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Diffusion Coefficients of Biomolecules Using Long-Lived Spin States

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The interactions of biomolecules and ligands in solution depend not only on their mutual affinity but also on their translational diffusion coefficients. Nuclear magnetic resonance (NMR) provides elegant ways to measure diffusion, both in vitro and in vivo.[1] Molecules in viscous or confined environments as well as large biomolecules and their complexes have very slow diffusion rates, so that spin memory must be preserved over sufficiently long time intervals to allow for a significant displacement between the pulsed field gradients (PFGs) that are used to encode their initial and final positions. Similar requirements hold for monitoring slow chemical exchange, bearing in mind that high-affinity interactions result in slow exchange rates. Until recently, longitudinal relaxation time constants (T_1) were believed to set an upper time limit for the study of slow processes by NMR. The magnetization of scalar-coupled spin-$^1/_2$ systems can be converted into long-lived states (LLS)[2−5] with lifetimes T_{LLS} that can be much longer than T_1. Ratios $T_{LLS}/T_1 > 36$ have been obtained in a partially deuterated sugar,[6] which may be useful for nucleic acids. In this Communication, we show that LLS can also be sustained in peptides and mobile parts of proteins. Slow diffusion coefficients can thus be determined using PFGs with weaker amplitudes and shorter durations.

The aliphatic CH^{α}_2 protons in monomeric glycine $NH_2CH^{\alpha}_2$-COOH are magnetically equivalent, but they become diastereotopic in peptides and proteins. Using methods described elsewhere[6,7] we have excited and exchanged LLS in the dipeptide Ala-Gly dissolved in a mixture of D_2O and deuterated DMSO which remains liquid at temperatures well below 273 K, thus allowing one to explore a wide range of rotational correlation times and translational diffusion coefficients (Figure 1). The lifetimes T_{LLS} may be extended by reducing the content of paramagnetic oxygen in the sample.

Table 1 shows ratios $T_{LLS}/T_1 > 7$, even when molecular tumbling is slowed down at 270 K, and even when paramagnetic oxygen content in the sample is not reduced. At 270 K, the diffusion coefficient $D = (0.91 \pm 0.09) \times 10^{-10}$ m^2 s^{-1} of Ala-Gly in D_2O/DMSO is similar to that of a small protein (∼10 kDa) in D_2O at 298 K.

These experiments can also be applied to mobile parts of proteins and other biomolecules. We measured the T_{LLS} of CH^{α}_2 pairs of protons in glycine residues in Ubiquitin. Only the signals of G75 are visible when the experiment is set up to match the shifts of this residue, while only those of G76 can be seen when the sequence is adapted to its chemical shifts, provided $\Delta_{LLS} > 500$ ms so that all other signals decay. Under these conditions, the T_{LLS} for Gly-75 and Gly-76 (Figure 2) were found to be $T_{LLS} = 6.3 \pm 0.3$ and 6.4 ± 0.4 s, respectively. The gain is $T_{LLS}/T_1 > 6$, since $T_1 \sim 1$ s for both residues. Other Glycine residues buried in the core of the protein were found to have $T_{LLS} \approx T_1 < 1$ s. A broadband

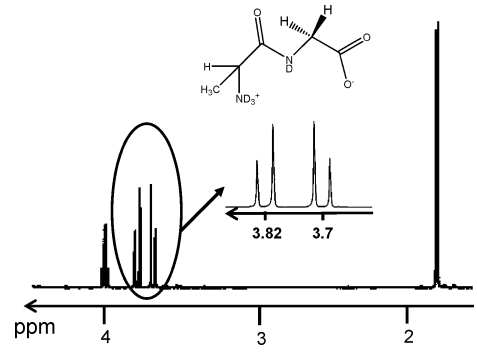

Figure 1. Proton NMR spectrum of DL-Ala-Gly in D_2O/DMSO at 500 MHz and 298 K. The resonances of the diastereotopic CH^{α}_2 protons of Gly are shown in the expanded region. The spins I and S ($I = H^{\alpha 1}$ and $S = H^{\alpha 2}$) have a geminal J-coupling $J_{IS} = -17.4$ Hz and a chemical shift difference $\Delta\nu_{IS} = 0.12$ ppm (61.4 Hz at 500 MHz).

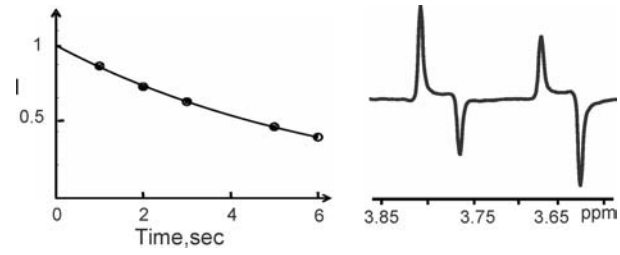

Figure 2. Exponential fit of the relaxation time constant $T_{LLS} = 6.4 \pm 0.4$ s of the long-lived states of the diastereotopic CH^{α}_2 protons in Gly-76 in Ubiquitin dissolved in D_2O at 600 MHz and 310 K after bubbling N_2 gas for 15 min (left) and signals of these protons recorded at 14.1 T (600 MHz) at the end of the LLS sequence (right) using a contiguous Sinc-shaped pulses[9] with a peak RF amplitude $\nu_1^{max} = 5.6$ kHz and a pulse length of 500 μs to sustain the LLS during a diffusion delay $\Delta = 5$ s. The CH^{α}_2 proton pair of Gly-76 has a chemical shift difference $\Delta\nu_{IS} = 55.7$ Hz at 500 MHz and a scalar coupling $J_{IS} = -17.3$ Hz.

experiment[6] including Thrippleton−Keeler filters[8] can excite all residues featuring LLS at the same time, independently of their chemical shifts, albeit at the cost of sensitivity.

