Journal of Biomolecular NMR, **12:** 361–372, 1998.
KLUWER/ESCOM

Characterization of magnetically oriented phospholipid micelles for measurement of dipolar couplings in macromolecules

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Received 6 March 1998; Accepted 12 May 1998

Key words: alignment, bicelle, dipolar coupling, liquid crystal, protein NMR, phospholipid

Abstract

Weak alignment of solute molecules with the magnetic field can be achieved in a dilute liquid crystalline medium, consisting of an aqueous mixture of dimyristoyl-phosphatidylcholine (DMPC) and dihexanoyl-phosphatidylcholine (DHPC). For a certain range of molar ratios, DMPC and DHPC can form large, disc-shaped particles, commonly referred to as bicelles (Sanders and Schwonek, 1992), which cooperatively align in the magnetic field and induce a small degree of alignment on asymmetrically shaped solute molecules. As a result, dipolar couplings between pairs of ^{1}H, ^{13}C or ^{15}N nuclei are no longer averaged to zero by rotational diffusion and they can be readily measured, providing valuable structural information. The stability of these liquid crystals and the degree of alignment of the solute molecules depend strongly on experimental variables such as the DMPC:DHPC ratio and concentration, the preparation protocol of the DMPC/DHPC mixtures, as well as salt, temperature, and pH. The lower temperature limit for which the liquid crystalline phase is stable can be reduced to 20 °C by using a ternary mixture of DHPC, DMPC, and 1-myristoyl-2-myristoleoyl-*sn*-glycero-3-phosphocholine, or a binary mixture of DHPC and ditridecanoyl-phosphatidylcholine. These issues are discussed, with an emphasis on the use of the medium for obtaining weak alignment of biological macromolecules.

Introduction

Residual one-bond dipolar couplings in high resolution NMR provide unique constraints in molecular structure determination (Saupe and Englert, 1963; Emsley and Lindon, 1975; Bastiaan et al., 1987; Bothner-By, 1996; Tolman et al., 1995; King et al., 1995; Tjandra et al., 1996). As the internuclear distances for such dipolar interactions are accurately known, the dipolar couplings provide direct information on the orientations of the corresponding bond vectors relative to the molecular alignment tensor. These constraints are therefore fundamentally different from the strictly local NOE and *J* coupling constraints, which constrain atom positions only relative to those in their immediate vicinity. Addition of only 90 such dipolar constraints, measured for a small protein complexed with a 16-basepair DNA fragment, resulted in a nearly two-fold reduction of ϕ/ψ pairs outside of the most-favored region of the Ramachandran map (Tjandra et al., 1997) and greatly improved the agreement between predicted and measured magnetic field dependence of ^{15}N shifts (Ottiger et al., 1997). Until recently, only the minute one-bond ^{1}H–^{15}N and ^{1}H–^{13}C dipolar couplings resulting from the molecular alignment induced by its own magnetic susceptibility anisotropy could be measured in macromolecules with particularly large anisotropies. The use of very dilute, lipid-based liquid crystalline media makes this method far more general (Tjandra and Bax, 1997), as the degree of molecular alignment can be adjusted to its optimum magnitude for measurement of dipolar couplings by changing the concentration of the lipid particles (Bax and Tjandra, 1997).

The use of binary detergent mixtures to form a liquid crystalline phase has been extensively explored by Prestegard and co-workers (Ram and Prestegard, 1988; Sanders and Prestegard, 1990; Sanders et al.,

1994). Mixtures of dimyristoyl-phosphatidylcholine (DMPC) and dihexanoyl-phosphatidylcholine (DHPC) in water were found to be particularly useful for this purpose because they form a stable liquid crystalline phase over a large range of concentrations (Sanders and Schwonek, 1992). These mixtures are believed to form disc-shaped particles of several hundred Ångstroms diameter and a thickness of ca. 40 Å, with the DMPC making up the plane of the discs, and the DHPC covering the edges (Sanders and Schwonek, 1992; Sanders et al., 1994; Vold and Prosser, 1996). These particles, commonly referred to as bicelles, can adopt a nematic liquid crystalline phase, where they cooperatively align with their normal orthogonal to the magnetic field direction. Addition of paramagnetic lanthanide ions can be used to 'flip' the bicelles by 90°, to an orientation where the bicelle normal is oriented parallel to the magnetic field (Prosser et al., 1996, 1998). Lipophilic molecules can be anchored in the oriented bicelles, and NMR spectra of such highly ordered absorbed molecules can provide unique structural information (Metz et al., 1995; Salvatore et al., 1996).

Alternatively, hydrophilic molecules can be dissolved in the aqueous solvent. The deviation from spherical symmetry of the aqueous domain available to these solute molecules, caused by the presence of the oriented particles, results in a small degree of molecular alignment. The extent of this alignment is a function of the asymmetry of the solute's three-dimensional shape and surface charge distribution, and of the density and degree of magnetic alignment of the bicelles.

Here we discuss practical considerations regarding sample preparation and stability of the bicelles and provide an NMR characterization of the bicelle medium over a range of conditions suitable for protein NMR. For most proteins, the concentration of the bicelles must be kept small to limit the solute ordering, so that severe line broadening, caused by multitudes of unresolved ^{1}H–^{1}H dipolar couplings, is avoided. Note that rotational diffusion rates, as monitored by a ^{15}N $T_{1\rho}$ experiment, are not significantly affected by the bicelles (Bax and Tjandra, 1997). However, under these dilute conditions the liquid crystal becomes more 'fragile' and the temperature range over which the samples form a stable nematic phase is a sensitive function of the DMPC:DHPC lipid ratio. The stability of the nematic phase is also modulated by the concentration and type of solute. Although most proteins can be dissolved in high concentrations, without any negative effect on the stability of the liquid crystalline phase, other proteins are only tolerated in submillimolar concentrations.

Experimental

All phospholipids were purchased as dry powders from Avanti Polar-Lipids, Inc. (Alabaster, AL) and used without further purification. Because the phospholipids are highly ordered in the liquid crystalline state, their proton resonances are very broad and in heteronuclear experiments do not significantly interfere with observation of the solute signals of interest. Purchase of the more expensive perdeuterated phospholipids is therefore not necessary. DHPC is very hygroscopic and hydrolyzes slowly in the presence of water. Therefore, DHPC was purchased in flame-sealed glass ampules, each containing 50–100 mg DHPC.

