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Adiabatic-passage cross polarization in N-15 NMR spectroscopy of peptides weakly associated to phospholipids: Determination of large RDC

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Abstract

Structural information can be extracted from one-bond residual dipolar couplings (RDC) measured in NMR spectra of systems in field-ordered media. RDC can be on the order of *J*-couplings if the anisotropy of alignment is $\sim 10^{-2}$, 10-fold stronger than that typically used for structural studies of water-soluble proteins. In such systems the performance of $^{1}H \rightarrow {}^{15}N$ polarization transfer methods of the INEPT type is not satisfactory. In this study we show the effectiveness of adiabatic-passage cross-polarization (APCP) in transferring the $^{1}H \rightarrow {}^{15}N$ polarization in the bicelle-associated peptide Leucine Enkephalin (Lenk). APCP is efficient both in static samples and in samples spun at the magic angle (MAS) or any other angle of the spinning axis to the magnetic field (variable-angle spinning, VAS). The anisotropic spectrum of an aligned static sample and the isotropic spectrum of the sample under MAS provide a set of possible values for the ^{1}H–^{15}N RDC of phospholipid-associated Lenk. The unambiguous determination of the ^{1}H–^{15}N RDC was accomplished by means of VAS experiments.

Introduction

The measurement of residual anisotropic NMR interactions in partially oriented media has become an important and indispensable NMR tool for the investigation of dissolved molecules. In the following we discuss the measurement of these quantities for peptides which associate dynamically with oriented lipid surfaces. Our system represents a case with an 'intermediate' degree of ordering with an anisotropy ΔA of the alignment tensor (Saupe, 1964) on the order of $\sim 10^{-2}$. Residual dipolar couplings (RDC) in liquid-state NMR are usually obtained in the regime of weak ordering ($\sim 10^{-3}$) where the spin–spin couplings are only slightly modified from their value in isotropic phase. Strong alignment ($\sim 10^{-1}$) is observed for strongly membrane-associated biomolecules (Sanders and Landis, 1995; Howard and Opella, 1996; Losonczi and Prestegard, 1998; Opella et al., 1999; Glover et al., 2001).

For structural studies of peptides the RDC deliver useful information about the angle of the ^{1}H–^{15}N bond with respect to the lipid surface. They can easily be determined from the splitting in the ^{15}N spectra which have little background signal from the lipids. In isotropic and weakly oriented systems, INEPT transfer (Morris and Freeman, 1979) via the one-bond ^{1}H–^{15}N *J*-coupling (J_{HN}) is the method of choice for $^{1}H \rightarrow {}^{15}N$ polarization transfer employed for sensitivity enhancement. Hartmann–Hahn cross-polarization (HHCP) techniques (Hartmann and Hahn, 1962; Pines et al., 1972; Bertrand et al., 1978; Levitt, 1991) are rarely applied.

In the presence of intermediate or strong orientational order, INEPT polarization transfer

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becomes inefficient due to transverse dephasing caused, e.g., by the strong coupling effects between the proton spins, making CP techniques more attractive (Levitt, 1991). However, CP as well as INEPT suffer from the fact that the polarization transfer is oscillatory with a frequency determined by the total coupling. $\Delta = J + D_{\mathrm{res}}$, where J is the scalar coupling and D_{res} the RDC (see Figure 1 for the case of CP). Because D_{res} depends on the orientation of the bond with respect to the magnetic field direction, no single time-period optimum for all bonds can be chosen, similar to the situation in a solid powder sample (Mehring, 1983). Again in analogy to solid-state NMR, it may be beneficial to use adiabatic CP schemes (Hediger et al., 1994; Zhang, 1994; Ernst and Meier, 2002), which promote efficient transfer essentially independent of the precise coupling strength. The application of adiabatic-passage cross-polarization (APCP) methods was indeed initially proposed for liquid samples by Chingas et al. (1980). APCP is also able to partially overcome the problem of matching the Hartmann–Hahn (HH) condition $|\omega_1(^1\mathrm{H}) - \omega_1(^{15}\mathrm{N})| = \mathrm{n}\,\omega_{\mathrm{r}}$ within the coupling Δ

