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Measurement of ^{1}H $T_{1\rho}$ in a Uniformly ^{15}N-Labeled Protein in Solution with Heteronuclear Two-Dimensional Spectroscopy

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In this Communication, we demonstrate the use of a pulse sequence that measures the ^{1}H $T_{1\rho}$ values for the resolved amide resonances in a two-dimensional heteronuclear correlation spectrum of a uniformly ^{15}N-labeled protein in solution. Rotating-frame spin–lattice relaxation times are of interest because they provide information about conformational or chemical exchange processes that occur on relatively slow time scales and are often difficult to monitor with other spectral parameters. A number of ways of measuring ^{15}N $T_{1\rho}$ of the heteronuclei in uniformly ^{15}N-labeled proteins have been described (*1, 2*). Even though the relaxation mechanisms for ^{1}H sites are not as clearcut as for ^{15}N (or ^{13}C) sites with directly bounded hydrogens, the measurement of ^{1}H $T_{1\rho}$ has advantages over ^{15}N $T_{1\rho}$. For example, much stronger spin-lock fields (12 kHz vs 2 kHz) can be generated in commercial triple-resonance probes, enabling events that occur over a wider range of time scales to be probed.

In the first half of the pulse sequence diagrammed in Fig. 1, the ^{1}H magnetization is spin-locked for the period τ_{SL}, during which time relaxation occurs at a rate determined by ^{1}H $T_{1\rho}$. The spin lock of the ^{1}H magnetization is accomplished with a $(\pi/2)_x$ pulse followed by repetitive $(\pi/2)_y$ pulses (*3*) with duration pw and amplitude $\omega_1 = \gamma B_1$, where *x* and *y* designate the relative phases. The spin-lock irradiation frequency is typically placed in the center of the amide resonance region. The delay between $(\pi/2)_y$ pulses (δ) establishes the duty cycle [dc = pw/(δ + pw)]. The effective spin-lock field strength is determined by $\omega_e = \omega_1$dc. This procedure yields a more accurate calibration of the effective field strength and lower phase distortions in the spectra than can be generally accomplished by spin-locking with continuous irradiation.

The second half of the pulse sequence consists of ^{15}N-edited HSQC as described by Mori *et al.* (*4*). A similar sequence was recently implemented by Talluri and Wagner (*5*) for three-dimensional NOESY–HSQC experiments. The water flip-back results in a dramatic improvement in sensitivity, especially for samples at relatively high pH. Baseline distortions in the resulting spectra are minimized by the combination of pulsed-field-gradient pulses in the second half of the sequence and an added phase cycle that inverts the spin-locked magnetization in alternate scans.

In order to verify the performance of the pulse sequence in Fig. 1, we compared measurements made with it to those with the method described by Blackledge *et al.* (*3*), which is similar to the first half of this pulse sequence. The test sample was a solution containing the tripeptide (Gly–^{15}N–Phe–^{15}N–Gly), which has two well-resolved amide resonances in its one-dimensional ^{1}H NMR spectrum. Figure 2 shows that the ^{1}H $T_{1\rho}$ values measured with these one- and two-dimensional methods are the same within experimental error.

The pulse sequence shown in Fig. 1 enables ^{1}H $T_{1\rho}$ measurements to be made for all resolved resonances in a two-dimensional heteronuclear correlation spectrum of a uniformly ^{15}N-labeled protein in solution. These data are especially valuable because they can be used to identify segments of the protein undergoing conformational exchange on rela-

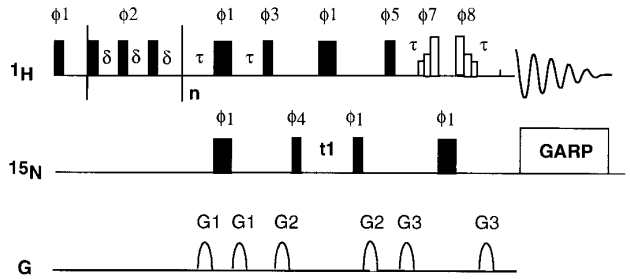

FIG. 1. Pulse sequence for two-dimensional ^{1}H $T_{1\rho}$ measurements. The narrow and wide solid bars represent $\pi/2$ and π pulses, respectively. The primary carrier frequency is placed on-resonance for water, and the spin-lock frequency is placed in the center of the amide resonance region. τ_{SL} and the spin-lock field strengths are calculated by setting *n* and δ using the equations in the text. The phase cycle consists of $\phi_1 = x, x, -x, -x$; $\phi_2 = y$; $\phi_3 = y, y, -y, -y$; $\phi_4 = x, -x, -x, x$; $\phi_5 = -x, -x, x, x$; $\phi_6 = y, y, -y, -y$; $\phi_7 = -y, -y, y, y$; receiver phase = $x, -x, -x, x$. ^{15}N decoupling during acquisition is performed using GARP (*8*). Quadrature detection in the indirect dimension was accomplished using the States–TPPI phase (*9*) incrementation of phase ϕ_4. The pulsed field gradient G1 is a 1 ms *y* gradient, approximately 6 G/cm. G2 is a 1 ms *x* gradient, approximately 6 G/cm, and a 1 ms *z* gradient, 10–20 G/cm.

