Measurement of Cross-Relaxation between Amide Protons in ^{15}N-Enriched Proteins with Suppression of Spin Diffusion

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A variant of two-dimensional nuclear Overhauser effect spectroscopy (NOESY) is described which allows one to observe cross-relaxation pathways between protons that have heteronuclear scalar couplings to nitrogen-15 or carbon-13. In ^{15}N-enriched proteins, it is possible to focus attention on Overhauser effects between amide protons that are due to a direct one-step transfer of longitudinal magnetization, e.g. $I_z(H^N_n) \rightsquigarrow I_z(H^N_{n+1})$, while eliminating two-step spin-diffusion pathways such as $I_z(H^N_n) \rightsquigarrow I_z(H^\alpha_n) \rightsquigarrow I_z(H^N_{n+1})$. This can be achieved by selective inversion of the longitudinal magnetization of all amide protons that are scalar-coupled to ^{15}N in the middle of the relaxation time τ_m. Selective inversion can be obtained by inserting a "bilinear rotation decoupling" (BIRD) pulse sandwich[1−3] in τ_m.

The mechanism of the resulting suppression of undesirable cross-relaxation pathways is closely related to the principle of "quenching undesirable indirect external trouble in nuclear Overhauser effect spectroscopy" (QUIET-NOESY).[4] The suppression relies on the doubly-selective inversion of the longitudinal magnetization of two chosen protons, a "source" A and "target" X.[4−6] Simultaneous inversion of A and X does not affect the flow of longitudinal magnetization between these two spins, e.g. the transformation $I_z(A) \rightsquigarrow I_z(X)$ continues unperturbed, while transfer processes via "clandestine" spins K are suppressed to first order. Thus, a partial migration $I_z(A) \rightsquigarrow I_z(K)$ will be followed by a flow of opposite sign for an equal amount of time. Likewise, the partial conversion $I_z(K) \rightsquigarrow I_z(X)$ is cancelled at the end of τ_m. As a result, two-step spin-diffusion processes such as $I_z(A) \rightsquigarrow I_z(K) \rightsquigarrow I_z(X)$ are largely quenched.

The QUIET-NOESY scheme has been adapted to two-dimensional experiments by inverting two frequency bands during the mixing time.[7] The scheme presented in this paper introduces inversion elements that depend on the topology of the (heteronuclear) scalar coupling network. All Overhauser effects between amide and other protons are eliminated by inserting a BIRD sequence[1−3] in the middle of the relaxation interval. Figure 1 shows a pulse sequence for QUIET-BIRD-NOESY.

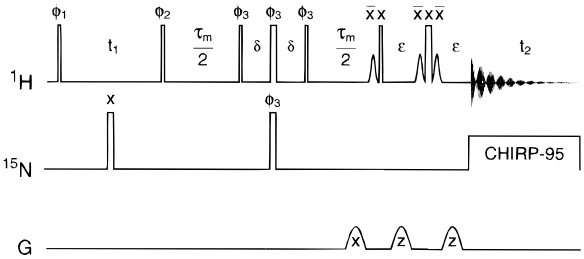

Figure 1. Pulse sequence for the QUIET-BIRD-NOESY experiment, with a bilinear rotation decoupling sequence (BIRD) inserted in the middle of the relaxation time τ_m, comprising two delays $\delta = (2J_{IS})^{-1}$. The heteronuclear scalar interactions are decoupled by applying a $(\pi)^S_x$ pulse in the middle of the t_1 period and a CHIRP-95 decoupling sequence in the detection period. Transverse magnetization in the second half of the mixing time is eliminated by inserting a G_x gradient. The water resonance is suppressed by the water flip-back implementation of WATERGATE using two G_z gradients. Time-proportional phase increments (TPPI) were employed in combination with an eight-step NOESY phase cycle: $\phi_1 = x, y, -x, -y, -x, -y, x, y$; $\phi_2 = x, y, -x, -y$; $\phi_3 = x, y, -x, -y$; acquisition $= x, x, x, x, -x, -x, -x, -x$.

