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Two-Dimensional Heteronuclear Chemical-Shift Correlation in Proteins at Natural Abundance ^{15}N and ^{13}C Levels

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Two-dimensional NMR spectroscopy is finding increasing application in the study of structure and function of proteins and nucleic acids. The development of 2D NOE spectroscopy (*1*) permits the measurement of interproton distances and provides a direct means of determining conformation. Unfortunately, the ^{1}H NMR spectra of macromolecules usually show severe overlap, even at the highest available field strengths. Assignment of a ^{1}H NMR spectrum can therefore be prohibitively complicated, which makes use of the valuable NOE information impossible. By correlation of ^{1}H chemical shifts with those of ^{15}N or ^{13}C, spectral overlap can largely be removed and the ^{15}N or ^{13}C chemical-shift value can be of additional help in assigning the ^{1}H spectrum. Early attempts to record such heteronuclear chemical-shift correlation spectra for proteins relied on ^{13}C detection and consequently had low sensitivity, requiring very large sample quantities and long measuring times or isotope enrichment (*2–4*). More recently, heteronuclear chemical-shift correlation schemes have been proposed that rely on ^{1}H detection and that offer up to two orders of magnitude improvement in sensitivity (*5–10*). This new approach has recently been demonstrated for isotopically labeled proteins (*11–13*) and nucleic acids (*14, 15*). Very recently, one report showed the feasibility of natural abundance ^{1}H–^{15}N correlation in small proteins (*16*). Here, we demonstrate that routine heteronuclear shift correlation in proteins is feasible and we outline procedures that optimize sensitivity for both ^{13}C and ^{15}N.

The main problems faced in the application of ^{1}H-detected heteronuclear chemical-shift correlation methods are the suppression of signals from protons that are not attached to ^{13}C or ^{15}N and the maximization of the sensitivity and resolution of the method. For ^{13}C, the best scheme for this purpose, we find, is

$$
\begin{array}{llll}
{}^{1}\mathrm{H}: & 90^\circ_x-\tau- & -t_1/2-180^\circ_\theta-t_1/2- & -\tau-\mathrm{Acquire}(\psi) \\
{}^{13}\mathrm{C}: & 90^\circ_\phi & & 90^\circ_x \ \ \mathrm{Decouple}
\end{array}
$$

where the time τ is chosen to be $1/2{}^1J_{\mathrm{CH}}$, or slightly (20%) shorter to minimize relaxation losses. The phase cycling employed is $\phi = x, y, -x, -y$; $\psi = x, x, -x, -x$. The

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phase θ is incremented by 90° and the receiver phase is inverted four times per t_1 value; i.e., if N scans per t_1 value are acquired, the phase θ is incremented after $N/4$ scans. Data for odd- and even-numbered scans are stored separately and processed in the standard way to generate a 2D absorption-mode spectrum (*17, 18*). To avoid significant perturbation of the absorption-mode lineshape by the effect of homonuclear scalar coupling and to maximize sensitivity of the experiment, the acquisition time in the t_1 dimension is kept relatively short (<30 ms). The ^{13}C frequency is adjusted to be at the center of the ^{13}C region of interest and WALTZ-16 modulation (*19*) is applied to maximize the ^{13}C region that can be decoupled. Use of ^{13}C decoupling during ^1H data acquisition is important because it doubles sensitivity, and it simplifies the final 2D spectrum by halving the number of resonances.

The same experimental scheme, preceded by a BIRD pulse unit for presaturation of protons not coupled to ^{13}C was demonstrated recently for heteronuclear shift correlation in small molecules ($\omega\tau_c < 1$) (*20*). For macromolecules this BIRD pulse cannot be used because during the period between the BIRD pulse and the beginning of the actual sequence the negative NOE effect would attenuate the signals from protons attached to ^{13}C. In practice, we find that for macromolecules the suppression of signals from protons not attached to ^{13}C presents no particular problem. There are two reasons for this: First, for macromolecules resonances are relatively broad and very small fluctuations in magnetic field strength affect the difference spectrum to a much lesser extent than for small molecules. Second, the molar concentration for macromolecules is necessarily low and a large number of scans is needed per t_1 value which also improves the difference spectrum.