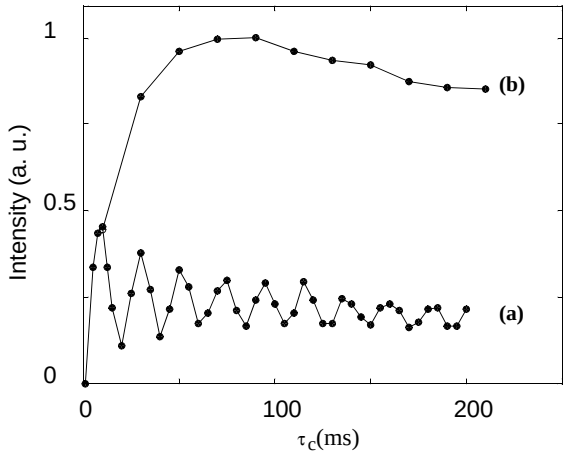

Figure 1. ^{15}N signal intensity of the Phe residue in HHCP (a) and APCP (b) experiments at different contact times. The sample was an aqueous solution of Lenk, [Lenk] = 60 mM. In the CP experiment the ^{15}N and ^{1}H rf fields were 2 kHz. In the APCP the ^{15}N rf field was 1 kHz and the ^{1}H was ramped up through the HH matching with a tangential sweep with the shape described by the constant d_{IS} (corresponding to the estimated coupling, see Equation 14 in the reference (Hediger et al., 1994)) of 220 Hz and the amplitude span of ±380 Hz (Hediger et al., 1995). The spectral intensity in the APCP experiments did not depend on the amplitude of the matched rf fields with $\omega_1(^{15}\mathrm{N})$ in the interval between 850 and 3200 Hz.

in the presence of the unavoidable rf-field inhomogeneity. This effect explains some of the gain of APHH over normal HHCP observed in Figure 1. For larger, slowly tumbling molecules cross-correlated relaxation could possibly be employed as an alternative transfer mechanism (Wimperis and Bodenhausen, 1989; Bruschweiler and Ernst, 1992; Riek et al., 1999; Khaneja et al., 2003).

This work describes the use of APCP polarization transfer in the ^{15}N NMR study of Leucine Enkephalin (Lenk), a membrane surface-associated peptide, in a system of oriented bicelles (Sanders and Landis, 1994; Rinaldi et al., 1997; Prosser et al., 1999; Zandomeneghi et al., 2003b). INEPT polarization transfer was ineffective except under MAS conditions while APCP is shown to be efficient in static samples and in samples under MAS or variable-angle-spinning (VAS). The ^{1}H–^{15}N RDC are unambiguously determined by means of a series of 1D VAS experiments, where the orientation of the liquid–crystalline director is varied.

Materials and methods

Sample preparation

The fully ^{15}N-labelled Lenk (labelling degree of 98%) with the sequence Tyr–Gly–Gly–Phe–Leu was prepared using conventional FMOC synthesis. The sample preparation is described in (Zandomeneghi et al., 2003b).

NMR experiments

Static and MAS NMR experiments were performed at 9.4 T on a Bruker DMX 400 spectrometer with a doubly-tuned Bruker 4 mm MAS probe. Proton decoupling was achieved using WALTZ-16 (Shaka et al., 1983) with an rf-field strength of 2.6 kHz. ^{15}N chemical shifts are indirectly referenced to external TMS with a ratio Ξ of 0.101329144 (Live et al., 1984), yielding ^{15}N shifts relative to liquid ammonia. The spectral width was 8 kHz, the acquisition time 127 ms, and the recycle delay 3 s. The ^{15}N and the ^{1}H rf fields were applied in the center of the amide ^{15}N and ^{1}H frequency intervals, respectively. In the APCP experiments the ^{1}H rf field

was ramped in a tangential sweep approximated by 1023 discrete amplitude steps.

