolved in 100 μL of TFA, 100 μL of formic acid, 200 μL of buffer B, and approximately 100−200 μL of buffer A (added to a level just prior to precipitate formation). The sample was mixed and centrifuged, and the supernatant was filtered through a Millipore Millex-FH$_{13}$ filter (Bedford, MA). Following injection, the peptide was eluted with a 20 min linear gradient (8 mL/min) of 30−65% buffer B, where buffer A is water/2-propanol/TFA (94.9:5:0.1) and buffer B is 2-propanol/water/TFA (94.9:5:0.1). The desired peak was saved and most 2-propanol removed by rotary evaporation, after which the remaining solution was frozen and lyophilized to yield pure Cor3 (verified by laser desorption mass spectroscopy) in about 20% yield from the crude material.

Isotopically labeled leucine enkephalin (Tyr-(1-^{13}C)Gly-(^{15}N)Gly-Phe-Leu) and P16 (K_2GL_7-(1-^{13}C)L-(^{15}N)LL_7KKA-amide) were both synthesized in-house using solid phase methods (FMOC- and tBOC-based chemistries, respectively). LENK was purified using standard reversed phase HPLC methods (C18, buffer A: water with 0.1% TFA; buffer B: acetonitrile with 0.1% TFA). Labeled P16 was purified and verified by mass spectroscopy exactly as described above for Cor3. The overall yield for the synthesis and purification of P16 was 8%.

Diacylglycerol kinase (DAGK) tagged with a poly-His affinity tag was purified from an overproducing strain of *Escherichia coli* using Ni^{2+} chelate affinity chromatography. Both the method (unpublished) and the overproducing strain were provided to us by Dr. James Bowie (University of California, Los Angeles).

Magnetically Orientable Detergent−DMPC Mixtures. CHAPSO-DMPC and DHPC-DMPC mixtures have been extensively characterized by NMR (reviewed in Sanders et al., 1994). Here we summarize only those previous results which are crucial to understanding the experiments presented in this paper. The bicellar assemblies orient with bilayer normals perpendicular to the direction of the magnetic field

[2] Actually this work does not represent the first report of magnetic orientation of a membrane protein for NMR study. Davis (1988) published a careful ^{2}H NMR study of amide ^{2}H-exchanged gramicidin D solubilized in an oriented lyotropic nematic liquid crystal composed of potassium laurate, decanol, potassium chloride, and water. While decanol and potassium laurate are also thought to assemble into bilayered discoidal micelles (Forrest & Reeves, 1981), it can be argued that there are advantages to the DMPC-based systems dealt with in this study: (i) bicelles are composed of phospholipids which are structurally, conformationally, and dynamically more similar to typical lipid bilayers than the surfactants composing the laurate/decanol system (Sanders, 1993; Sanders & Schwonek, 1992), (ii) the bicellar systems have been subjected to a much higher level of characterization with respect to their suitability as model lipid bilayers (see Sanders et al., 1994), and (iii) well-developed methods exist for scaling overall bicellar assembly order and for exploiting this capability to facilitate spectral analysis and to provide a method to control for detergent-specific artifacts (this work, Sanders, 1994, and Sanders et al., 1994). On the other hand, it must be pointed out that while numerous attempts to orient gramicidin in bicelles have failed (this study), Davis' oriented-sample ^{2}H NMR spectra of gramicidin are of extremely high quality. Thus, the potential utility of the laurate/decanol system for some applications should not be discounted.

Table 1: Summary of Reconstitution Tests

peptide, reconst method,[a] pH	peptide:DMPC (mol:mol)	detergent:DMPC (mol:mol)	temp (°C)	sample appearance	NMR of DMPC + detergent phase[b]	NMR of peptide
Proteins for Which "Normal" Membrane Location Is Integral						
gramicidin D, 1−3, various pH	1:50−1:10	both, numerous	30−50	thick milk	P31: broad isotropic peak on top of powder pattern	NR[c]
P16, 2, pH = 6.3	1:65	DHPC, 1:2.7−1:2.0	38	clear with slight haze	C13: oriented normally	C13: peak is observed from the labeled Leu
P16, 2, pH = 6.3	1:65	CHAPSO, 1:3.5−1:3.0	35	clear with slight haze	C13: oriented normally	C13: peak is observed from the labeled Leu
unlabeled P16, 1, in 0.1 M KCl	1:57	DHPC, 1:3.0	40	clear	C13: oriented normally	C13: NO[c]
P24, 1, pH = 7.0	1:53	DHPC, 1:3.3−1:2.6	30−40	cloudy	P31: powder pattern plus isotropic, or multiple phases	NR
bacteriorhodopsin, 1,[d] pH = 5.5	1:360	DHPC, 1:2.8	40	dark purple	lock signal: initially oriented, but orientation lost after 1 h; C13: multiple phases	C13: NO
cor3, 2, pH 6.3	1:65	CHAPSO, 1:3.5	34	clear with particles	C13: oriented normally	C13: peaks from labeled carbonyls are very broad
cor3, 2, pH 6.3	1:62	DHPC, 1:2.5	36	clear	C13: oriented normally	C13: peaks from labeled carbonyls are very broad
Proteins For Which "Normal" Membrane Location Is Peripheral						
Leu enkephalin, 3, pH = 6−7.3	<1:8	DHPC, 1:3.5−1:2.0; CHAPSO, 1:4.0−1:3.0	33−50	clear	C13: all oriented normally	C13: protein peaks observed and exhibit $S_{bilayer}$-scalable anisotropy
cytochrome *c*,[e] 3, pH 6.2	1:34	DHPC, 1:2.5	38	dark purple	C13: oriented normally	H1 and C13: protein peaks observed and are isotropic
cytochrome *c*, 3, pH 6.2	1:35	DHPC, 1:2.5; +SDS[f] 1:10	38	dark purple	C13: oriented normally	H1 and C13: protein peaks observed and exhibit $S_{bilayer}$-scalable anisotropy
cytochrome *c*, 3, pH 6.2	1:30	DHPC, 1:2.7−1:2.0; +SDS,[f] 1:12	40	dark purple	C13: all oriented normally	H1 and C13: protein peaks observed and exhibit $S_{bilayer}$-scalable anisotropy
cytochrome *c*, 3, pH 6.2	1:35	DHPC, 1:2.8; + DMPA,[g] 1:10	40	dark purple	C13: sample oriented slowly but otherwise normally	H1 and C13: protein peaks observed and exhibit $S_{bilayer}$-scalable anisotropy
myelin basic protein, 3, pH 7.0	1:190	DHPC, 1:2.8	35	clear	P31: axially symmetric powder pattern ($\delta_0 - \delta_{90} = +29$ ppm)	H1: broad aromatic peaks are visible
CBz-fFG, 3, pH 7.3	1:10	DHPC, 1:2.8	40	very cloudy	C13 and P31: multiple phases present	C13: broad and noisy aromatic/carbonyl
CBz-fFG, 2, pH 7.3	1:10	CHAPSO, 1:3.5	40	cloudy, silky	NR	NR
CBz-fFG, 3, pH 7.3	1:10	DHPC, 1:2.8	35	slightly cloudy	C13: oriented almost normally	C13: aromatic peaks just barely visible
CBz-fFG, 3, pH 7.3	1:60	DHPC, 1:2.8	40	clear	C13: oriented normally	C13: NO
FFG-Bz, 3, pH 7.3	1:9	DHPC, 1:2.8	40	very cloudy	P31: multiple phases present	NR
FFG-Bz, 3, pH 7.3	1:9	DHPC, 1:2.8	35	NR	P31 + C13: oriented normally	C13: somewhat broad aromatic and carbonyl peaks
FFG-Bz, 3, pH 7.3	1:50	DHPC, 1:2.8	40	clear	C13: oriented normally	C13: NO
Proteins For Which "Normal" Membrane Location Is Variable or Ambiguous						
melittin, 2, pH = 6.3	1:50	CHAPSO, 1:3.5	35	clear	C13: oriented normally	C13: peak from (1−^{13}C)Ile$_{20}$ is very broad
alamethicin, 1, pH = 5.5	1:36	DHPC, 1:2.9	40	clear	C13: normal, high quality sample orientation	C13: somewhat broad and noisy aromatic resonances
β-amyloid-(1−39), 1,[h] pH = 7.0	1:120	DHPC, 1:3.1	38	clear with slight texturing	C13: substantially oriented, but powder pattern characteristics are detected	C13: peak from labeled Gly-α is present (linewidth 40 Hz), Met-CH_3 obscured by lipid

