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Article

Applications of variable-angle sample spinning experiments to the measurement of scaled residual dipolar couplings and ^{15}N CSA in soluble proteins

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Abstract

NMR spectra of ubiquitin in the presence of bicelles at a concentration of 32% w/v have been recorded at 700 MHz under sample spinning conditions at the magic angle (54.7°) and at an angle of 45.5°. At the magic angle, the ^{1}H–^{15}N HSQC spectrum of ubiquitin in bicelles is virtually indistinguishable from the one recorded on the protein in solution. Spinning the sample at the magic angle creates an isotropic environment with no preferred bicelle orientations, thus allowing the determination of scalar coupling constants. For an angle of rotation of 45.5°, the bicelles orient with their normal perpendicular to the spinning axis leading to the observation of strong residual dipolar couplings and chemical shift variations of the ^{15}N resonances.

Introduction

Residual dipolar couplings (RDCs) have become an invaluable tool to study the structure and dynamics of proteins by NMR (Tolman et al., 1995; Tjandra and Bax, 1997; Prestegard et al., 2000; Bax et al., 2001). This new class of structural constraints allows to characterize long range order in proteins and complements efficiently classical NOE data (Blackledge, 2005). Extracting reliable RDCs requires however two samples containing the protein in an isotropic and in a partially aligned state. This procedure clearly complicates the task of the experimentalist and can introduce some errors in the measurement of the RDCs since the two samples are in different media and/or at different temperatures. Another potential problem is the actual magnitude of the RDCs. Since it is difficult to predict how a protein will behave in an anisotropic environment, the resulting RDCs might end up being too large leading to an overall degradation of the resolution and sensitivity of the spectrum. Another drawback of the current methodology is that RDCs of different magnitude are usually measured on the same sample. While the alignment might be adequate for strong $^{1}D_{\mathrm{NH}}$ RDCs, it might be too weak to measure with sufficient accuracy smaller RDCs like $^{1}D_{\mathrm{C'C\alpha}}$, $^{1}D_{\mathrm{C'N}}$ and $^{2}D_{\mathrm{C'HN}}$.

We have recently shown (Lancelot et al., 2004) that a promising solution to these problems is to employ variable angle sample spinning (VASS) experiments on bicellar systems (Sanders and

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Schwonek, 1992). VASS (Courtieu et al., 1982, 1994; Tian et al., 1999; Kishore and Prestegard, 2003) allows to scale the dipolar couplings from zero to their maximum value thereby allowing the measurement of RDCs of different magnitude on a single sample.

In this paper, we elaborate further on this topic by investigating the practical aspects of variable angle sample spinning experiment applied to a soluble protein in a bicellar environment. We show that the magic angle spectra of ubiquitin in a DMPC/DHPC bicelle system at a concentration of 32% w/v are virtually indistinguishable from the spectra recorded on ubiquitin in solution. The isotropic $^1J_{NH}$ scalar couplings obtained from these data agree, to a very high degree of confidence, to those obtained in an isotropic ubiquitin solution. This methodology allows to measure some of the largest $^1D_{NH}$ detected on ubiquitin in undoped DMPC/DHPC bicelles (from -24 to $+34$ Hz) and makes it possible to extract ^{15}N CSA contributions. Since the correct alignment of the bicelles is always a concern in these experiments, we present new ^{1}H-coupled carbon data recorded on bicelles that exhibit strong relaxation interference effects that depend directly on the angle of rotation of the sample.

Material and methods

Sample preparation

Bicelles were prepared by mixing 64 mg of lipids (1,2-Dihexanoyl-*sn*-glycero3-phosphocholine (DHPC) and 1,2-Ditetradecanoyl-*sn*-glycero3-phosphocholine (DMPC), q = [DMPC]/[DHPC] = 3) with 140 µl of phosphate buffer (10 mM phosphate buffer, 0.15 mM sodium azide, 90% H_2O, 10% D_2O, pH = 6.6). They were then carried through the following steps 5 times: vortexing (2 min), heating to 37 °C (20 min), vortexing (2 min), cooling to 0 °C (20 min). 3.5 mM of ^{15}N-labelled ubiquitin solution in buffer was prepared and 60 µl were added to the bicelle solution. The sample was again carried through the procedure of vortexing, heating to 37 °C, vortexing and cooling to 0 °C (one time). The final concentrations of ubiquitin and bicelles were 1.05 mM and 32% w/v respectively. 50 µl of this solution were then transferred into a 4 mm HRMAS rotor fitted with a Teflon insert in order to position the entire sample within the detection volume of the solenoid coil. Therefore, the total amount of ubiquitin present in the rotor was estimated to be 0.45 mg. ^{15}N-labelled ubiquitin was purchased from VLI Research Inc. and the lipids from Avanti Polar Lipids. Under these experimental conditions, the life time of the sample in the rotor was about 6 months.