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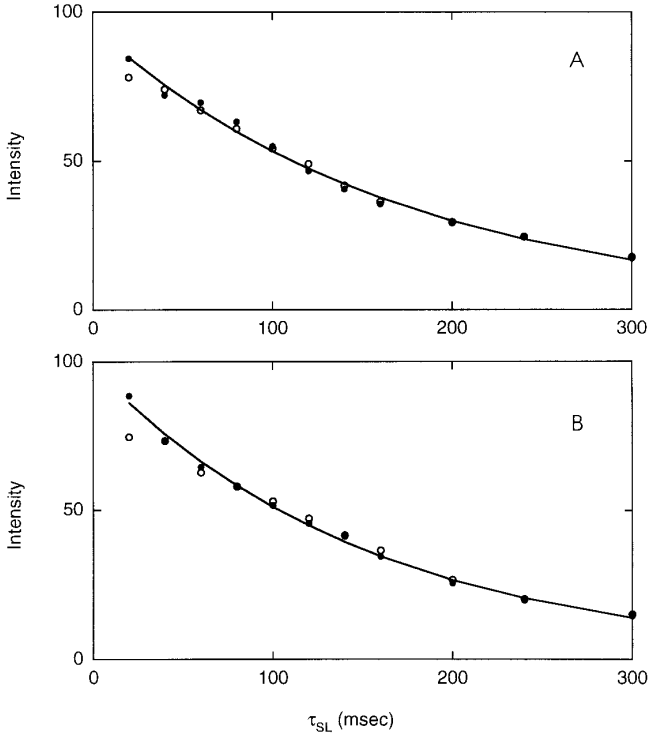

FIG. 2. Comparison of the ^{1}H $T_{1\rho}$ decay curves of Gly–^{15}N–Phe–^{15}N–Gly measured by two different methods with a spin-lock field strength of 10 kHz. The sample contained 40 m*M* peptide in a 30% glycerol/water solution. The open circles represent data obtained using the method described by Blackledge *et al.* (*3*), and the filled circles represent data obtained using the pulse sequence diagrammed in Fig. 1. (A) Decay curve for the ^{1}H on ^{15}N–Phe. (B) Decay curve for the ^{1}H on ^{15}N–Gly.

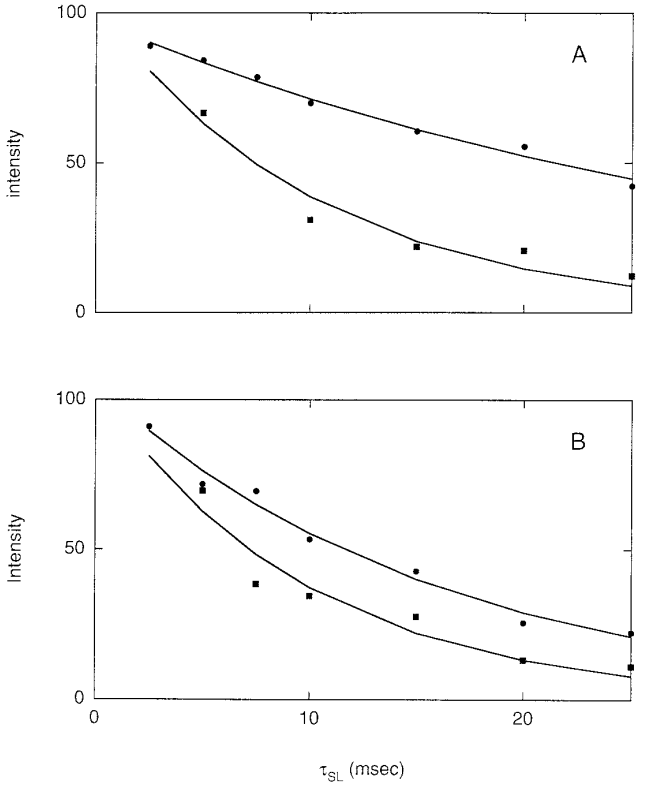

FIG. 4. Comparison of ^{1}H $T_{1\rho}$ decay curves at two different spin-lock field strengths for sites on fd coat protein in SDS micelles at 25°C using the pulse sequence diagrammed in Fig. 1. (A) Decay of curves for the ^{1}H on the indole nitrogen of the Trp26 side chain. (B) Decay curves for ^{1}H on the amide nitrogen of Ile22. The squares represent data obtained with a 2 kHz field strength, and the circles represented data at a 10 kHz field strength for the spin lock.