The BIRD sequence consists of three pulses $(\pi/2)^I_x - \delta - (\pi)^I_x - \delta - (\pi/2)^I_x$ applied to I = ^{1}H and separated by intervals $\delta = (2J_{IS})^{-1}$ (which amounts to 5.5 ms for typical amide protons with $J_{NH} \approx 91$ Hz). A $(\pi)^S_x$ pulse is applied to S = ^{15}N simultaneously with the $(\pi)^I_x$ pulse so that the heteronuclear coupling is not refocused by the $(\pi)^I_x$ pulse. For protons that are not coupled to ^{15}N, the BIRD sequence has no effect, e.g. $I_z(H^\alpha) \rightarrow I_z(H^\alpha)$, since the three pulses applied to I amount to a 360° pulse. On the other hand, the amide protons are inverted, e.g. $I_z(H^N) \rightarrow -I_z(H^N)$, because the precession of the two proton doublet components under J_{NH} (which is not refocused by the two simultaneous π pulses) through 180° in the interval 2δ leads to a change in sign.

In the ω_1 and ω_2 dimensions of the two-dimensional spectra, the heteronuclear scalar interactions are decoupled by applying a $(\pi)^S_x$ pulse in the middle of the t_1 period and a broadband CHIRP-95 sequence[8,9] in the detection period. The water resonance is suppressed by the water flip-back[10] implementation of WATERGATE.[11,12]

The principle of QUIET-BIRD-NOESY is illustrated by application to a ^{15}N-labeled mutant of the FK506 binding protein (FKBP). This mutant, C22A,[13] was expressed and purified essentially as described previously for wild-type FKBP.[14] Figure 2a shows the low-field region of the conventional NOESY spectrum. The corresponding region of the QUIET-BIRD-NOESY spectrum is shown in Figure 2b. Four peaks are emphasized for the sake of illustration: (1) the cross-peak H^N(R13) $\rightsquigarrow$ H^N(T14) appears in both spectra, since it is due to a one-step Overhauser effect between two amide protons located 2.06 Å apart in the average solution structure obtained

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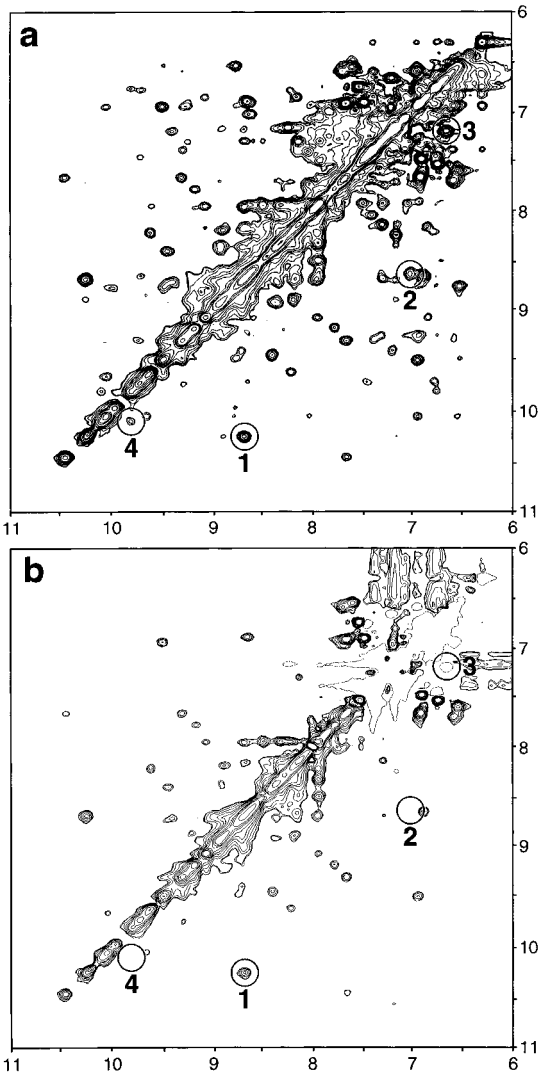