Table 1 (Continued)

peptide, reconst method,[a] pH	peptide:DMPC (mol:mol)	detergent:DMPC (mol:mol)	temp (°C)	sample appearance	NMR of DMPC + detergent phase[b]	NMR of peptide
Proteins For Which "Normal" Membrane Location Is Variable or Ambiguous						
gramicidin S, 3, pH = 7.0	1:8	DHPC, 1:2.5	40	2 phases separated	NR	NR
gramicidin S, 3, pH = 7.0	1:8	DHPC, 1:2.5	33	clear	C13: well-oriented sample	C13: noisy and somewhat broad aromatic and carbonyl
gramicidin S, 3, pH = 7.0	1:10	CHAPSO, 1:4.5	40	NR	C13: orients normally (some residual powder pattern)	C13: noisy and somewhat broad

[a] Reconstitution methods: 1: peptide dissolved in detergent solution; 2: peptide and DMPC first codissolved in organic solvent; 3: addition of peptide to detergent–DMPC mixture (see Materials and Methods for additional details). [b] Examples of C13 and P31 spectra of "normally oriented" bicellar samples are illustrated elsewhere (Sanders, 1993; Sanders & Schwonek, 1992; Sanders & Prestegard, 1990). [c] NR: not recorded; NO: not observed (spectrum was acquired, but peptide resonances were not clearly observable above the noise). [d] 10 mg of BR was mixed with 0.085 mL of 25% DHPC, pH 5.5; the protein dissolved completely after about 3 h of incubation at room temperature and maintained a clear purple appearance even after adding DMPC, mixing, and spectral acquisition at 40 °C for 12 h. [e] All cytochrome *c* studies were carried out at 20% (w/v) total amphiphile rather than 25%. [f] SDS:DMPC molar ratio. [g] DMPA:DMPC molar ratio; DMPA is *sn*-1,2-dimyristoyl-3-glycerophosphate. [h] Most, but not all of the amyloid peptide dissolved.

of the spectrometer. Magnetic orientation occurs over a wide range of total amphiphile (detergent + DMPC) contents (5–40%, w/v) and temperatures (ca. 32–50 °C). Within a pH range of 5–8 and total salt + buffer concentrations of 50–200 mM, orientational behavior has not been observed to be sensitive to exact buffer/salt composition. At 25% total amphiphile, CHAPSO-DMPC mixtures orient at CHAPSO:DMPC molar ratios from about 1:4 to 1:2.8, while for DHPC:DMPC the range is ca. 1:3.5 to 1:2.0. Outside of these ranges, NMR spectra show residual powder patterns (at lower detergent:DMPC ratios) or become isotropic (at higher ratios). On the basis of previous studies of bile salt–PC mixtures (for review, see Sanders & Schwonek, 1992) and the extreme similarity of the DHPC-DMPC and CHAPSO-DMPC systems, it appears that even in the isotropic limit the bilayered disc micelle aggregate morphology is maintained. From an NMR standpoint, optimal oriented sample spectra are obtained at CHAPSO:DMPC of about 1:3.5 for 25% samples, while for DHPC-containing samples optimal spectra are obtained when the DHPC:DMPC ratio is near 1:2.75. Well-oriented samples are usually clear and viscous in the 30–50 °C range, with birefringence sometimes being observed. Finally, it should be pointed out that isotropic DHPC-DMPC mixtures yield sharper resonances than isotropic CHAPSO-DMPC mixtures and that below 25 °C most magnetically orientable samples undergo a phase transition to a fluid, isotropic phase.

NMR Sample Preparation. Twenty-five percent (weight total amphiphile/total volume) DHPC-DMPC and CHAPSO-DMPC samples were prepared in 5 mm NMR tubes by mixing solid DMPC, solid CHAPSO, or 25% DHPC solution and buffer according to methods previously described (Sanders & Schwonek, 1992). Studies conducted at pD of 5.5 employed a 15 mM acetate buffer containing 85 mM KCl in D_2O. Studies conducted in the pD range of 6.3–7.3 employed phosphate buffer (25–100 mM) with added KCl or NaCl such that the total buffer + salt concentration was 100 mM. Peptides were reconstituted by one of three methods: (i) dissolution of the peptide by 25% DHPC (in buffer) followed by addition of solid DMPC and buffer and (finally) mixing, (ii) codissolution of the peptide and DMPC by 95:5 (v/v) benzene–ethanol (Moll & Cross, 1990) followed by freeze-drying to yield a white powder, addition of this powder, detergent, and buffer to an NMR tube, and subsequent mixing, or (iii) addition of solid peptide directly to a premixed bicellar sample, followed by additional mixing with mild sonication, heating/cooling cycles, and physical agitation (vortexing and centrifuging) to disperse and dissolve the peptide within the liquid crystal. The method used for each peptide is listed in Table 1.

NMR Methods. All oriented-sample NMR spectra were acquired using an MR Resources-refurbished Bruker AC-270 NMR spectrometer. A standard variable temperature control unit was utilized with the flow of air through the probe being fairly high in order to ensure rapid dissipation of decoupler-induced heat between scans. Samples were generally incubated in the NMR probe for 15–30 min prior to spectral acquisition to allow the process of magnetic field-induced sample orientation to be completed. Most samples contained D_2O such that the process of sample orientation could be followed by observing the appearance of two symmetric components in the sweeping lock signal (representing the two components of the quadrupolar doublet from D_2O which is exchanging rapidly onto and off of the oriented membrane surface). Following orientation, the field frequency deuterium lock was activated. Methods for acquiring ^{13}C and ^{31}P spectra have been described previously (Sanders & Schwonek, 1992).

The 2-D $^1H/^{13}C$ heteronuclear multiple quantum (HMQC) spectrum (Bax et al., 1983) of an isotropic DHPC–DMPC–cytochrome *c* mixture was acquired using an inverse-detection probe and a Varian UNITYplus 600 MHz NMR spectrometer. This spectrum was acquired with the assistance of Dr. George Gray of Varian Associates (Palo Alto, CA) and was acquired with presaturation of the water signal. 128 *t*1 points, representing 64 scans each, were acquired.

