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Measuring the Signs of $^1H^\alpha$ Chemical Shift Differences Between Ground and Excited Protein States by Off-Resonance Spin-Lock $R_{1\rho}$ NMR Spectroscopy

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Protein dynamics are of fundamental importance for many biological processes, including folding, binding, catalysis, and molecular recognition.[1,2] Often these dynamics involve conformational rearrangements whereby a highly populated conformer exchanges with one or more low-populated, transiently formed states. Such states can be of functional significance and hence are of interest for detailed study, although their low populations and short lifetimes render them "invisible" to many of the techniques of structural biology. In cases where the exchange occurs on the millisecond time scale with excited states populated at 0.5% or higher, Carr−Purcell−Meiboom−Gill (CPMG) relaxation dispersion NMR spectroscopy is a sensitive technique for characterizing the kinetics and thermodynamics of the exchange process.[3] Structural information is also forthcoming in the form of the absolute values of the chemical shift differences between probes in the ground and excited states ($|\Delta\tilde{\omega}|$). The signs of the shift differences and hence the chemical shifts of the excited state, $\tilde{\omega}_E$, can be obtained in many cases by a comparison of peak positions in HSQC/HMQC data sets recorded at a number of static magnetic fields.[4] To date, isotopic labeling strategies and NMR experiments that exploit these labeling approaches have been developed for the measurement of backbone ^{15}N, $^1H^N$, $^{13}C^\alpha$, and ^{13}CO signed $\Delta\tilde{\omega}$ values as well as $^1H^\alpha$ and $^{13}C^\beta$ $|\Delta\tilde{\omega}|$ values.[5−10] We are particularly interested in $^1H^\alpha$ $\tilde{\omega}_E$ values, since the $^1H^\alpha$ chemical shift is sensitive to both secondary and tertiary structure[11] and thus provides important restraints in structure calculations for the excited state. In principle, the sign of $^1H^\alpha$ $\Delta\tilde{\omega}$ could be obtained from analysis of $^1H^\alpha$−$^{13}C^\alpha$ double- and zero-quantum CPMG relaxation dispersion profiles once the sign of $^{13}C^\alpha$ $\Delta\tilde{\omega}$ is known, as is currently done in the case of $^1H^N$ (see refs 12 and 13). In practice, this would require protein samples with isolated $^1H^\alpha$ and $^{13}C^\alpha$ spins, and such samples would be challenging to produce. Herein we describe an alternative and very simple approach in which off-resonance $^1H^\alpha$ decay rates under conditions of spin-locking, $R_{1\rho}$, are measured via one-dimensional spectroscopy using very weak spin-lock fields. Comparison of a pair of decay curves measured with the radio-frequency field applied on opposite sides of the ground-state peak provides the necessary sign information. The utility of the methodology is first established using an exchanging system for which the signed $\Delta\tilde{\omega}$ values are available, and subsequently, an application to the A39V/N53P/V55L Fyn SH3 domain is presented, in which an on-pathway folding intermediate converts with the folded state.[14]

For an exchange reaction between a ground state (G) and an excited state (E) (i.e., G $\rightleftarrows$ E, with rate constants $k_{G\to E}$ and $k_{E\to G}$) in which the G population is much greater than the E population (i.e, $p_G \gg p_E$), Trott and Palmer[15] have shown that $R_{1\rho}$ is given by

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

where R_1 and R_2 are longitudinal and intrinsic transverse relaxation rates, respectively, and R_{ex} is the exchange contribution to $R_{1\rho}$, given by

$$R_{ex} = \frac{p_E \Delta\omega^2 k_{ex}}{\omega_{E,eff}^2 + k_{ex}^2} = \frac{p_E \Delta\omega^2 k_{ex}}{(\delta_G + \Delta\omega)^2 + \omega_1^2 + k_{ex}^2} \quad (2)$$

In eqs 1 and 2, ω_1 is the strength of the applied field (rad/s), $\delta_G = \Omega_G - \Omega_{SL}$ and $\delta_E = \Omega_E - \Omega_{SL}$ are resonance offsets from the spin-lock (SL) carrier for the G and E states, respectively, $\theta = \arctan(\omega_1/\delta_G)$, $\omega_{E,eff}^2 = \omega_1^2 + \delta_E^2$, $\Delta\omega = \Omega_E - \Omega_G$, and $k_{ex} = k_{G\to E} + k_{E\to G}$. Because the maximum in R_{ex} occurs when the spin-lock field is resonant with the frequency of the minor state (eq 2), recording a pair of $R_{1\rho}$ decay curves with $\delta_G \approx \pm\Delta\omega$ allows the sign of $\Delta\omega$ to be determined, since $R_{1\rho}$ values recorded for $\delta_G = -\Delta\omega$ will be larger than those for $\delta_G = \Delta\omega$.

