Measuring $^{13}\text{C}/^{15}\text{N}$ chemical shift anisotropy in [^{13}C , ^{15}N] uniformly enriched proteins using CSA amplification

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ABSTRACT

Extended chemical shift anisotropy amplification (xCSA) is applied for measuring $^{13}\text{C}/^{15}\text{N}$ chemical shift anisotropy (CSA) of uniformly labeled proteins under magic-angle spinning (MAS). The amplification sequence consists of a sequence of π -pulses that repetitively interrupt MAS averaging of the CSA interaction. The timing of the pulses is designed to generate amplified spinning sideband manifolds which can be fitted to extract CSA parameters. The $^{13}\text{C}/^{13}\text{C}$ homonuclear dipolar interactions are not affected by the π -pulses due to the bilinear nature of the spin operators and are averaged by MAS in the xCSA experiment. These features make the constant evolution-time experiment suitable for measuring CSA of uniformly labeled samples. The incorporation of xCSA with multi-dimensional $^{13}\text{C}/^{15}\text{N}$ correlation is demonstrated with a GB1 protein sample as a model system for measuring $^{13}\text{C}/^{15}\text{N}$ CSA of all backbone ^{15}NH , ^{13}CA and ^{13}CO sites.

Keywords: chemical shift anisotropy, CSA, magic-angle spinning, MAS, spinning sidebands, magic-angle turning, MAT, CSA amplification, extended chemical shift modulation, protein, GB1

1. Introduction

Magic-angle spinning (MAS) is an ubiquitous method for obtaining high-resolution nuclear magnetic resonance (NMR) spectra of solids. Rapid sample spinning averages line broadening arising from anisotropic spin interactions, such as chemical shift anisotropy (CSA) and dipolar coupling, yielding high-resolution spectra of powder samples. These anisotropic spin interactions are of great interest because of their direct

relation to molecular structure and local electronic environment. In the presence of molecular motion, measurement of the scaled chemical shift anisotropy and dipolar coupling can also yield order parameters from the molecular dynamics. Numerous NMR techniques have been developed to reintroduce/recouple CSA or dipolar coupling under fast MAS, which is particularly useful for uniformly labeled proteins in conjunction with multi-dimensional correlation experiments because it can be used under high resolution conditions. However, the presence of ^{13}C homonuclear coupling among the directly bonded carbons can interfere with recoupling of the anisotropic interactions of interest. For CSA measurement, only a few recoupling pulse sequences, namely ROCSA [1] and R18_1 [2-4], have been designed specifically for uniformly labeled samples by minimizing the effects from homonuclear dipolar interaction. In this work, we show that a simple method of CSA amplification can be applied directly to uniformly labeled systems. CSA amplification is a class of pulse sequences that uses π -pulses to repeatedly interrupt the averaging of CSA by MAS. The timing of pulses can be designed in a way such that the effective CSA evolution yields spinning sideband manifolds equivalent to those at a much lower spinning frequency. As shown in early works by Maricq and Waugh [5], and Herzfeld and Berger [6], spinning sidebands are a direct result from modulation of the CSA by MAS. For spinning rates that are several times smaller than the breadth of the anisotropy, the spectral intensity is concentrated into a handful of narrow spinning sidebands that can be fitted to determine the CSA tensor parameters. This ‘slow spinning’ method has become a method of choice for CSA measurement of dilute spin systems wherein CSA is the only spin interaction present and the line width is usually independent of the spinning frequency. For example, it has been possible to

measure the ^{15}N CSA in uniformly ^{15}N labeled GB1 protein samples [7, 8], since homonuclear coupling among ^{15}N nuclei is weak. Nevertheless, dipolar coupling among covalently bonded ^{13}C requires fast MAS to obtain high spectral resolution, making the slow spinning method unsuitable. The CSA amplification method presented here makes the measurement of spinning sideband intensities possible under fast MAS high-resolution conditions.

In the following, a brief theory of our CSA amplification method [9] (dubbed xCSA) is presented, as well as why homonuclear dipolar coupling does not affect it. This key feature, essential for application to uniformly ^{13}C labeled systems, is examined experimentally using an alanine sample. A comparison of ^{13}C sideband intensities of $[^{13}\text{C}, ^{15}\text{N}]$ and $[^{15}\text{N}]$ labeled alanine verifies that the homonuclear couplings have indeed negligible effects on the CSA measurement. For application to uniformly labeled proteins and macromolecules, the xCSA pulse sequence needs to be combined with two-dimensional $^{13}\text{C}/^{15}\text{N}$ correlation to achieve site resolution. Three-dimensional xCSA experiments with NCA and NCO correlation for $^{13}\text{C}/^{15}\text{N}$ CSA measurement of the backbone NH, CA, and CO sites are demonstrated with a sample of $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ labeled GB1 protein.