NMR Theory. The detailed equations for chemical shift anisotropy and quadrupolar and dipolar coupling are given elsewhere (Sanders et al., 1994; Sanders, 1993). For the purposes of this paper it should be sufficient to note that in an oriented bilayer system the observed dipolar/quadrupolar couplings and chemical shifts can be thought of as "variables" which are the sums of isotropic and anisotropic components:

$$\text{(observed variable)} = \text{(intrinsic isotropic variable)} + S_{\text{bilayer}} f\langle\text{orientation}\rangle \quad (1)$$

where the "intrinsic isotropic variable" is equal either to 0 (for dipolar and quadrupolar coupling) or to the isotropic chemical shift. $f\langle$orientation$\rangle$ is an anisotropic function which includes a description of the time-averaged orientation of the relevant spin tensor (chemical shift, dipolar, or quadrupolar) with respect to the motional director axis of the system (i.e., the bilayer normal). In this paper, the term "chemical shift anisotropy" (CSA) refers to the anisotropic component of the observed shift and is equal to the oriented sample shift minus the isotropic shift. $S_{bilayer}$ is defined to be an order parameter which varies from 1.0 to 0.0 describing the degree of rapid fluctuation of the orientation of the bilayer normal axes of the bicelles with respect to the magnetic field. $S_{bilayer}$ is equal to 1.0 for bilayers which are fixed with respect to the field and is equal to 0.0 when rapid bilayer motions result in effectively isotropic tumbling on the NMR time scale. For CHAPSO-DMPC and DHPC-DMPC systems, $S_{bilayer}$ can be systematically varied from about 0.6 to 0.0 by varying the detergent:DMPC ratio[3] (see above section). In the studies of this paper, $S_{bilayer}$ for each sample was determined *in situ* by measuring the $^{31}P-^{13}C$ dipolar couplings for DMPC's headgroup α and glycerol-2 carbons as described previously (see footnote to Table 1 in Sanders, 1994).

Diacylglycerol Kinase Assay. Catalytic (5 μg) quantities of purified DAGK were added from ca. 1.5 mg/mL stock solutions containing 3% β-octyl glucoside and 10% glycerol (as a stabilizing agent) into NMR tubes containing 0.45 mL, 25% w/v amphiphile, DHPC:DMPC (mol:mol), and 19 mM ATP in pD 6.6 DAGK assay buffer (Walsh & Bell, 1992). Following incubation of the DAGK-containing bicellar mixtures, Mg^{2+} was added (to 28 mM) and DAGK reactions were initiated by adding *sn*-1,2-dihexanoylglycerol (DHG) to a level of 24 mM. The reaction was followed at 33 °C over a period of several hours by acquiring ^{1}H-decoupled ^{31}P spectra and quantitating the disappearance of MgATP and appearance of MgADP resonances (data not shown).

RESULTS AND DISCUSSION

Reconstitution from the Standpoint of the Model Membranes: Can Membrane Proteins Be Reconstituted without Grossly Disrupting the Discoidal Bilayered Morphology and/or Ability of Bicellar Assemblies To Be Magnetically Oriented? The first step of this study was to screen a number of integral and peripheral proteins and peptides for their affects upon bicellar orientation and morphology. If proteins cannot be reconstituted without grossly disrupting the model membrane system, the possible advantages to be gained by using such media would be negated. Two general classes of perturbation are possible. First, orientation could be disrupted or altered because the diamagnetic susceptibility of associated proteins might promote bilayer fragment orientation with normals parallel to the field (Worcester, 1978). Depending upon the balance of summed susceptibilities from the lipid aggregates (favoring 90° orientation) and from the reconstituted proteins, aggregate orientation could remain unperturbed, lose orientational preference to yield either powder patterns or isotropic spectra (depending upon assembly motions), or align with bicelle normals parallel to the field, yielding a dramatically altered oriented sample spectrum (e.g., see Sanders et al., 1993; Van Echteld et al., 1982). A second possible class of perturbations is morphological in nature. Reconstituted proteins could distort the disc shape of the bicelles, induce phase changes or separations, and/or promote disc aggregation or fusion, all of which would likely be manifested in the form of an NMR-detectable loss of sample orientation and/or in a change in the optical properties of the samples.

Table 1 summarizes the results of peptide screening from the standpoint of model system disruption. It should be pointed out that many of the proteins which were examined were available in very limited quantities, precluding extensive variations in test conditions for each. Determination of the exact physical nature of some of the observations listed in Table 1 would require much additional investigation and would add little to address the crucial issues of this paper. However, a number of useful observations can be made.

1. Some proteins can be reconstituted with little or no perturbation of the oriented phase or formation of aggregate.

2. Some proteins cannot be reconstituted even at relatively low concentrations. In each case where this was observed, there is a reasonable *a priori* explanation. While gramicidin D appears to have a reasonably good "hydrophobic match" with DMPC bilayers (Van Echteld et al., 1982), its shape deviates considerably from an ideal cylinder and might distort the bilayered discs (Ketchem et al., 1993). In the case of P24, comparison with P16 suggests that hydrophobic matching of the bilayer thickness with the length of the hydrophobic domain of the peptide may sometimes be important for optimal reconstitution. Assuming that P16 and P24 span the bilayer as α-helices, the polyleucine stretch of P16 (24 Å in length) would closely match the hydrophobic thickness of DMPC bilayers (23 Å; Lewis & Engelman, 1983) while the corresponding domain of P24 is too long to incorporate (36 Å) without substantial tilt. Disruption of morphology by myelin basic protein may relate to a role this peripheral membrane protein likely plays *in vivo*: binding and appropriately spacing juxtaposed bilayer surfaces in myelin sheaths (Smith, 1992). Finally, the fact that bacteriorhodopsin appeared to initially reconstitute successfully but induced a time-dependent disruption of orientation may be related to an oligomerization process (Kahn et al., 1992).

3. In no case was peptide-induced sample disorientation clearly a result of a perturbation in discoidal aggregate diamagnetic susceptibility. Cases where peptides disrupted sample orientation were inevitably accompanied by changes in the visual appearance of the bicellar sample, indicative of bulk morphological changes. This suggests a tendency of peptides to disrupt bicelle morphology well below the threshold needed to change the sign of aggregate diamagnetic susceptibility.

4. Lowering the peptide:DMPC ratio and/or sample temperature is sometimes necessary to reconstitute a given peptide without disrupting sample orientation. The peptide-concentration dependence of orientational perturbation is not surprising. The fact that some samples oriented at 35° which would not orient at 40° is less easy to explain since optimal orientation normally occurs for protein-free samples near the higher temperature.

5. Some peptides appear to partition between homoaggregated and discoidal bilayer-associated states. A number

[3] At $S_{bilayer} > 0.6$ residual powder patterns appear, reflecting residual sample disorientation. NMR spectral resolution is substantially reduced in such samples.