Bicelle preparation

An effective and convenient recipe for preparing the bicelles makes use of a buffer solution containing 5–10 mM phosphate buffer, pH 6.6, 0.15 mM sodium azide, 93% H_2O (HPLC grade, Aldrich), 7% D_2O (99.9%, CIL). Below, this solution will simply be referred to as buffer. DMPC/DHPC stock solutions containing a total of 15% w/v (150 mg lipid/ml) are prepared as follows: first, the DHPC is carefully weighed in a dry atmosphere, and dissolved in buffer by briefly vortexing it, followed by centrifugation to remove bubbles and slow, sideways rotation of the tube to ensure sample homogeneity. DHPC is a detergent that is readily soluble up to high concentrations in water. Second, a fraction of this DHPC stock solution is added to a vial containing a predetermined amount of DMPC (also weighed in a dry box), sufficient to yield the desired molar ratio, q = [DMPC]:[DHPC]. Additional buffer is added to give a final total lipid (DHPC + DMPC) concentration of 150 mg/ml. The mixture is briefly vortexed every few hours and is kept at about 18 °C. Depending on q, the DMPC dissolves over a period of less than 8 ($q \leq 3$) to 24 ($q \approx 3.5$) hours. After all powder has dissolved, the sample is heated to ca. 40 °C for several minutes, followed by cooling to 0 °C. This cycle is repeated several times to ensure sample homogeneity. Subsequently it is vortexed once more and spun down to remove bubbles. The same procedure is used for preparing stock solu-

tions of binary mixtures of DHPC and ditridecanoylphosphatidylcholine (DTPC), except that the sample is kept at 0 °C during vortexing.

A ternary mixture of 1-myristoyl-2-myristoleoyl-*sn*-glycero-3-phosphocholine [(14:0) (14:1n5) PC, which differs from DMPC by the fifth bond on the *sn*2 chain, starting from the methyl group, being unsaturated and *cis*], DMPC and DHPC, in a molar ratio of 0.75:2.25:1, is made most conveniently by first combining [(14:0) (14:1n5) PC] and DMPC as dry powder in a 1:3 molar ratio, and subsequently adding the appropriate amount of DHPC stock solution, followed by several cycles of vortexing, heating and cooling. The sample is kept in a nitrogen atmosphere to prevent slow oxidation of the double bond in (14:0) (14:1n5) PC. Above 15 °C, the ternary mixture is very viscous and it is dissolved most easily in a cold environment.

In aqueous solution, the phospholipids are subject to hydrolysis of the four ester bonds, with the two carboxy esters connecting the aliphatic chains to the glycerol part being hydrolyzed faster (Grit and Cromelin, 1993). This hydrolysis is catalyzed both by acid and by base (see below). When preparing the sample it is therefore important to ensure that the solution pH is $\geq$5, and preferably in the 6–7 range. DMPC/DHPC bicelle stock solutions may be used immediately or, for long-term stability, can be stored frozen, preferably at −80 °C.

NMR sample preparation

Dilute liquid crystalline NMR samples are obtained by adding buffer to the 15% w/v stock solution, followed by vortexing and centrifugation.

Protein samples, containing the commercially obtained uniformly $^{13}C/^{15}N$-enriched ubiquitin (VLI Research, Southeastern, PA; 0.7 mM) or unlabeled BPTI (ICN Biomedicals Inc., Aurora, OH; 8.5 mM), were prepared by first dissolving them in the buffer solution. After pH adjustment, this solution was subsequently used to dilute the 15% bicelle stock solution to the desired concentration.

In order to reduce the required sample quantities, all experiments on BPTI and ubiquitin were carried out in thin-wall Shigemi microcells (Shigemi Inc., Allison Park, PA). As the DMPC/DHPC liquid crystalline phase is only persistent above room temperature, air bubbles tend to form under the plunger in these microcells. To avoid formation of these bubbles, which have an adverse effect on magnetic field homogeneity, the complete microcell with its plunger in the final position and no air bubbles below it, but not properly sealed at the top, is placed in a low pressure (ca. 50 millibar) flask for 15 min. Bubbles newly formed under the plunger are subsequently removed in the normal manner, prior to sealing the sample at the top of the tube with Parafilm. In our experience, samples prepared in this manner stay bubble-free even after multiple-day measurements at temperatures as high as 40 °C.

NMR spectra were recorded on Bruker DMX spectrometers operating at ^1H resonance frequencies of 750, 600 and 500 MHz. ^2H spectra were acquired with the deuterium field frequency lock turned off. A broad-band inverse probehead was used for acquiring ^{31}P spectra; triple resonance, three-axis pulsed field gradient probeheads were used for all other experiments. 2D NMR spectra were processed using the NMRPipe software package (Delaglio et al., 1995) and peak locations were determined with the program PIPP (Garrett et al., 1991). Resonance assignments for BPTI were taken from Wagner and Brühwihler (1986). The chemical shifts of the ubiquitin amide resonances at pH 6.6 differ somewhat from those published for lower pH values (Wang et al., 1995; Wand et al., 1996) and were kindly given to us by N. Tjandra.

Results and discussion

The dipolar coupling between two nuclei, A and B, in a solute macromolecule of fixed shape is given by:

$$D^{AB}(\theta, \phi) = D_a^{AB}\{(3\cos^2\theta - 1) + {}^3/_2 R(\sin^2\theta \cos 2\phi)\} \quad (1)$$

where R is the rhombicity defined by D_r^{AB}/D_a^{AB} and is always positive; D_a^{AB} and D_r^{AB} (in units of Hertz) are the axial and rhombic components of the traceless second rank diagonal tensor $\boldsymbol{D}$ given by ${}^1/_3[D_{zz}^{AB} - (D_{xx}^{AB} + D_{yy}^{AB})/2]$ and ${}^1/_3[D_{xx}^{AB} - D_{yy}^{AB}]$, respectively, with $|D_{zz}^{AB}| > |D_{yy}^{AB}| \geq |D_{xx}^{AB}|$; θ the angle between the A–B interatomic vector and the z axis of the tensor; and ϕ the angle which describes the position of the projection of the A–B interatomic vector on the x–y plane, relative to the x axis. D_a^{AB} subsumes various constants, including the gyromagnetic ratios of the two nuclei γ_A and γ_B, the inverse cube of the distance between the two nuclei, $<r_{AB}^{-3}>$, where the brackets indicate vibrational averaging, and the generalized order parameter $\boldsymbol{S}$ for fast angular fluctuations of the internuclear vector (Lipari and Szabo, 1982) which provides a first-order correction for the effect of rapid

internal motion on D^{AB} (Tjandra et al., 1996; Tolman et al., 1997). Approximate values of D_a^{AB} and D_r^{AB} are readily obtained from the distribution of measured dipolar couplings (Clore et al., 1998). In the liquid crystal bicelle medium

$$D_a^{AB} = -(\mu_o h/16\pi^3)\boldsymbol{S}\gamma_A\gamma_B < r_{AB}^{-3} > A_a, \qquad (2)$$

where A_a is the unitless axial component of the molecular alignment tensor $\boldsymbol{A}$.