As an example, Fig. 1 presents a ^1H–^{13}C correlation spectrum of a 40 mg sample of hen egg white lysozyme (M_r 14,400) dissolved in 0.4 ml D_2O, recorded at 500 MHz ^1H frequency. The measuring time was 20 h. Sensitivity was sufficient to permit the use of relatively strong resolution enhancement digital filtering. With the exception of Phe-3, our resonance assignment is in agreement with the earlier ^1H assignment made at pH 5.3 by Redfield *et al.* (*21*) and Hore and Kaptein (*22*). Because our experiments were recorded at pH 3.8, 2D NOE and 2D HOHAHA (*23*) spectra were recorded to trace small changes in ^1H chemical shifts relative to the earlier studies. Comparison of the ^{13}C chemical shifts with those typically found in amino acids in short peptides also is helpful for assignment purposes. For example, the three intense resonances near F_1 = 118 ppm must correspond to the C_ϵ signals of tyrosine residues; the sequence-specific assignment then follows from the earlier ^1H assignment. All resonances from protonated aromatic carbons could be identified in this spectrum.

Correlation of ^1H with ^{15}N signals is more difficult than correlation with ^{13}C signals because the natural abundance of ^{15}N is three times lower than that of ^{13}C and because the spectrum must be recorded in H_2O instead of D_2O solution. Suppression of signals not coupled to ^{15}N is a particularly difficult problem for amide protons that overlap with the intense Tyr and Phe signals. Also, suppression of the H_2O resonance is critical to avoid dynamic range problems. In practice, the sequence described above for ^{13}C is not very suitable for ^{15}N, mainly because the 180° pulse at the center of the evolution period makes effective water suppression difficult and also because it decreases the suppression of non-^{15}N-attached protons. After experimental comparison of a number of different pulse schemes, we find that the best sequence for correlating ^1H and ^{15}N chemical shifts in H_2O solution is

FIG. 1. Two-dimensional absorption-mode heteronuclear chemical-shift correlation of the aromatic residues in hen egg white lysozyme. The spectrum results from a 2 × 150 × 1024 data matrix; acquisition times in the t_1 and t_2 dimensions were 30 and 85 ms, respectively. The delay time between scans was 1.5 s and 320 scans were recorded for every t_1 value. Data accumulation time was 20 h, using a 40 mg sample in 0.4 ml D_2O, p^2H 3.8, 60 m*M* NaCl, 45°C. Chemical shifts are relative to internal TSP.

$$
\begin{array}{ll}
{}^{1}\mathrm{H}: & 45^{\circ}_{x} - \tau - \quad - t_1 - \quad - \tau - \mathrm{Acquire}(\phi) \\
{}^{15}\mathrm{N}: & \qquad\quad 90^{\circ}_{\phi} \quad 90^{\circ}_{x} \quad \mathrm{Decouple}
\end{array}
$$

The 45° ^1H pulse is either of the Redfield type (*25*) or otherwise adjusted so that excitation of the H_2O resonance is minimized. The delay τ is again set somewhat shorter than $1/2^1J_{NH}$; we used a value of 4.5 ms. The phase ϕ is cycled along all four axes. Again data acquired for odd- and even-numbered scans are stored separately. In this case, ^1H signals are modulated by true zero- and double-quantum frequencies. By coadding the signals from odd and even scans the double-quantum contribution is eliminated, and 2D Fourier transformation gives a signal at $(F_1, F_2) = (\delta_H + \delta_N, \delta_H)$. Computer manipulation can be used to subtract the F_2 coordinate from the F_1 coordinate (*7*), resulting in a peak at (δ_N, δ_H). The data are phase-modulated in this

experiment and an absolute-value mode calculation has to be made before display of the spectrum. By subtracting the signals of even-numbered scans from the odd scans, a double-quantum spectrum is obtained. After removal of the δ_H contribution from the F_1 dimension, the correlation appears at $(-\delta_N, \delta_H)$. This spectrum is then reversed and coadded to the spectrum resulting from the zero-quantum data, yielding a $\sqrt{2}$ improvement in sensitivity, i.e., making this scheme nearly as sensitive as the phase-sensitive $^1H-^{13}C$ correlation experiment described above. The advantage of the pulse scheme used for ^{15}N is the high degree of suppression for protons not coupled to ^{15}N; the main disadvantage is the loss in resolution due to the absolute-value mode lineshape.

