Spin-State-Selective Polarization or Excitation for Simultaneous E.COSY-Type Measurement of $^3J(C',H^\alpha)$ and $^3J(H^N,H^\alpha)$ Coupling Constants with Enhanced Sensitivity and Resolution in Multidimensional NMR Spectroscopy of $^{13}C,^{15}N$-Labeled Proteins

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Received September 25, 1997

Three-bond *J* coupling constants are very important in protein NMR because of their correlation with dihedral angles through Karplus relations.[1] However, due to ambiguities in these relations, a single *J* is insufficient for a given dihedral angle. A prominent example is the backbone dihedral angle ϕ in proteins which is correlated with, e.g., $^3J(H^N,H^\alpha)$ and $^3J(C',H^\alpha)$.

We have recently introduced new pulse sequence elements, spin-state-selective excitation[2] (S^3E) and spin-state-selective coherence transfer[3] (S^3CT), which in combination with E.COSY-type techniques[4] yield two edited subspectra corresponding to the two possible spin states of a spin (e.g., H^N or N) involved in the *J* coupling constant of interest. Hence, the latter can be determined from the relative peak displacements in the two edited subspectra and the effective spectral resolution is doubled because the number of peaks in the spectra is halved. In this way, $^3J(H^N,H^\alpha)$ coupling constants can be measured conveniently as demonstrated elsewhere,[2,3] but the S^3E or S^3CT pulse sequence elements can also be combined with other E.COSY-type methods available.[5]

Two approaches[6,7] have been presented for E.COSY-type measurement of $^3J(C',H^\alpha)$. Wang and Bax[6] have proposed to apply HCAN[C′] E.COSY in D_2O solution. Since this method requires resolution of $^1J(C^\alpha,C')$ in a constant-time period with transverse C^α magnetization, it is relatively insensitive given the notoriously short T_2 relaxation times for $^{13}C^\alpha$ in proteins. Moreover, this adds to the relaxation loss in a further constant delay of the same length also with transverse C^α magnetization. Löhr and Rüterjans[7] proposed a more sensitive approach, H^α-coupled H(N)CA,CO, only requiring to resolve the larger $^1J(C^\alpha,H^\alpha)$ in the C^α evolution period and without constant delays with transverse C^α magnetization.

The purpose of this paper is to extend the ideas of S^3E and S^3CT to the experiment of Löhr and Rüterjans in order to improve the effective spectral resolution and the sensitivity by reducing the time of transverse $^{13}C^\alpha$ magnetization. This reduction is made possible by the fact that the S^3-based methods do not have to resolve coupling constants in periods with transverse $^{13}C^\alpha$ magnetization. The first approach, spin-state-selective polarization (S^3P), circumvents one of the fundamental tenets of E.COSY,[4] namely, the requirement of two spins A and B active in two different evolution periods and a mixing period between them, which effects the A ↔ B coherence transfer while leaving a passive spin unperturbed in order to measure either J_{AC} or J_{BC} in an E.COSY-type way. Actually, in S^3P both spins A and B no longer have to be active in evolution periods: only the one involved in the *J* coupling constant of interest. However, the S^3P approach requires that the associated coherence transfer processes be very selective (vide infra). The basic idea behind S^3P in the present context is to edit the C′ spectrum according to the two possible spin states of H^α so that $J(C',H^\alpha)$ can be measured as relative displacements in two edited subspectra. Prior to the