2. Theory

CSA amplification is a class of pulse sequences for rotating solids pioneered by Griffin *et al.* [10] and Gullion [11], which utilizes π -pulses to interrupt the averaging of CSA under MAS. The timing of the π -pulses can be designed such that the effective evolution yields sidebands that are exactly the same as if the spinning rate is reduced or

the CSA is magnified, hence these methods have been dubbed extended chemical shift modulation (XCS) or CSA amplification. The relative sideband intensities are the same when multiplying the anisotropy or reducing the spinning frequency by a factor κ . A number of CSA amplification pulse sequences have been developed to obtain the largest amplification factor, while using the minimum number of π -pulses and shortest total duration. A recent review on this topic provides a thorough explanation and comparison of these sequences [12]. In the following, we use our recent CSA amplification sequence [9], dubbed extended CSA amplification (xCSA), to describe the theory of CSA amplification and the effect from homonuclear dipolar couplings.

Under MAS, the chemical shift interaction can be separated into the constant isotropic shift and the modulating components from the CSA,

$$\omega(t) = \omega_{iso} + \omega_{CSA}(t), \quad \omega_{CSA}(t) = \sum_{m=\pm 1, \pm 2} \omega_m \exp(im\omega_r t) \quad (1)$$

The signal phase evolution can be obtained by integration of the modulated chemical shift over time $\omega_{CSA}(t)$, in terms of the indefinite integral $\xi(t)$,

$$\phi_{CSA} = \int_{t_a}^{t_b} \omega_{CSA}(t) dt = \xi(t) \Big|_{t_a}^{t_b} \quad (2)$$

Application of π -pulses alternates the sign of the accumulated signal phase. At the end of a sequence of n π -pulses, the total phase becomes

$$\phi_{total} = \sum_{q=0}^n (-1)^{n+q} \xi(t) \Big|_{t_q}^{t_{q+1}} \quad (3)$$

Fig. 1a shows our recent CSA amplification sequence designed based on the magic-angle turning (MAT) experiment [13, 14]. The sequence consists of a basic amplification unit (Fig. 1b) with four π -pulses spanning two rotor periods. The π -pulses are separated by

time units of $\tau_r/3$, where $\tau_r (= 1/\nu_r)$ is the rotor period. The total evolution for the four evolution segments of the basic amplification unit can be written out explicitly

$$\begin{aligned}\phi_{total} &= \left[\xi\left(t_0 + \frac{2\tau_r}{3}\right) - \xi(t_0) \right] - \left[\xi(t_0) - \xi\left(t_0 + \frac{2\tau_r}{3}\right) \right] \\ &\quad + \left[\xi\left(t_0 + \frac{\tau_r}{3}\right) - \xi(t_0) \right] - \left[\xi(t_0) - \xi\left(t_0 + \frac{\tau_r}{3}\right) \right] \\ &= -6\xi(t_0) + 2\xi(t_0) + 2\xi\left(t_0 + \frac{\tau_r}{3}\right) + 2\xi\left(t_0 + \frac{2\tau_r}{3}\right)\end{aligned}\quad (4)$$

Here t_0 is the time at the start of the pulse sequence. Invoking the MAT condition for a second-rank tensor [9, 13, 14], such as CSA,

$$\xi(t) + \xi\left(t + \frac{\tau_r}{3}\right) + \xi\left(t + \frac{2\tau_r}{3}\right) = c \quad (5)$$

where c is a constant, Eqn. 4 becomes

$$\phi_{total} = -6\xi(t_0) + 2c \quad (6)$$

The complete CSA amplification sequence consists of two basic amplification units. The second unit is shifted by a t_1 evolution time (Fig. 1a). The last π -pulse of the first basic unit is eliminated such that total phase evolution takes the form

$$\phi_{xCSA}(t_1) = 6[\xi(t_0) - \xi(t_0 + t_1)] \quad (7)$$

The result shows that the CSA evolution is amplified by a factor $\kappa = 6$. In addition, it can be seen from the coherence transfer diagram in Fig. 1a that the total duration of the $p = +1$ and -1 segments remain equal while t_1 is incremented. Hence, the isotropic shift is kept refocused while the CSA is amplified. Refocusing of the isotropic chemical shift is necessary to ensure that only the periodic modulation of the amplified CSA is present during t_1 . Thus, Fourier transform of t_1 increments spanning one rotor period yields spinning sideband intensities corresponding to amplified CSA. More amplification units (Fig. 1b) can be inserted into the positions denoted by arrows in the pulse sequences of

Fig. 1 to obtain larger amplification factors. In general, xCSA sequences consist of $(8 \cdot n - 1)$ π -pulses covering $4 \cdot n$ rotor periods to yield amplification factors of $\kappa = 6 \cdot n$.