tively slow time scales. The ^{1}H $T_{1\rho}$ values measured for nearly all of the backbone amide sites of the 50-residue fd coat protein in SDS micelles at 35°C with a spin-lock field strength of 10 kHz are plotted in Fig. 3. The results are consistent with the dynamics of the protein characterized with conventional relaxation measurements (*6*). The protein has a hydrophobic α helix between residues 27 and 44 that reorients with a correlation time of 9 ns at 50°C and an amphipathic helix between residues 7 and 16 that has some additional motion. The loop region connecting the two helices (residues 17–26) has about the same mobility as the amphipathic α helix. The ^{1}H $T_{1\rho}$ values for the amphipathic α helix and the loop region are somewhat longer than those for the hydrophobic α helix. No differences were observed in the values of ^{1}H $T_{1\rho}$ for the amide resonances when the measurements were performed at 35°C with a spin-lock strength of 2 kHz. However, the resonance from the indole N–H site of the Trp26 side chain had a decreased ^{1}H $T_{1\rho}$ value when measured with a 2 kHz field compared to a 10 kHz field.

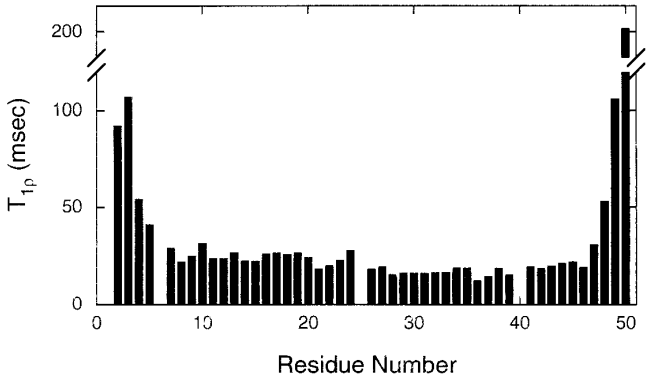

FIG. 3. ^{1}H $T_{1\rho}$ for the backbone amide sites of uniformly ^{15}N-labeled fd coat protein in SDS micelles at 35°C. The spin-lock irradiation was performed at 8.5 ppm in the center of the amide resonance with a field strength corresponding to 10 kHz.

More pronounced differences were observed in ^{1}H $T_{1\rho}$ values as a function of spin-lock field strength with the sample at 25°C. Substantial differences can be observed in the data shown in Fig. 4 for the backbone amide site of Ile22 as well as the indole site of the side chain of Trp26. The

more pronounced difference in ^{1}H $T_{1\rho}$ observed for the side chain of Trp26 at 25°C does not necessarily mean that τ_{ex} is higher, since the results at 25°C could reflect differences in resonance frequencies or populations. ^{15}N $T_{1\rho}$ measurements for the same sites do not show differences as a function of spin-lock field strength at either temperature. Thus, the ^{1}H $T_{1\rho}$ measurements provide valuable qualitative support for the conclusion that the loop region of fd coat protein in micelles has more than one conformation. Structures calculated from solution NMR data for fd coat protein in micelles indicated that there were multiple conformations in a loop connecting two α helices (*6*). Time-resolved fluorescence also provided some evidence of conformational variability in this region of the protein (*7*).

The pulse sequence diagrammed in Fig. 1 enables ^{1}H $T_{1\rho}$ measurements to be made on all resolved resonances in two-dimensional correlation spectra of uniformly ^{15}N-labeled proteins. The measurements are shown to be in good agreement with those obtained with one-dimensional methods. ^{1}H $T_{1\rho}$ measurements were shown to be sensitive to the pressure of exchange processes which could not be detected with ^{15}N $T_{1\rho}$ measurements. In addition, quantitative interpretations of $T_{1\rho}$ as a function of spin-lock field strength (*2, 3*) are more precise for ^{1}H than ^{15}N since the value of $\Delta\omega$ can be determined over a larger range of spin-lock field strengths.

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