VAS ^{15}N NMR spectra were obtained on a Varian Infinity-Plus 500 spectrometer at a magnetic field of 11.7 T with a doubly-tuned home-built probe using 6 mm Chemagnetics MAS rotors. The orientation of the rotation axis was controlled by a servo motor (Schneider Automation, North Andover, MA), connected via Kevlar strings to the stator containing the Helmholtz coil. The setting of the spinning angle was precise to 1°. The spectral width was 5 kHz, the acquisition time 102 ms, and the recycle delay 4 s. In the APCP experiments the ^{1}H rf field was constant during the contact time while the ^{15}N rf field was ramped up with a tangential sweep approximated by 714 amplitude steps. During the acquisition, CW proton decoupling with a field of 6 kHz was applied. The bicelle order parameter S_{Bic} was determined from the ^{31}P NMR spectra of the bicelle/Lenk sample and DMPC/Lenk as described earlier (Zandomeneghi et al., 2003b).

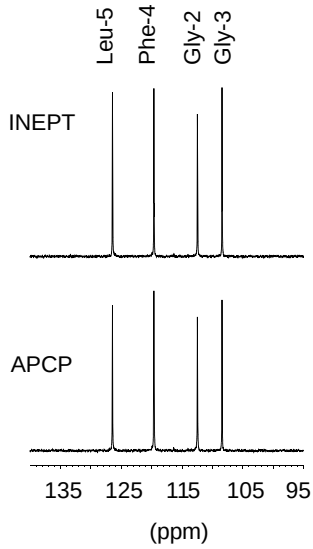

Figure 2. ^{1}H-decoupled refocused INEPT and APCP ^{15}N NMR spectra of Lenk in aqueous solution. Number of transients was 400. In the refocused INEPT in-phase magnetization is transferred in a time τ. The ^{1}H 90° pulse was 9 μs, ^{15}N 90° pulse was 11 μs and τ_C delay was 10.64 ms ($\tau_C = 1/|J_{HN}|$). In the APCP spectrum the ^{15}N rf field was 1 kHz, the ^{1}H was ramped up with a tangential sweep with the amplitude span of ± 380 Hz and a shape with the constant $d_{IS} = 220$ Hz. The contact time τ_c was 100 ms. The proton 90° hard pulse was 9 μs.

Results and discussion

Isotropic systems: Lenk in aqueous solution

The ^{15}N spectra of Lenk in an isotropic aqueous solution obtained with refocused INEPT and APCP are shown in Figure 2. The resonance assignments, obtained by HSQC, are reported in Table 1. We may conclude from the spectra that, under isotropic conditions, the two methods provide 1D spectra with comparable intensities, as expected. The length of the APCP contact time τ_c was 70 ms, though the efficiency of the transfer was rather insensitive to τ_c between 50 and 100 ms (see Figure 1). The length of the contact time (imposed by the requirement of adiabaticity) is a drawback of the APCP method compared to refocussed INEPT (with $\tau_C = 10.6$ ms and $\tau_C = 4\tau$ where $\tau = 1/4J_{HN}$) and, to keep sample heating in an acceptable range, the amplitude of the irradiation should be minimized. On the other hand, sufficiently high rf fields must be used to cover the required spectral bandwidth. Here we have applied a ^{15}N rf field of 1 kHz and we have varied the proton rf field by ± 380 Hz around the Hartmann–Hahn condition, thus, being able to cover both the ^{15}N and ^{1}H spectral width for the amide signals (about 35 ppm for ^{15}N and 3 ppm for ^{1}H). Due to the relatively long $T_{1\rho}$relaxation time of the sample investigated, the details of the pulse shape during CP are not very critical. For samples with faster relaxation this becomes more of an issue and the considerations discussed in (Hediger et al., 1994, 1995) become important. For the calculation of the best shape the smallest $|\Delta|$ is relevant, corresponding to d_{IS} in (Hediger et al., 1994), for the initial offset from the Hartmann–Hahn condition,

Table 1. NMR parameters of Lenk in aqueous solution

Residue	^{15}N isotropic chemical shift (ppm)	J_{HN} coupling (Hz)
Gly-2	112.44(1)	−94.4(2)
Gly-3	108.39(1)	−94.4(2)
Phe-4	119.60(1)	−93.0(2)
Leu-5	126.43(1)	−93.0(2)

the largest $|\Delta|$ should be considered. A detailed study of the optimum shape in the presence of different relaxation active processes has, however, not yet been undertaken.