Fig. 1. (a) Pulse sequence and coherence transfer pathway for a $\kappa = 6$ xCSA experiment. (b) Basic amplification unit that can be inserted at the positions denoted by red arrows if additional CSA amplification is desired. (c, d) 3D xCSA pulse sequences for measurement of (c) ^{15}N and (d) ^{13}C CSA. In (d), the $^{15}\text{N}/^{13}\text{C}$ CP and the ^{13}C carrier frequency can be changed to obtain NCA or NCO correlation. Narrow solid and empty rectangles in the pulse sequence denote $\pi/2$ - and π -pulses, respectively. A cogwheel phase cycle is employed during the CSA amplification sequences to select the desired alternating

coherence transfer pathway: 0 for all odd-numbered pulses starting from the excitation or CP pulse, $\{0, 1, 2, \dots, 2a + 1\} \times \pi/(a + 1)$ for all even-numbered pulses, and $\{0, \pi\}$ for the receiver phase, where a is the total number of π -pulses.

For uniformly ^{13}C and ^{15}N labeled samples, both homo- and hetero-nuclear interactions are present in addition to the CSA being measured. For homonuclear coupling, the effect of π -pulses can be described in a toggling frame. A π -pulse applied about the x -axis transforms the spin operators such that $I_z \rightarrow -I_z$, $I_y \rightarrow -I_y$, $I_x \rightarrow I_x$, effectively reversing the sign of the chemical shift Hamiltonian. However, the spin operators of the homonuclear dipolar coupling are bilinear and therefore their signs remain unchanged by the π -pulses throughout the whole xCSA sequence. At the end of the constant evolution time the homonuclear dipolar evolution becomes null due to the averaging of the dipolar coupling spatial components by MAS at complete rotor cycles. Hence, homonuclear dipolar coupling has no first-order effect on the CSA amplification. This is why xCSA can be applied directly to ^{13}C uniformly enriched systems without any modification. It should be mentioned that the homonuclear J -coupling remains constant under MAS leading to a signal attenuation $\cos(\pi J \tau)$ for a constant-time xCSA experiment of duration τ . The short evolution time of xCSA usually makes this signal attenuation negligible. Most importantly, the constant attenuation does not introduce any t_1 modulation and therefore does not affect the CSA measurement. Similarly, heteronuclear dipolar coupling can be decoupled by applying π -pulses that are synchronous with the xCSA π -pulses, as illustrated in the pulse sequences of Figs. 1c and 1d. In this way, the spin part of the heteronuclear dipolar interaction remains unchanged because synchronous π -pulses are applied to both nuclei and the fast MAS averages out the spatial component of the second-rank dipolar interaction. Again the heteronuclear scalar J -

coupling remains, but it is usually small for the constant evolution times typical used for the xCSA experiment.

With complete refocusing of isotropic shift and amplification of CSA in t_1 , the 2D signal of the xCSA experiment can be generally expressed as

$$\begin{aligned} s(t_1, t_2) &= \left\langle e^{-i\phi_{\text{xCSA}}(t_1)} e^{-i\phi_{\text{CSA}}(t_2) - i\omega_{\text{iso}} t_2} \right\rangle \\ &= \left\langle e^{-i\kappa[\xi(t_1) - \xi(0)]} e^{-i[\xi(t_2) - \xi(0)] - i\omega_{\text{iso}} t_2} \right\rangle \end{aligned} \quad (8)$$

where $\langle \rangle$ denotes a powder average. Under fast spinning, the CSA modulation during t_2 can be neglected and the time-domain data can be simplified as

$$s(t_1, t_2) = \left\langle e^{-i\kappa[\xi(t_1) - \xi(0)]} \right\rangle e^{-i\omega_{\text{iso}} t_2} \quad (9)$$

Since $\xi(t)$ is cyclic with respect to the rotor period, t_1 increments spanning only one rotor period are sufficient to encode the complete CSA modulation/evolution. A Fourier transformation in t_1 yields a spinning sideband intensity profile corresponding to a CSA that has been scaled up by a factor κ , as illustrated in Fig. 2. It is evident from Eqn. 9 and Fig. 2a that over one rotor period the real and imaginary parts of the t_1 time-domain data are symmetric and antisymmetric, respectively,

$$s(t_1, f_2) = s^*(\tau_r - t_1, f_2) \quad (10)$$

Fig. 2b illustrates the modulation of $s(t_1, f_2)$ for the CH site of $[^{15}\text{N}]$ alanine. Typically, phase-modulated signals from t_1 -evolution of a single coherence transfer pathway is associated with phase-twisted line shapes [15]. In this case, though the $s(t_1, f_2)$ slices have mixed-phase (Fig. 2b), the final 2D spectrum $s(f_1, f_2)$ is completely absorptive (Fig. 2c). This is similar to the case of the phase-adjusted spinning sidebands (PASS) experiment [16-18]. Both xCSA and PASS have a periodic t_1 -evolution that spans one rotor period.