NMR experiments

NMR experiments were carried out on a 700 MHz Bruker Avance spectrometer equipped with a standard gradient ^{1}H/^{13}C/^{15}N/^{2}H HRMAS probe (Lippens et al., 1999). By adjusting the micrometer screw used to adjust precisely the magic angle, it becomes possible to access a range of angles between 45° and 57°. NMR experiments on the ubiquitin/bicelle system were recorded at a temperature of 308 K and at a speed of 0 Hz (static conditions) or 941 Hz in order to obtain a stable homogeneous liquid crystal phase. ^{1}H–^{15}N HSQC experiments were recorded using the sensitivity improved acquisition scheme (Kay et al., 1992). The ^{1}H-coupled ^{1}H–^{15}N HSQC experiment differed from the ^{1}H-decoupled experiment simply by the omission of the 180° (^{1}H) pulse during the t1 evolution delay of the ^{15}N magnetization. 1 k complex points were acquired in the acquisition dimension (acquisition time 122 ms) and 128 complex points were required in the indirect dimension (acquisition time 45 ms). Sine-bell shaped gradient pulses of length 1.5 ms and of strength 40 G cm^{-1} and 4.04 G cm^{-1} were used to select the correct coherence order pathway. The length of the proton 90° pulse was 11.25 µs at $\theta = 54.7°$ and 12.5 µs at $\theta = 45.5°$.

The residual dipolar coupling under variable-angle sample spinning

The expression of the residual time-independent dipolar coupling under variable angle spinning of an oriented medium is given by the following expression (Lancelot et al., 2004):

$$D_{\mathrm{Res}} = \left(\frac{\mu_0}{4\pi}\right)\left(\frac{h}{2\pi}\right)\frac{\gamma_N\gamma_H}{\pi r_{NH}^3}S \times \left[\frac{1}{2}(3\cos^2\beta_{D_{AV}A}-1)A_A + \frac{3}{4}A_R\sin^2\beta_{D_{AV}A}\cos(2\gamma_{D_{AV}A})\right] \times \left[\frac{1}{4}(3\cos^2\theta-1)(3\cos^2\beta_{DR}-1)\right] \quad (1)$$

where μ_0 is the magnetic permittivity of vacuum, h is the Planck's constant, γ_N and γ_H are the magnetogyric ratio, r_{NH} is the distance between the two spins ^{15}N and ^{1}H, S is an internal order parameter, A_a and A_r are respectively the axial and rhombic component of the alignment tensor, θ is the angle between the rotation axis and B_0, β_{DR} is the angle between the director and the rotation axis, $\beta_{D_{AV}}A$ and $\gamma_{D_{AV}}A$ are structural angles relating the PAS of the dipolar interaction to the alignment tensor.

Equation 1 shows that the values of the dipolar coupling constants under VASS are equal to the RDCs classically obtained on oriented non-spinning samples multiplied by a scaling factor λ given by:

$$\lambda = \frac{1}{4}(3\cos^2\theta - 1)(3\cos^2\beta_{DR} - 1) \quad (2)$$

Equation 2 shows that the scaling factor is affected by the angle of rotation θ and by the angle β_{DR} that the director makes with the rotor axis.

Results and discussion

The goal of the present study is to obtain RDCs of large magnitude using a strongly orienting medium under VASS conditions. Therefore, the bicelle concentration was increased from 25 to 32% w/v compared to our previous study (Lancelot et al., 2004). The magnitude and the quality of the alignment of the 32% w/v sample were checked under static conditions by monitoring the ^{2}H quadrupolar splitting of the D_2O molecules. The 700 MHz deuterium spectrum shown in Figure 1a exhibits a fairly sharp doublet of 55 Hz which proves that the sample is properly oriented. An identical sample containing only 25% w/v of bicelles exhibited a coupling of 36.5 Hz. This observation reflects the fact that increasing the bicelle concentration from 25% to 32% increases the strength of the alignment tensor. The intensity of the dipolar ^{1}H–^{1}H and ^{1}H–^{15}N interaction is such that no useful ubiquitin spectra can be obtained under static conditions.