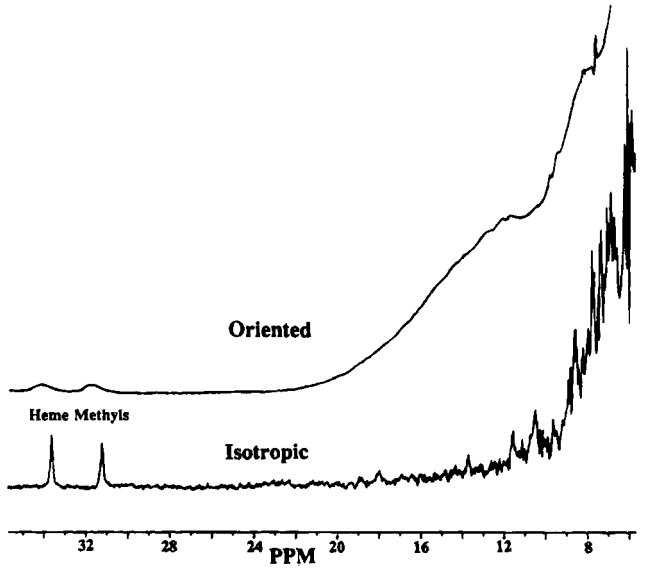

FIGURE 1: ^{1}H NMR spectra (270 MHz) of the downfield region of DHPC-DMPC mixtures containing horse heart ferricytochrome *c* at 38 °C and pD = 6.3. Both samples contained 22% total lipid (w/v) in 50 mM phosphate and 50 mM KCl in D_2O, pD = 6.5, and contained 7 mM CytC and a lauryl sulfate:DMPC molar ratio of 1:10. The oriented sample exhibits an $S_{bilayer}$ of 0.62 and has a DHPC:DMPC mol:mol ratio of 1:2.5. The isotropic spectrum ($S_{bilayer}$ = 0) contains a DHPC:DMPC ratio of 1:1.6. The absolute scales of the two spectra are not equal (if compared at the same absolute scales, the oriented sample spectrum would be considerably reduced in intensity compared to what is illustrated). The oriented and isotropic spectra were produced following exponential multiplication of the free induction decays by line-broadening factors of 0.5 and 1 Hz, respectively. In both cases the HDO resonance (not shown) was used as a rough chemical shift reference (4.65 ppm).

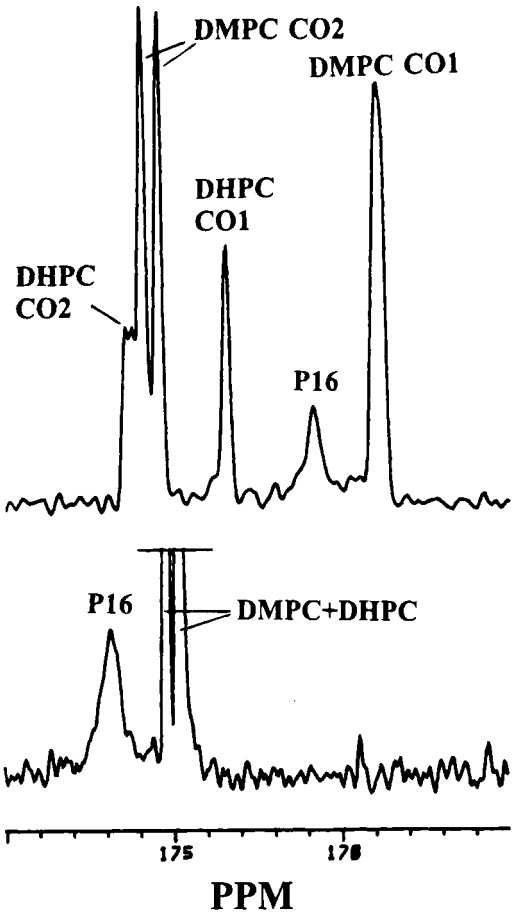

FIGURE 2: 67.8 MHz ^{13}C (^{1}H-decoupled) spectra of bicelle-associated (1-^{13}C)Leu$_{11}$(^{15}N)Leu$_{12}$-P16 under isotropic (bottom) and oriented-sample (top) conditions at pD = 6.3 and 38 °C. The samples contained 25% w/v total amphiphile, a P16:DMPC ratio of 1:65, and represent DHPC:DMPC ratios of 1:2.7 (top, $S_{bilayer}$ = 0.58) and 1:1.6 (bottom). 14−75K scans were acquired and Fourier transformed following a small degree of Gaussian apodization of the free induction decay. Assignment of the lipid resonances has been described elsewhere (Sanders, 1993).

of cases were observed in which a solid suspension was present, but in which the bicellar assemblies appeared to orient normally. These suspensions are most likely aggregated peptide or peptide−lipid complexes. The fact that aggregates were sometimes observed in cases where reconstitution was carried out following organic cosolubilization of DMPC and polypeptide suggests that the bicelles are sometimes saturatable with polypeptide, with excess polypeptide forming aggregates rather than incorporating into bicelles at levels at which a morphological transition is induced.

Reconstitution from the Standpoint of Proteins: Can an Integral Membrane Protein be Functionally Reconstituted into Bicelles? Diacylglycerol kinase is a 13 kDa enzyme which has been demonstrated to contain three transmembrane segments (encompassing roughly 80% of its sequence) in native membranes (Smith et al., 1994b). This enzyme can be functionally reconstituted into β-octyl glucoside micelles but is rather unstable in that medium ($t_{1/2}$ of 230 min at 25 °C; Walsh & Bell, 1986). This is in contrast to its impressive stability in native membranes ($t_{1/2}$ of 20−60 min at 100 °C; Raetz et al., 1981; Russ et al., 1988).

DAGK was reconstituted into DHPC-DMPC bicelles containing ATP, incubated, and then assayed in the bicelles following the addition of Mg^{2+} and dihexanoylglycerol (see Materials and Methods). When the assay was carried out immediately after reconstitution (no incubation), the specific activity was observed to be equal to that for the enzyme in the standard octyl glucoside-based mixed micellar assay system: 12 μmol of ADP produced/(mg·min). When DAGK was first incubated in DHPC-DMPC bicelles for 24 h at 38 °C and then assayed, its specific activity was actually observed to *increase* by a factor of about 2. This suggests that the bicelles provide an environment in which not only is active DAGK stable, but which allows inactivated DAGK present in the micellar stock DAGK solutions to be slowly reactivated, perhaps by facilitating refolding or formation of the appropriate oligomeric state (which is currently not known with certainty). In any case, these results demonstrate that at least one integral membrane protein can be *functionally* reconstituted into bicellar systems. Furthermore, the fact that DAGK was observed to be substantially more stable in bicelles than in octyl glucoside micelles suggests that bicelles provide a more optimal approximation of native bilayers.[4] Finally, it should be pointed out that while DAGK could possibly be reconstituted into lipid multilamellae, even the very simple kinetic study described here would be very difficult to carry out in such a medium because of the fact that one of the substrates (MgATP) is highly polar and would not be able to diffuse freely throughout multilamellae.