Fig. 2. (a) Plots of the real and imaginary parts of the t_1 time-domain signal for the CH peak of $[^{15}\text{N}]$ alanine and stacked plots of the $s(t_1, f_2)$ (b) and (c) $s(f_1, f_2)$ xCSA spectrum before and after t_1 Fourier transform.

Interpretation of xCSA as a direct amplification of CSA is only strictly valid as in Eqn. 9 when the sideband intensities in f_2 are negligible, or are all summed. For sites with large CSA, like protein backbone ^{13}CO , there can be significant spinning sidebands in f_2 . In practice, it is often easier and advantageous in terms of signal-to-noise to measure only the center-band peak intensity. Hence, it is necessary to derive the general expressions for the 2D xCSA spinning sideband intensities. The equations derived in the remainder of this section are used to perform numerical simulations and fit xCSA data by measuring

only the centerband intensities. A MATLAB [19] script for the computation is given in the Appendix.

The indefinite integral $\zeta(t)$ and Fourier expansion coefficients of $e^{i\zeta(t)}$ and $e^{i\kappa\zeta(t)}$ can be numerically computed quickly using a Fast Fourier Transformation (FFT)

$$\begin{aligned} e^{i\zeta(t)} &= \sum_k a_k e^{ik\omega_r t} \\ e^{i\kappa\zeta(t)} &= \sum_k b_k e^{ik\omega_r t} \end{aligned} \quad (11)$$

We can then express the general xCSA signal of Eqn. 8 as a Fourier series

$$s(t_1, t_2) \propto \left\langle \sum_{k,l,m,n} b_k^* b_l a_m^* a_n e^{-ik\omega_r t_1} e^{-im\omega_r t_2} e^{-i(k-l+m-n)\gamma} \right\rangle_{\alpha,\beta,\gamma} e^{-i\omega_{iso} t_2} \quad (12)$$

where $\langle \rangle_{\alpha,\beta,\gamma}$ denotes a powder average over the three Euler angles (α, β, γ) that rotate the molecular axis frame into the rotor frame. The average over the rotor angle γ can be obtained by simply keeping only the terms with $k - l + m - n = 0$, leading to the general expression for the 2D xCSA sideband intensities in terms of the Fourier coefficients,

$$s(k, m) \propto \left\langle \sum_l b_k^* b_l a_m^* a_{k-l+m} \right\rangle_{\alpha,\beta} \quad (13)$$

noting that the powder average is reduced to two polar angles (α, β) . By setting $m = 0$, we obtain the formula for calculating the f_1 spinning sideband manifold obtained by measuring only the intensities from the f_2 isotropic centerband

$$s(k, 0) \propto \left\langle \sum_l b_k^* b_l a_0^* a_{k-l} \right\rangle_{\alpha,\beta} \quad (14)$$

We can examine the case of fast spinning, where there are no spinning sidebands in f_2 , i.e., $a_0 = 1$ and $a_{k-l} = 0$ for $k \neq l$,

$$s(k) \propto \left\langle |b_k|^2 \right\rangle_{\alpha,\beta} \quad (15)$$

Since b_k are the Fourier expansion coefficients of $e^{i\kappa\zeta(t)}$, the xCSA sideband intensities in f_1 are the same as the spinning sidebands of a regular 1D MAS spectrum with its CSA scaled up by factor κ . The main contribution to the sideband intensities in Eqn. 14 comes from terms with k close to l . In the MATLAB script given in the Appendix, the variable `ks` defines how many of these terms are summed.