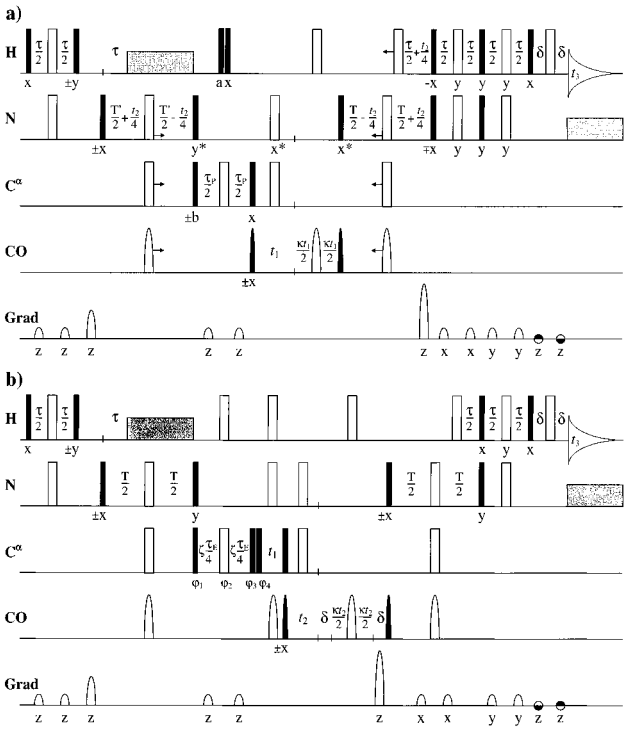

Figure 1. S^3P and S^3E pulse sequences for simultaneous measurement of $^3J(C',H^\alpha)$ and $^3J(H^N,H^\alpha)$ in $^{13}C,^{15}N$-labeled proteins. $\tau = (2^1J_{NH})^{-1}$, $\tau_P = \tau_E = (2^1J_{CH})^{-1}$, δ gradient delay. Filled and open bars indicate $\pi/2$ and π pulses, respectively, and phases are included below the pulses. Pulse phases with the prefix ± indicate independent two-step phase cycles with alternating receiver phase while the $\mp x$ pulse is used for echo or antiecho selection in combination with the shaded pulsed field gradients. The CO chemical shifts are scaled by a factor $(1 + \kappa)^{-1}$ relative to the *J* coupling constants. The complementary E.COSY-type spectra can be obtained by a π phase shift of the final $\pi/2$ ^1H pulse. (a) ω_{CO}-scaled 3D S^3P HN(CA),CO; the three pulse phases marked with an asterisk must be phase cycled for selection of zero change in coherence order in a concerted manner.[9] The concerted S^3P editing cycle consists of two steps $(a,b) = (-x,x)$ or (x,y) where the resulting two data sets are stored separately and subsequently added and subtracted to yield the two subspectra. (b) ω_{CO}-scaled 3D S^3E H(N)CA,CO. Two data-sets with the phase vectors $(\varphi_1, \varphi_2, \varphi_3, \varphi_4)$ are recorded, i.e., (*A*) $\{(\pi/4, 0, 0, 0) - (\pi/4, 0, \pi/2, \pi/2)\}$, (*B*) $\{(\pi/4, 0, 0, \pi) - (5\pi/4, 0, \pi/2, 3\pi/2)\}$. The linear combinations $A + B$ and $A - B$ yield the edited subspectra with a relative phase shift of $\pi/2$ in t_1. The S^3E editing procedure is described in detail elsewhere.[2] In both experiments, the final element for excitation of H^N magnetization at the end ideally does not perturb the H^α protons.[10,11] ζ is a scaling factor for an alternative scheme for suppression of relaxation-induced cross-talk.[12]

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S0002-7863(97)03358-1 CCC: $15.00
Published on Web 04/02/1998

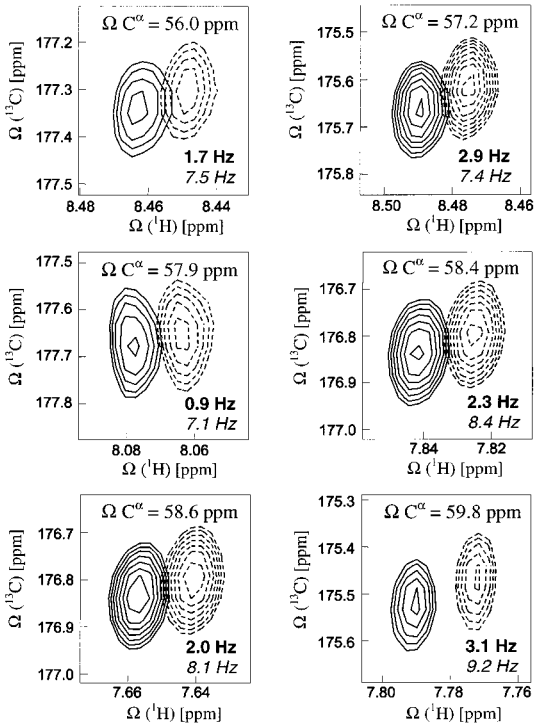

Figure 2. Representative cross-peaks from edited S^3E H(N)CA,CO spectra of $^{13}C,^{15}N$-NCAM 213−308 (90% H_2O/10% D_2O) recorded with the sequence in Figure 1b on a Varian Unity Inova 500 MHz spectrometer. The two edited subspectra (solid and dashed contours, respectively) have been overlaid using the software package PRONTO.[16] Parameters: relaxation delay 1.5 s, t_1(max) = 5.5 ms; 32 scans; τ = 5.56 ms; τ_E = 3.57 ms; T = 28.6 ms; ζ = 1.0; κ = 1.0 (scaling factor = 2.0). DIPSI-2[13] was used for proton decoupling while WALTZ-16[14] decoupling was used for ^{15}N in t_3. EBURP[15] shape was applied for selective CO $\pi/2$ pulses with duration of 563.2 μs and rectangular shape for selective CO π pulses with a duration of 200 μs. A data matrix of 20 × 128 × 4096 points covering 2000 × 1500 × 6200 Hz was zero-filled to 32 × 256 × 4096 prior to Fourier transformation. Cosine square in t_1 and t_2 and 7 Hz exponential line broadening in t_3 was applied. States-TPPI mode was employed in t_1 and echo-antiecho mode in t_2/t_3. The edited subspectra ($A + B$ and $A - B$) where combined in a ratio of 1:−0.08 for suppression of cross-talk signals. The coupling constants were estimated from 1D sections with a precision of about ±0.2 Hz. A higher accuracy can be obtained by taking the entire 2D peakshapes into account. The determined $^3J(C',H^\alpha)$ (bold) and $^3J(H^N,H^\alpha)$ (italic) coupling constants are indicated next to the peaks, while the chemical shifts of C^α in F_1 is given on top of the peaks. The ppm scale in F_2 refers to the ^{13}C chemical shifts so that the $^3J(C',H^\alpha)$ coupling constants appears scaled with a factor of 2.0. The NMR spectra of NCAM 213−308 have not been assigned as yet.

