Journal of Magnetic Resonance 203 (2010) 208–212

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

A practical implementation of cross-spectrum in protein backbone resonance assignment

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ARTICLE INFO

Article history:
Received 7 October 2009
Revised 16 December 2009
Available online 24 December 2009

Keywords:
Backbone assignment
Cross-spectrum
Heteronuclear experiments
Automation

ABSTRACT

The concept of cross-spectrum is applied in protein NMR spectroscopy to assist in the backbone sequential resonance assignment. Cross-spectrum analysis is used routinely to reveal correlations in frequency domains as a means to reveal common features contained in multiple time series. Here the cross-spectrum between related NMR spectra, for example HNCO and HN(CA)CO, can be calculated with point-by-point multiplications along their common C′ carbon axis. In the resulting higher order cross-spectrum, an enhanced correlation signal occurs at every common *i*-1 carbon frequency allowing the amide proton H^N (and nitrogen N) resonances from residues *i* and *i*-1 to be identified. The cross-spectrum approach is demonstrated using 2D spectra H(N)CO, H(NCA)CO, H(NCO)CACB, and H(N)CACB measured on a $^{15}N/^{13}C$ double-labeled Ubiquitin sample. These 2D spectra are used to calculate two pseudo-3D cross-spectra, H_i–H_{i-1}–C'_{i-1} and H_i–H_{i-1}–$CA_{i-1}CB_{i-1}$. We show using this approach, backbone resonances of H, C′, CA, and CB can be fully assigned without ambiguity. The cross-spectrum principle is expected to offer an easy, practical, and more quantitative approach for heteronuclear backbone resonance assignment.

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1. Introduction

In protein NMR spectroscopy the backbone resonance assignment is a key step in the characterization of protein structure and dynamics. Numerous efforts have been made to expedite this critical, time-consuming, and labor-intensive task by improvements to the NMR data collection schemes as well as the analysis methods. Novel data acquisition methods were developed to reduce the sampling space, thus experimental time, for multi-dimensional experiments. Approaches to reduce measurement time include non-uniform sampling [1], G-matrix Fourier-transform (GFT) NMR [2], Projection–Reconstruction (PR) NMR [3,4], Covariance NMR [5], and Automated Projection Spectroscopy (APSY) [6]. For the purpose of resonance assignments, additional correlations among lower dimensional (mostly 2–4D) data sets have to be established. In protein NMR spectroscopy one relies on approaches, either visual discrimination or computer algorithm or both, to identify paired *i*-1 carbon resonances from a pair of spectra, acquired using either conventional or reduced-sampling methods [7–10]. We should note experimentally Kupce and Freeman [11,12] have demonstrated the applicability of Hyperdimensional NMR spectroscopy, which correlates up to 10 spin nuclei, by further exploiting PR-NMR. In theory Hyperdimensional NMR excludes the need for peak-matching assignments, but up to now this effort is still under development. Nevertheless, all of the above developments illustrate the demand for better and faster resonance assignment approaches.

At the outset, it is noted that, computational methods to automatically correlate pairs of lower dimensional NMR data sets through their common axis have been demonstrated by calculating the linear correlation coefficient between two time domain series, such as COBRA (COrrelation Based Reconstruction Algorithm) [13], or the covariance between two frequency or time domains, e.g. Generalized Indirect Covariance (GIC) NMR [14]. Both involve sophisticated formulas and algorithms. Our objective here is to provide an alternative that is easier to implement using existing common NMR processing and analysis tools.

Here we present a rather simple and practical idea for protein sequential spin assignments based on the principle of a cross-spectrum calculation to replace the resonance-matching step. Instead of calculating the cross-correlation from two time series [13], we employ a fast-Fourier-transform algorithm which allows the equivalent cross-correlation calculation using frequency domain spectra [15]. The cross-spectrum is very useful in revealing correlations in spectra of related time series [16]. If a common frequency component exists in all of the related time series, a peak will result at that given frequency in the Fourier-transform of the cross-correlated time series. This simplifies identification of the

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1090-7807/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.jmr.2009.12.018

common frequencies. Sequential backbone assignment generally requires identification of common frequencies such as CA and CB for a given residue in multiple time function experiments, for example, as in inter-residue correlations expressed in HNCACB and HN(CO)CACB spectra. Therefore it seems fitting to employ the cross-spectrum approach for sequential assignment purposes.

