Monitoring Macromolecular Motions on Microsecond to Millisecond Time Scales by $R_{1\rho}-R_1$ Constant Relaxation Time NMR Spectroscopy

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Dynamic processes on microsecond to millisecond (μs−ms) time scales are important for the functions of proteins, including recognition, allostery, and catalysis.[1,2] Intramolecular motions on μs−ms time scales contribute to nuclear magnetic relaxation through adiabatic dephasing of coherent states and are exhibited as conformational exchange phenomena in solution-state NMR spectroscopy.[3] Nuclear magnetic relaxation in the rotating frame (i.e., in the presence of a radiofrequency (rf) field) constitutes a unique source of information on chemical and conformational exchange processes.[4] This communication presents a new rotating frame technique for studying intra- and intermolecular exchange in proteins[5−8] that overcomes several difficulties associated with existing spin-lock and spin-echo experiments. First, rotating frame and laboratory frame relaxation rate constants are averaged during a novel constant relaxation time (CRT) period in order to simplify the off-resonance effects normally encountered in spin-lock experiments. Second, an off-resonance spin-lock rf field[9−11] is used to increase the magnitude of the effective magnetic field in the rotating frame in order to access faster dynamic processes. The off-resonance $R_{1\rho}-R_1$ CRT nuclear magnetic relaxation experiment allows determination of conformational exchange times at least as short as 25 μs in proteins.

The effect of conformational (or chemical) exchange on the off-resonance rotating frame relaxation rate constant, $R_{1\rho}^{\text{off}}$, is described by[8]

$$R_{1\rho}^{\text{off}} = R_1 \cos^2\theta + R_2 \sin^2\theta + R_{\text{ex}} \sin^2\theta \qquad (1)$$

in which $\theta = \arctan(\omega_1/\Delta\omega)$ is the "tilt angle" between the directions of the reduced static field, $\Delta\omega = \omega - \omega_0$, and the effective field, $\omega_e = (\omega_1^2 + \Delta\omega^2)^{1/2}$, in the rotating frame; ω is the spin-lock rf frequency; ω_1 is the spin-lock field strength in units of rad/s; ω_0 is the population-averaged chemical shift; R_1 and R_2 are the spin−lattice and spin−spin relaxation rate constants, respectively; and R_{ex} is the contribution to the transverse relaxation rate from exchange processes. For exchange between two sites *A* and *B*,[8]

$$R_{\text{ex}} = (\delta\omega)^2 p_A p_B \tau_{\text{ex}}/(1 + \tau_{\text{ex}}^2 \omega_e^2) \qquad (2)$$

in which p_i is the population of spins in site *i*, $\delta\omega = \omega_A - \omega_B$ is the chemical shift difference between the two sites, and $\tau_{\text{ex}} = 1/k_{\text{ex}} = p_B/k_{A\to B} = p_A/k_{B\to A}$ is the time constant for the exchange process. The off-resonance spin-lock experiment allows ω_e to be varied by changing ω while keeping ω_1 constant in order to minimize sample heating effects. In addition, complications that arise because the resonance frequencies for the (two) individual conformations (e.g., ω_A and ω_B) differ from ω_0 are mitigated because $\Delta\omega \gg \delta\omega$. The desired exchange parameters, $(\delta\omega)^2 p_A p_B$ and τ_{ex}, cannot be determined in practice simply by measuring $R_{1\rho}^{\text{off}}$ as a function of ω_e, because $\cos^2\theta \to 1$ and $\sin^2\theta \to 0$ as ω is shifted off-resonance.

The pulse sequence for the off-resonance $R_{1\rho}-R_1$ CRT experiment is illustrated in Figure 1. Following an initial refocused INEPT[12,13] polarization transfer from *I* (^{1}H) to *S* (^{15}N) spins (point a), the *S* spin coherence is aligned along the direction of the effective field of the off-resonance spin lock (point b). After a spin-locking period of length *t* (point c), the *S* spin coherence is returned to the *z*-axis (point d) for a laboratory frame relaxation period $T - t$, in which *T* is the total CRT period (point e). Finally, the *S* spin coherence is returned to the transverse plane, frequency-labeled during t_1, and transferred back to the *I* spins for detection. The signal intensity is given by[14]

$$\begin{aligned} I(t) &= I_0 \exp[-R_{1\rho}^{\text{off}} t] \exp[-R_1(T-t)] \\ &= I_0 \exp[-R_1 T] \exp[-(R_{1\rho}^{\text{off}} - R_1)t] \\ &= \tilde{I}_0 \exp[-R_{\text{eff}} t] \end{aligned} \qquad (3)$$

in which $\tilde{I}_0 = I_0 \exp[-R_1 T]$ is the signal intensity at $t = 0$, I_0 is a constant, and

$$R_{\text{eff}} = R_{1\rho}^{\text{off}} - R_1 = (R_2 - R_1 + R_{\text{ex}}) \sin^2\theta \qquad (4)$$