In order to average out the dipolar interactions, the sample was subjected to a rotation at the magic angle at a speed of 941 Hz (Zandomeneghi et al., 2001; Lancelot et al., 2004). These conditions provide an isotropic medium with no preferred bicelle director alignment which is suitable to measure isotropic $^{1}J_{NH}$ couplings. When starting from a static bicelle sample, already oriented with its director perpendicular to the magnetic field B_0, it is important to spin the sample for a sufficiently long period of time (about 1 h) to obtain a true powder pattern distribution of the directors. Bicelle metastable phases with a preferred alignment of the directors can indeed survive for a short period of time when spinning at the magic angle (Zandomeneghi et al., 2001; Zandomeneghi et al., 2003a, b). The 2D ^{1}H–^{15}N HSQC experiment acquired under these conditions (Figure 2b)

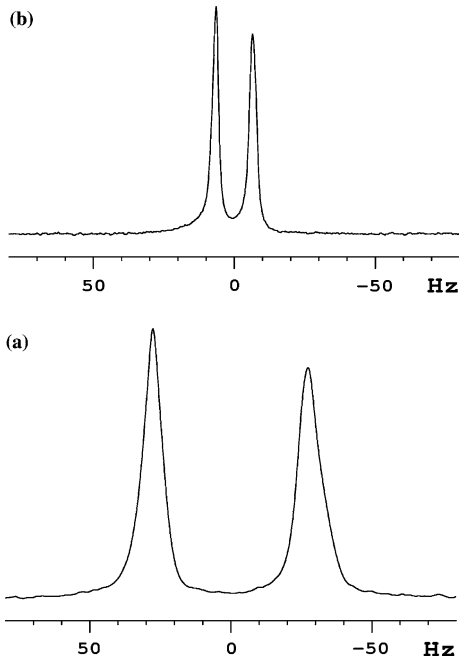

Figure 1. 1D deuterium spectrum of D_2O molecules in a sample of ubiquitin in the presence of DMPC/DHPC bicelles (32% w/v) at 700 MHz and at a temperature of 308 K. (a) static sample, $\Delta\nu_{Static}$ = 55 Hz (b) spinning at a speed of 941 Hz and at an angle of 45.5°, $\Delta\nu_{Res}$ = 13 Hz.

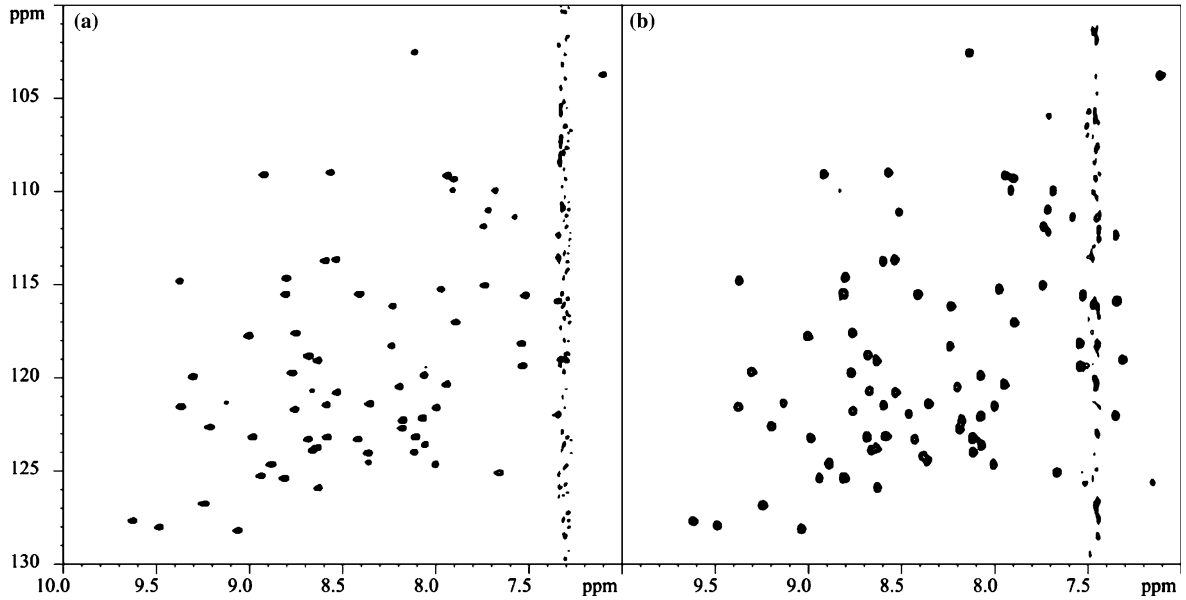

Figure 2. ^{1}H-decoupled ^{1}H/^{15}N HSQC spectra of ubiquitin at 700 MHz spinning at the magic angle (54.7°) and at a temperature of 308 K. (a) in solution at a speed of 890 Hz. Experimental time 1.2 h using 8 scans per t1 increment. (b) in the presence of DMPC/DHPC bicelles at a concentration of 32% w/v and at a speed of 941 Hz. Experimental time 1.2 h using 8 scans per t1 increment.