For accurate measurement of one-bond ^{13}C–^{13}C and ^{13}C–^{15}N dipolar couplings it is desirable that $A_a \geq \sim 10^{-3}$. However, for alignments significantly larger than $\sim 10^{-3}$, the observed ^1H signals will increase considerably in line width as a result of the multitude of residual ^1H–^1H dipolar interactions. Therefore, alignment values of $\sim 10^{-3}$ are optimal for the purpose of extracting heteronuclear couplings in fully protonated proteins. For perdeuterated proteins, considerably higher degrees of alignment can be tolerated, although in the latter case dipolar couplings over multiple bonds can result in undesirable ^{13}C and ^{15}N line broadening. Higher degrees of alignment can also result in poor INEPT magnetization transfer for spin pairs where the dipolar contribution is comparable in magnitude to the one-bond J coupling. All discussions below are aimed at the characterization of the very dilute liquid crystalline samples, needed for obtaining the small degree of alignment discussed above for fully protonated macromolecules.

Initial characterization of the liquid crystalline phase

At room temperature, the lipids are in a gel phase and optically the sample is perfectly clear, usually indistinguishable from pure water. When the temperature is increased to a level where a stable liquid crystalline phase persists (~37 °C), the sample first turns white, but after a few minutes it becomes homogeneous and translucent, although less clear than water. The degree of remaining turbidity increases with increasing values of q.

The deuterium solvent signal, normally used for field-frequency lock purposes, provides a very convenient probe for monitoring the liquid crystalline state. The rapid exchange of water molecules between the partially aligned hydration shell of oriented bicelles and bulk solvent results in incomplete averaging of the ^2H quadrupole splitting (Salsbury et al., 1972; Finer and Darke, 1974). When the bicelles form a homogeneous nematic liquid crystalline phase, the ^2H solvent signal shows a doublet with line widths narrower than 0.7 Hz. Above a given threshold, there is no significant effect of the magnetic field strength on the degree of bicelle alignment, and no difference in solvent ^2H splitting or protein alignment is detected between measurements at 500 and 750 MHz. Note that the small degree of magnetic alignment, which results from the protein's diamagnetic susceptibility, typically is negligible relative to the much larger alignment induced by the liquid crystal.

If the density of bicelles varies across the sample, this will lead to inhomogeneous line broadening of the ^2H doublet, with regions that have a higher density of bicelles showing a larger splitting than regions of lower density. Thus, if the magnetic field is shimmed to homogeneity by selecting shim values which maximize the lock signal, this can actually introduce an incorrectly shimmed static magnetic field. This is illustrated in Figure 1, which shows ^2H solvent spectra of an incompletely homogenized 4% w/v ($q = 3.5$) liquid crystalline sample, (A) after optimizing the lock signal when locked on the downfield doublet component, and (B) when locked on the upfield doublet component. Besides a variation in bicelle density, a small temperature gradient across the sample can also affect the magnitude of the ^2H splitting (see below), and therefore can have the same effect when shimming the sample. Shimming procedures which are based on mapping the solvent ^1H signal as a function of the position in the sample ('gradient-shimming') are not sensitive to this distribution of quadrupole splittings and therefore provide a better alternative for optimizing magnetic field homogeneity. On the other hand, the asymmetry in doublet intensity, observed after shimming to maximize the lock signal, can be used as a sensitive indicator for monitoring the homogeneity of the liquid crystalline phase.

For most proteins tested so far, A_a values of 10^{-3} can be achieved at bicelle concentrations of 3–5% w/v, which correspond to a ^2H lock signal doublet splitting of 5–10 Hz at 37 °C. However, to obtain sufficient alignment for more spherically shaped molecules, or smaller ones such as carbohydrates, we have used considerably higher bicelle concentrations (up to 15% w/v), corresponding to a solvent ^2H splitting of 27 Hz at 37 °C.

Effect of DMPC:DHPC ratio

When using high bicelle concentrations (>10% w/v), stable liquid crystalline bicelle samples can be made over a wide range of DMPC:DHPC ratios. However, at the low concentrations typically required to obtain

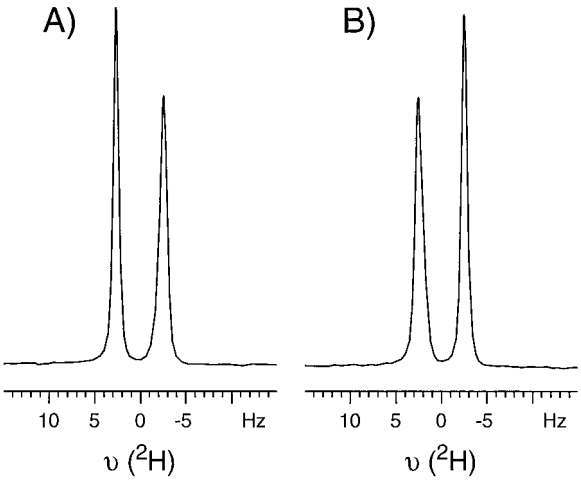

Figure 1. Effect of sample inhomogeneity on shimming. Solvent ^{2}H spectra of an inhomogeneous 4% w/v, $q = 3.5$ (DMPC/DHPC) liquid crystalline sample after maximizing the lock signal: (A) when locked on the downfield doublet component; and (B) when locked on the upfield doublet component.

the desirable degree of solute alignment, the range of ratios yielding a stable liquid crystalline phase is narrower. For example, a lipid mixture at a ratio of $q = 2.5$ does not yield a stable liquid crystalline phase at a concentration of 5%, whereas it does at 15%. At the low bicelle concentrations typically required for protein solution NMR, optimum q values fall in the range of 2.9 to 3.7.

Figure 2 shows the solvent ^{2}H and the lipid ^{31}P spectra as a function of temperature for two samples, each containing 5% w/v lipid, for DMPC:DHPC ratios of (A) 3.0 and (B) 3.5. It is important to note that this temperature series is obtained by first causing the temperature to jump to 38 °C, by inserting a cold sample into a preheated probe, subsequently followed by a stepwise increase or decrease of the sample temperature. However, at 5% w/v ($q = 3.0$) concentration, if the sample temperature is increased very slowly from room temperature to 38 °C, macroscopic phase separation occurs just around the temperature where the onset of the liquid crystalline phase takes place, i.e., at ca. 27 °C. This phase separation is irreversible on a time scale of hours, and is characterized by a clear solution upon cooling but with a heterogeneous distribution of optical refraction indices, visible to the eye. Cooling to 4 °C, followed by vortexing, can then be used to restore the homogeneity of the sample. At higher temperatures this phase separation does not occur, even on a time scale of many days, and the liquid crystalline phase is therefore assumed to be thermodynamically stable. Also, when raising the temperature of a sample which has started to phase separate microscopically, as evidenced by the simultaneous presence of a ^{2}H singlet and a ^{2}H doublet (second trace in Figure 2A), it will slowly return to the homogeneous liquid crystalline phase. This indicates that the homogeneous liquid crystalline phase is truly stable and not merely 'kinetically trapped'.