The cross-spectrum can be calculated most easily in the frequency domain, as the simple product of the frequency functions. Specifically, the cross-spectrum is formed via point-by-point multiplication between the common axes where the frequencies are to be compared. For example, the cross-spectrum of two 2D spectra H(N)CO and H(NCA)CO will be a pseudo-3D spectrum whose axes are H^N of residue *i*, H^N of residue *i*-1, and the common C′ chemical shift of residue *i*-1 (nuclei such as nitrogen contained within the parenthesis in the description of the NMR experiment are involved in magnetization transfer but do not evolve under chemical shift). The strong cross-peaks in the cross-spectrum will have their coordinates corresponding to H^N chemical shifts of both residues *i* and *i*-1, which unambiguously provides the backbone assignment. Furthermore, the cross-spectrum of two 3D spectra, e.g. HNCO and HN(CA)CO, will be a pseudo 5D spectrum whose axes are H^N and N of residue *i*, H^N and N of residue *i*-1, and the common C′ chemical shift of residue *i*-1. Similarly, the sequential connection can also be established by calculating the cross-spectrum where the carbon axes containing CA and CB chemical shifts are convolved, leading to the complete backbone resonance assignments.

2. Methods and results

We demonstrated the applicability of the cross-spectrum approach in protein backbone sequential assignments using 2D H(N)CO, H(NCA)CO, H(NCO)CACB, and H(N)CACB spectra collected on a $^{15}N/^{13}C$ double-labeled Ubiquitin sample. NMRPipe [17] was used for all data processing and spectral multiplication. Figures of NMR spectra were prepared using NMRWish scripts within the NMRPipe package.

2.1. Cross-spectrum through carbonyl carbon

Shown in Fig. 1A and B are small regions of the H(N)CO and H(NCA)CO 2D spectra, respectively. The through-bond *J*-correlations are between H_i (H now denotes H^N) and C'_{i-1} within the H(N)CO spectrum, and primarily between H_{i-1} and C'_{i-1} within the H(NCA)CO spectra. For example both resonances in red in Fig. 1A and B have the same C′ chemical shift of 176.0 ppm from residue Q2. They indicate connection between amide protons of I3 (Fig. 1A) and Q2 (Fig. 1B) at chemical shifts of 8.30 and 8.95 ppm, respectively. The cyan resonance in Fig. 1B yields the same *i*/*i*-1 correlation as H(N)CO in Fig. 1A does, but it usually bears weaker intensity than the intra-residue connection. This is due to the smaller two-bond $J_{NC\alpha}$ coupling. The objective is to calculate the cross-spectrum through the carbonyl carbon C'_{i-1} so that we can correlate sequential proton resonances. The pseudo-3D cross-spectra, H_i–H_{i-1}–C'_{i-1}, were calculated using Eq. (1) and the real part of the two Fourier-transformed 2D spectral matrices, $S_{H(N)CO}$ and $S_{H(NCA)CO}$. In Eq. (1) *i* and *k* index the H_i and C'_{i-1} axes of the 2D H(N)CO matrix ($S_{H(N)CO}$), respectively, while *j* and *k* index H_{i-1} and C'_{i-1} axes of the 2D H(NCA)CO matrix ($S_{H(NCA)CO}$), respectively. Since the same carbon indexing *k* is used in both 2D spectra, it is essential to keep the measurement conditions, chemical shift range and digital resolution identical for each related axis by suitable acquisition and processing. The calculated 3D matrix S_{cross} kept both proton dimensions (*i* and *j*) and obtained a new crossed carbon dimension (*k*) through point-by-point multiplication. As a result, axes H_i and H_{i-1} were preserved from both spectra of H(N)CO and H(NCA)CO, respectively, and the new C'_{i-1} dimension encodes the cross-correlated or convoluted axis.