displays very sharp cross-peaks and the overall spectrum is almost indistinguishable from a spectrum recorded under identical conditions on ubiquitin in solution (Figure 2a). This observation tends to prove that no interaction exists between ubiquitin and the bicelles and that the rotational correlation time of ubiquitin is not significantly affected by the bicelle medium at the magic angle. The fact that cross-peaks belonging to T9, R74 and G75 are absent from Figure 2a and present in Figure 2b is most likely due to a pH difference between the two samples. Scalar $^{1}J_{NH}$ couplings (from −90.2 to −95.9 Hz) were readily extracted from a ^{1}H-coupled HSQC spectrum and were found to be in good agreement with values published on ubiquitin in solution (Tjandra et al., 1996). The components of the doublets in the ^{15}N dimension of the ^{1}H-coupled HSQC spectrum (Figure 3a) show no relaxation interference effects (Pervushin et al., 1997) which is again consistent with the fact that the correlation time of ubiquitin in the bicelle medium spinning at the magic angle is similar to the one observed in solution.

While the sample was spinning, the axis of rotation was slowly changed to an angle of 45.5° with respect to the magnetic field. The bicelle directors, which were in a random orientation, undergo at this stage, a complete reorganization to point on average along a direction which is perpendicular to the axis of rotation of the rotor. This process has a slow dynamics and the complete rearrangement usually takes over an hour. The bicelle orientation is then probed by recording 1D deuterium spectra until the quadrupolar coupling reaches a constant time-independent value. Since the stator is now in a different position, a set of shims adapted to the new angle of rotation is essential to obtain a correct lineshape. Using a 1% $CHCl_3$ sample in acetone is particularly convenient to obtain this new set of shims (Piotto et al., 2005). Compared to the magic angle shims, the largest variations are observed for the shims Z^1, Y^1, $Z(X^2-Y^2)$ and YZ^1 . The spectrum obtained under these off-magic angle conditions displays a very sharp doublet with a scaled quadrupolar coupling of 13 Hz (Figure 1b). By comparing the quadrupolar values obtained under static and off-resonance sample spinning conditions, it is possible to compute the actual angle of rotation using the following formula which is valid for an angle of rotation $\theta < 54.7°$ (Lancelot et al., 2004):

$$\frac{\Delta\nu_{Res}}{\Delta\nu_{Static}} = \frac{(3\cos^2\theta - 1)}{2} \qquad (3)$$

where $\Delta\nu_{Static}$ and $\Delta\nu_{Res}$ are the static and the spinning deuterium quadrupolar splitting