Reconstitution from a Spectroscopic Standpoint: Do Reconstituted Membrane Proteins Yield High Quality Oriented-Sample NMR Spectra? ^{1}H NMR spectra were acquired for most of the samples listed in Table 1. ^{1}H NMR provides a simple means of determining whether or not the reconstituted protein is isotropic in solution or whether it is associated with bicelles and/or aggregated. In the isotropic case,

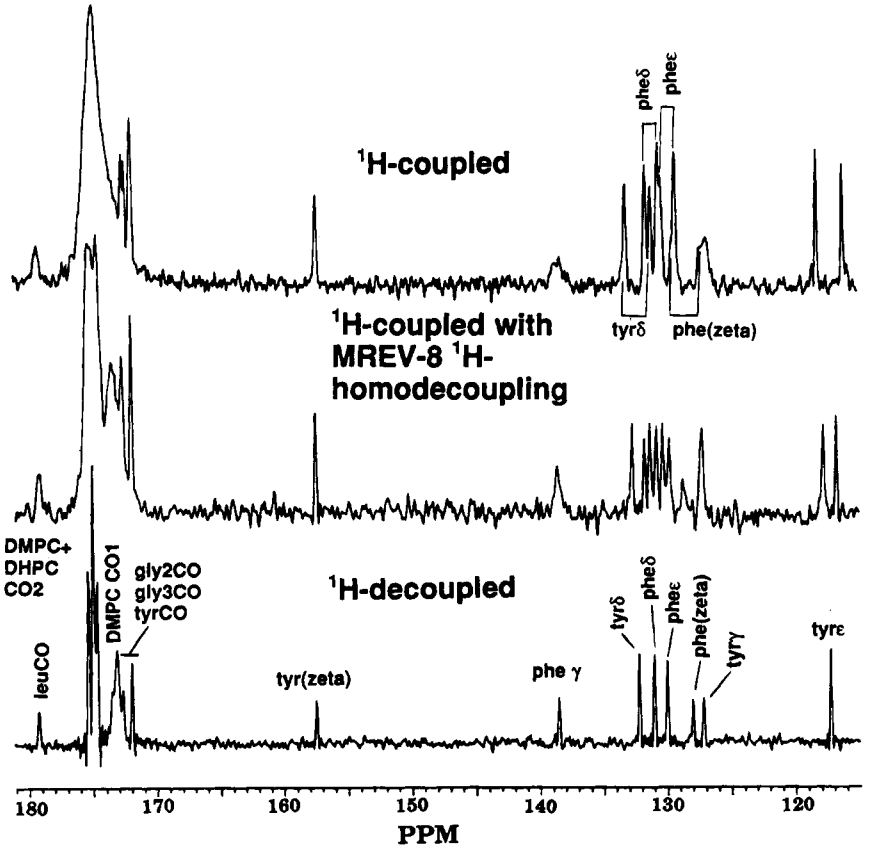

FIGURE 3: ^{13}C NMR spectra of unlabeled leucine enkephalin in a oriented ($S_{bilayer}$ = 0.17) DHPC-DMPC (1:2.1 mol:mol) mixture at 42 °C (carbonyl and aromatic regions only). The bottom and top spectra were acquired with and without ^{1}H-decoupling, respectively. The middle spectrum was acquired with protons coupled to ^{13}C but $^{1}H-^{1}H$ homodecoupled using the MREV-8 pulse sequence (which also scales down the magnitude of the observed $^{1}H-^{13}C$ dipolar couplings; see Sanders & Prestegard, 1991). Both samples contained 25% (w/v) total lipid (DHPC + DMPC) and were buffered with 70 mM sodium phosphate and 30 mM KCl in D_2O at pD = 7.0. The DMPC:LENK mol:mol ratio was roughly 10:1. The Phe carbonyl peak in the oriented spectrum appears as a left shoulder to the DMPC *sn*-1 carbonyl resonance, while the right shoulder of this peak represents Tyr. These and all of the other assignments were made using $S_{bilayer}$-variation plots as described in the text and in Sanders and Landis (1994).

resolvable ^{1}H signals are normally discernible, particularly if the polypeptide contains aromatic residues. In the latter cases, the onset of extensive $^{1}H-^{1}H$ dipolar coupling leads to broad, usually unresolvable ^{1}H spectra. Thus, at least in the absence of special isotopic enrichment or pulse techniques dedicated to reducing strong homonuclear dipolar coupling, ^{1}H NMR is of practical utility primarily as a way of determining whether a given protein may exist in an isotropic state within a sample in which the bicelles are oriented. An example of ^{1}H NMR spectra from isotropic and oriented bicellar samples appears in Figure 1.

^{13}C NMR offers a more facile route to structurally interpretable NMR data. Strong $^{13}C-^{1}H$ dipolar coupling can be removed by high power ^{1}H-decoupling. $^{13}C-^{13}C$ coupling is not present unless high levels of multisite ^{13}C isotopic enrichment has been employed. The results of attempts to observe ^{13}C spectra from proteins reconstituted into bicelles are summarized in the last column of Table 1.

In some cases proteins yielded no observable ^{13}C spectra even after 10−100K scans, either because the protein was too dilute or because resonances were broad. Broad lines could ensue when molecular motions are slow or in the case that the protein exists in a motional or aggregational state which leads to powder patterns. For some proteins, ^{13}C spectra *could* be detected, although isotopic labeling was sometimes necessary. Key examples are illustrated in Figures 2−4.

Melittin and Cor3 both could be reconstituted without disrupting the orientation or morphology of the bicellar phase. However, ^{13}C NMR resonances from labeled carbonyl positions on these reconstituted polypeptides were just barely detectable above the noise (spectra not shown) and were extremely broad and asymmetric. These results provide examples of peptides which appear to be successfully reconstituted into the bicellar systems, but which apparently do not execute motions therein which allow a high resolution oriented-sample spectrum to be observed (see Sanders et al., 1994).[5] The use of bicelles as model membranes would appear to offer little advantage for proteins of this class.

[4] Incubation of DAGK in bicellar mixtures at temperatures of >70 °C were also carried out. In these cases, loss of activity was substantial, but not total. However, interpretation of these results was complicated by the fact that a morphological transition occurs in the bicellar systems at T > 70 °C, as evidenced by a change in appearance of the samples (clear to cloudy). In addition, some studies were carried out at lower DHPC:DMPC ratios and in 1:3.5 CHAPSO:DMPC samples. In these cases, DAGK stability results were fully consistent with the results reported in the text. However, interpretation of these results was not as straightforward because in these cases diacylglycerol disrupted the bicellar morphology as evidenced both by NMR and by changes in sample appearance. Interestingly, DAGK-catalyzed conversion of diacylglycerol to phosphatidic acid actually led to restoration of the oriented bicellar phase for at least one CHAPSO-DMPC mixture, providing a rare example of an *in vitro* enzyme-catalyzed morphological phase transition.