Figure 1 illustrates the one-dimensional NMR pulse scheme that has been developed to measure the signs of $^1H^\alpha$ $\Delta\tilde{\omega}$ values. The sequence is similar to one previously developed by Korzhnev et al.[16] for studies of exchange in proteins by ^{15}N off-resonance $R_{1\rho}$ and a subsequent experiment by Hansen et al.[17] quantifying ^{13}C $R_{1\rho}$ values in nucleic acids and closely follows previously developed schemes for measuring ^1H relaxation rates in proteins.[18] Two-dimensional NMR experiments have also been proposed,[19,20] but the present approach is very efficient when only a subset of residues (those for which $\Delta\tilde{\omega} \neq 0$) must be queried and when very weak spin-lock fields are desired. In the present case, selective Hartmann−Hahn magnetization transfer[21] between $^1H^\alpha$ and $^{13}C^\alpha$ (a to b in Figure 1) of a chosen residue and subsequently from $^{13}C^\alpha$ back to $^1H^\alpha$ (c to d) is used so R_ρ values can be measured from a one-dimensional ^1H spectrum containing in general only the peak of interest. For the uniformly ^{13}C- and fractionally ^2H-labeled samples used [in both these experiments and those measuring $|\Delta\tilde{\omega}|$ (ref 10a)], simulation and experiment have established that a 130−150 Hz continuous-wave field ($\omega_{1,CW}/2\pi$) ensures excellent transfer without losses due to $^{13}C^\alpha$−^{13}CO or $^{13}C^\alpha$−$^{13}C^\beta$ couplings

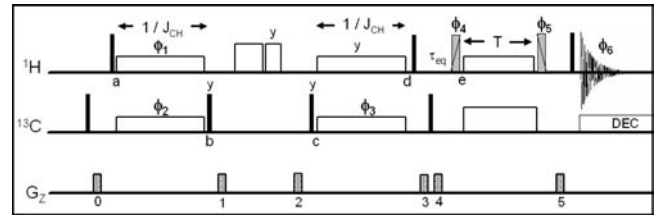

Figure 1. Pulse scheme for measuring $^1H^\alpha$ off-resonance $R_{1\rho}$ relaxation rates in proteins. All of the solid pulses have flip angles of 90° and are applied along the *x* axis, unless indicated otherwise. ^1H pulses of phase $\phi4/\phi5$ (shaded pulses) are applied with a flip angle θ for which $\tan\theta = \omega_1/|\delta_G|$, where δ_G and ω_1 are optimized as described in the text. See the SI for details.

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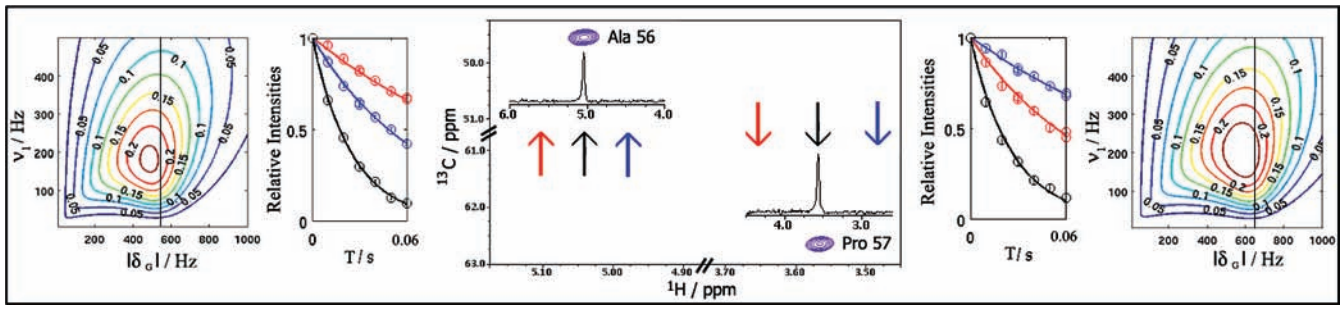

