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Bicelle-based liquid crystals for NMR-measurement of dipolar couplings at acidic and basic pH values

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Abstract

It is demonstrated that mixtures of ditetradecyl-phosphatidylcholine or didodecyl-phoshatidylcholine and dihexyl-phosphatidylcholine in water form lyotropic liquid crystalline phases under similar conditions as previously reported for bicelles consisting of dimyristoyl-phosphatidylcholine (DMPC) and dihexanoyl-phosphatidylcholine (DHPC). The carboxy-ester bonds present in DMPC and DHPC are replaced by ether linkages in their alkyl analogs, which prevents acid- or base-catalyzed hydrolysis of these compounds. ^{15}N-^{1}H dipolar couplings measured for ubiquitin over the 2.3–10.4 pH range indicate that this protein retains a backbone conformation which is very similar to its structure at pH 6.5 over this entire range.

Weak alignment of solute macromolecules with the magnetic field can be achieved in dilute lyotropic liquid crystalline media (Bax and Tjandra, 1997; Tjandra and Bax, 1997). Dipolar couplings in the isotopically enriched macromolecules can be measured accurately using conventional high resolution NMR techniques and contain unique structural information (Saupe and Englert, 1963; Emsley and Lindon, 1975; Bastiaan et al., 1987; Tolman et al., 1995; Tjandra et al., 1997; Bewley et al., 1998; Ottiger and Bax, 1998b). For macromolecular solute alignment, a lyotropic liquid crystal consisting of a binary mixture of dimyristoyl-phosphatidylcholine (DMPC) and dihexanoyl-phosphatidylcholine (DHPC) in water is commonly used. These phospholipids form magnetically orientable disc-shaped particles, commonly referred to as bicelles (Sanders and Schwonek, 1992). The somewhat limited temperature range over which these bicelle mixtures are stable can be extended by using a ternary mixture of DHPC, DMPC, and 1-myristoyl-2-myristoleoyl-*sn*-glycero-3-phosphocholine, or by using shorter chain phospholipids instead of DMPC (Wang et al., 1998; Ottiger and Bax, 1998a). Long-term stability of such phospholipid based liquid crystals can restrict their applicability, however, as they are susceptible to acid- and base-catalyzed hydrolysis of the two carboxy-ester bonds connecting the aliphatic chains to the glycerol backbone (Grit and Cromelin, 1993; Ottiger and Bax, 1998a).

Here, we report on liquid crystalline media which are stable over a very wide pH range. In these new media, the diacyl phospholipids of regular bicelles are replaced by their hydrolysis-resistant dialkyl analogs, where the carbohydrate chains are connected to the glycerol part by ether instead of ester bonds, i.e. ditetradecyl-phosphatidylcholine (IUPAC name: 1,2-O-ditetradecyl-*sn*-glycero-3-phosphocholine, short: ditetradecyl-PC) and dihexyl-PC instead of DMPC and DHPC, respectively. Samples were prepared as described previously (Ottiger and Bax, 1998a), except that the solution was not phosphate buffered. All liquid crystal samples tested were prepared in a molar ratio of lipid to detergent (dihexyl-PC) of 3:1.

The dialkyl-PC bicelle mixture forms liquid crystals over the range of at least pH 1.0 to 12.5 and re-

Supporting Information available from the authors: One table with the alignment tensor orientation and magnitude of ubiquitin in the liquid crystalline phase as a function of pH with respect to the average NMR structure (1 page).

mains chemically stable for many weeks at room temperature. The temperature range over which the samples form stable liquid crystals varies with pH. At neutral pH, the overall temperature dependence qualitatively mimics that of the corresponding DMPC/DHPC mixture. However, the temperatures at which a liquid crystalline phase is observed are shifted to somewhat higher values: at pH 6.5 this temperature range is 32–46 °C, but slow phase separation occurs below 38 °C. The corresponding temperature values for the DMPC/DHPC mixture are 27–45 °C and 33 °C, respectively (Ottiger and Bax, 1998a). At pH 2.5, a stable liquid crystalline phase is obtained from 33 to 46 °C. In the 35±0.5 °C range, the doublet components observed for the deuterium HDO signal, which result from partial alignment of the water molecules, become somewhat broader over the course of about an hour. Presumably, this is caused by microheterogeneity of the sample. This broadening is reversible as soon as the temperature is increased or lowered. At pH 1.0 the liquid crystal is fully stable between 34 and 41 °C.