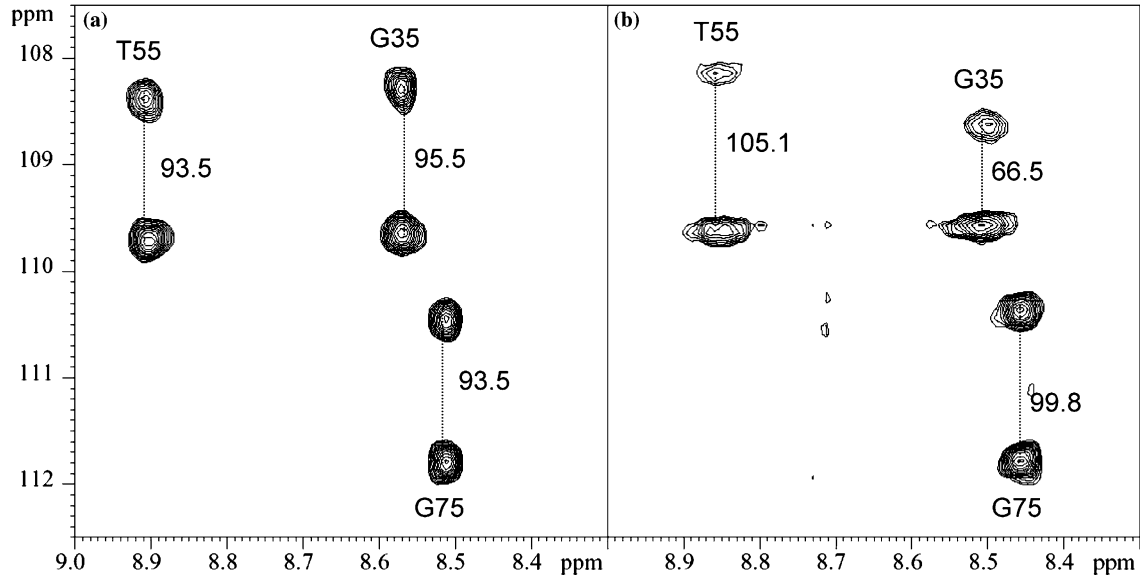

Figure 3. Ubiquitin spectra recorded in the presence of bicelles at a concentration of 32% w/v and at a temperature of 308 K recorded at 700 MHz. (a) Expansion of a ^{1}H-coupled ^{1}H/^{15}N HSQC spectrum obtained while spinning at the magic angle at a speed of 941 Hz and at an angle of 54.7° showing characteristic scalar J-couplings $^{1}J_{NH}$. Experimental time 1.2 h using 8 scans per t1 increment. (b) Expansion of a ^{1}H-coupled ^{1}H/^{15}N HSQC spectrum obtained while spinning at the magic angle at a speed of 941 Hz and at an angle of 45.5° showing characteristic $^{1}J_{NH} + {}^{1}D_{NH}$ coupling constants. Experimental time 2.4 h using 16 scans per t1 increment.

respectively. With our experimental results, $\Delta\nu_{Static} = 55$ Hz and $\Delta\nu_{Res} = 13$ Hz, θ is computed to be equal to 45.5°. This value was confirmed by physically opening the probe and measuring the actual angle of rotation. The estimated precision of this measurement is of the order of ±0.5°.

The ^{1}H-decoupled HSQC spectrum obtained at this angle of 45.5° displays cross-peaks that are similar to those obtained at the magic angle, but that are slightly broadened because of ^{1}H–^{1}H dipolar couplings. The ^{1}H-coupled HSQC experiment (Figure 3b) exhibits doublets in the f1 dimension that are markedly different from those recorded at the magic angle, ranging from 59.7 to 116.5 Hz. D_{NH} values obtained from this spectrum range from −23.5 to +34.5 Hz and are about twice as large as those obtained on ubiquitin in a 5% DMPC/DHPC bicelle solution using traditional liquid state techniques (Cornilescu et al., 1998). Under our experimental conditions, $^{1}D_{NH}$ values always remain inferior to $^{1}J_{NH}$ and therefore can always be extracted by simply taking the difference (splitting − $^{1}J_{NH}$). For more strongly oriented media, an inversion in the sign of the splitting is possible and the use of multiple angles of rotation can help resolve the ambiguity (Tian et al., 1999). The results of Figure 3 show that there is no significant line broadening of the data in the ^{15}N dimension and that the accuracy of the data is not compromised. The experiment recorded at 45.5° has about half of the signal to noise of the experiment recorded at the magic angle. A point of interest is that the components of the ^{15}N doublet exhibit this time some relaxation interference (Pervushin et al., 1997) contrary to what is observed at the magic angle. This observation implies that, when the sample is spinning at 45.5°, with the bicelle directors aligned perpendicular to the axis of rotation, the correlation time of ubiquitin is slightly longer than when the sample is spinning at the magic angle. The general ordering of the bicelles seems to have some effect on the ability of ubiquitin to rotate freely. The exact nature of the dynamics of ubiquitin under these conditions should be investigated further. Since the previous HSQC experiments were recorded off-magic angle, the pulsed-field gradients are not anymore collinear to the rotation axis, and a slight modulation of the intensity of the gradients will be present during a gradient pulse as the spins rotate. In the experiments reported in Figure 3b, no precautions were taken to counteract this effect, however solutions based on rotor-synchronized pulsed-field gradient pulses exist and should be

employed (Wieruszeski et al., 2001; Elbayed et al., 2005). Most of the $^{1}H/^{15}N$ dipolar coupling constants could be extracted from the spectrum of Figure 3 and the general aspect of the distribution of the RDCs versus the amino acid sequence (Figure 4) is in close agreement with the one previously published on ubiquitin in a 5% w/v bicelle solution (Bax and Tjandra, 1997; Cornilescu et al., 1998). The correlation graph of these two data sets (Figure 5) shows that the VASS technique provides results that are strongly correlated to the ones obtained using classical alignment media ($R = 0.98$). An alternative method that can be used to recover the dipolar coupling under MAS or VASS conditions is to recouple the time-dependent terms of the dipolar interaction using dedicated pulse sequences (Glaubitz et al., 2001; Trempe et al., 2002, 2003). However, for reasons unclear to us, we were unable to implement this approach successfully for the ubiquitin/bicelle system.