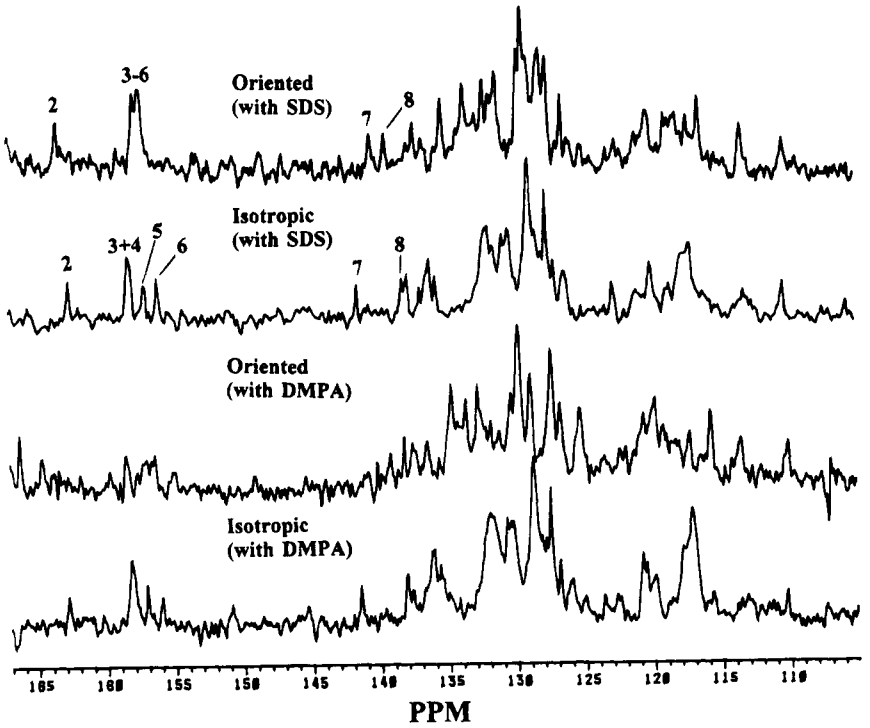

FIGURE 4: Aromatic and partial carbonyl regions of ^{13}C (^1H-decoupled) NMR spectra of CytC in pD = 6.3 DHPC-DMPC mixtures containing either lauryl sulfate (SDS, top two) at 34 °C or DMPA (bottom two) at 40 °C. The oriented and isotropic DMPA-containing samples represent DHPC:DMPC ratios of 1:2.8 ($S_{bilayer}$ = 0.68) and 1:1.6 , respectively, and contain CytC:DMPC ratios of about 1:35 and DMPA:DMPC = 1:10. The oriented and isotropic lauryl sulfate-containing samples contain SDS at a level of 1:12, represent DHPC:DMPC ratios of 1:2.7 ($S_{bilayer}$ = 0.66) and 1:1.6 (respectively), and contain CytC:DMPC = 1:33. All four spectra were produced following exponential multiplication of the free induction decay (8 Hz line broadening) and represent 75 000−125 000 scans each. It should be noted for future reference that sodium is the preferred counterion in these studies because of the propensity of K^+ to induce precipitation of lauryl sulfate. Most of the CytC studies of this work were carried out with SDS as the negatively charged amphiphile in the bicelles. However, because SDS does not resemble a natural lipid, spectra were acquired for samples in which DMPA was used instead (shown here). It would not be expected that the DMPA spectra should be equivalent to their SDS counterparts because the affinity of CytC for the bicelle surface and also possibly its motional dynamics are expected to vary for different lipid compositions (Devaux et al., 1986; Reitveld et al., 1983).

P16 offers a more favorable contrast to the melittin and Cor3 results. The ^{13}C spectrum from a single carbonyl located about midway through the Leu_{16} sequence exhibits a line width of about 50 Hz in the oriented bicelles (Figure 2, top). This appears to be more narrow by a factor of about 3 than carbonyl-^{13}C resonances previously observed from transmembrane peptides reconstituted in bilayers oriented by sandwiching between glass plates (Wang et al., 1992; Smith et al., 1994a). This result is encouraging from the standpoint of enhanced oriented sample spectral resolution. Unfortunately, it must be pointed out that the resultant gain in NMR *sensitivity* is not fully realized relative to corresponding spectra from glass plate-oriented multilamellae because of the higher peptide:lipid ratios attainable (without orientational disruption) in the latter system (e.g., see Moll & Cross, 1990; Smith et al., 1994).

P16 is thought to traverse bilayers as an α-helix (Bloom et al., 1991). The difference in oriented and isotropic chemical shifts for the carbonyl (Figure 2) is about −5.8 ppm. Given that the $S_{bilayer}$ for this sample is about 0.58, this CSA is close to that predicted for a transmembrane helix well-aligned with the bilayer normal (−4 to −5 ppm; Sanders & Schwonek, 1993).[6]

Gramicidin S, β-amyloid (1−39), and FFG-Bz all yielded ^{13}C linewidths (data not shown) comparable to that observed for P16. While relatively little is known about the exact mode of interaction of these peptides with membrane bilayers, it is unlikely that any of them interact with bilayers as transmembrane α-helices. Most likely, their modes of interaction with membranes involve partial insertion into bilayers in a manner such that their translational, rotational, and conformational motions are substantially dampened, leading to moderately broad ^{13}C linewidths similar to those from P16.

Leucine enkephalin (Figure 3) and cytochrome *c* (Figure 4) appear to represent a spectroscopically distinct class of proteins (see also Figures 1 and 3 of Sanders & Landis, 1994). The breadth of their ^1H NMR resonances (Figure 1 and additional data not shown) and the fact that their ^{13}C chemical shifts scale with $S_{bilayer}$ (see next section) clearly indicate bicelle association. However, the extremely high

[5] Failure to execute rapid axial rotation about the bilayer normal is one obvious possibility. However, we are hesitant to forward even a tentative interpretation of these data in terms of possible modes of interaction with the bicelles. In the case of melittin, it is known that interactions with bilayers are varied and complex (see Dempsey, 1990).

[6] It should be noted that no $^{15}N-^{13}C$ dipolar coupling is observed in the ^{13}C signal from P16, despite the proximity of the labels. This could be because the coupling is small (<30 Hz) or because of an extreme case of differential line broadening due to interference effects between the dipolar tensor and the CSA relaxation mechanism (Sanders, 1993). Since the dipolar coupling expected in this case for a transmembrane helix is fairly large (>200 Hz; Sanders & Schwonek, 1993), the latter interpretation seems more probable.

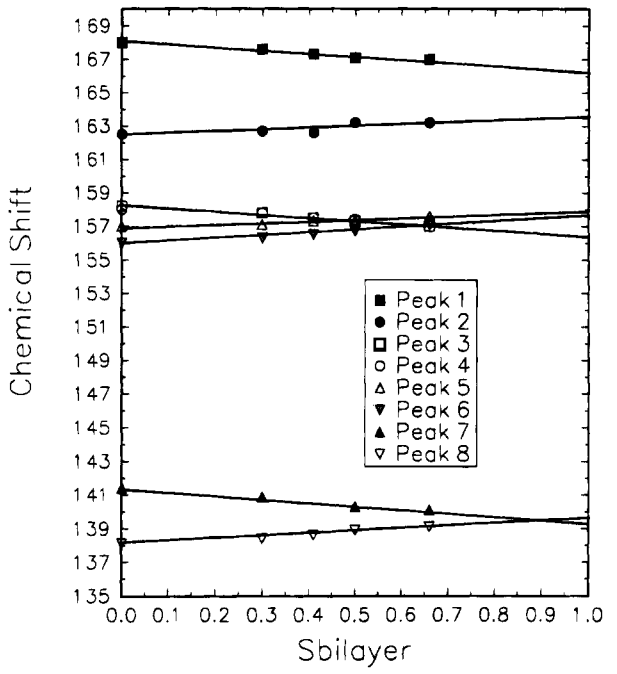

FIGURE 5: Dependence of ^{13}C resonance positions for selected CytC peaks in oriented DHPC-DMPC mixtures containing lauryl sulfate as a function of $S_{bilayer}$ at 40 °C. The numbering of the resonances corresponds to that shown in the top two spectra of Figure 4 (peak 1 is not shown here, but appears in Figure 3 of Sanders & Landis, 1994). The uncertainty of most points is ±0.15 ppm. The linear fit for peak 4 is not drawn. The samples represented by these data contained a fixed molar ratio of DMPC:lauryl sulfate (12:1) and variable DHPC:DMPC ratios (1:2.8 to 1:1.6) at a total amphiphile concentration of 20%. The CytC concentration was typically 8 mM, and the buffer was 50 mM sodium phosphate and 50 mM NaCl, pD = 6.3.