$$S_{cross}[i][j][k] = S_{H(N)CO}[i][k] \times S_{H(NCA)CO}[j][k] \quad (1)$$

Shown in Fig. 1C is a region of the 2D projection of the pseudo-3D cross-spectrum H_i–H_{i-1}–C'_{i-1} onto the H_i and H_{i-1} dimensions, displaying the same proton chemical shift range as the spectra in Fig. 1A and B. The two red peaks in Fig. 1A and B are now correlated in a single resonance also indicated in red in Fig. 1C, which correlates proton chemical shifts of Q2 and I3 along H_{i-1} and H_i axes, respectively. The cyan diagonal peak of Fig. 1C originates from the red peak in Fig. 1A and the cyan peak in Fig. 1B, and it is much less enhanced after multiplication. The cyan diagonal peak provides the chemical shifts of H–I3 and C′–Q2 only and does not carry any assignment connectivity. The correct cross-peak providing connectivity can be identified by its strong intensity in the cross-spectrum. In this example, since we only use the 2D versions of these heteronuclear NMR experiments, diagonal peak coordinates are needed to identify a 1D vector along the H_{i-1} dimension for resonance intensity evaluation. For instance, the position of the diagonal peak, C'_{i-1} at 176.0 ppm and H_i at 8.30 ppm (the red peak in Fig. 1A) was used to identify the proper 1D vector/slice (Fig. 1D) from the 3D cross-spectrum, that readily shows the resonance at 8.95 ppm for H_{i-1} (identified in red) to be the strongest cross-peak (Fig. 1D). In this C′-only example with Ubiquitin, this simple "strongest cross-peak" criterion produces the correct sequential assignment for about 75% of the residues. Exceptions occur due to resonance overlap in C'_{i-1} dimension, as well as relaxation effects which change peak intensities and linewidths. One exception example is shown in Fig. 2A, where the correct cross-peak for T7/L8 (colored red) is not the strongest one.

2.2. Verification using cross-spectrum through CA and CB

The resonance overlap problem can be reduced substantially if a suitable complementary cross-spectrum exists. To illustrate this, 2D spectra of H(NCO)CACB and H(N)CACB were collected and multiplied similarly to produce pseudo-3D cross-spectrum, H_i–H_{i-1}–$CA_{i-1}CB_{i-1}$. Shown in Fig. 2B and C are the 1D-slices corresponding to CA and CB cross-spectrum for T7/L8 connectivity, respectively. The correct cross-peaks, shown in red (Fig. 2), from all three slices of the cross-correlation spectra yield the same H_{i-1} chemical shift. Ambiguities such as the one illustrated in Fig. 2A can be resolved immediately. In fact when both H_i–H_{i-1}–C'_{i-1} and H_i–H_{i-1}–$CA_{i-1}CB_{i-1}$ cross-spectra were employed together, all observed peaks from Ubiquitin could be assigned correctly.

3. Discussions

We have demonstrated a simple, general-purpose cross-spectrum method for protein heteronuclear backbone sequential resonance assignment on ^{13}C and ^{15}N labeled Ubiquitin. This approach replaces peak-matching steps commonly used during manual- and auto-assignment. Four Fourier-transformed 2D spectra were used to calculate two pseudo-3D cross-correlated spectra (cross-spectra). Here a single Tcl-tk script within NMRPipe converts two real-FT spectra into one cross-spectrum in the same format as any regular NMR spectrum. We then directly read out the chemical shift of H_{i-1} spins from strong cross-peaks at the proper 1D-slice given by the H_i–C_{i-1} position within the pseudo-3D spectra. The use of two cross-spectra through C′ and CA/CB allows valid cross-peaks to be differentiated from accidental correlations, so that assignments can be generated unambiguously. The signal to noise (*S*/*N*) of the cross-peak of interest ($S/N_{cross} = S/N_A \times S/N_B$) is significantly improved compared to its parent spectra. This feature always