Recently, doping of bicelle mixtures with cetyltrimethylammonium bromide (CTAB) has been shown to prevent phase separation (Losonczi and Prestegard, 1998). After addition of CTAB to our alkyl-bicelle sample, prepared at pH 6.5 in a molar ratio of 1:30 CTAB versus the total lipid concentration ([ditetradecyl-PC] + [dihexyl-PC]), this sample forms a stable liquid crystalline phase over the whole temperature range. However, at around 35 °C the HDO deuterium doublet signals again show increased line widths, analogous to the CTAB-free sample at pH 2.5. The absence of an isotropic deuterium signal indicates that there is no macroscopic phase separation and the sample looks opaque but homogeneous. Addition of more CTAB (1:20) helped only partially. Therefore, these samples behave very similarly to the undoped sample at pH 2.5. These results suggest that partial titration of the lipids' phosphate groups with protons affects the stability of the liquid crystalline phase in the same way as doping of the bicelles with CTAB, in both cases adding a net charge on the bicelle surface. High pH values of up to 12.5, however, do not prevent phase separation.

Interestingly, the 20 Hz deuterium quadrupole splitting observed for HDO in a 5% (w/w) liquid crystal at 40 °C is about twofold larger than in a similar medium of regular diacyl bicelles. At the same time, the alignment of solute macromolecules is virtually the same as for regular bicelles (Table 1). The stronger alignment of water molecules presumably results from a different distribution of hydration waters on the surfaces of the two types of bicelles. Note that on the size scale of a water molecule, the bicelle surfaces are very rough, whereas relative to the dimensions of a protein the surfaces are rather smooth and subtle variations of their surface characteristics are not expected to affect macromolecular alignment.

A mixture of didodecyl-PC and dihexyl-PC in a 3:1 molar ratio has also been evaluated. At pH 6.5 a stable liquid crystalline phase is obtained for 28–54 °C. When doping this sample with CTAB in a molar ratio of 1:25 of total lipid (or presumably also at low pH values, see above) this range can be extended down to about 20 °C, where phase separation occurs in the absence of CTAB. Interestingly, no microscopic heterogeneity of the type described above for the ditetradecyl-PC/dihexyl-PC bicelles at 35 °C is

Table 1. Alignment tensor orientation and magnitude of ubiquitin in the liquid crystalline phase as a function of pH[a]

pH	θ (°)	ϕ (°)	ψ (°)	D_a (Hz)	D_r/D_a	Number of D_{NH}	Q (%)
2.3	26	46	29	−3.9	0.44	60	26.4
2.8	46	57	−2	−4.7	0.12	62	28.3
3.4	55	59	−61	−7.9	0.30	65	26.3
3.8	53	58	−56	−9.4	0.25	65	25.0
4.5	46	54	−24	−9.7	0.12	64	20.6
4.8	43	53	−11	−9.6	0.12	63	19.4
5.5	38	47	17	−7.9	0.14	63	18.1
6.5[b]	33	41	51	−9.1	0.16	62	15.9
7.6	34	43	41	−8.9	0.14	57	18.5
8.4	34	42	41	−8.0	0.15	55	18.3
9.5	33	43	41	−8.5	0.14	48	18.1
10.4	31	33	46	−8.7	0.18	37	13.2

[a]The Euler angles θ, ϕ and ψ define the alignment tensor relative to the coordinate frame of the X-ray structure (Vijay-Kumar et al., 1987). See text for experimental details. The measurements were made in the following order: pH 4.8–2.3, 5.5, 8.5–10.4, 7.6. Therefore, the measurements made at pH 5.5 and 7.6 serve as controls that the observed effects are reversible. Note that dilution effects due to pH adjustments are reflected in the magnitude of the alignment, but they are accounted for by the normalized Q-factors. Also, due to volume losses during pH measurement, new liquid crystal stock solution had to be added for the last measurement at pH 7.6 such that the total bicelle concentration for that measurement was slightly higher than for the previous ones.