Equation 4 can be recast using eq 2 as

$$R_{\text{eff}}/\sin^2\theta = R_2 - R_1 + (\delta\omega)^2 p_A p_B \tau_{\text{ex}}/(1 + \tau_{\text{ex}}^2 \omega_e^2) \qquad (5)$$

The resonance offset dependence is reduced to a scaling factor $\sin^2\theta$ that is determined solely by the known parameters ω, ω_1, and ω_0. The number of free parameters is also reduced compared with eqs 1 and 2, because R_2 and R_1 appear only as the difference $R_2 - R_1$. Experimentally, R_{eff} is measured as a function of ω_e by varying ω, and the parameters $(\delta\omega)^2 p_A p_B$, τ_{ex}, and $R_2 - R_1$ are determined by nonlinear curve-fitting to eq 5.

The off-resonance $R_{1\rho}-R_1$ CRT experiment is demonstrated for a 1 mM uniformly ^{15}N-labeled sample of the fibronectin type III domain of the extracellular matrix protein tenascin (M_r = 10.1 kDa).[15,16] Representative relaxation curves for the backbone amide ^{15}N spin of residues D30 (δ = 118.81 ppm), R45 (δ = 123.11 ppm), and N55 (δ = 120.35 ppm) are shown in Figure 2. Values for $R_{\text{eff}}/\sin^2\theta$ determined at eight effective field strengths are shown in Figure 3. For maximum precision, $R_2 - R_1$ were fixed at values calculated from laboratory frame R_1 and NOE measurements (P. A. Carr and A. G. Palmer, unpublished results) using the model free formalism of Lipari and Szabo[17] and a rotational correlation time of 4.4 ns. The

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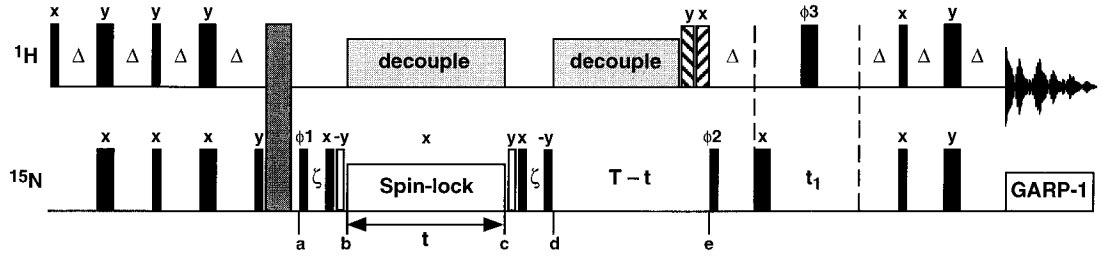

Figure 1. Pulse sequence for the off-resonance $R_{1\rho}-R_1$ CRT experiment. The narrow and wide solid bars depict 90° and 180° pulses, respectively. The narrow empty bars depict pulses with a flip angle 90° - arctan($\omega_1/\Delta\omega_c$). High-power pulses are applied with a field strength ω_{1c} and with the transmitter frequency ω_c centered in the ^{15}N spectrum. The wide gray bar depicts a homospoil gradient pulse. The wide hatched bars depict high-power purge pulses of 0.5−1.0 ms duration. ^{1}H decoupling is applied during the relaxation delays using the WALTZ-16 sequence[18] or a train of ^{1}H 180° pulses. ^{15}N decoupling during acquisition is performed using GARP-1.[19] The off-resonance spin-lock field is applied as continuous irradiation with a constant field strength ω_1 and offset $\Delta\omega_c = \omega - \omega_c$. The frequency is switched phase-coherently from ω_c to ω at point b and switched back at point c. The elements between a and b and between c and d serve to rotate the coherences between the *z*-axis and the directions of effective field for the ^{15}N spins. Numerical solutions of the Bloch equations indicate that the delay $\zeta = \omega_1/(\delta\omega_c^2 + \omega_1^2) - 2/\omega_{1c}$ is optimal for $\omega_{1c} \gg |\omega_0 - \omega_c|$; $\zeta = 0$ is satisfactory when this formula yields a negative value, provided that $\omega_1 \geq |\omega_o - \omega_c|$. The first term of ζ is determined by the contribution to θ due to chemical shift offset,[20,21] and the second term compensates for evolution during pulses.[22,23] The phase cycle is $\phi1 = y,-y$; $\phi2 = y,y,-y,-y,y,y,-y,-y$; $\phi3 = 4(x),4(-x)$; receiver $= x,-x,-x,x,-x,x,x,-x$. Quadrature detection in the indirect dimension uses the States-TPPI phase cycling scheme.[24] The sequence can be elaborated to include sensitivity enhancement,[25] gradient coherence selection,[26] or coherence transfer using cross polarization.[27]