Oriented systems: Lenk associated to phospholipids surface in aligned bicelles

We have previously observed that Lenk associates to bicelles (Zandomeneghi et al., 2003b). Between 303.5 and 323.0 K, bicelles self-orient in the magnetic field $\mathbf{B}_0$ with the bicelle director aligned orthogonal to $\mathbf{B}_0$ (Sanders and Landis, 1995). At 311 K and with $B_0 = 9.4$ T the bicellar order parameter S_{Bic} for the system bicelle/Lenk was measured to be $S_{\mathrm{Bic}} = 0.62 \pm 0.06$.

The proton-decoupled and coupled ^{15}N APCP spectra of bicelles-associated Lenk in a static sample are shown in Figures 3a and b, respectively. An APCP contact time of 50 ms was chosen. The ^{1}H–^{15}N couplings Δ observed in the spectrum of Figure 3b, the ^{15}N chemical shifts obtained from the spectra in Figure 3a and the assignment of the resonances are reported in Table 2. The signals in the proton-coupled spectrum, especially the Phe-4 and Leu-5 ones, are characterized by broad lines predominantly due to ^{1}H–^{1}H RDC. This explains why the INEPT $^{1}\mathrm{H} \rightarrow {}^{15}\mathrm{N}$ polarization transfer works poorly with an transfer efficiency down by an order of magnitude (data not shown). In addition, a distribution in the bicelles director orientation (mosaic spread) produces a distribution in the ^{1}H–^{15}N RDC and ^{15}N residual chemical-shift anisotropy and, thus, can contribute to the broadening of the lines.

Figure 4 reports the ^{15}N spectra of bicelle-associated Lenk under MAS of 270 Hz. The ^{15}N isotropic chemical shifts are very close to the ones in water and can be readily assigned (Table 2). Under MAS conditions, the efficiency of the polarization transfer via refocused INEPT is comparable to the one in the isotropic solution. The magnitude of the ^{1}H–^{15}N J couplings can be determined from Figure 4b and their sign is known to be negative (Bovey, 1988).

When the spectral lines are too broad, ^{1}H homonuclear decoupling must be applied in order to obtain resolved splittings in the ^{15}N spectrum and determine the heteronuclear couplings. An example is the Leu-5 signal around