resolution of the ^{13}C spectra are unprecedented for oriented membrane proteins. The observed high resolution likely derives from either or both of two classes of motions. First, it is quite possible that when bound to the bicelle surfaces these proteins exhibit considerable conformational dynamics. This is supported by the experimentally-based hypothesis that surface association of CytC actually induces partial unfolding of this protein (de Jongh et al., 1992; Spooner & Watts, 1991) and by molecular dynamics simulations of interface-associated leucine enkephalin (C. Sanders and J. Schwonek, unpublished data), suggesting considerable conformational heterogeneity. A second class are whole-protein motions. These would include on-surface wobbling and diffusion and also rapid dissociation of the protein into isotropic solution followed by tumbling and reassociation. Both possibilities are supported by binding data which indicate that CytC and LENK bind to lipid surfaces with relatively weak affinities and low specificities (Devaux et al., 1986; Reitveld et al., 1983; Deber & Benham, 1984; Takeuchi et al., 1992). The presence of extensive motional averaging is also supported by the relatively small magnitudes of CSAs and dipolar couplings observed for both bicelle-associated proteins (see next section). These proteins appear to represent examples of an entire class of membrane proteins for which the bicellar systems may be especially well-suited as media for structural studies.

Exploitation of Unique Properties of Bicelles as a Tool for NMR Structural Study of Reconstituted Membrane Proteins: (i) Screening for Detergent-Specific Artifacts. One of the properties of the DHPC-DMPC and CHAPSO-DMPC

Table 2: Dipolar Couplings and Carbon-13 CSA for Surface-Associated Leucine Enkephalin

coupled spins	dipolar coupling[a]	carbon atom	CSA ($\delta_{obs} - \delta_{isotropic}$, in ppm)[a]
$Tyr^1H_\alpha/Tyr^{13}C1$	|72| ± 35	TyrC1	+2.8 ± 0.5
$Gly2^1H_\alpha/Gly2^{13}C1$[b]	<|40|	Gly2-C1	+0.7 ± 0.5
$Gly3^1H_\alpha/Gly3^{13}C1$[b]	<|40|	PheC1	+5.1 ± 1.0
$Tyr^1H_\epsilon/Tyr^{13}C_\zeta$	<|25|	LeuC1	−3.5 ± 0.5
$Tyr^1H_\epsilon/Tyr^{13}C_\delta$[c]	<|40|	$TyrC_\gamma$	+2.1 ± 0.3
$Tyr^1H_\delta/Tyr^{13}C_\epsilon$[d]	<|40|	$TyrC_\delta$[c]	+0.7 ± 0.3
$Tyr^1H_\delta/Tyr^{13}C_\delta$	<70[e]	$TyrC_\epsilon$[d]	+0.9 ± 0.3
$Tyr^1H_\epsilon/Tyr^{13}C_\epsilon$	< 70[e]	$TyrC_\zeta$	+1.6 ± 0.3
$Phe^{13}C_\gamma$ to Phe^1H_δ and/or Phe^1H_β	>|70|	$PheC_\gamma$	+1.4 ± 0.3
$Phe^{13}C_\epsilon/Phe^1H_\delta$	<|60|	$PheC_\delta$[c]	+1.8 ± 0.3
$Phe^{13}C_\epsilon/Phe^1H_\zeta$	<|60|	$PheC_\epsilon$[d]	+1.7 ± 0.3
$Phe^1H_\epsilon/Phe^{13}C_\delta$	<|60|	$PheC_\zeta$	+1.0 ± 0.3
$Phe^1H_\epsilon/Phe^{13}C_\zeta$	>|50|	Gly3-C1	−1.1 ± 0.5
$Tyr^{13}C_\gamma$ to Tyr^1H_δ and/or Tyr^1H_β	>|50|		
$Phe^1H_\epsilon/Phe^{13}C_\epsilon$	−382 ± 75		
$Phe^1H_\delta/Phe^{13}C_\delta$	−417 ± 75		
$Phe^1H_{zeta}/Phe^{13}C_\zeta$	<|75|		
$Leu^1H_\alpha/Leu^{13}C{=}O$	<|75|		
$Leu^1H_\beta/Leu^{13}C{=}O$	<|75|		
$Gly3^1H_{\alpha1}/Gly3^{13}C'$	+770 ± 100		
$Gly3^1H_{\alpha2}/Gly3^{13}C_\alpha'$	+45 ± 30		
$Gly2^{15}N/Gly3^{13}C_1$	|8| ± 4[g]		
$Gly3^{13}C_1/Gly3^{13}C_\alpha$	|16| ± 6[g]		

[a] These CSAs and dipolar couplings have been normalized for $S_{bilayer}$ = 1.0 (see Sanders, 1994) and thus represent measurements made for binding of the peptide to immobile bilayers which are oriented perpendicular to the direction of the magnetic field. In addition to the listed uncertainties associated with each measurement, the data should be regarded as possibly containing a few gross errors in assignment or measurement. In the modeling carried out in our lab, we have regarded as consistent with the experimental data any structures (or ensembles) which satisfy 90% of the measurements. [b] Coupling to *either* α-proton. [c] The two δ-carbons are degenerate. [d] The two ε-carbons are degenerate. [e] Sign is probably negative. [f] Proton could not be stereospecifically assigned, coupling constant must be regarded as tentative due to the possibility of complication from strong $^1H-^1H$ coupling (no MREV-8 experiment was run). [g] J and D have the same sign.

systems is the ability to gradually decrease oriented bicelle order from high ($S_{bilayer}$ = 0.6) to the isotropic limit ($S_{bilayer}$ = 0) by varying the detergent:DMPC ratio. In previous work (Sanders & Prestegard, 1992; Sanders, 1993) this has been demonstrated to be useful as a means for screening out detergent-specific artifacts in NMR measurements made in bicellar systems: if CSAs, dipolar, and quadrupolar couplings scale linearly as a function of $S_{bilayer}$, this indicates that the measurements are independent of the detergent content in the sample. Furthermore, even in cases where spectral parameters do not scale linearly, by extrapolating $S_{bilayer}$ plots to 1.0, a reasonable estimate of the intrinsic value of the CSA or coupling in a detergent-free bilayer may be obtained.

In this work we undertook DHPC titrations of both CytC and LENK samples and followed chemical shift positions as a function of $S_{bilayer}$. The plot for the 5 carbonyl carbons of LENK appears elsewhere (Sanders & Landis, 1994) and shows that the shifts for all 5 scaled linearly with $S_{bilayer}$. Figure 5 illustrates a similar plot for selected aromatic resonances of CytC and also exhibits linear scaling. These data rule out significant detergent-induced perturbations of the structures of bicelle-associated LENK and CytC.