3. Experimental

The samples of $[^{15}\text{N}]$ and $[^{13}\text{C}, ^{15}\text{N}]$ labeled alanine were purchased from Cambridge Isotope Laboratories, Inc. (CIL) and used without further purification. $[\text{U-}^{13}\text{C}, ^{15}\text{N}]\text{GB1}$ was expressed in E.coli BL21- (DE3) cells that grow in minimal media (1 g/L $^{15}\text{NH}_4\text{Cl}$, 2 g/L ^{13}C glucose), protein expression was induced with 1 mM isopropyl- β -D-thiogalactoside for 4 hours. Purification of protein was carried out by anion exchange on a Q-Sepharose FF column, followed by gel filtration on a Superdex75 column. Peak fractions were pooled and concentrated with Centriplus 3 kDa MWCO filters, and the buffer thoroughly exchanged to 20 mM sodium phosphate, pH 8.0. The GB1 solution was concentrated to 25 mg/ml and then a 2 ml aliquot was combined with a (66.7% MPD + 33.3% 2-propanol) precipitation solution by vortex mixing in aliquots of 2, 2, 1, and 1 ml and then incubated at 4 °C for durations of 7 min, 7 min, 7 min, and 18 hours after each successive addition. The resulting solution was centrifuged at 5000 rpm for two minutes and the precipitated microcrystalline sample was transferred into the NMR rotor.

All NMR experiments were performed on a wide bore 600 MHz Bruker Avance I spectrometer using a 3.2 mm low- E MAS probe [20] designed and built at the NHMFL.

The *rf* fields employed for (^{13}C and ^{15}N) π -pulses and ^1H heteronuclear decoupling are 50 and 100 kHz, respectively. Both ^{13}C and ^{15}N π -pulse lengths are 10 μs . ^{13}C chemical shift were externally referenced to tetramethylsilane (TMS) by setting the high-frequency resonance of solid adamantane to $\delta_{iso} = 38.48$ ppm [21]. In the acquisition of multi-dimensional xCSA spectra, the t_1 increments span exactly one rotor and t_1 Fourier transformation is applied without any line broadening or first-point scaling. The xCSA evolution dimension is acquired in a phase-modulated manner without hypercomplex phase cycling, while the ^{15}N t_2 dimension in the sequences of Figs. 1c and 1d was acquired using the method of States [22].

4. Results and discussion

We first examine experimentally the effect of homonuclear dipolar coupling to the CSA amplification sequence. Fig. 3 compares spinning sideband manifold intensities obtained with xCSA under 12 kHz MAS between [^{13}C , ^{15}N] and [^{15}N] enriched alanine samples. The two samples have the same ^{13}C CSA and ^{13}C - ^{15}N heteronuclear coupling. The ^{13}C - ^{13}C homonuclear couplings in the [^{13}C , ^{15}N] enriched sample are sizeable, whereas they can be neglected in the [^{15}N] enriched sample due to the low ^{13}C natural abundance. The experimental results show nearly identical xCSA spinning sideband manifolds for the three sites of alanine acquired with various amplification factors. The CO has the largest CSA and an amplification of $\kappa = 6$ at 12 kHz MAS generates sufficient spinning sidebands to measure its CSA tensor (Fig. 3b). Hodgkinson and Emsley [23] have studied the behavior of spinning sideband manifolds and their analysis for extracting CSA parameters. They have observed from direct one-dimensional

measurements that too many sidebands can introduce greater errors in CSA fitting. Hence, the use of MAS frequencies that only generate a handful of sidebands, such as for the CO site with $\kappa = 6$, is recommended for optimal CSA measurement. For the CA site, $\kappa = 6$ amplification generates only small first-order sidebands (Fig. 3a). Larger amplification factors of $\kappa = 12$ or 18 (Figs. 3c and 3d) are needed to obtain a sufficient number of sidebands to determine CSA parameters accurately. The methyl peak requires $\kappa = 18$ to measure the smallest ^{13}C CSA among the three sites (Fig. 3e). In all cases, the spectra of the $[^{13}\text{C}, ^{15}\text{N}]$ and $[^{15}\text{N}]$ enriched samples are nearly identical, indicating a negligible effect from ^{13}C - ^{13}C homonuclear dipolar coupling in the $[^{13}\text{C}, ^{15}\text{N}]$ sample. The xCSA results for both samples are also in excellent agreement with the slow spinning 1D MAS sideband profiles acquired with their corresponding spinning frequencies ν_r/κ for $[^{15}\text{N}]$ alanine.

Fig. 3. Comparison of xCSA f_1 projections acquired at $\nu_r = 12$ kHz with different κ scaling factors for ^{15}N alanine (blue dashes) and $^{13}\text{C}, ^{15}\text{N}$ alanine (red lines) with 1D ^{13}C slow MAS spectra of ^{15}N alanine at the corresponding spinning frequencies $\nu_r = 12/\kappa$ kHz (black circles).