For the $q = 3.0$ sample, Figure 2 shows that the ^{2}H splitting increases with temperature, until the liquid crystalline phase collapses at ∼45 °C. The ^{31}P spectrum provides additional insight in this remarkable behavior. Two ^{31}P resonances are observed in the liquid crystalline phase, corresponding to DHPC (downfield) and DMPC, with relative integrated intensity ratios corresponding to the molar ratio of the two phospholipids. As a result of the bicelle alignment with the field, the ^{31}P chemical shift anisotropy (CSA) no longer averages to zero but results in an upfield shift which reflects the degree of alignment of the bicelle with the magnetic field. If the order parameter of the bicelles is given by S_{bicelle}, the DMPC ^{31}P chemical shift of the oriented bicelles is approximated by $\delta_i + S_{\text{bicelle}} \times (\delta_\parallel - \delta_\perp)/3$, where δ_i is the isotropic DMPC shift and $\delta_\parallel - \delta_\perp$ is the ^{31}P chemical shift anisotropy in the case of rapid rotation of DMPC around its long axis. Considering that $\delta_\parallel - \delta_\perp \approx 45$ ppm (Gawrisch et al., 1992), the ^{31}P NMR spectra indicate that S_{bicelle} slowly changes from -0.6 to -0.7 when the temperature rises from 29 °C to 41 °C. At 45 °C, the bicelles no longer exist and a narrow isotropic DHPC resonance is seen, together with an even more strongly upfield-shifted DMPC resonance, indicative of highly ordered DMPC. This high temperature phase is heterogeneous and contains white flakes which precipitate slowly over time. The upfield-shifted ^{31}P DMPC resonance, together with the lack of a quadrupole splitting in the bulk ^{2}H solvent signal, indicates that the DMPC surface hydration water is no longer undergoing rapid exchange with bulk solvent. Although a hexagonal-II phase has never been reported for DMPC, to the best of our knowledge, the above observations would be compatible with such a phase for the dense, white phospholipid particles.

At low temperature (29 °C), the ^{2}H signal for the $q = 3.0$ sample shows the onset of phase separation, characterized by a ^{2}H singlet (isotropic phase), superimposed on a doublet structure which contains a higher concentration of ordered bicelles and an increase in ^{2}H splitting relative to the homogeneous 32 °C sample. This splitting increases slowly with

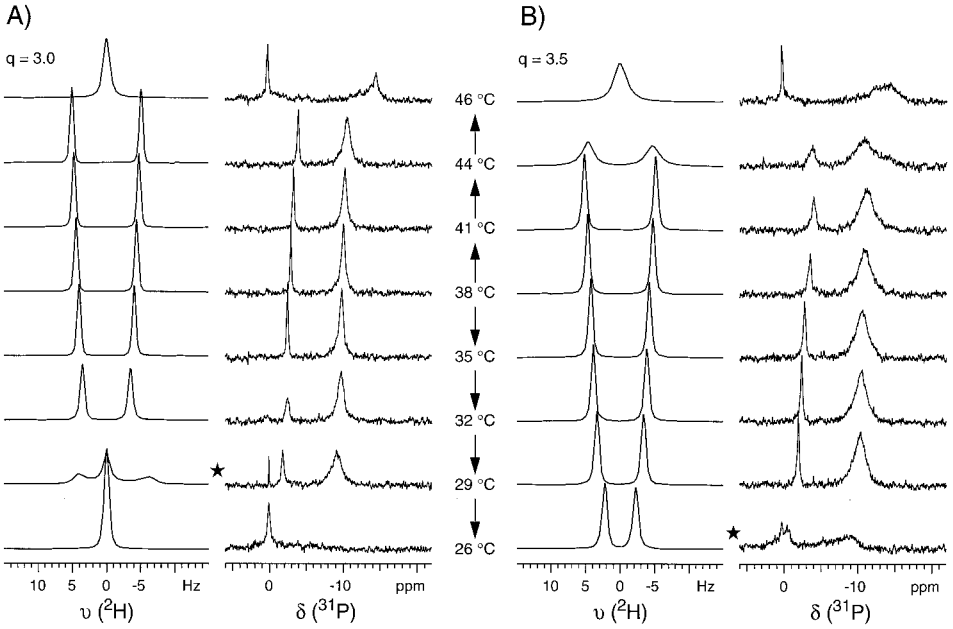

Figure 2. Influence of temperature on the ^{2}H and solvent ^{31}P spectra of 5% w/v DMPC/DHPC mixtures. (A) for $q = 3.0$ and (B) for $q = 3.5$. Note that the series were obtained by first causing the temperature of the samples, initially homogenized at 4 °C, to jump to 38 °C before either increasing or decreasing the temperature in steps of one degree, as indicated by the arrows. After reaching the temperature, the sample was allowed to equilibrate for 4 min prior to recording the spectrum. Asterisks denote spectra of unstable phases, which are subject to slow phase separation. All ^{2}H and ^{31}P spectra were recorded at 600 MHz ^{1}H frequency with the deuterium field frequency lock turned off. For ^{31}P, WALTZ-16 ^{1}H decoupling with a 4 kHz RF field was used. In order to avoid a strong signal near 0 ppm, samples were prepared without addition of phosphate buffer.

time, accompanied by a decrease in doublet and an increase in singlet intensity (data not shown). The ^{31}P spectrum of this heterogeneous sample shows a narrow resonance at the isotropic shift position, together with the ^{31}P resonances corresponding to magnetically aligned bicelles (Figure 2A). The temperature at which phase separation occurs is found to be a function of both q and total lipid concentration (Table 1). When cooling the sample from the liquid crystalline to the isotropic phase, macroscopic phase separation can be minimized by quickly cooling the sample tube in ice-water.

For a higher DMPC:DHPC ratio of $q = 3.5$, Figure 2B shows that the behavior is qualitatively the same as for the $q = 3.0$ sample. An increase in the ^{2}H quadrupole splitting and an increase in the upfield shift of the DHPC and DMPC ^{31}P resonances is observed when the temperature is increased from 29 °C to 41 °C. The temperature range over which the liquid crystalline phase is stable is shifted by ca. 3 °C to lower temperature, however, and the DMPC ^{31}P resonance is broadened considerably relative to the $q = 3.0$ sample. The reason for this increase in ^{31}P line width is not understood. The increase in ordering with increasing temperature, characterized by the upfield change in ^{31}P shift and increase in ^{2}H splitting, contrast with what is observed for most other liquid crystals and may result from an increase in bicelle size with temperature (see below).

