New Principle for the Determination of Coupling Constants That Largely Suppresses Differential Relaxation Effects

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The determination of coupling constants in biomacromolecules provides information about local conformations and complements the structural information obtained from NOEs.[1] A number of different methods for the determination of coupling constants have been introduced over the years. Among these are E.COSY-derived methods,[2] methods relying on fitting procedures,[4] and techniques based on quantitative J correlations.[4,5] All these methods either measure directly or let evolve during a limited amount of time a relatively small coupling constant (~10 Hz) of interest. Due to the faster relaxation of antiphase terms compared to in-phase terms, the observed value J^{eff} tends to be smaller than the actual coupling constant J.[6] This effect is also known as scalar relaxation of the second kind.[7] From the formula derived for differential relaxation[6] and its Taylor series expansion one obtains for $J \gg \Delta\rho/2\pi$

$$J^{eff} = \sqrt{J^2 - \left(\frac{\Delta\rho}{2\pi}\right)^2} = J - \frac{\Delta\rho^2}{(2\pi)^2(2J)}$$

where $\Delta\rho$ is the difference of the relaxation times for antiphase and in-phase transverse coherences.

Since the absolute error of J, $(J - J^{eff})$, is inversely proportional to J, the larger J, the smaller the error due to differential relaxation. Since coupling constants cannot be changed in size, we propose to measure in an appropriate three-spin system ABC the sum and the difference of a large ^1J(A,C) and a small coupling ^1J(B,C) of interest.[8] Double quantum (DQC) and zero quantum coherences (ZQC) have the desired property to evolve sums and differences of coupling constants[9] (Figure 1b). If J(A,C) is of the order of 100 Hz, as is the case for the ^1J(C,H) or ^1J(N,H) couplings, this results in an increase in the measured size of the coupling by roughly a factor of 10. By contrast to the E.COSY methodology (Figure 1a), where the interesting J(B,C) coupling is measured in an orthogonal dimension to J(A,C), the interesting coupling J(B,C) is measured in the same frequency dimension as J(A,C) in the DQ/ZQ method. Both methods make the relative signs of the coupling constants J(A,C) and J(B,C) available.

We demonstrate the new approach on the measurement of $^3J(H^N,H_\alpha)$ coupling constants which are the most widely measured in proteins and allow the restriction of the backbone torsional angle ϕ. The pulse sequence (Figure 2) is derived from a CT HNCA experiment[10] and uses a heteronuclear

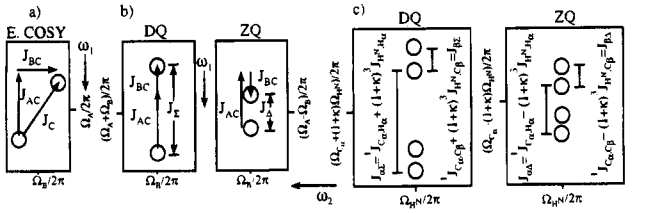

Figure 1. (a) Schematic representation of an E.COSY pattern for a three-spin system A, B, C, in which A is correlated with B and the interesting J(B,C) coupling can be measured in ω_2 due to the resolution of the submultiplet components by J(A,C) in ω_1. (b) Schematic representation of a DQ/ZQ experiment in which the sum J_Σ and the difference J_Δ of J(A,C) and J(B,C) are used to extract J(B,C) from the difference of the splittings in the two spectra. (c) In DQ/ZQ+SQ-HNCA a doublet of doublets is observed yielding $^3J(H^N,H_\alpha)$ and $^3J(H^N,C_\beta)$ couplings as indicated.