For proteins, the isotropic shift resolution in a one-dimensional experiment is typically not sufficient to resolve overlap among the large number of sites. The 3D pulse sequences in Figs. 1c and 1d combine xCSA with commonly used 2D $^{15}\text{N}/^{13}\text{C}$ correlation experiments, which in this instance provide near-complete site resolution for the GB1 protein sample. Fig. 4 illustrates the extraction of $^{15}\text{N}/^{13}\text{C}$ xCSA sideband intensities from

three 3D experiments for the three backbone CO-NH-CA sites of the amide bond between residues A48-T49. Amplification of $\kappa = 6$ was used for the ^{15}NH and ^{13}CO sites, while $\kappa = 12$ was used for the ^{13}CA sites. For each site, the xCSA sideband intensity profiles are fitted by comparing with numerical simulations using the centerband only formula in Eqn. 14 and the MATLAB script in the Appendix. The Appendix also shows the corresponding two-dimensional root-mean-square difference between the experimental and simulated sideband intensities as a function of the CSA fitting parameters. All fitted CSA parameters are tabulated in Table 1. It should be noted that the GB1 sample used here was prepared at pH 8.0, which differs from the pH 5.5 samples used in the literature for CSA measurement [24]. The ^{15}NH , ^{13}CA and ^{13}CO isotropic shifts for some of the residues have significant deviation with reported values. Hence, 3D NCACX and NCOCX spectra (not shown) were acquired and used to assign all backbone resonances.

Fig. 4. Representative ^{13}CO , ^{13}CA and ^{15}NH xCSA sideband manifold patterns obtained from 3D NCO/xCSA (^{13}CO), NCA/xCSA (^{13}CA) and NCA/xCSA (^{15}NH) spectra, respectively, using the pulse sequences in Figs. 1c and 1d. All experiments were performed at 14.1 T with 10 kHz MAS, 0.5 s recycle delay, and a maximum ^{15}N t_2 evolution time of 16 ms. The amplification factor, number of xCSA t_1 points, scans and total experimental time for each experiment are: (^{13}CO : 6, 32, 32, 11.4 hours), (^{13}CA : 12, 16, 64, 11.4 hours), (^{15}NH : 6, 16, 32, 5.7 hours).

Fig. 5 shows plots of the three principal CSA components (δ_{11} , δ_{22} , δ_{33}) measured using xCSA for the GB1 backbone ^{13}CA , ^{13}CO and ^{15}NH sites. CSA parameters of GB1 have been measured previously using slow spinning and ROCSA methods [8, 25, 26] and are also plotted in Fig. 5. The comparison shows good agreement with reported values. The ^{13}CA CSA shows significant variation through the protein sequence. In addition to the difference in residue type, the ^{13}CA CSA is also sensitive to peptide dihedral angles and protein secondary structure [27-29], and has been used for structure refinement [25]. The ^{13}CO and ^{15}NH CSA tensor components show less variation relative to their span. Since they are less sensitive to protein secondary structure, ^{13}CO and ^{15}NH CSA are often used to probe protein dynamics. In the present case, minimal scaling (due to dynamics) of the ^{13}CO and ^{15}NH CSA is observed since GB1 is a fairly rigid protein, as shown by the ~ 0.95 order parameters reported for most sites [26].

Fig. 5. Comparison of the GB1 backbone (a) ^{13}CA , (b) ^{13}CO and (c) ^{15}NH CSA tensors determined in this work using xCSA (red traces) with values reported in the literature (blue traces).

The constant-time xCSA experiment has the advantage of not requiring line broadening when fitting spinning sideband intensities, as with ROCSA, to extract CSA

tensor parameters. It has been noted in the literature that line broadening and CSA parameters are correlated when fitting CSA powder patterns [7]. For example, the CSA tensors of amide ^{15}NH sites usually have asymmetry factors near to zero (i.e., $\delta_{22} \approx \delta_{33}$), and applying line broadening tends to make the CSA patterns appear to have a larger difference between δ_{22} and δ_{33} . This may lead to the tendency to smaller differences between δ_{22} and δ_{33} for amide ^{15}NH sites measured by xCSA (Fig. 5c) than with experiments such as ROCSA.

Non-ideal π -pulses arising from inhomogeneous *rf* fields, frequency offset and finite pulse lengths can lead to signals of coherence transfer pathways other than the one shown in Fig. 1a. The employed cogwheel phase cycle removes these signals, ensuring the acquisition of only the desired alternating pathway. As a result, distortions due to non-ideal π -pulses are avoided by the filtering of undesired signals. However, the efficiency of the xCSA experiment decreases, especially when a large number of π -pulses are needed for large amplification factors. For example, the efficiencies of the xCSA experiments in Figs. 3a ($\kappa = 6$, $7 \times \pi$ -pulses), 3c ($\kappa = 12$, $15 \times \pi$ -pulses) and 3d ($\kappa = 6$, $23 \times \pi$ -pulses) are approximately 80, 70 and 60%, respectively, compared to a conventional CP/MAS spectrum.