The degree of alignment of 0.7 mM ubiquitin dissolved in a 5% w/v DMPC/DHPC solution ($q = 3.0$), pH 6.6, has been measured as a function of temperature. This is done by determining the θ, ϕ, ψ, D_a, and R values of Equation 1 which yield the best fit between the measured dipolar couplings and the X-ray structure, as described previously (Tjandra et al., 1996). The value of the axial component of the alignment tensor (A_a) is extracted from D_a, using Equation 2 with $r_{NH} = 1.02$ Å and assuming an order parameter $\boldsymbol{S} = 1$. An A_a value of 1 would correspond to perfect orientation of all protein molecules along a single axis parallel to the magnetic field. As can be seen in Figure 3, A_a is nearly independent of temperature over the range of 33 to 39 °C, but decreases by 15% when the temperature is lowered to 29 °C. This result contrasts with the quadrupole splitting observed for the solvent

Table 1. Conditions for stable and unstable liquid crystalline bicelle phases[a]

Conc. (w/v)	q	Temp. (°C)	Salt (NaCl) (mM)
Stable:			
15%	2.5–3.0	28–45	≤ 200
7.5%	3.0	29–30	nd
5%	4.0	26–32	nd
5%	3.5	28–41	≤ 300
5%	3.0	33–45	< 100[b]
4%	3.5	27–31	nd
3%	3.5	26–31	nd
3%	3.0	29–42	≤ 200
3%	2.7	32–45	nd
5%[c]	3.0	30–37	nd
3%[c]	3.0	23–36	nd
5%[d]	3.0	28–50	nd
3%[d]	3.0	22-50	nd
Unstable (phase separation):[e]			
7.5%	3.0	32–46	nd
5%	3.0	27–32	nd
5%[c]	3.0	21–28	nd
5%	2.7	33–45	nd
2.5%	3.0	25–45	nd
5%[d]	3.0	18–27	nd

[a] Unless stated otherwise, values are reported for mixtures of (fully saturated) DMPC, DHPC. Only tested ranges are given, stable conditions may also exist outside given ranges. nd = 'not determined'.
[b] At higher NaCl concentrations, very long equilibration times are needed, causing phase separation with the top 10% of the sample being isotropic.
[c] The DMPC is a mixture of 75% fully saturated DMPC and 25% (14:0) (14:1n5) PC.
[d] A mixture of DTPC and DHPC.
[e] An oriented liquid crystalline phase forms initially, but phase separates in minutes to hours.

deuterons, which shows a considerably larger temperature dependence over this range. The magnitude of the alignment tensor is roughly proportional to the upfield shift (relative to the isotropic value) of the ^{31}P resonance, and presumably reflects the higher degree of alignment of bicelles with increasing temperature. The stronger dependence observed for ^{2}H presumably results from a slightly different distribution of hydration waters relative to the bilayer normal with increasing temperature (Finer and Darke, 1974; Gawrisch et al., 1978). Note that, on the size scale of a water molecule, the bicelle surface is very rough, whereas relative to the dimensions of a protein its surface is

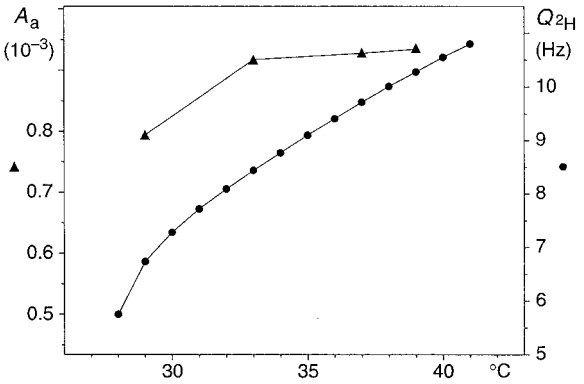

Figure 3. Plots of the axial components of the molecular alignment tensor, A_a (triangles), and the ^{2}H quadrupolar splittings, Q_{2H} (dots), as a function of the temperature. All spectra were recorded with a 4-month-old 0.7 mM ubiquitin sample, pH 6.6, 5% w/v DMPC/DHPC, $q = 3.0$. A_a values were determined by fitting of at least 51 dipolar couplings extracted from well resolved doublets of [^{15}N,^{1}H]-HSQC spectra against the X-ray structure of ubiquitin (Vijay-Kumar et al., 1987) as described elsewhere (Tjandra et al., 1996).

rather smooth and different effects of temperature on the average alignment are not surprising.

Liquid crystalline phase at lower temperature

Although several of the DHPC/DMPC mixtures retain their liquid crystalline phase to temperatures as low as 26 °C (Table 1), the temperature range over which each of these samples is fully stable is relatively narrow. In our experience, the stability of the liquid crystalline phase of such samples tends to be more sensitive to the addition of solutes than samples which retain their liquid crystalline phase over a wider range of temperatures. Presumably, the lower temperature limit at which the bicelles can adopt nematic liquid crystalline ordering is dictated by the gel-to-liquid-crystal phase transition of the DMPC fatty acid chains, which occurs at 24 °C. We have tested this hypothesis by using a DMPC/1-myristoyl-2-myristoleoyl-*sn*-glycero-3-phosphocholine [(14:0) (14:1n5) PC] mixture instead of pure DMPC. The phospholipid (14:0) (14:1n5) PC is a mono-unsaturated analog of DMPC, with one *cis* double bond in the *sn*2 chain. This analog itself has a very low gel-to-liquid-crystal phase transition temperature (< 0 °C) and one can mix it with DMPC to lower the gel-to-liquid-crystal phase transition temperature of the mixture considerably. Similarly, ditridecanoyl-phosphatidylcholine (DTPC) has a lower gel-to-liquid-crystal phase transition temperature (14 °C) than DMPC, and we here report

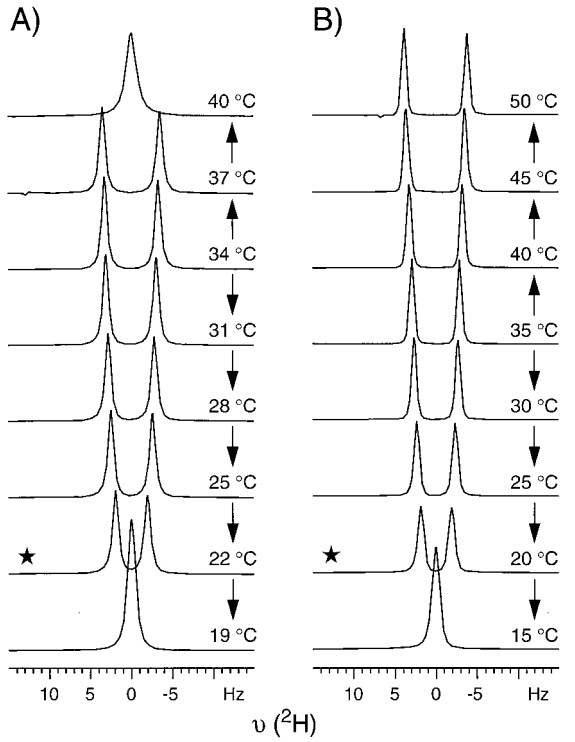

Figure 4. Influence of temperature on the solvent ^{2}H quadrupole splitting for (A) a 3% w/v, (14:0) (14:1n5) PC, DMPC, and DHPC mixture with molar ratios of 0.75:2.25:1, and (B) for a 3% w/v DTPC/DHPC mixture with a molar ratio of 3.0:1. See Figure 2 for further explanation.

some preliminary results on a liquid crystalline phase consisting of a DTPC/DHPC mixture.