C′ evolution period, editing according to the spin state of H^α must take place in a state of transverse C^α magnetization and this separation is then passed on to the subsequent C′ coherences. The new experiment that we dub S^3P HN(CA),CO is outlined in Figure 1a. The actual S^3P phase cycle consists of only two steps, namely, concerted cycling of $(a,b) = (-x,x)$ or $(a,b) = (x,y)$, the data sets of which are stored separately and added and subtracted in order to give the two edited subspectra. The time of transverse $^{13}C^\alpha$ magnetization is kept as low as $(^1J_{C^\alpha H^\alpha})^{-1} = 3.57$ ms which compares to an evolution time of 14.1 ms in H^α-coupled H(N)-CA,CO[7] and a total of 57.12 ms in HCAN[C′] E.COSY.[6] The success of this experiment hinges on magnetization from ^{15}N being transferred exclusively to neighboring $^{13}C^\alpha$ and not to those $^{13}C^\alpha$ two bonds away. The desired contribution is proportional to $\sin(\pi^1J_{N-C^\alpha}T')\cos(\pi^2J_{N-C^\alpha}T')\sin(\pi^1J_{N-C^\alpha}T)\cos(\pi^2J_{N-C^\alpha}T)$ while the undesired one is proportional to the same expression with $^1J_{N-C^\alpha}$ and $^2J_{N-C^\alpha}$ permuted. Thus delays of $(^1J_{N-C^\alpha})^{-1}$ or $(^2J_{N-C^\alpha})^{-1}$ will favor the desired contributions at the expense of the undesired ones. However, when the coupling constants

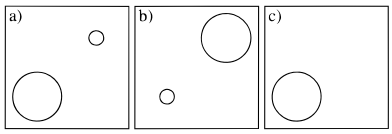

Figure 3. Schematic S^3 E.COSY-type cross-peak multiplets illustrating the technique for suppression of cross relaxation or pulse imperfection cross-talk. The main peaks are the desired ones while the smaller ones represent cross-talk. (a) S^3-edited E.COSY pattern ($A + B$), (b) S^3-edited E.COSY pattern ($A - B$), (c) S^3-edited E.COSY pattern with cross-talk suppressed by appropriate linear combination of the spectra in a and b.

involved deviate significantly from their typical values of $^1J_{N-C^\alpha}$ = 11 Hz or $^2J_{N-C^\alpha}$ = 7 Hz, the differentiation between the two contributions becomes insufficient.

This situation calls for the pulse sequence in Figure 1b where the two contributions are separated on the basis of the associated different $^{13}C^\alpha$ chemical shifts in a short evolution period. That pulse sequence includes a constant S^3E delay of about $(4^1J_{C^\alpha H^\alpha})^{-1}$ = 1.79 ms also with transverse $^{13}C^\alpha$ magnetization. Both experiments in Figure 1 yield spectra where $^3J(H^N,H^\alpha)$ and $^3J(C',H^\alpha)$ can be determined simultaneously as the coordinates of 2D displacement vectors. However, in contrast to conventional E.COSY, the two pertinent clusters of peaks are not in the same spectrum but in two separate ones.

The new S^3E H(N)CA,CO pulse sequence in Figure 1b was tested on the $^{13}C,^{15}N$-labeled domain 3 (residues 213−308) of the protein neural cell adhesion molecule (NCAM). Representative cross-peaks sorted according to $^{13}C^\alpha$ chemical shifts in F_1 are shown in Figure 2 along with values of $^3J(C',H^\alpha)$ and $^3J(H^N,H^\alpha)$ coupling constants measured from appropriate 1D sections. An 8% postprocessing correction for cross-talk arising from relaxation of the passive H^α spins[6,8]or pulse imperfections was applied to the 3D spectra—the problem is illustrated in Figure 3. Ideally, only the large peak should be present in the 2D spectrum of Figure 3a, and the presence of the other component influences the accuracy with which the interesting J coupling constants can be measured. Fortunately, the other S^3P or S^3E subspectrum (Figure 3b) exhibits the same problem but with opposite intensity ratio of the two signals so that, e.g., subtraction of a few percent of the spectrum in Figure 3b from the one in Figure 3a results in a clean subspectrum as shown in Figure 3c. A similar linear combination cleans up the other subspectrum.

In conclusion, we have introduced novel multidimensional NMR experiments for simultaneous measurement of $^3J(H^N,H^\alpha)$ and $^3J(C',H^\alpha)$ and demonstrated one of them, S^3E H(N)CA,CO, by way of a $^{13}C,^{15}N$-labeled protein. The pulse sequence elements S^3P and S^3E can be combined with a wide range of NMR experiments for protein structure determination.

Acknowledgment. Use of the facilities of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules at Carlsberg Laboratory is acknowledged. We thank Flemming M. Poulsen and Vladik Soroka for the loan of the NCAM 213-308 sample. A.M. is supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

JA973358C

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