Another parameter of interest which is accessible when studying aligned proteins is the ^{15}N chemical shift anisotropy (CSA) tensor. The knowledge of the magnitude of the tensor ($\Delta\sigma$) is particularly important for the interpretation of ^{15}N relaxation data (Boyd and Redfield, 1999). Similarly to the RDCs, the ^{15}N resonance frequency of the different amino acids depends on the angles between the principal axis of the CSA tensor and the alignment tensor (Cornilescu et al., 1998). Classically, this measurement is performed using either one of the following two approaches. The first one requires two samples: one containing the protein in an anisotropic medium and the second the protein in an isotropic medium. The second approach makes use of a single protein sample in a bicellar environment studied at different temperatures to create anisotropic and isotropic conditions (Cornilescu et al., 1998; Cornilescu and Bax, 2000). Clearly, these two procedures introduce some errors in the measurement since the chemical shift is particularly sensitive to experimental conditions like pH, ionic strength, temperature and sample concentration. Characterizing ^{15}N chemical shifts that differ by less than 1 ppb, using these methods, is therefore particularly difficult (Cornilescu and Bax, 2000). Recently, a third approach was proposed using a single protein sample studied successively in a bicellar environment spun at the magic angle to generate isotropic conditions and in a high resolution probe under static conditions (Kurita et al., 2003). This approach is related to the VASS technique described in the following.

Since under our experimental VASS conditions, ubiquitin is more strongly oriented than in classical media, chemical shift variations induced by the ^{15}N CSA are larger. By comparing the ^{15}N chemical shifts of the HSQC spectra recorded at

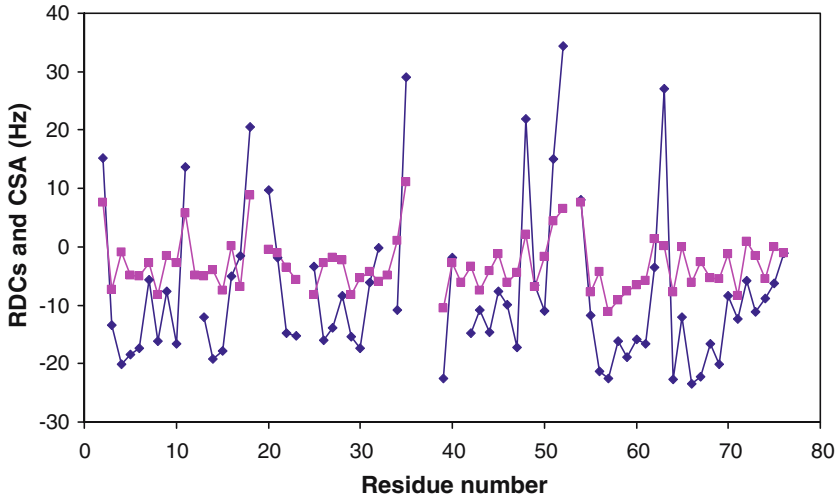

Figure 4. Dipolar coupling constants $^{1}D_{NH}$ (blue) and chemical shift anisotropy of ^{15}N (purple) observed in the $^{1}H/^{15}N$ HSQC of ubiquitin in the presence of DMPC/DHPC bicelles at a concentration of 32% w/v spinning at a speed of 941 Hz and at an angle of 45.5° with respect to the magnetic field. Using the data obtained from several measurements, the uncertainty of the $^{1}D_{NH}$ and CSA values is estimated to be about ±0.3 Hz and ±0.6 Hz respectively.

the magic angle (isotropic conditions) and at an angle of 45.5° (anisotropic conditions), the chemical shift variations of the different amino acids of ubiquitin can be extracted in a straightforward manner. The results of Figure 4 (in purple) show that these frequency differences range from roughly −11.1 to 11.1 Hz and that the pattern of their variation along the amino acid sequence follows the pattern of the RDCs. The VASS technique has therefore the unique advantage of being able to provide accurate ^{15}N CSA data using a single sample at a constant temperature in a straightforward manner. One should point out these ^{15}N CSA data are (to our knowledge) the first ones reported on ubiquitin in undoped DMPC/DHPC bicelles.