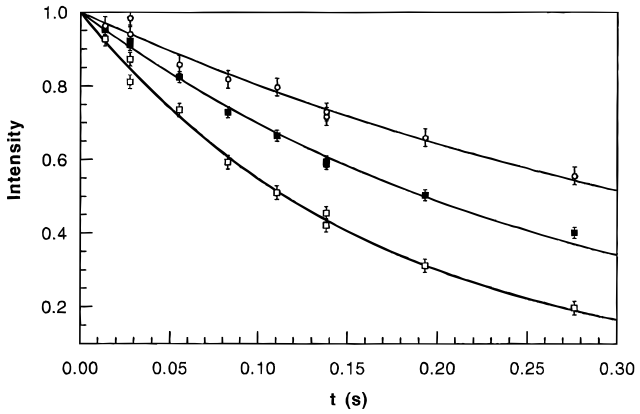

Figure 2. Representative relaxation curves for the ^{15}N off-resonance CRT $R_{1\rho}-R_1$ experiment obtained using a spin-lock field strength of 2330 ± 30 Hz and an offset of 46 ppm from the center of the ^{15}N spectrum. Residue D30 (○, $\theta = 45.1°$), R45 (■, $\theta = 42.9°$), and N55 (□, $\theta = 44.2°$). The decay was monitored using eight spin-lock periods, two of which were obtained in duplicate in order to assess the peak intensity error.

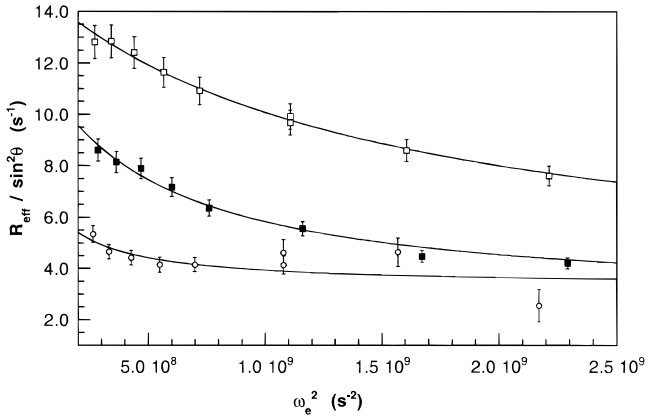

Figure 3. Curve fits of eq 5 to the experimental $R_{eff}/\sin^2\theta$ data for residues D30 (○), R45 (■), and N55 (□) obtained at eight different effective fields. The spin-lock field strength was 2330 ± 30 Hz. The offsets from the center of the ^{15}N spectrum were 138, 115, 92, 69, 57, 46, 34, and 23 ppm (corresponding to nominal tilt angles of 18.4, 21.8, 26.6, 33.7, 38.7, 45.0, 53.1, and 63.4°). A duplicate data set was acquired for an offset of 92 ppm.

optimized values of τ_{ex} and $(\delta\omega)^2 p_A p_B$ are D30, 88 ± 68 μs and (61 ± 24) × 10^3 s^{-2}; R45, 46 ± 5 μs and (2.15 ± 0.05) × 10^5 s^{-2}; and N55, 27 ± 2 μs and (4.5 ± 0.2) × 10^5 s^{-2}. Assuming equally populated conformers ($p_A = p_B = 0.5$), $\delta\omega$ = 492 ± 308 s^{-1} (1.5 ± 1.0 ppm), 927 ± 141 s^{-1} (2.9 ± 0.4 ppm), and 1342 ± 264 s^{-1} (4.2 ± 0.8 ppm) for D30, R45, and N55, respectively. For comparison, if $R_2 - R_1$ is treated as a free parameter, the optimized values of τ_{ex} and $(\delta\omega)^2 p_A p_B$ are R45, 35 ± 9 μs and (2.8 ± 0.8) × 10^5 s^{-2}; N55, 24 ± 9 μs and (5.5 ± 3.7) × 10^5; data for D30 could not be fit reliably.

In conclusion, the off-resonance $R_{1\rho}-R_1$ CRT relaxation experiment offers significant advantages for characterizing exchange processes on μs−ms time scales in complex biological macromolecules: (i) the dynamics of multiple sites in a complex molecule can be investigated in a single experiment, (ii) only moderate spin-lock field strengths are required, and (iii) differences between the effective fields experienced by a nuclear spin in different conformations are minimized.

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