Figure 2. $R_{1\rho}$ decay curves for Ala56 and Pro57 of the A39V/N53P/V55L Fyn SH3 domain (800 MHz), with the spin-lock carrier positioned downfield (red), on-resonance (black), or upfield (blue) of the position of the major-state peak. Each point of each decay profile was recorded in 6 min (room temperature probe head), giving a total measurement time of 0.9 h for each curve (9 points, including a pair of repeats). Contour plots of $\Delta(\delta_G, \nu_1)$ (where $\nu_1 = \omega_1/2\pi$) simulated for 800 MHz are also shown; the vertical line indicates $|\delta_G| = |\Delta\omega|$. The sample (1 mM protein dissolved in D_2O) was prepared with 50% D_2O supplemented with 1 g/L $^{15}NH_4Cl$ and 3 g/L [$^{13}C_6$,$^{2}H_7$]-glucose as the sole nitrogen and carbon sources, respectively, as described previously.[10]

and with essentially no excitation of spins resonating at frequencies outside of a window extending beyond $\pm\omega_{1,CW}$ from the $^1H/^{13}C$ carriers. The $^1H^\alpha$ magnetization at point e is subsequently locked along its effective field for a time T, during which relaxation occurs as $I = I_0 \exp(-R_{1\rho}T)$, prior to recording the spectrum.

As a test of the method, we used an Abp1p SH3 domain-ligand exchanging system with only a small mole fraction of added ligand, as described previously.[22] In this case, the ground state is the apo form of the protein, and the signs of the previously measured $^1H^\alpha$ $\Delta\tilde{\omega}$ values[10] can be obtained using the pulse scheme in Figure 1 and subsequently compared with the "correct" signs from chemical shift values measured directly in the spectra of the apo and fully ligand-bound domains. Previous $^1H^\alpha$ CPMG dispersion measurements at 25 °C established that $k_{ex} = 300$ s^{-1} and $p_E = 6\%$, with $\Delta\tilde{\omega}$ in the range 0.05−0.55 ppm for 17 residues.[10]

For each of these residues, values of δ_G and ω_1 were chosen on the basis of k_{ex}, p_E, and residue-specific $|\Delta\tilde{\omega}|$ values from CPMG measurements using a grid search that maximized

$$\Delta = \left|\exp(-R_{1\rho}^{+}T) - \exp(-R_{1\rho}^{-}T)\right| \qquad (3)$$

where $T = 50$ ms and, according to eqs 1 and 2,

$$R_{1\rho}^{\pm} = R_1 \cos^2\theta + \left(R_2 + \frac{p_E\Delta\omega^2 k_{ex}}{(\pm\delta_G + |\Delta\omega|)^2 + \omega_1^2 + k_{ex}^2}\right)\sin^2\theta \qquad (4)$$

and the optimum δ_G and ω_1 values were subsequently used in the experiments. Notably, although Δ depends on R_1 and R_2, the position of the maximum does not. For 14 of the 17 residues, the signs of $\Delta\tilde{\omega}$ ($|\Delta\tilde{\omega}| \geq 0.05$ ppm) could be determined correctly, while for the remaining three, the $R_{1\rho}$ values for $\delta_G \approx \pm\Delta\omega$ were not sufficiently different to establish the sign [see the Supporting Information (SI)]. Not surprisingly, the $\Delta\tilde{\omega}$ values for these residues were small (0.01−0.03 ppm).

Encouraged by these results on a test system, we next turned to the A39V/N53P/V55L Fyn SH3 domain,[14] for which $k_{ex} = 780$ s^{-1} and $p_E = 1.4\%$ (20 °C). $^1H^\alpha$ $R_{1\rho}$ values were measured for 24 residues, and the sign of $\Delta\tilde{\omega}$ was unambiguously determined for 19 of them ($\Delta\tilde{\omega} \geq 0.17$ ppm; see the SI). Figure 2 shows $R_{1\rho}$ decay curves (along with an on-resonance measurement) for residues Ala56 and Pro57. The larger $R_{1\rho}$ value when the irradiating field was applied upfield of the peak from the major conformer of Ala56 indicates that $\Delta\omega = \Omega_E - \Omega_G$ is negative; conversely, the sign of $\Delta\tilde{\omega}$ for Pro57 must be positive. As expected from eq 1, the decay curve for the on-resonance spin-lock case (black) was always below those generated when the spin-lock was applied off-resonance. Figure 2 also shows contour plots of Δ (eq 3) used to generate the optimized experimental δ_G and ω_1 values.

In summary, we have presented a simple method for measuring the signs of $^1H^\alpha$ $\Delta\tilde{\omega}$ values, allowing the determination of $^1H^\alpha$ chemical shifts of invisible, excited conformers. It is anticipated that these shifts will be important restraints for defining conformational ensembles characterizing intermediates that are both transiently formed and short-lived but nevertheless play important roles in biological function.

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Supporting Information Available: Relaxation curves for all of the measured residues in the proteins studied, tables of $^1H^\alpha$ $\Delta\tilde{\omega}$ values, simulations of decay profiles for different offsets and ω_1 values, and additional pulse sequence information. This material is available free of charge via the Internet at http://pubs.acs.org.

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