Exploitation of the Unique Properties of Bicelles as a Tool in NMR Structural Studies of Reconstituted Membrane

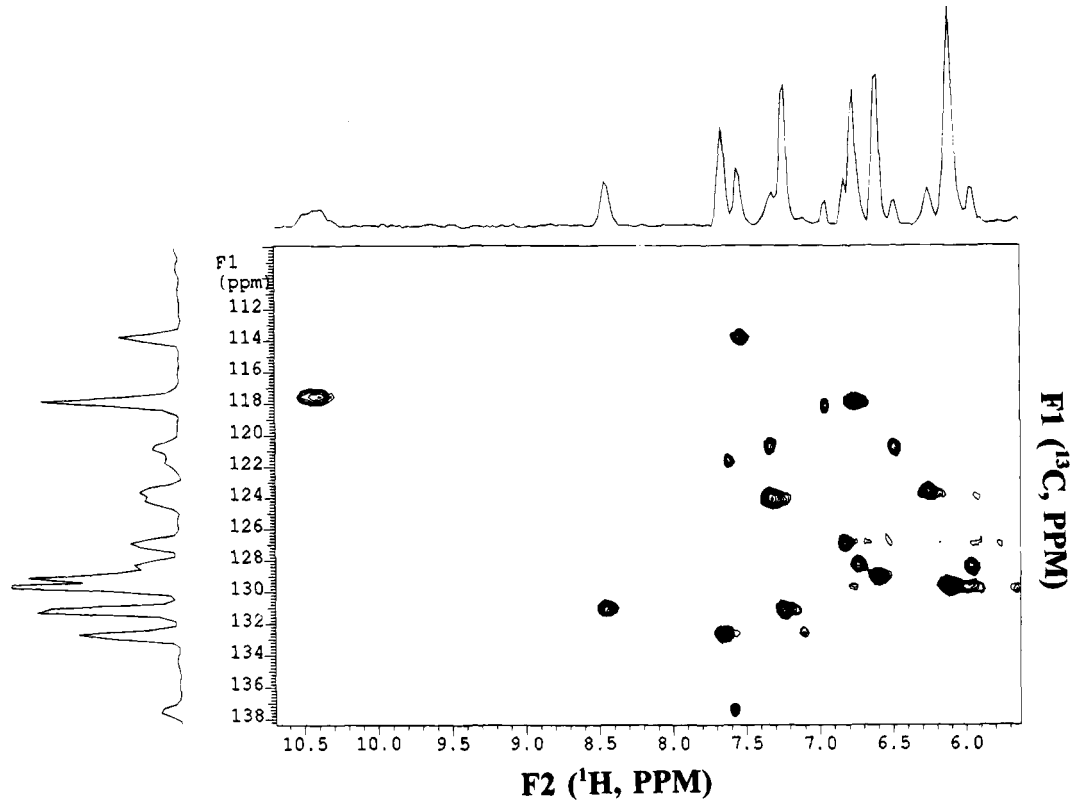

FIGURE 6: $^{1}H-^{13}C$ HMQC of ferricytochrome *c* in an isotropic 1:1.6 DHPC:DMPC mixture containing SDS:DMPC = 1:12 at 40 °C. Additional conditions for sample and spectral acquisition are presented in the caption to Figure 4 and in the Materials and Methods section. The ^{13}C (*F*1) 1-D projection is illustrated to the left of the contour plot while the ^{1}H (*F*2) projection is shown above.

Proteins: (ii) Correlating Oriented-Sample ^{13}C Resonances with Isotropic Resonances. Plots such as Figure 5 are also of use in assigning oriented-sample spectra. ^{13}C dipolar couplings and CSAs obtained from oriented-sample spectra can be used most effectively only if the relevant peaks can be assigned to specific carbons within the protein. Because dipolar coupling is through-space in nature (not absolutely related to chemical connectivities) and because oriented-sample ^{1}H NMR spectra are often uninterpretable, *direct* assignment of oriented ^{13}C resonances via standard multidimensional, multinuclear correlation NMR methods is not generally feasible. However, $S_{bilayer}$ plots such as Figure 5 (see also Figure 2 in Sanders & Landis, 1994) can be used to follow oriented-sample peaks to their isotropic positions, at which point it may be possible to assign peaks using standard solution NMR methods.[7]

In the case of LENK, isotropic ^{13}C resonances have previously been assigned (Khaled et al., 1979). Using $S_{bilayer}$ plots (Figure 2 in Sanders & Landis, 1994, and additional plots not shown), it was possible to assign all oriented sample aromatic and carbonyl peaks and determine their CSAs. Furthermore, additional ^{13}C spectra acquired either in the absence of ^{1}H-decoupling or in the presence of $^{1}H-^{1}H$ *homo*decoupling (while leaving ^{1}H and ^{13}C coupled, Figure 3) facilitated the determination of a number of dipolar couplings. These data are presented in Table 2 and may be employed in future quantitative structural analysis (which lies beyond the scope of this paper).

Exploitation of the Unique Properties of Bicelles as a Tool in NMR Structural Studies of Reconstituted Membrane Proteins: (iii) Correlating Isotropic ^{13}C Resonances with ^{1}H Resonances. The small size of LENK and previous assignment of the ^{13}C NMR resonances obviated the need to directly assign isotropic ^{13}C resonances in the bicellar samples. However, in the case of CytC complete isotropic ^{13}C assignments have not been made. Even if they had, it might be improper to assume that peak positions in simple aqueous solution would necessarily correspond to those from CytC associated with isotropic bicelles. Since ^{13}C NMR resonances of relatively large proteins are normally assigned by correlating ^{13}C resonances with assigned ^{1}H resonances, we tested to see whether a standard $^{13}C-^{1}H$ correlation experiment could be successfully executed in a bicellar sample. A subsection of a ^{1}H-detected HMQC spectrum of CytC in isotropic DHPC-DMPC bicelles is illustrated in Figure 6 and clearly shows a number of well-resolved cross-peaks. No $^{1}H-^{1}H$ correlation experiment has been carried out because of the huge background expected from the nondeuterated bicellar phase, although the high resolution of the ^{1}H spectrum from bicellar CytC (Figure 1) indicates that such experiments should be quite possible in the presence of an appropriate labeling and isotope-filtering pulse scheme.[8] While complete spectral assignment of bicelle-associated CytC would likely require isotopic labeling and is a very

[7] It must be acknowledged that oriented/isotropic correlation of *all* CytC resonances would require an $S_{bilayer}$ plot having many more $S_{bilayer}$ points than that illustrated in Figure 5 and better signal to noise than observed for the spectra of Figure 4. This is entirely feasible if low percentage (ca. 20%) uniform ^{13}C enrichment can be carried out. The spectra represented in Figures 4 and 5 represent natural abundance ^{13}C and required >48 h each for acquisition.

[8] Either the detergent and lipid could be perdeuterated or the protein could be isotopically labeled with ^{15}N and/or ^{13}C, followed by ^{15}N or ^{13}C filtering to enhance protein resonance intensities relative to the background spectrum from the detergent and lipid components.

large task requiring many additional $^{1}H-^{1}H$ and $^{13}C-^{1}H$ experiments, the results of this paper indicate that this approach is feasible.

CONCLUSIONS

This work was driven by the hypothesis that bicellar model membrane systems: (i) may sometimes allow certain difficulties associated with the use of multilamellar or classical micellar systems to be circumvented in studies of membrane proteins and (ii) offer unique properties which can be exploited to actually *facilitate* NMR analysis. The results of this study clearly bear these out for at least two surface-associating proteins. Although total structural analysis was not attempted, the work of this paper would appear to bring the continuing development of bicellar systems to a point where an attempt at such an analysis for a surface-associating protein may be a logical next step.

The merits of using bicelles in studies of integral membrane proteins or proteins whose mode of surface interaction probably extends well into the hydrophobic domain of bilayer surfaces appears to be a matter for case-by-case consideration in which a number of factors must be weighed. All model membrane systems have their merits and drawbacks. However, it can be hoped that the bicellar systems may fill a useful niche in the broad spectrum of methods used to mimic bilayer environments in studies of membrane proteins.

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