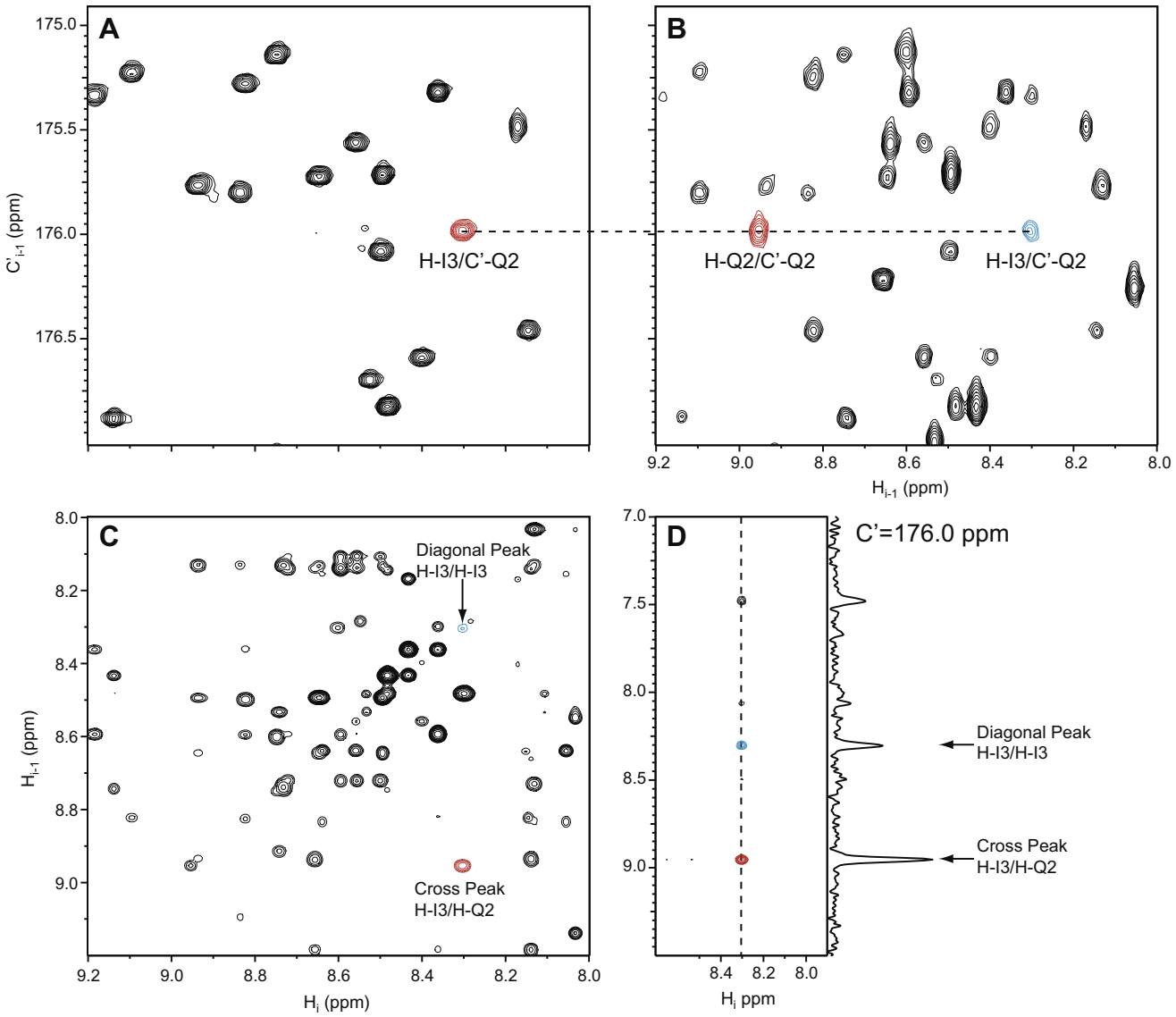

Fig. 1. Illustrations of cross-spectrum based sequential resonance assignment using 2D spectra of H(N)CO (A) and H(NCA)CO (B). The resonances in red correlate H–I3 and C′–Q2, and H–Q2 and C′–Q2 in A and B, respectively. The cyan resonance in B is the inter-residue cross-peak and provides the same correlation as the red peak in A. Both 2D spectra were multiplied according to Eq. (1) to produce the pseudo-3D cross-spectrum H_i–H_{i-1}–C'_{i-1}. Part of the 2D projection of the cross-spectrum onto H_i and H_{i-1} dimensions is shown (C). The red resonance peak in C represents the cross-peak yielding correlation between H–I3 and H–Q2. The cyan resonance peak in C is the diagonal peak at chemical shifts of H–I3. The 1D-slice at C′–Q2 chemical shift of 176.0 ppm and H–I3 chemical shift of 8.30 ppm from the 3D cross-spectrum illustrates the correct diagonal peak (cyan) and the cross-peak (red) for Q2–I3 sequential proton assignment (D). The 2D experiments were collected on a 0.5-mM $^{15}N/^{13}C$ labeled Ubiquitin sample at 22.5 °C using a Bruker DMX600 spectrometer equipped with a room-temperature TXI probe equipped with a xyz-gradient. Pulse sequences for HNCO [20] and HN(CA)CO [21] experiments were used without chemical shift evolution on nitrogen dimension. Proton and carbonyl carbon carrier were at 4.76 and 175.46 ppm, respectively. Spectral widths of 6602 and 1786 Hz were used for proton and carbon, respectively. Data matrices used were 640* × 256* (* denotes complex points) for both experiments. Total experimental time were 2 and 10 h for H(N)CO and H(NCA)CO, respectively. Proton and carbon dimensions were apodized with cosine-squared and cosine functions respectively, the data matrices were zero-filled to 2048* × 512* points prior to Fourier transformation, and only the data corresponding to the proton chemical shift range of 9.7–5.7 ppm was retained. The multiplications between the two spectra were carried out using NMRPipe. The final size of the 3D cross-spectrum was 793 × 793 × 512 for H_i, H_{i-1}, and C'_{i-1}, respectively.

distinguishes the strongest peaks from the parent spectra that are capable of yielding the right cross-peak. For instance a weak resonance peak in one experiment can be significant if multiplied by a relatively intense resonance peak in the other.