The long lifetimes T_{LLS} of CH^{α}_2 pairs in glycine residues of the highly mobile C-terminus occur because, on average, these protons are relatively remote from the bulk of the protein, so that dipolar interactions with spins belonging to the compactly folded part of the protein are partly averaged out. It is noteworthy that the methods presented here are not limited to glycine residues (work in progress in our laboratory). In particular, the narrow proton resonances of His-tags appear suitable to sustain LLS.

Diffusion studies were carried out for Ubiquitin to compare the gradient strengths required for STE[10,11] and LLS.[12] It can be seen from Figure 3 that a long diffusion interval $\Delta_{LLS} = 2$ s was made possible[13] by the lifetime $T_{LLS} = 5$ s, while a shorter interval Δ_{STE}

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Table 1. Longitudinal Relaxation Time Constants T_1 and Lifetimes T_{LLS} of the Long-Lived State Associated with the Diastereotopic CH^{α}_2 Protons of Gly in Ala-Gly[a]

temp (K)	T_1 (s)[b]	T_{LLS} (s)[b]	T_{LLS}/T_1[b]	T_1 (s)[c]	T_{LLS} (s)[c]	T_{LLS}/T_1[c]	$D_{STE} \times 10^{10}$ (m^2 s^{-1})
294	0.842 ± 0.002[d]	11.6 ± 0.2[d]	14[d]	0.948 ± 0.002[d]	16.3 ± 0.1[d]	17[d]	2.17 ± 0.09[d]
294				1.702 ± 0.002[e]	42.1 ± 0.1[e]	25[e]	
270	0.653 ± 0.002[d]	4.8 ± 0.1[d]	7[d]	0.647 ± 0.002[d]	8.2 ± 0.2[d]	13[d]	0.91 ± 0.09[d]

[a] The translational diffusion constants D_{STE} were determined using conventional stimulated echoes (STE) with a diffusion interval $\Delta = 0.5$ s and pulsed field gradients of strength $5 < G_z < 45$ G cm^{-1} and durations $\delta = 650$ and 1500 μs at 294 and 270 K, respectively. [b] Without removing paramagnetic oxygen. [c] O_2 concentration reduced by bubbling N_2 gas. [d] In 70/30% D_2O/deuterated DMSO. [e] In D_2O.

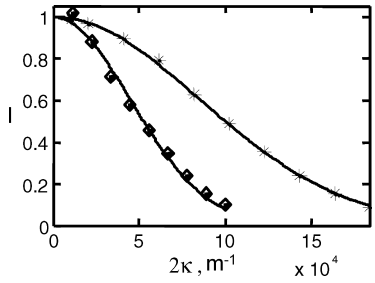

Figure 3. Fits of translational diffusion coefficients of Ubiquitin dissolved in D_2O at $T = 298$ K and $B_0 = 11.7$ T using LLS (diamonds) and conventional stimulated echoes (stars). The gradient amplitudes were $3 < G < 45$ G cm^{-1} with durations $\delta = 650$ and 1200 μs, respectively. The diffusion intervals were $\Delta = 2$ and 0.55 s, respectively. Very similar diffusion coefficients $D = (1.264 \pm 0.002) \times 10^{-10}$ m^2 s^{-1} were obtained by both methods (less than 1% deviation between the two measurements).

$= 0.55$ s had to be used for STE since $T_1 \approx 1$ s. The LLS method allows one to limit the range of the abscissa $2\kappa = (2\delta)\gamma psG$, where γ is the proton gyromagnetic ratio, $p = 1$ is the coherence order, δ is the gradient duration, and $s = 2/\pi$ is a shape factor that describes the sine-shape of the gradients. The range of κ can be reduced by a factor of $(\Delta_{LLS}/\Delta_{STE})^{1/2}$.

In conclusion, long-lived states associated with diastereotopic proton pairs in mobile parts of proteins can have long lifetimes, provided that the nuclei are relatively remote from other spins with high gyromagnetic ratios. This finding opens the way to measuring diffusion coefficients of macromolecules without isotope labeling using field gradients with moderate strengths and durations.[13,14] Similar results may also be obtained using the long T_1 lifetimes of heteronuclei,[15,16] albeit at the expense of incorporating ^{15}N or ^{13}C isotopes. The LLS method to study diffusion may be applied to intrinsically unstructured proteins, which are found to be encoded by a significant part of the genome,[17] even in the absence of resonance assignments. Since LLS lifetimes are modulated by the mobility of the loops, T_{LLS} may be used as a probe for motions in natively unstructured or denatured proteins.

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