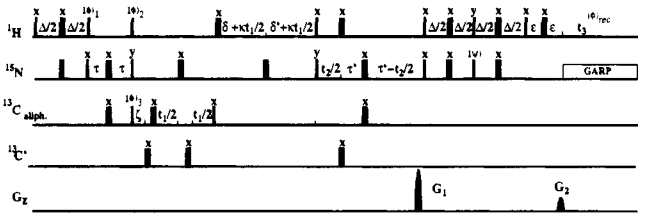

Figure 2. Pulse sequence for the determination of the $^3J(H^N,H_\alpha)$ coupling constant in a 3D DQ/ZQ+SQ-HNCA experiment. The 2D version ($t_2 = 0$) was recorded with the following parameters: The ^{15}N spins were decoupled using the GARP[14] sequence during t_3. The delays were $\Delta = 5.2$ ms, $\tau = 13.7$ ms, $\tau' = 13.5$ ms, $\delta = 1.3$ ms, $\delta' = 0.55$ ms, $\epsilon = 2.303$ ms, $\zeta = 34$ μs (=duration of 180°(^{15}N) + $t_1(0)$). The phase cycling employed for the sum of DQ and ZQ (difference of DQ and ZQ) is as follows: $\phi_1 = y, y, -y, -y$; $\phi_2 = x, -x$ $(y, -y)$; $\phi_3 = x, -x$ $(y, -y)$; $\phi^{rec} = x, x, -x, -x$. The two sets (DQ+ZQ and DQ−ZQ) were stored separately. States-TPPI[15] on ϕ_3; eight FIDs for each set of DQ+ZQ and DQ−ZQ experiment and t_1 value; 200 t_1 values, spectral width in ω_1, ω_2 4167 Hz, 4 kHz; 2048 complex points in t_2. The duration, strengths of the gradients, and phase ψ were as follows: G_1 = (3.8 ms, ± 49.5 G/cm, 300 μs recovery), G_2 = (1.8 ms, 10.1 G/cm, 200 μs recovery), $\psi = \pm y$. For the 3D version, t_2 is incremented for ^{15}N chemical shift evolution. For the DQ/ZQ+SQ version, $\kappa = 1$ is recommended.

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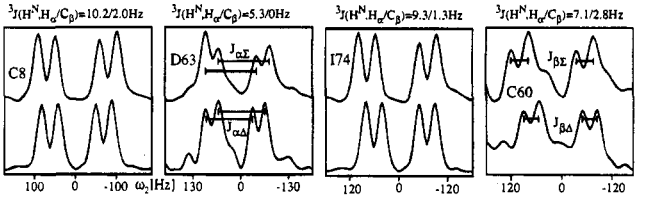

Figure 3. Traces obtained by summation over four representative cross peaks in the DQ and ZQ spectra resulting from the 2D version of the pulse sequence shown in Figure 2 with $\kappa = 0$. The experiment took 11 h on a 1.5 mM sample of ^{13}C, ^{15}N labeled rhodniin (103aa) in a Shigemi tube at 600 MHz. The traces show the characteristic DQ and ZQ pattern. The $^3J(H^N,H_\alpha)$ coupling can be extracted as $(1/2)(J_{\alpha\Sigma} - J_{\alpha\Delta})$ twice as indicated on D63. $^3J(H^N,C_\beta)$ couplings can be obtained from $(1/2)(J_{\beta\Sigma} - J_{\beta\Delta})$ as indicated on C60. Data processing was accomplished with FELIX by forming the sum and difference of the the DQ+ZQ and DQ−ZQ spectra. The final matrices consisted of 2048 × 8192 real points, resulting in a resolution of 0.51 Hz in the ω_1 dimension.

gradient echo and COS-HSQC for the N → H transfer.[11] C_α,H^N DQC and ZQC evolve during t_1 while H_α is the passive spin. Since the H^N,C_α DQC and ZQC have a shorter T_2 time than the H^N single quantum coherence (SQC), the sensitivity and resolution can be increased by evolution of the latter during another time κt_1. The splitting in such a DQ/ZQ+SQ spectrum is $^1J(H_\alpha,C_\alpha) \pm (1 + \kappa)^3J(H^N,H_\alpha)$, which provides better resolution of the coupling constant of interest. In the proposed experiment the C_β is also a passive spin coupled to the C_α with a large coupling of $^1J(C_\alpha,C_\beta) = 35$ Hz. Therefore the $^3J(H^N,C_\beta)$ coupling can be obtained by inspection of the same spectrum. Figure 3 shows slices through the 2D version of the DQ/ZQ-HNCA with $\kappa = 0$ of four representative residues recorded on rhodniin[12] together with the coupling constants as extracted from two peak displacements each in the traces. The difference of the two coupling constant values was ≤0.5 Hz.