Conclusions

In summary, CSA amplification by xCSA is a robust method for measuring $^{13}\text{C}/^{15}\text{N}$ CSA of uniformly ^{13}C and ^{15}N enriched samples. Both theory and experimental results show that ^{13}C homonuclear dipolar coupling does not interfere with xCSA amplification. With xCSA, the amplification factor can be varied depending on the

applied spinning speed and magnitude of the CSA to obtain the optimal number of spinning sidebands for measuring CSA tensors accurately. The xCSA pulse sequence uses a short constant evolution-time of $4 \cdot n$ rotor periods and $(8 \cdot n - 1) \pi$ -pulses to achieve amplification factors of $\kappa = 6 \cdot n$. The amplified spinning sideband manifolds can be fitted in a straight forward manner without the implications of line broadening and scaling factors present in other recoupling methods. As illustrated with a model protein sample, the xCSA experiment can be combined with multi-dimensional experiments to measure $^{13}\text{C}/^{15}\text{N}$ CSA parameters of proteins and provide valuable information of their structure and dynamics.

Table 1 Isotropic shifts^a and CSA tensor elements^b determined in this study for the GB1 backbone ^{15}NH , ^{13}CA and ^{13}CO sites.

residue	^{15}NH				^{13}CA				^{13}CO			
	δ_{11} (ppm)	δ_{22} (ppm)	δ_{33} (ppm)	δ_{iso} (ppm)	δ_{11} (ppm)	δ_{22} (ppm)	δ_{33} (ppm)	δ_{iso} (ppm)	δ_{11} (ppm)	δ_{22} (ppm)	δ_{33} (ppm)	δ_{iso} (ppm)
Y3	242	70	58	123.3	73	58	40	56.8	250	185	90	174.8
K4	237	77	51	121.6	72	55	40	56.0	239	187	95	173.4
L5	236	70	70	125.3	72	54	33	53.0				
I6	236	77	63	125.2	78	60	43	60.1	243	186	97	175.4
I7	240	80	58	126.2	74	55	36	55.1	249	186	89	174.4
N8	237	69	69	125.2	71	52	30	51.0	258	165	103	175.6
G9	224	52	52	109.3	69	44	20	44.6	250	171	100	173.5
K10	240	63	63	121.7	74	60	44	59.7	247	179	109	178.2
T11	223	53	52	109.3	80	62	46	62.8	241	179	99	172.9
L12	248	67	67	127.2	72	53	36	53.7	248	172	101	173.7
K13	240	77	52	122.7	71	54	35	53.3	238	182	106	175.4
G14	219	57	42	106.2	64	47	28	46.1	239	171	102	170.5
E15	242	60	60	120.3	71	55	38	54.7	245	177	99	173.8
T16	225	59	58	113.9	77	59	44	60.2	243	175	98	172.2
T18	225	61	61	115.5	77	62	48	62.1	239	176	98	171.0
E19	234	68	68	122.9	73	54	36	54.3				
A20	236	68	68	124.0	73	51	29	51.0	253	192	88	177.4
V21	231	70	52	117.4	77	62	52	63.8	246	174	101	174.0
D22	239	53	53	115.2	71	50	36	52.4	246	191	89	175.2
A24									247	198	97	180.5
T25	234	58	58	116.2	83	66	51	67.0				
A26	246	73	52	123.7	71	55	39	55.0	251	185	95	177.0
E27	236	57	57	116.6	75	58	46	59.4				
V29	239	60	80	119.6	80	67	52	66.4	253	191	93	179.0
F30	235	61	61	118.9	72	57	43	57.5				
K31	245	60	60	121.6	75	60	45	60.3	251	201	86	179.4
Q32	243	61	61	121.3	74	58	46	59.4	244	192	94	176.7
Y33	242	60	60	120.8	75	60	48	61.2	251	196	89	178.5
A34	242	62	62	121.9	74	56	38	56.0	251	199	87	178.9
N37	221	58	58	112.4	75	52	33	53.4	245	176	104	175.2