Figure 4A shows that at a 0.75:2.25:1 molar ratio of (14:0) (14:1n5) PC, DMPC, and DHPC the bicelles form a stable liquid crystalline phase from 23 to 36 °C. However, the liquid crystalline phase is very dilute (3% w/v). At intermediate concentrations (5% w/v) macroscopic phase separation occurs at the lower end of this temperature range, analogously to what is observed at the lower end of the $q = 3.0$, 5% w/v DMPC:DHPC mixture (Figure 2, Table 1). Testing of the range of ternary mixture of phospholipids was restricted by the quantity of (14:0) (14:1n5) PC available to us. However, the results obtained so far suggest that the behavior of the ternary mixture of phospholipids is analogous to the binary DMPC:DHPC mixture, but with the temperature range over which the bicelles adopt a nematic liquid crystalline ordering shifted to lower temperature by an amount which depends on the molar ratio of [(14:0) (14:1n5) PC]:DMPC.

Figure 4B shows that a 3% w/v mixture of DTPC and DHPC with a molar ratio of 3.0 retains a stable liquid crystalline phase over the entire range of about 22 to 50 °C. However, at 5% w/v, the liquid cryatalline phase is stable only between 28 and 50 °C, and phase separation occurs between 18 and 28 °C (data not shown). The DTPC/DHPC and ternary mixtures thus enable measurements at ca. 10 °C lower temperature compared to the (pure) DMPC/DHPC bicelles, making it an attractive medium for molecules that are unstable at higher temperatures. However, a disadvantage of this system is that it is not as easy to measure the *J* couplings in the isotropic phase by simply lowering the temperature because the solution viscosity is rather high at the temperature where the solution becomes isotropic (∼15 °C). Instead, preparation of a sample without bicelles is preferred in this case.

Bicelle size

Vold and Prosser (1996) proposed a simple relation between the bilayer diameter, $2R_{\text{bicelle}}$, and the DMPC:DHPC ratio, q:

$$2R_{\text{bicelle}} = rq[\pi + (\pi^2 + 8/q)^{1/2}] \qquad (3)$$

where r is half the bilayer thickness (∼20 Å). This predicts R_{bicelle} values of 200 and 232 Å for $q = 3.0$ and $q = 3.5$, respectively. As argued below, relative to the average bicelle spacing this size appears to be too small to form a stable liquid crystalline phase at the low lipid/solvent volume fractions: the liquid crystalline phase requires cooperative alignment of the individual bicelles (note that the magnetic susceptibility anisotropy of a single 200-Å radius bicelle is much too small to yield the observed high magnetic alignment, particularly at lower magnetic fields). However, at 30 mg lipid/ml ($q = 3.0$), Equation 3 corresponds to 5×10^{15} bicelles/ml, or 1 bicelle per 2×10^8 Å^3. This means that each bicelle would fit in its own cube of 585×585×585 Å. Intuitively, this makes it difficult to explain the cooperativity of the alignment of these nearly neutral particles, needed to form a liquid crystalline phase.

Equation 3 was derived for application to relatively concentrated systems, where the fractions of free DHPC and DMPC in isotropic solution are very small relative to the total concentration. For the very dilute lipid concentrations used in our application, this approximation is no longer valid, at least not for DHPC. The solubility of DMPC in water is extremely low, and the fraction of monomeric DMPC in isotropic

solution may safely be neglected. If the free DHPC concentration, $[DHPC]_{free}$, is accounted for, one can define an effective q ratio, q_{eff}:

$$q_{eff} = [DMPC]/\{[DHPC] - [DHPC]_{free}\} \quad (4)$$

which represents the DMPC:DHPC molar ratio in the bicelles. Substitution of q_{eff} for q in Equation 3 increases the bicelle diameter.

If the free DHPC is undergoing rapid exchange with the bicelle-bound DHPC, only a single ^{31}P resonance is observed. An estimate of $[DHPC]_{free}$ is obtained in the following manner: when a 15% w/v, $q = 3.5$ solution is diluted 20-fold by adding DHPC-containing water, the solution turns milky for DHPC concentrations $\leq$ 2 mM at 20 °C, and $\leq$5 mM at 37 °C. When using [DHPC] = 3 mM at 20 °C, the solution remains clear. Similarly, at 37 °C, when diluting with [DHPC] = 6 mM, the solution stays clear. Thus, the $[DHPC]_{free}$ needed to prevent the formation of large, light-scattering DMPC particles is between 2 and 3 mM at 20 °C, and between 5 and 6 mM at 37 °C. It is interesting to note that these concentrations are both lower than the DHPC critical micelle concentration (CMC), which is 14 mM (Burns et al., 1982).

Using Equation 4 and $[DHPC]_{free} = 5$ mM at 37 °C, $q = 3.0$ corresponds to $q_{eff} = 4.2$, and $q = 3.5$ yields $q_{eff} = 5.1$ for a 5% w/v solution. Upon dilution to 30 mg/ml, these q_{eff} values further increase to 5.6 and 7.2, respectively, resulting in a dramatic increase in bicelle size. Note, however, that the 3% w/v, $q = 3.5$ solution is rather milky and does not yield a stable liquid crystalline phase above 31 °C (Table 1), which is consistent with the presence of large particles. The increase in bicelle size that occurs on dilution explains the remarkably low concentration at which the cooperativity of the bicelle alignment can be maintained. The strong increase in $[DHPC]_{free}$ with temperature also appears to explain the unusual increase in bicelle order parameter with temperature, as judged by the upfield shift of the DMPC ^{31}P resonance. It is important to note, however, that the above discussion of bicelle size is all based on a presumably somewhat oversimplified model of an 'ideal' bicelle; it is anticipated that more unequivocal information to resolve this issue of bicelle size will soon become available from X-ray and neutron scattering experiments.