We do need to point out that other methods, COBRA [13] and covariance [14], reveal correlations between two spectra as well, and all share the same principle of cross-correlation. In fact, the summation over k in Eq. (1) would yield covariance NMR. Here we chose not to collapse the correlation dimension k, the carbon axis, because it is essential in our cross-spectrum method to use the C_{i-1} chemical shift values for choosing the proper diagonal and cross-peaks. In addition, the cross-spectrum implementation described here is free of any adjustable parameters that need to be taken care of in sophisticated manners. For example, in COBRA, the optimal phase value in calculating phase-weighted correlation coefficient has to be empirically determined; in the GIC method, the matrix exponential factor λ needs optimization.

The cross-spectrum approach holds further potential in automatic peak assignment. Accuracy of peak position will depend on the signal to noise of a given spectrum. Therefore when one compares two spectra with different signal to noise profiles, it is rather difficult to use a single value for deviation of peak positions to

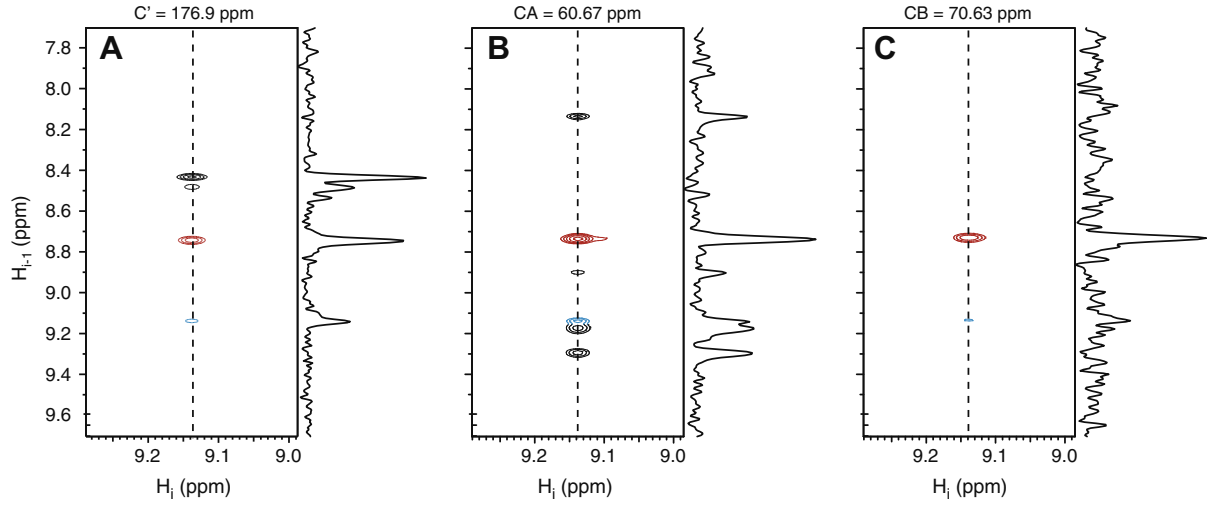

Fig. 2. Examples of T7–L8 amide proton sequential assignment using three cross-peaks from two pseudo-3D cross-spectra, H_i–H_{i-1}–C'_{i-1} and H_i–H_{i-1}–$CA_{i-1}CB_{i-1}$. The 1D-slices were extracted at chemical shifts of H of L8 and that of C′(A), CA (B), and CB (C) of T7. Cross-peaks in red correlate T7–L8 amide proton chemical shifts. Cyan peaks are diagonal peaks of H of L8. Noting the consensus agreement among them for the proton chemical shift of T7 at H_{i-1} axes. Pulse sequences for HNCACB [22] and HN(CO)CACB [23] experiments were used without chemical shift evolution on the nitrogen dimension. Proton and carbon carrier were at 4.75 and 43.98 ppm, respectively. Spectral widths of 7212 and 10,000 Hz were used for proton and carbon, respectively. The size of the data matrices were 640* × 180* for both experiments. Total experimental time were 8 and 9 h for H(N)CACB and H(NCO)CACB, respectively. Data processing details were similar to those for H(N)CO and H(NCA)CO given in Fig. 1. The final size of the 3D cross-spectra were 811 × 811 × 512 for H_i, H_{i-1}, and $CA_{i-1}CB_{i-1}$, respectively.