Figure 2. (a) Low-field region (5-ppm window in both dimensions) of the conventional NOESY spectrum of the ^{15}N-labeled C22A mutant of FKBP. (b) Corresponding region of the QUIET-BIRD-NOESY spectrum. Both spectra were recorded at 300 K and 7 T with a Bruker DMX 300 spectrometer. The parameters used for acquisition were the following: spectral width 4500 Hz (512 points) in both dimensions, 64 scans, mixing time $\tau_m = 200$ ms, BIRD-delay $\delta = 5.5$ ms, sine-shaped pulsed field gradients of 1-ms duration and amplitudes $G_{x,z} \approx$ 20 G/cm, the recovery time being 160 μs for all three gradients. Selective 90° Gaussian pulses of 3-ms duration were used for selective water irradiation. Decoupling was achieved by inserting a 180° ^{15}N-pulse at $t_1/2$ and a CHIRP-95 sequence in t_2 with 1-ms CHIRP units of ~1.5-kHz amplitude and 15-kHz sweep width embedded in an 80-step phase cycle. Four cross peaks are emphasized: (1) the cross peak H^N(R13) [8.66 ppm] ⇝ H^N(T14) [10.25 ppm] survives; (2) H^N(F36) [8.65 ppm] ⇝ H^δ(F36) [7.01 ppm] is eliminated; (3) H^δ(Y82) [7.19 ppm] ⇝ H^ϵ(Y82) [6.65 ppm] remains unperturbed; (4) H^N(K73) [9.80 ppm] ⇝ H^N(L74) [10.09 ppm] is strongly attenuated (see text for details).

by Rosen *et al.*[15] The peak is positive in NOESY and negative in QUIET-BIRD-NOESY due to the inversion of the amide protons; (2) the cross-peak connecting the amide proton H^N(F36) and the aromatic H^δ(F36) is absent from the QUIET-BIRD-NOESY spectrum although their distance is 1.77 Å. This results from the fact that this cross-peak links an inverted proton, H^N(F36), and a non-inverted proton, H^δ(F36); (3) the aromatic H^δ(Y82) ⇝ aromatic H^ϵ(Y82) nOe survives unperturbed (the distance is 2.44 Å), as do all other cross peaks between protons that are not affected by the BIRD element; and (4) the cross peak H^N(K73) ⇝ H^N(L74) is strongly attenuated in QUIET-BIRD-NOESY. This may be regarded as evidence that the cross-peak observed in conventional NOESY arises in part from spin diffusion and that the direct effect that survives is rather small, consistent with an internuclear distance of 3.94 Å.

Although similar in its objectives, our scheme is only remotely related to the "BD-NOESY" and "CBD-NOESY" schemes described by Markley, Macura, and co-workers,[16−19] which suppress spin diffusion by exploiting the fact that cross-relaxation rates in the rotating and laboratory frames have opposite signs, while re-introducing the desired nuclear Overhauser effects in a selected spectral region. Local variations of correlation times makes it necessary to adjust the laboratory and rotating frame intervals empirically. In the QUIET scheme, by contrast, the efficiency of the cancellation of spin diffusion does not depend on the correlation times.

For very long mixing times, where the QUIET principle may break down because spin diffusion may already occur in the first half of τ_m, it may be necessary to insert two BIRD sandwiches[4] at $(1/4)\tau_m$ and $(3/4)\tau_m$. However, there is no evidence in Figure 2a that this is necessary for a mixing time of 200 ms and a correlation time estimated to be $\tau_c \geq 12$ ns. Amide exchange will tend to attenuate the Overhauser cross-peaks, making it necessary to measure the exchange rates separately before embarking on a quantitative structural analysis.[20] If one records build-up curves where cross-peak amplitudes due to one-step processes $I_z(H^N_n)$ ⇝ $I_z(H^N_{n+1})$ are monitored as a function of τ_m, this should allow very accurate estimates of the distances between amide protons to be obtained.[21−23] If the signal-to-noise ratio is sufficient, it might be possible to detect valuable long-range amide−amide interactions which are very helpful for structural studies.

The method can also be applied to ^{15}N-labeled RNA and DNA and to fully ^{13}C-labeled molecules. In the latter case, using a ^{13}C BIRD sequence, possibly with a selective $(\pi)^S_x$ pulse applied either to the aromatic carbons, or, in favorable cases, to the C^α or C^β regions, allows one to say "be quiet!" to a given subset of ^{13}C-bound protons. The QUIET-BIRD-NOESY method is perhaps the simplest experiment suggested to date that can significantly enhance the accuracy of structures of macromolecules in solution.

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