Stability of the liquid crystalline phase

The ^{2}H solvent splitting and the ^{31}P NMR spectrum of the dilute (5% w/v) oriented bicelles are virtually unaffected by pH over the range of 4 to 10, and are relatively tolerant to salt. However, the salt-dependence of the phase diagram appears to be very complex (Table 1) and because it is a function of many variables (concentration, q factor, temperature, ion type, etc.) it has not been completely investigated. For example, stable samples containing up to 300 mM NaCl have been prepared at 5% w/v, $q = 3.5$. However, at $q = 3.0$ (5% w/v), addition of salt results in a longer equilibration time (up to several hours) before the liquid crystalline phase is fully homogeneous. When the salt concentration of this latter sample is raised to 200 mM, phase separation occurs, the bottom 90% of the sample forming a liquid crystalline phase, and the top 10% being isotropic. In contrast, both 3% w/v or 15% w/v samples at $q = 3.0$ and 200 mM NaCl behave like salt-free samples.

Stabilization of a homogeneous liquid crystalline phase does not require the presence of a magnetic field and therefore is most conveniently accomplished by incubating the liquid crystal sample in a water bath of regulated temperature. Formation of the liquid crystalline phase is easily followed by eye, and is characterized by a slightly opaque appearance, which must be uniform across the sample. Upon insertion of this pre-equilibrated sample into the preheated probehead in the magnet, the sharp solvent ^{2}H doublet appears within seconds.

Although the bicelles' degree of alignment is not significantly influenced by pH, their long-term chemical stability is a steep function of pH. The long-term stability is limited by hydrolysis of the ester bonds connecting the saturated fatty acid chains to the glycerol-phosphatidylcholine headgroup. This hydrolysis is catalyzed both by base and by acid, and is therefore a strong function of pH, with a minimum at pH 6.5 (Grit and Cromelin, 1993). The 50 mg lipid/ml sample ($q = 3.0$), containing 0.7 mM ubiquitin, pH 6.6, which was used for collecting the data shown in Figure 3 has maintained a stable liquid crystalline phase for over six months now. However, hydrolysis becomes a serious problem at high or low pH values. Figure 5 shows the ^{2}H solvent spectra obtained for two 5% w/v samples ($q = 3.0$) as a function of time. For the pH 3.4 sample, the ^{2}H quadrupole splitting starts to decrease considerably after as little as 18 h, and the liquid crystalline phase collapses after 24 h. The pH 9.9 sample remains stable for only 12 h. Although the pH 9.9 sample maintained a ^{2}H splitting even after 15 h, the liquid crystalline phase was unstable and could not be restored after the sample was cooled, vor-

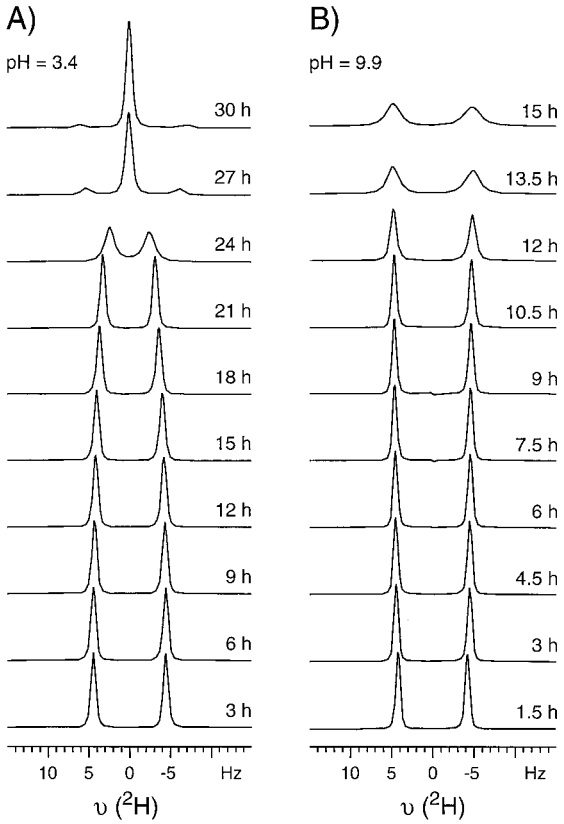

Figure 5. pH dependence of the long-term (chemical) stability of liquid crystalline samples, as monitored by the solvent ^{2}H splitting. Samples contain 5% w/v phospholipid ($q = 3.0$), with pH values adjusted to (A) 3.4 and (B) to 9.9. Sample (B) also contains 67 mM glycine buffer. Sample (A) contains no buffer but the pH remained nearly constant as the pH is close to the pK of the hydrolyzed fatty acid. Sample temperature is maintained at 38 °C throughout.

texed and reinserted into the magnet. Note that as a result of the hydrolysis the sample pH of unbuffered lipid solutions drops during the course of the experiment, further accelerating its decomposition when the pH is below 6. For long-term (several months) stability of the liquid crystalline protein samples, it is therefore essential to buffer the pH in the 6–7 range at all times.

Samples may be stored frozen, refrigerated, or at room temperature with no noticeable effect on the reproducibility of the liquid crystalline phase once the samples are reheated to 38 °C. However, frozen samples hydrolyze much more slowly, as judged by the unchanged pH of an unbuffered control sample (pH 6.5; 7.5% w/v, $q = 3.0$) kept at −80 °C for 6 months. Over the same time, the pH of an identical sample stored at 20 °C dropped from 6.5 to 5.3, indicating significant hydrolysis. In general, we have observed a slow decrease in the upper transition temperature as the sample ages. Presumably this reflects an increase of q_{eff} over time, resulting from a more effective hydrolysis of DHPC relative to DMPC. As seen in Figure 2, the upper level transition temperature is lower for higher q values.

Most water-soluble proteins may be added to the bicelle medium without any adverse effect on the liquid crystalline phase. For reasons of cost, only low concentrations of isotopically labeled proteins were used here and in earlier studies (Bax and Tjandra, 1997; Tjandra and Bax, 1997). Since the measurement of dipolar couplings in isotopically labeled samples is very sensitive, and therefore does not require high concentrations, there is generally no need to make highly concentrated samples. However, as shown in Figure 6, measurement of one-bond dipolar couplings can also be carried out in concentrated solutions at natural abundance. Figure 6 shows a small region of the F_1-coupled [^{1}H,^{13}C]-HSQC spectrum of 8.5 mM basic pancreatic trypsin inhibitor (BPTI), (A) in the oriented phase at 31 °C (5% w/v; $q = 3.5$), (B) in the oriented phase at 35 °C (3% w/v; $q = 3.0$; 200 mM NaCl) and (C) in the absence of lipids at 31 °C. As can be seen, the change in $^1J_{\text{CH}}$ splitting when shifting from the isotropic to the 5% w/v liquid crystalline phase is quite large, and for several $^{13}\text{C}^{\alpha}$–$^1\text{H}^{\alpha}$ sites it exceeds 40 Hz. For the more dilute liquid crystalline phase of Figure 6B, the $^1J_{\text{CH}}$ splittings are intermediate. As will be shown elsewhere, the dipolar couplings obtained from these differences in $^1J_{\text{CH}}$ splittings are useful not only for improving the quality of solution structures, but can also be used for evaluating previously solved structures and for identifying real differences between the structures in the solution and crystalline states.