[b]The dipolar couplings at pH 6.5 were measured previously at very high S/N, using more concentrated protein and longer acquisitions, in DMPC/DHPC bicelles (Ottiger and Bax, 1998b).

observed. Unfortunately, the low melting temperature of didodecyl-PC makes handling of the liquid crystal less easy. After dissolving the compounds by vigorous vortexing, heating (>50 °C) and cooling cycles and ultra-sonication, the resulting solution remains quite viscous even at temperatures of 0 to −8 °C and is best handled at about 0 °C or, for short periods of time, above 55 °C. Based on these results and on our previous experience with a bicelle mixture of ditridecanoyl-PC (DTPC) and DHPC (Ottiger and Bax, 1998a), we expect that a mixture of ditridecyl-PC and dihexyl-PC will form a liquid crystalline phase above ca. 27 °C. Unfortunately, this system could not yet be tested because ditridecyl-PC is not yet commercially available.

Figure 1 shows an example of the superimposed upfield and downfield ^{15}N-{^{1}H} doublet components from a small region of the IPAP [^{15}N,^{1}H]-HSQC spectrum (Ottiger et al., 1998) obtained for U-^{15}N human ubiquitin in 5% w/w didodecyl-PC/dihexyl-PC bicelles, containing also CTAB in a molar ratio of 1:15 versus total lipids. The spectrum is recorded at 22 °C, and at this low temperature the contribution from dipolar couplings to the ^{15}N-^{1}H doublet splitting is 2.2-fold smaller compared to 42 °C (data not shown). At 20 °C, the liquid crystal shows increased deuterium line width for the HDO signal, again indicative of microheterogeneity, and the ubiquitin alignment is about 8 times weaker compared to 42 °C (data not shown).

In order to evaluate the influence of pH on the alignment of a protein and to detect and follow possible conformational changes as a function of pH, we performed a pH titration with 0.5 mM U-^{15}N ubiquitin in a 5% ditetradecyl-PC/dihexyl-PC bicelle solution (molar ratio 3:1). At each pH value a set of N-H^{N} dipolar couplings was measured and fit against the X-ray (Vijay-Kumar et al., 1987) and average NMR (Cornilescu et al., 1998) structures of ubiquitin using a Powell minimization procedure (Tjandra et al., 1996). Dipolar couplings were obtained from the differences in the *J*-splittings in the aligned (42 °C) and isotropic (27 °C) states, measured with IPAP [^{15}N,^{1}H]-HSQC experiments. The results for the X-ray coordinates are listed in Table 1. A similar table for the average NMR structure is available from the authors as Supporting Information. As can be seen from this table, orientation, magnitude and rhombicity of the alignment tensor all are functions of the pH. For example, when decreasing the pH from 6.5 to 3.4 the orientation of the principal *z* axis of the alignment tensor changes by 23° and the rhombicity increases, after an initial