decide whether two resonances are related. Ideally an evaluation that takes into account relative peak intensities is preferred. Our cross-spectrum implementation inherently affords the quantification of correlations in the cross-spectra from intensity and line width information of the parent spectra. Fig. 3 shows the simulated cross-peak intensities as a function of frequency offsets between two peaks. The cross-spectrum intensity provides a possible function for assignment evaluation (e.g. the use of a intensity cutoff as a criteria). The further cross (multiplication) of cross-spectra (for instance spectra in Fig. 2 along the proton axis) can differentiate between valid cross-peaks and accidental correlations because only the real (red resonance peak in Fig. 2) cross-peak will be further enhanced. This is in contrast to the conventional assignment procedure, where the resonance intensity and line width are often not used to assist in evaluation.

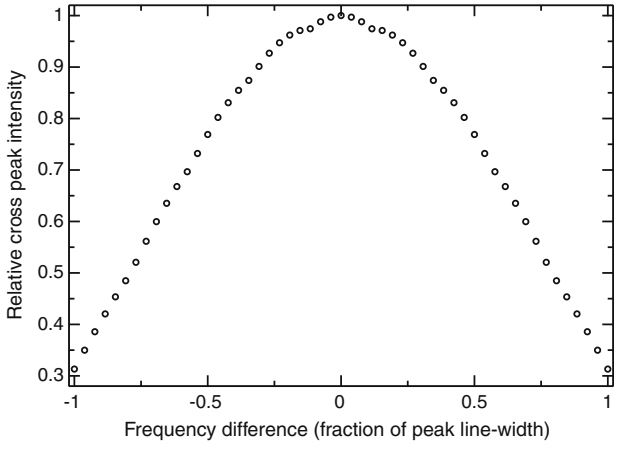

Fig. 3. Simulated cross-peak intensity as a function of frequency difference between two resonances. Simulation parameters were set to follow spectral width typically used for the carbonyl carbon in real experiments at a 600 MHz spectrometer. Time series ($e^{-i\omega t}$) were simulated with 64 pairs of complex points at a sampling interval of 667 μs. The reference peak was at zero frequency. FID was then zero-filled to a total of 512 points and Fourier-transformed. The reference peak line-width at half-height was 26 Hz. The other peak carried an offset ranging from −26 to 26 Hz and was identically processed as the reference spectrum. The cross-spectrum was calculated with point-by-point multiplication between two 1D spectra. All intensities were normalized to the reference peak. Each point in the figure corresponds to relative intensity of the cross-spectrum peak versus the offset expressed in fractions of peak line-width. MATLAB 2007b (Mathworks, MA) was employed for the simulation.

Previously, Szalma and Pelczer [18] proposed a reconstruction of a 3D solid from a pair of 2D spectra using Boolean algebra. This was done to recover the full *J*-coupled spin network from 2D H,H-COSY and H,C-COSY. The 2D spectra were converted to Boolean matrices that identify peak positions. The method then searches for correlations of peak positions in these matrices that allow the reconstruction of the full 3D solid correlation matrix. By contrast, our cross-spectrum method generates correlations directly without the need for systematically searching through spectra and matching peak positions.

The cross-spectrum method can easily be extended to accommodate larger and more complicated molecular systems. For example, it is clear in the illustration used here that the diagonal peak identification would be much easier to perform if an additional nitrogen dimension is used. In this analysis, the cross-spectrum will be five dimensions, H_i–N_i–H_{i-1}–N_{i-1}–C_{i-1}, with cross-peaks readily identifying assignments. The cross-spectrum approach can also be extended to other assignment tasks. For instance inclusion of side chain experiments such as CC(CO)NH [19] and its cross-spectrum with CA and CB resonances in the typical backbone experiments could assist with side chain assignments. Potentially, this can also be applied to the combination of NOESY and TOCSY types of experiments to extract distance information, unambiguously. In all these experiments, we can envision general-purpose spectral viewing and analysis schemes which generate and display suitable regions of the cross-spectra "on the fly" rather than pre-computing entire spectral products with five or more dimensions.

Acknowledgments

We thank Xiongwu Wu, Melvin J. Hinich, Robert Gahl and Yang Shen for helpful discussions. This work was supported by the Intra-

mural Research Program of the NIH, National Heart, Lung, and Blood Institute.

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