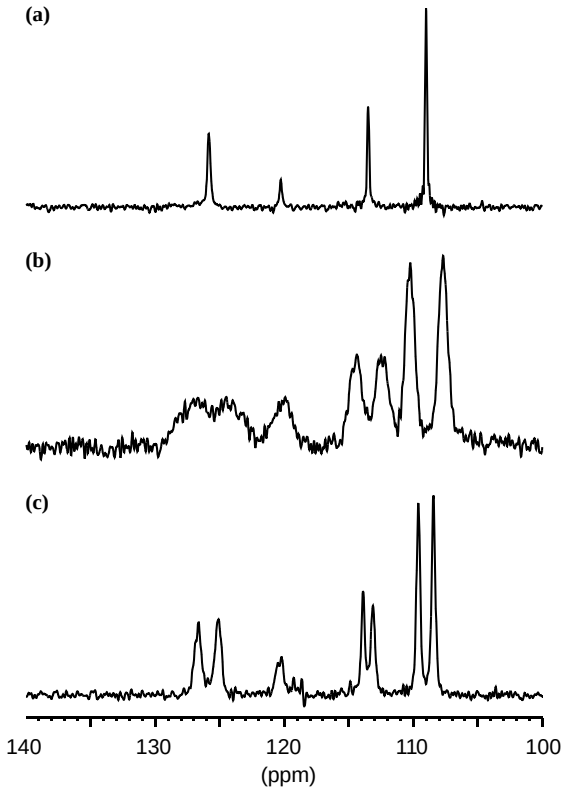

Figure 3. ^{15}N NMR spectra of Lenk in bicellar solution. The temperature was $T = 311$ K and the sample was static with $S_{\mathrm{Bic}} = 0.62$. The polarization transfer is obtained via APCP with the same rf fields as in Figure 2 and $\tau_c = 50$ ms. (a) ^{1}H-decoupled with 4000 scans; (b) ^{1}H-coupled spectrum with 10,000 transients measured; (c) ^{1}H–^{1}H homodecoupled spectrum with 15,000 scans. Homodecoupling was obtained with the BLEW-48 sequence and a rf ^{1}H field of 4 kHz. The splittings of Gly-2 and Gly-3 in (c) can be compared to the ones in (b) and their ratio (0.34 ± 0.12, 0.45 ± 0.03, respectively) corresponds, within error, to the theoretical scaling in the limit of the infinitely short pulses, 0.424. The unresolved splitting relative to Leu-5 in (b) can be calculated from (c) and from the average experimental scaling: $|\Delta| = 150 \pm 20$ Hz.

126 ppm in the static spectrum (Figure 3b), where the splitting is difficult to evaluate. However, under BLEW-48 proton homonuclear decoupling (Burum et al., 1981), the scaled heteronuclear coupling frequency is clearly resolved (Figure 3c). From the proton-coupled ^{15}N spectra it is not possible to determine unambiguously the contribution of the dipolar couplings to the splittings measured, since the doublets recorded provide only the absolute values $|\Delta| = |J + D_{\mathrm{res}}|$. From the residual chemical shift we can infer that all D_{res} values must be positive. We find that for the residue Leu-5 only one solution,

Table 2. NMR parameters of Lenk in bicelle solution

Residue	^{15}N isotropic chemical shift[a] (ppm)	J_{HN} coupling[a] (Hz)	^{15}N chemical shift[b] (ppm)	^{1}H–^{15}N splitting[b,c] (Hz)	^{15}N residual chemical shift anisotropy[b] (ppm)	^{1}H–^{15}N residual dipolar coupling[b] (Hz)
Gly-2	112.96(2)	−94(1)	113.52(2)	82(15)	0.56(4)	176(16) or 12(16)
Gly-3	108.28(2)	−94(1)	109.03(2)	110(10)	0.75(4)	204(11) or −16(11)
Phe-4	119.04(2)	−91(1)	120.27(2)	30(10)	1.23(4)	121(11) or 61(11)
Leu-5	124.18(2)	−91(1)	125.87(5)	150(20)	1.69(7)	240(20)

[a]Determined from MAS spectra.
[b]Determined from static spectra, with $S_{Bic} = 0.62$.
[c]Absolute value.