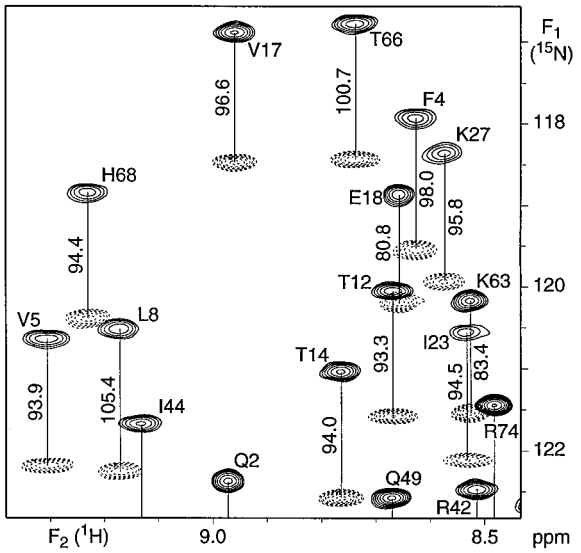

Figure 1. Region of a ubiquitin IPAP [^{15}N,^{1}H]-HSQC spectrum recorded in the liquid crystalline phase of a 5% w/w didodecyl-PC/dihexyl-PC/CTAB mixture (molar ratio of 30:10:2.5) at 22 °C. The sum and the difference of the in-phase and antiphase spectra are overlaid and represented with solid (upfield doublet component) and dashed lines (downfield component), respectively. Assignments are given for the upfield doublet components, and the $^{1}J + {}^{1}D$ splittings are indicated in Hz. Note that the individual sum and difference spectra are free of resonance overlap, but that a standard F_1-coupled [^{15}N,^{1}H]-HSQC spectrum would cause serious overlap for several doublets.

decrease, by a factor of two while the magnitude of the alignment stays more or less constant when accounting for rhombicity and dilution effects between subsequent measurements (see footnotes a and b of Table 1). When the pH is further decreased to 2.8 and below, the magnitude of the alignment drops quickly while the orientation becomes again more similar to that observed at pH 6.5. In order to exclude possible bias caused by the different numbers of measured dipolar couplings and by the effect of potential outliers at the different pH values, we also performed a series of fits where only residues were included for which the measured dipolar coupling was in good agreement with the structure (determined at pH 6.5) at all pH values. The results of these fits are very similar to the ones listed in Table 1.

The rms difference between measured and predicted couplings provides a measure for the structural changes induced by pH. However, it is important to realize that, depending on the orientation of a bond vector relative to the alignment tensor, dipolar couplings from some bond vector orientations are more sensitive than others to changes in orientation rela-

tive to the remainder of the protein. Nevertheless, the dipolar couplings provide direct insight into how closely a protein's true structure agrees with a given set of coordinates. They therefore can be used to rapidly screen for changes in protein conformation induced by solvent conditions or ligand binding. In general, the orientation, magnitude, and rhombicity of the alignment tensor are also modulated by these factors (Ramirez and Bax, 1998). This needs to be taken into account when comparing the difference between measured and predicted couplings. Previously, a quality factor, Q, has been defined which provides a normalized measure for the agreement between a set of coordinates and measured changes in chemical shift between isotropic and aligned states (Cornilescu et al., 1998). Analogously, here we define

$$Q = [\Sigma_{i=1,..,N}(D_{\mathrm{NHi}}^{\mathrm{meas}} - D_{\mathrm{NHi}}^{\mathrm{pred}})^2/N]^{1/2}/D^{\mathrm{rms}} \quad (1)$$

where the summation extends over all N residues for which ^{15}N-^{1}H$^{\mathrm{N}}$ dipolar couplings (D_{NHi}) could be measured. D^{rms} refers to the root-mean-square value of D_{NHi} for randomly distributed N-H$^{\mathrm{N}}$ vectors when using an alignment tensor obtained from best fitting the experimental data to the ubiquitin structure (Tjandra et al., 1996). As the N-H$^{\mathrm{N}}$bond vector orientation distribution in ubiquitin is considerably non-uniform (Lee et al., 1997), we here use $D^{\mathrm{rms}} = \{D_{\mathrm{a}}^2[4 + 3(D_{\mathrm{r}}/D_{\mathrm{a}})^2]/5\}^{1/2}$ (Clore et al., 1998), instead of the root-mean-square value of $D_{\mathrm{NHi}}^{\mathrm{meas}}$.

In Figure 2, this Q-factor is plotted as a function of pH. From pH 6.5 to 2.8 Q increases monotonically, indicating that the average backbone conformation increasingly diverges from the one at pH 6.5 with decreasing pH. However, it is important to point out that Q is very sensitive to small changes in structure, and Q-factors less than 30% still correspond to good agreement between the protein's true structure and the coordinates used for calculating $D_{\mathrm{NHi}}^{\mathrm{pred}}$. For example, the rmsd between the coordinates of the backbone atoms of residues 1–71 of the X-ray and NMR structures is only 0.33 Å, even while Q-factors are considerably lower for the NMR structure. Therefore, the slight decrease in Q observed at pH 2.3 is probably insufficient evidence for concluding that the pH 2.3 structure is closer to the pH 6.5 structure than is the pH 2.8 structure. Measurement of other types of dipolar couplings (^{13}C-^{1}H, ^{13}C-^{15}N, ^{13}C-^{13}C) could answer this question. Thus, the data shown in Figure 2 indicate that the structural differences in the backbone conformation induced by lowering the pH are quite small.