G38	227	50	50	108.8	72	46	22	46.7	250	184	89	174.3
V39	247	62	62	123.3	77	61	48	61.9	248	187	89	174.4
D40	242	71	71	127.8	72	53	36	53.6	243	174	104	174.1
G41	208	69	50	108.6	64	47	26	45.4	244	178	93	171.8
E42									259	169	104	177.4
W43	239	69	69	125.9	79	56	37	57.4	254	177	99	177.0
T44	215	65	46	108.9	81	61	41	61.0	244	179	97	173.2
Y45									246	184	84	171.5
D46									253	195	82	176.6
D47	240	78	53	123.7	70	55	41	55.6	240	191	100	177.1
A48	243	58	58	119.3	73	53	37	54.0	250	190	95	177.9
T49	219	43	43	101.8	80	60	42	60.7	244	186	95	174.8
T51	224	56	56	111.8	80	62	46	62.9	247	181	95	174.3
F52	246	71	71	129.3	74	57	40	56.8	245	181	100	175.2
T53	226	67	43	111.6	77	60	44	60.2	245	182	89	172.2
V54	235	75	42	117.3	77	58	40	58.1	242	185	90	172.5
T55	239	67	67	124.2	78	61	46	61.5	243	188	90	173.5
E56	244	82	69	131.7	78	57	38	57.6				
Y3	242	70	58	123.3	73	58	40	56.8	250	185	90	174.8

^a the isotropic shift is defined as $\delta_{iso} = (\delta_{11} + \delta_{22} + \delta_{33})/3$

^b the CSA tensor component are defined such that $\delta_{11} \geq \delta_{22} \geq \delta_{33}$

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Appendix

```
% xcsa2d.m - calculation of 2D xCSA sideband intensities
% Zhehong Gan & Ivan Hung, c. 2012

vr=12000;      % spinning rate [Hz]
delta=-3000;   % reduced anisotropy(delta) [Hz]
eta=0.1;       % asymmetry(eta), 0 <= eta <= 1
kappa=12;     % amplification factor, 6*n
ns=16;        % number of ssbs in t1
ks=5;         % number of shifts, k=0 no shift,
               % k=1 three terms, ...

% Fibonacci number 1 1 2 3 5 8 13 21 34 55 89 144 233
% 377 610 987 1497 2484 3981 6465 10446 16911 27357
% (JCP V47 p5307 and v59 p3992)
fb2=610; fb1=233;

wrt=(0:ns-1)'/ns*2*pi; % wrt, 2*pi rotor period
cb=zeros(ns,1);        ss=zeros(ns,1);

% powder average
for ip=0:1:fb2-1;
```

```

beta=ip*pi/fb2;  alfa=mod(ip*fb1/fb2,1)*pi;

% the 1st and 2nd cosine and sine coefficients
c1=-sin(2*beta)/sqrt(2)*delta*(1+cos(2*alfa)*eta/3);
s1=sqrt(2)/3*sin(beta)*sin(2*alfa)*delta*eta;
c2=delta/2*(sin(beta)^2- ...
    (1+cos(beta)^2)/3*cos(2*alfa)*eta);
s2=cos(beta)/3*sin(2*alfa)*delta*eta;

% phase integration in t1 and t2
pht1=kappa*(c1*sin(wrt)-s1*(cos(wrt)-1)+ ...
    c2*sin(2*wrt)/2-s2*(cos(2*wrt)-1)/2)/vr;
pht2=(c1*sin(wrt)-s1*(cos(wrt)-1)+ ...
    c2*sin(2*wrt)/2-s2*(cos(2*wrt)-1)/2)/vr;

% expansion coefficient in t1 and t2
fid=exp(-1i*pht1);    b=swap(fft(fid))/ns;
fid=exp(-1i*pht2);    a=swap(fft(fid))/ns;

% sum terms with different shift, cb=centerband only
for k=-ks:1:ks
    bs=lsh(b,k); as=lsh(a,k);
    cb=cb+conj(b).*bs*a(ns/2+1)* ...
        conj(as(ns/2+1))*sin(beta);
end

% sum of all ssbs in f2
ss=ss+b.*conj(b)*sin(beta);
end

% plot spectra
ax=(-ns/2:1:ns/2-1);    % axis as sideband order =
                        %          f*kappa/vr
ssbs=real(cb/max(cb)); % normalized xCSA sideband
intensities
plot(ax,ssbs,'k');
axis([ min(ax) max(ax) -0.1 1.1 ]);

```

Fig. A1. Contour plots of the root-mean-square difference from the least-square fits in Fig. 4 as a function of the CSA parameters. The three traceless CSA principal components used in least-square fitting and the plots are defined as $\chi \cdot \sqrt{2/3}[\cos(\xi - 120^\circ), \cos(\xi + 120^\circ), \cos\xi]$, with $\chi = \sqrt{(\delta_{11} - \delta_{iso})^2 + (\delta_{22} - \delta_{iso})^2 + (\delta_{33} - \delta_{iso})^2}$ and angle ξ representing the anisotropy and asymmetry of the CSA tensor. For the ^{13}CO and ^{13}CA plots, the double minima are due to a change of the order of the three principal components.

Graphical Abstract