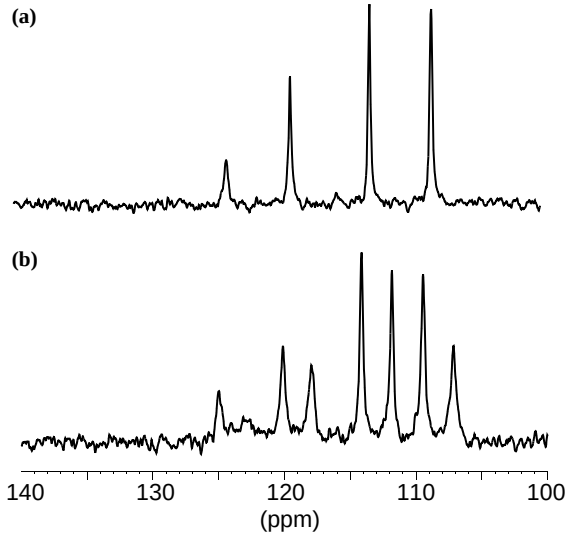

Figure 4. ^{15}N NMR spectra of Lenk in a bicellar solution. The temperature was 311 K and the sample was spun at the magic angle with a spinning frequency of 270 Hz. The polarization transfer is obtained via APCP with identical experimental conditions as in Figure 3. (a) ^{1}H-decoupled spectrum with 3000 transients accumulated; (b) ^{1}H-coupled spectrum, with 10,000 scans.

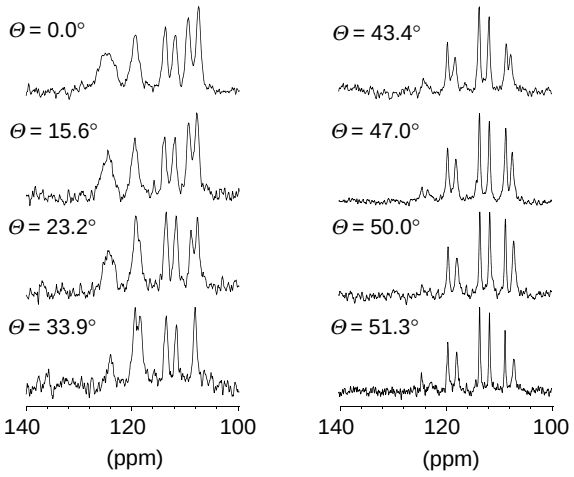

Figure 5. ^{15}N NMR spectra of Lenk in bicellar solution spinning at different angles Θ. Spinning frequency was between 800 and 650 Hz, stable at each angle, and temperature was 313 K, not corrected for the effect of the bearing air at lower temperature. $S_{Bic} = 0.65$. The APCP polarization transfer was obtained with a ^{1}H rf field of 1 kHz, a tangential sweep of the ^{15}N rf field with an amplitude span of ±400 Hz and $d_{IS} = 90$ Hz (Hediger et al., 1994, 1995) and $\tau_c = 50$ ms. Number of transients was between 2400 and 12,000.

$D_{res} = 240 \pm 20$ Hz, is likely. For the other residues the experimental data are consistent with two values of the dipolar couplings for each splitting (Table 2). In particular, for Gly-3 the value $D_{res} = -16 \pm 11$ Hz, which is negative but close to zero, was not directly excluded.

Variable-angle spinning experiments

The ^{1}H–^{15}N dipolar coupling constants of bicelle-associated Lenk could be determined with a series of VAS experiments where ^{15}N spectra are recorded as a function of the angle Θ between the rotor axis and the magnetic field direction (Tian et al., 1999). The experiments are based on the observation that the orientation of the bicelle liquid–crystalline director can be reoriented by sample-spinning. For spinning at angles $0 \leq \Theta < 54.7°$ the bicellar director is oriented orthogonal to the rotor axis. (Tian et al., 1999; Zandomeneghi et al., 2001) It is worthwhile to point out that for $\Theta = 0°$ bicelles orient as in the static sample and, therefore, the static spectrum and the spectrum under spinning with $\Theta = 0°$ are identical. A selection of the ^{15}N VAS spectra measured with $0 \leq \Theta \leq 54.7°$ is presented in Figure 5.