In order to evaluate the influence of differential relaxation in various methods for the determination of the $^3J(H^N,H_\alpha)$ coupling constant, the spin dynamics were simulated for a large number of amino acid residues in BPTI for correlation times varying between 5 and 40 ns for the DQ/ZQ-HNCA experiment, the DQ/ZQ+SQ-HNCA with $\kappa = 1$, the HNCA-E.COSY[13] experiment, and the HNHA experiment[6] (Figure 4). The effective coupling constant observed in the two DQ/ZQ-HNCA experiments is always larger than that found in the HNCA-E.COSY and the HNHA experiment. In both DQ/ZQ-HNCA experiments, the effective coupling increases for increasing correlation times in contrast to all other methods. This remarkable increase of J^{eff} despite the scalar relaxation of the second kind comes from the fact that the $^3J(H^N,H_\alpha)$ coupling is derived from a difference of two large couplings. The larger of the two is affected less by differential relaxation than the smaller one; therefore the difference increases with increasing differential relaxation.

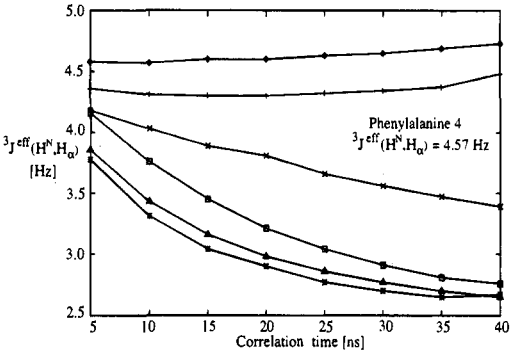

Figure 4. Simulations of the effective $^3J(H^N,H_\alpha)$ coupling constant for Phe-4 of BPTI for a range of correlation times between 5 and 40 ns. The dihedral angles and proton−proton distances used in the simulations were derived from the combined X-ray and neutron diffraction structure of BPTI after adding hydrogen atoms with the program INSIGHT (PDB5PTI).[16] The Karplus curve according to ref 17 was used for the relation between ϕ and $^3J(H^N,H_\alpha)$. A four-spin system containing the H^N, H_α, C_α spin of Phe-4 was used for the spin dynamic simulations with the program wtest.[18] The fourth spin is a pseudospin P that is not coupled to either of the other three spins and reflects the influence of dipolar relaxation of all other protons H_i. Isotropic tumbling with a unique correlation time τ_c is assumed. The distance of the pseudospin P is calculated according to $r^{-6}(P,H_\alpha) = \sum_i r^{-6}(H_i,H_\alpha) = (1.95\ Å)^{-6}$ for Phe-4. The other parameters used are $r(H^N,H_\alpha) = 2.72$ Å, $^1J(H_\alpha,C_\alpha) = 145$ Hz, and $^3J(H^N,H_\alpha) = 4.57$ Hz. Pulses were represented as ideal rotations. The effective $^3J(H^N,H_\alpha)$ coupling constants obtained from the simulations are shown for (◇) DQ/ZQ-HNCA; (|) DQ/ZQ+SQ-HNCA; (□) HNCA-E.COSY; (×) HNHA with a defocusing time $2\zeta^5 = 5$ ms and a ratio of cross to diagonal peak $S_c/S_d = 1/71$; (△) HNHA with a defocusing time $2\zeta =$ 26 ms and $S_c/S_d = 1/3.5$; (∗) HNHA with 2ζ chosen such that the cross and diagonal peaks have identical intensity.

In conclusion, we have introduced a new principle for the measurement of coupling constants in large isotopically labeled biomacromolecules that reduces the effects of differential relaxation by approximately a factor of 10. We therefore expect that the new methodology should yield reliable coupling constant values J^{eff} for molecules up to three times as large as the molecules investigated so far.

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