When performing VASS experiments on bicelles, the state of alignment of the bicelles is always of prime concern. In particular, when switching from the magic angle to the off-magic angle position, it is very convenient to have an experimental parameter representative of the state of alignment of the bicelles.

As mentioned in this paper, the classical method to probe bicelle orientation under variable-angle spinning (VAS) is to monitor the ^{2}H quadrupolar splitting of the water molecules present in the sample. Monitoring bicelle orientation through the observation of the alignment of the water molecules is however only an indirect method. Observing ^{31}P signals of DMPC/DHPC, provides a more direct access to bicelle alignment through the analysis of the spinning sidebands present in the spectrum (Zandomeneghi et al., 2003a, b). This analysis requires however a complete simulation of the spectrum and affords an evaluation of the mosaic spread of the sample. A more straightforward method of monitoring bicelle alignment is to use selectively deuterated DMPC/DHPC samples. The quadrupolar splitting observed for each site provides unambiguous information on the state of alignment of the bicelles. However, this procedure is costly and time-consuming.

In the process of looking for alternative ways of probing bicelle orientations, we have recorded ^{1}H-coupled carbon spectra at several angles of rotation. The spectra consist of fairly broad peaks originating from the bicelles. Spinning at the magic angle and at high speeds (8 kHz) does not lead to a sharpening of the spectra which is consistent with the fact that the bicelles undergo slow rotational diffusion. Among these peaks, methyl groups are markedly sharper than the remaining

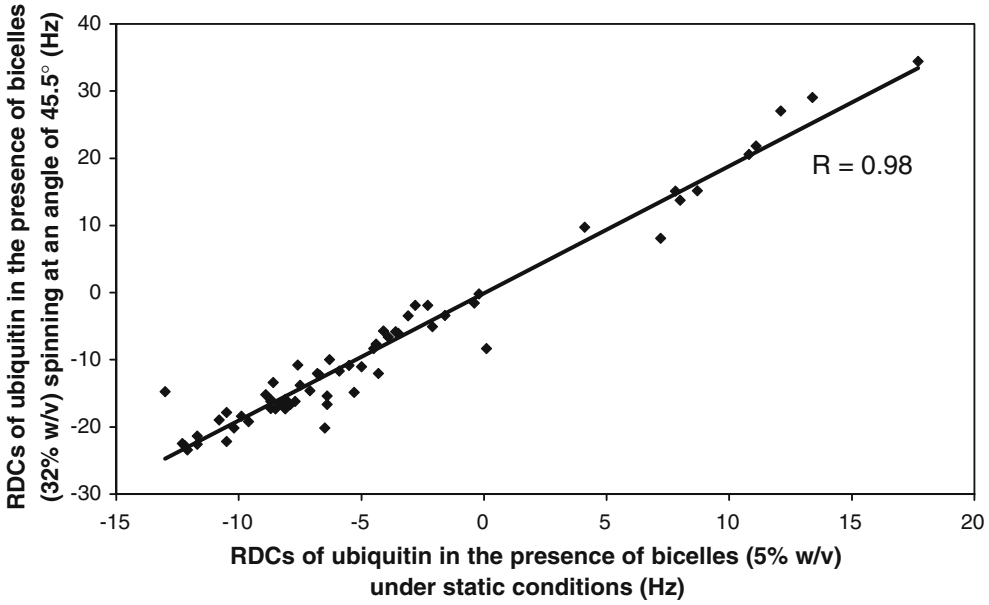

Figure 5. Residual dipolar coupling constants $^{1}D_{NH}$ observed in the ^{1}H/^{15}N HSQC of ubiquitin in the presence of 32% w/v DMPC/DHPC bicelles spinning at an angle of 45.5° versus the $^{1}D_{NH}$ obtained in a 5% w/v bicelle sample under static conditions (Cornilescu et al., 1998).