Although the lack of adverse effect of dissolved protein on bicelle stability applies to the vast majority of proteins for which we have tested the use of liquid crystal NMR, we have also found some exceptions. In particular, for a Vα domain of a T-cell inhibitor (Plaksin et al., 1996) a decrease of protein signal intensity over a 12 h period was observed, accompanied by a considerable decrease in ^{2}H lock level intensity and the sample's turning completely opaque (J.-S. Hu, unpublished results). Although the period over which the sample remained stable was sufficient for measuring the ^{1}H–^{15}N dipolar splittings, adequate measurement of the valuable ^{1}H–^{13}C and ^{13}C–^{15}N couplings could not be accomplished within such a short time span. Similar problems were encountered by Cai and Clore

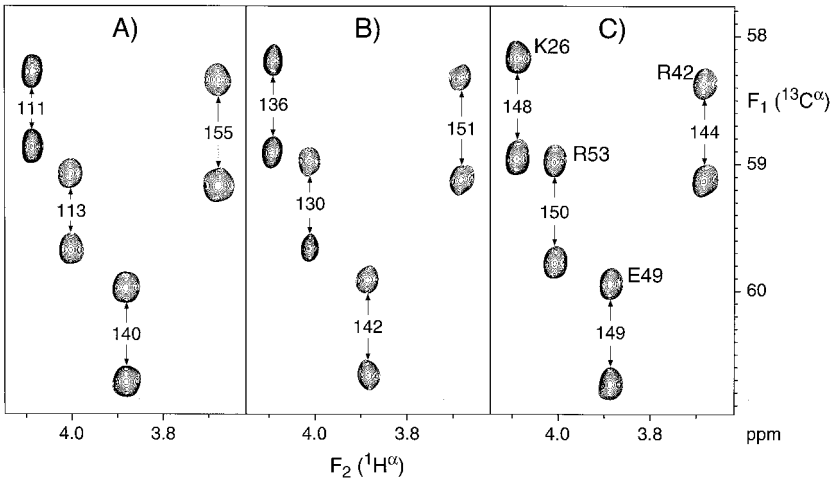

Figure 6. Small sections of the 750 MHz C^{α}–H^{α} region of ^{13}C-coupled [^{13}C, ^{1}H] CT-HSQC spectra of 8.5 mM BPTI, pH 6.7 recorded at natural abundance. (A) In the aligned, liquid crystalline state using a solution of 5% w/v DMPC/DHPC, $q = 3.5$, in 93% H_2O, 7% D_2O, 31 °C; (B) 3% w/v DMPC/DHPC, $q = 3.0$, 200 mM NaCl, 35 °C; (C) in the isotropic state, using only 93% H_2O, 7% D_2O, 31 °C. Values for the $^{1}J_{CH} + {}^{1}D_{CH}$ doublets (Hz) are marked between arrows and the residue assignments are given in (C).

(unpublished results) for a domain which required measurement at pH 4.5. A possible reason why some proteins adversely affect the stability of the liquid crystalline phase might be that a hydrophobic patch on their surface serves as a nucleation site for the formation of DMPC vesicles. Alternatively, weak affinity for DHPC could increase q_{eff} and thereby destabilize the bicelles. Clearly, more work is needed to characterize such systems and to find media which retain a stable liquid crystalline phase in the presence of such proteins.

Conclusions

Phospholipid bicelle based liquid crystals offer the opportunity to obtain the weak degree of solute alignment ($A_a \approx 10^{-3}$) needed for measuring one-bond dipolar couplings in a convenient and sensitive manner. These dipolar couplings provide unique information on the protein structure because, in contrast to NOE and *J* coupling based restraints, they align all interactions relative to a single axis system. As discussed above, preparation of the samples and evaluation of their stability and suitability for measurement of dipolar couplings is quite straightforward. For reasons not completely clear to us, it has happened on occasion that a new preparation of liquid crystal did not show the desired stability of the liquid crystalline phase. In one such case, the cause could be traced to a malfunctioning water purification system. Small but consistent differences in the onset of DMPC precipitation upon dilution of a bicelle solution with water are also observed when different sources of deionized, carbon-filtered water are used. HPLC-grade, bottled water appears to yield the most consistent results. It is recommended that the stability of a liquid crystalline stock solution, when diluted to the desired concentration, is tested for a period of at least 24 h before adding expensive labeled protein. Testing can be done in an incubator or temperature controlled water bath. In our experience, the visual appearance of the sample is a sufficient indicator of the stability of the liquid crystalline phase, but it can also be inserted briefly into a magnet in order to assess the solvent ^{2}H splitting. The pH of the solution is critical for long-term stability and for this reason the use of solutions buffered at pH 6–7 is preferred. We also recommend that the sample pH be checked every few months and readjusted if necessary.

Most proteins can be separated efficiently from the lipids by either of two procedures: first, shaking of the bicelle/protein solution with five volume fractions of a 2:1 (v/v) chloroform:methanol solution drives the DMPC and most DHPC into the chloroform phase. Dialysis of the aqueous fraction of this mixture can then be used to remove the methanol and residual DHPC. For proteins which precipitate in the presence of methanol, a second procedure simply uses 20-fold dilution of the bicelle/protein solution with water, followed by centrifugation at 4 °C to spin down

the precipitated DMPC. After concentrating this sample in a centricon, dialysis is used to remove DHPC. Small amounts of residual DMPC can then be spun down.

Most applications of the liquid crystalline medium for the measurement of dipolar couplings have focused on highly soluble proteins and nucleic acids. The temperature range over which macromolecules can be studied in the dilute bicelle medium now extends from 22 to 50 °C, and in all likelihood an even larger range can be covered by suitably chosen (ternary) lipid mixtures. The liquid crystalline medium appears to be reasonably tolerant to ionic strength and can therefore be used for the vast majority of proteins and nucleic acids that are commonly studied with high-resolution NMR methods to date. However, it is expected that macromolecules which have an affinity for binding to membranes, or those that affect the chemical stability of phospholipids, will be less amenable for study in the bicelle medium. On the other hand, it is anticipated that different, non-phospholipid-based liquid crystalline media may be useful for the study of such molecules. The utility of several such liquid crystals is currently under evaluation.

Acknowledgements

We thank Nico Tjandra, John Louis, Jin-Shan Hu, John Marquardt, Ben Ramirez, Mengli Cai, Marius Clore, Dennis Torchia, Chuck Sanders and Jill Trewhella for helpful discussions and their sharing results; Frank Delaglio and Dan Garrett for assistance and use of their software; and Klaus Gawrisch for advice and the sample of (14:0-d27) (14:1n5) PC. This work was supported by the AIDS Targeted Anti-Viral Program of the Office of the Director of the National Institutes of Health.

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