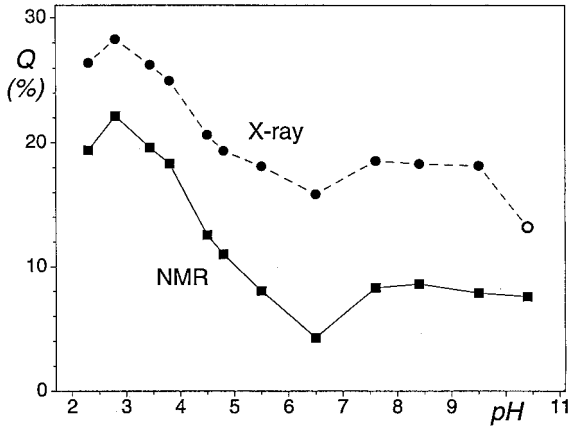

Figure 2. Plots of the quality factor, Q (Equation 1), versus the sample pH. Q-factors with respect to the average NMR structure of ubiquitin (Cornilescu et al., 1998) are represented by squares and are connected by solid lines, those with respect to the X-ray structure (Vijay-Kumar et al., 1987) are shown as circles and are connected by dashed lines. The open circle (pH 10.4) represents a result which is biased by the small number of amides for which dipolar couplings could be measured (see text). D_{NH} dipolar couplings, previously measured at pH 6.5 in regular DMPC/DHPC bicelles (Ottiger and Bax, 1998b), were included in the NMR structure determination (Cornilescu et al., 1998). The lower Q values of the NMR structure therefore cannot be interpreted to indicate that the NMR structure provides a better description of the solution conformation than the X-ray structure. However, independent information confirming that the NMR structure is closer to the solution structure is obtained from better agreement with predicted changes in ^{13}C′ shift (Cornilescu et al., 1998).

When increasing the pH from 6.5 to 10.4 the changes in Q are even smaller. This is consistent with the absence of ionizable groups with a pK_{a} between 7 and 9.5 in ubiquitin. It should be noted, however, that due to faster exchange of the backbone amide protons at high pH, dipolar couplings can only be measured for protected amide groups, which are involved in secondary structure elements. Their conformation is therefore less likely to be influenced by pH changes. However, even when using identical sets of residues the resulting fits are still very close to the ones reported in Table 1, except for the drop in Q at pH 10.4 when compared to the X-ray structure (Figure 2, open circle). This drop results from the smaller number (37) of amides for which dipolar couplings could be measured, compared to 65 at pH 3.4 and 3.8. When plotting the measured dipolar couplings versus the values predicted based on the best fits there are no large outliers (data not shown), indicating that the protein remains fully intact.

In conclusion, we have shown that dialkyl-PC bicelles allow measurement of dipolar couplings at

extremely acidic or basic pH values. Dialkyl-PCs are phospholipase-resistant, thus also providing a system for measurement of dipolar couplings in proteins with phospholipase activity. Furthermore, dipolar couplings provide a very sensitive and convenient method for monitoring structural changes relative to a set of molecular coordinates as a function of sample conditions. The pH-dependent changes in the ubiquitin backbone conformation remain quite small over the entire pH range from 2.3 to 10.4, testifying to the high stability of this protein.

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Note added in proof

A 5% w/w mixture of ditridecyl-PC and dihexyl-PC in a molar ratio of 3:1 forms a liquid crystalline phase between 23 and 49 °C. In the absence of CTAB, the HDO doublet is very broad below 28 °C. This temperature range and behavior are very similar to those for dodecyl-PC bicelles, described above, but the easier preparation and handling of the ditridecyl-PC bicelles make them an attractive alternative.

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