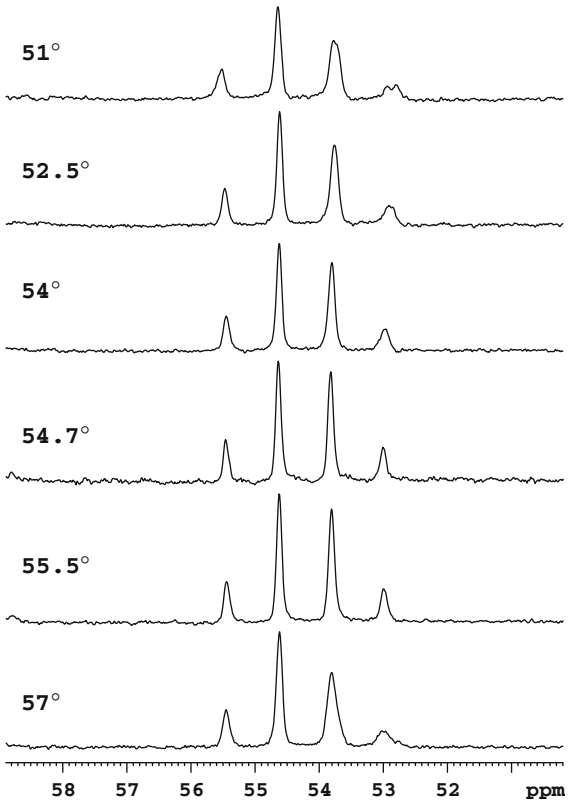

Figure 6. Expansion of the ^{1}H-coupled 700 MHz carbon spectrum of the choline $-N^{+}-(CH_3)_3$ methyl group of the DMPC/DHPC bicelle sample at a concentration of 32% w/v containing 1.05 mM ubiquitin at different angles of rotation and at a speed of 914 Hz.

peaks because of the fast rotation of the methyl group around its C3 axis of symmetry. This property has been used extensively in the study of very large proteins by NMR. Among the two methyl groups present in a bicelle system, the choline $-N^{+}-(CH_3)_3$ group at 54 ppm is the sharpest signal. At the magic angle, its ^{1}H-coupled carbon spectrum exhibits a sharp quadruplet whose individual components all possess the same linewidth and height. When changing the angle of the axis of rotation, the results of Figure 6 show that the structure of the quadruplet changes substantially. The four components of the quadruplet are altered, the most affected peak being the high field component that becomes significantly broader. We are in the presence of a phenomenon known as differential line broadening (DLB) which originates from relaxation interferences between carbon–proton dipolar couplings or carbon CSA/carbon–proton dipolar couplings. This phenomenon was first observed on methanol bound to sol–gel silica (Hartzell et al., 1989) and was explained by the strongly anisotropic motion of methanol in this system. Similar effects were also noticed and studied in the ^{1}H-coupled carbon spectra of polymers and lipid-water systems under MAS conditions (Oldfield et al., 1991, 1992; Chung et al., 1992). Our data suggest that the DLB effects observed might reflect the actual organization of the bicelle domains and the way in which different domains interact. At the magic angle, there is no global organization of the different domains and bicelles are allowed to rotate with respect to each other. When the angle of rotation is progressively moved away from the magic angle position, the different bicelles domains begin to align in a cooperative manner which has the consequence of restricting the mobility of each bicelle unit thereby leading to strong anisotropic motions and DLB effects. This effect is potentially very useful to characterize the degree of alignment of the bicelles, however the complexity of the phenomenon is such that more experiments using deuterium labeled bicelles have to be performed.

Conclusions

The variable-angle sample spinning methodology described in this paper has the potential to measure rapidly ^{15}N CSA and RDCs of various magnitudes in a straightforward manner on a single protein sample by simply changing the axis of rotation by a few degrees around the magic angle position. The use of a bicelle system at a concentration of 32% w/v combined with VASS has allowed to measure some of the strongest RDCs and ^{15}N CSA published on ubiquitin in undoped bicelles. When studying more anisotropic proteins with this methodology, it is likely that the magnitude of the alignment tensor will increase even further and that the bicelle concentration should be reduced. The key to the success of the method is that the protein under study should not interact with the aligning bicelle medium. Provided that the rotational diffusion of the protein is not significantly affected, the line broadening observed in the spectrum will be essentially due to residual dipolar interactions and, to a lesser extent, to magnetic susceptibility

effects. Using the VASS technique in the vicinity of the magic angle has the great advantage of reducing significantly these effects leading to reasonably sharp line widths.

Supporting Information Available

The following electronic supplementary material is available at http://dx.doi.org/10.1007/s10858-005-y-3210-1: One table containing $^{1}D_{NH}$ residual dipolar couplings and ^{15}N chemical shift anisotropy of ubiquitin in a 32% w/v bicelle medium obtained at 308 K while spinning at 941 Hz and at an angle of 45.5°.

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