As an example, the method is demonstrated for a 50 mg sample of bovine pancreatic trypsin inhibitor (BPTI) (M_r 6500), dissolved in 0.4 ml 90% H_2O/10% D_2O. The spectrum, resulting from coaddition of the zero- and double-quantum derived correlation spectra, is shown in Fig. 2. The total measuring time was 11 h. Resolution enhancement digital filtering has been used in both dimensions.

The present study demonstrates that heteronuclear chemical-shift correlation spectra of proteins can be obtained without the need for ^{15}N or ^{13}C enrichment. For ^{13}C, the sensitivity limits the applicability of the method to protein concentrations of about 5

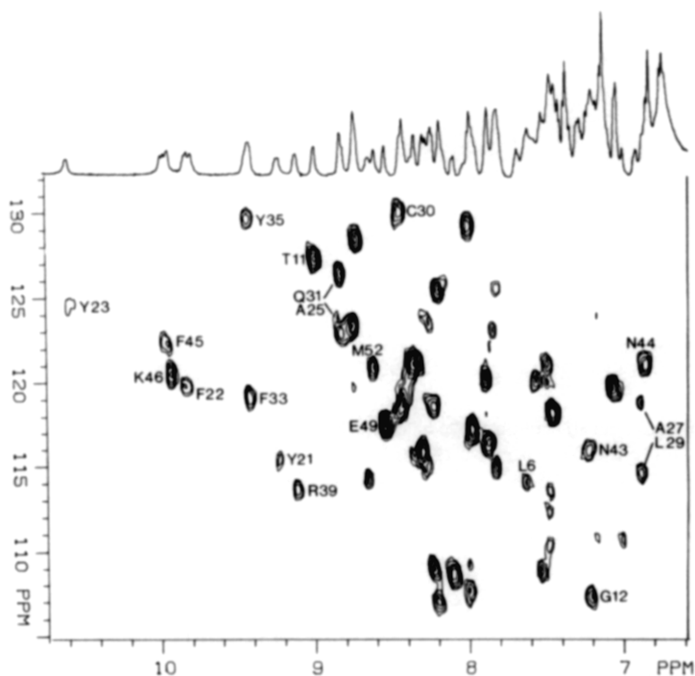

FIG. 2. Two-dimensional absolute-value mode $^1H-^{15}N$ chemical-shift correlation spectrum of the amide region in BPTI. ^1H and ^{15}N chemical shifts are relative to internal TSP and liquid NH_3 at 25°C, respectively (*27*). Data accumulation time was 11 h, using a 50 mg sample in 0.4 ml 90% H_2O/10% D_2O, pH 4.6, 35°C. The spectrum results from the coaddition of the zero- and double-quantum derived correlation spectra, as discussed in the text. Spectral assignments are based on ^1H chemical shifts reported by Wagner and Wüthrich (*26*) and on a 2D HOHAHA spectrum. The spectrum results from a 2 × 100 × 1024 data matrix; acquisition times in the t_1 and t_2 dimension were 40 and 85 ms, respectively. The delay time between scans was 400 ms and 768 scans were recorded per t_1 value. Standard Nicolet software was used for all data manipulations.

mM for an overnight experiment in a 5 mm sample tube. Of course, by using larger sample-tube volumes more dilute protein solutions can be studied. Note also, that in regular ^{13}C-detected heteronuclear shift correlation spectra the sensitivity for methyl and methine signals are nearly identical because for methyl groups only about one-third of the ^{1}H magnetization can be transferred to the ^{13}C nucleus. In ^{1}H-detected experiments, three protons are used to detect the presence of a single methyl carbon, increasing the sensitivity of the experiment by another factor of 3. For methyl groups heteronuclear shift correlation spectra can therefore be obtained in an order of magnitude less time than for methine signals (*28*). Similarly, methylene signals offer an extra factor of 2 relative to the ^{13}C-detected experiment. For ^{15}N, the sensitivity of the ^{1}H-detected experiment is about a factor of three lower than for ^{13}C because of the lower natural abundance. Very recently, Wagner (*29*) demonstrated the use of slightly different methods for correlating ^{1}H with ^{13}C and ^{15}N chemical shifts in proteins.

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