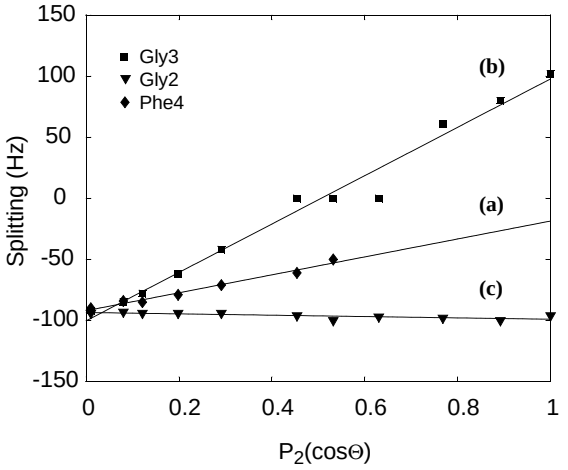

Figure 6. For each VAS experiment (Figure 5) the ^{1}H–^{15}N splittings are reported for the residue Phe-4 (◆), Gly-3 (■) and Gly-2 (▼) at the corresponding value of $P_2(\cos\Theta)$. Line (a) represents $(-92 + 73P_2(\cos\Theta))$, Line (b) represents $(-100 + 198P_2(\cos\Theta))$ and Line (c) $(-93-5P_2(\cos\Theta))$.

A series of VAS experiments (or, alternatively a 2D SAS experiment, Zandomeneghi et al., 2003b) relate the isotropic spectrum at $\Theta = 54.7°$ to the one at $\Theta = 0°$, and allow for the assignment of the resonances in the static sample (Table 2) and resolve the ambiguity in the determination of D_{res}. The dependence of the observed line splittings Δ on Θ is described by $\Delta = J + D_{\text{res}} \cdot P_2(\cos\Theta)$, with the second-order Legendre polynomial $P_2(\cos\Theta) = (3\cos^2\Theta - 1)/2$. Due to the averaging of the susceptibility tensor, the mosaic spread of the bicellar liquid crystal increases close to the magic angle (Zandomeneghi et al., 2003a) and partial powder patterns (of hetero- and homonuclear interactions) can be expected to determine the line shape. Therefore, SAS methods may be necessary for accurate measurements (Zandomeneghi et al., 2003b). In the VAS spectra in Figure 5, the heteronuclear couplings are large near the magic angle and the resolution turned out to be sufficient to perform the technically simpler VAS experiment.

The splittings corresponding to the Phe-4, Gly-3 and Gly-2 residues are reported in Figure 6 as a function of $P_2(\cos\Theta)$. The experimental data can be described with the linear function characterized by the coupling constants (J, D_{res}), reported in Table 3. The values determined are consistent, within statistical errors, with one of the possible two solutions given in Table 2 (obtained at different field using a different sample).

Table 3. Coupling constants from the VAS spectra[a]

Residue	J_{HN}(Hz)	D_{res}(Hz)
Gly-2	−93(1)	−5(2)
Gly-3	−100(5)	198(9)
Phe-4	−92(1)	73(5)

[a]Data from Figure 4, $S_{\text{Bic}} = 0.65$.

Conclusions

Adiabatic-passage cross polarization is shown to be efficient in peptides associated with isotropic and ordered bicelles. Under the moderately oriented conditions described, APCP is a more efficient polarization transfer method than INEPT. It is easy to implement and quite robust against mismatching of the Hartmann–Hahn condition. For a system with an anisotropy of the alignment in the order of 10^{-2}, like the sample Lenk/bicelles, low-power rf fields are sufficient to excite the amide spectral region.

Variable-angle spinning experiments have been used to assign the resonances from the static, oriented sample of bicelle-associated Lenk and to determine the ^{1}H–^{15}N dipolar couplings. A series of 1D VAS experiments allows to correlate the anisotropic spectrum recorded at $\Theta = 0°$ (identical to the static spectrum) to the isotropic one, measured under MAS at $\Theta = 54.7°$, facilitating the assignment of the resonances. Besides, the VAS spectra recorded with $0° \leq \Theta \leq 54.7°$ permit to determine unambiguously the residual dipolar couplings which provide information about the orientation of the N–H bonds of Lenk with respect to the bilayer surface. We are presently investigating whether the information obtained in the present study, together with other residual anisotropic spin interactions and together with ^{1}H–^{1}H distances from NOE measurements in an isotropic bicelle sample (Marcotte et al., 2004), is sufficient for a precise structure determination of the membrane-associated Lenk molecule.

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