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Probing Transient Conformational States of Proteins by Solid-State $R_{1p}$ Relaxation-Dispersion NMR Spectroscopy**

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Abstract: The function of proteins depends on their ability to sample a variety of states differing in structure and free energy. Deciphering how the various thermally accessible conformations are connected, and understanding their structures and relative energies is crucial in rationalizing protein function. Many biomolecular reactions take place within microseconds to milliseconds, and this timescale is therefore of central functional importance. Here we show that $R_{1p}$ relaxation dispersion experiments in magic-angle-spinning solid-state NMR spectroscopy make it possible to investigate the thermodynamics and kinetics of such exchange process, and gain insight into structural features of short-lived states.

Solution-state NMR spectroscopic techniques, and in particular so-called relaxation-dispersion (RD) NMR approaches, have proven very successful for studying $\mu$-ms motion and characterizing the exchanging short-lived conformational exchange processes. RD NMR techniques exploit the effect of conformational-exchange processes on line broadening, that is, on the relaxation rates of nuclear spin coherence ($R_2, R_{1p}$). By quantifying spin relaxation rates in the presence of a variable radiofrequency (rf) field, RD approaches provide information about relative populations and exchange rates, as well as chemical shifts of short-lived conformational states, and thus about local structure. In the case of very large assemblies or insoluble aggregates, where solution-state NMR is severely challenged, magic-angle-spinning solid-state NMR (MAS ssNMR) is rapidly emerging as a tool for the study of structure and dynamics. However, the characterization of conformational-exchange dynamics in the solid state remains challenging. Herein, we show a ssNMR approach in which amide-$^{15}N$ $R_{1p}$ data, that is, the rate of coherence decay under $^{15}N$ spin-lock fields of variable field strengths, are quantitatively analyzed and interpreted in terms of conformational dynamics, providing insight into short-lived states in terms of chemical shifts and bond vector orientations. We investigate the robustness of this approach by studying a conformational flip in crystalline ubiquitin.

Conformational fluctuations between different states expose a given nuclear spin in a protein to different local environments, which are characterized by different bond geometries. A simple case, exchange between two states, is shown in Figure 1a. As a result of conformational-exchange dynamics, a given spin will experience a fluctuation of its chemical shift (CS), as well as of its bond vector orientations and, thus, dipolar coupling interactions with neighboring spins and CS anisotropies (CSA). In solution-state NMR, dipolar coupling and CSA interactions are averaged to zero by Brownian movement (molecular tumbling). Consequently, only fluctuations of the isotropic CS are relevant when considering conformational dynamics on the microsecond to millisecond timescale. $R_{1p}$ RD experiments in solution can thus only pick up conformational dynamics if they involve a change in the isotropic CS. The relevant theory is well established, and the effects of exchange can be described by the Bloch–McConnell equations.

In MAS ssNMR, where stochastic molecular tumbling is absent, anisotropic interactions (dipolar couplings and CSA) are periodically modulated by magic-angle sample spinning (MAS), and are averaged out over a sample rotation period (in the $\mu$s range). This averaging leads to the line narrowing required for high-resolution studies. However, when considering molecular dynamics and rf irradiation, as is the case in $R_{1p}$ RD experiments, the various time-dependent processes (sample rotation, rf irradiation, and conformational exchange) may interfere, leading to different decay processes. Consequently, the situation is more complex than in solution-state NMR experiments. Before considering experimental

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**Figure 1.** The effect of conformational exchange processes on $^{15}N$ $R_{1p}$ relaxation rates, as revealed by numerical simulations. a) Schematic representation of a two-spin N–H system that is in dynamic exchange between a minor and a major conformation, which differ in the $^{15}N$ chemical shift and the orientation of the NH bond vector. The $^{15}N$ CSA tensor is assumed to be collinear with the NH bond. b,c) Simulated $^{15}N$ $R_{1p}$ RD profiles obtained for this two-spin system, assuming relative populations of $p_b = 10\%$, $p_c = 90\%$. Different scenarios were assumed, regarding the $^{15}N$ isotropic CS (solid/dotted lines), and the change of the bond angle orientation, $\theta$, occurring during exchange (red/black), as indicated. More simulations are shown in Figure S1. The inserts in (b,c) show enlargements of the low-rf field regime.

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implementations of MAS ssNMR $R_{ip}$ RD experiments, we thus investigate the properties of $^{15}$N $R_{ip}$ decay in an exchanging system undergoing MAS by numerical simulations.

Figure 1b, c shows simulated $^{15}$N $R_{ip}$ RD profiles for a $^{1}$H–$^{15}$N spin pair that undergoes exchange between two states, populated to 90% and 10%, respectively, and which is subject to MAS and a $^{15}$N spin-lock rf field of variable amplitude. We assumed different exchange scenarios that involve either only a fluctuation of the $^{15}$N spin’s isotropic CS, or a change of the NH bond orientation within the molecular frame of reference, or both. The results of these simulations can be summarized as follows: 1) If exchange takes place between two states that differ only in their isotropic CS, but not in bond orientations, $R_{ip}$ RD profiles can be described by the Bloch–McConnell formalism (black curves in Figure 1b, c), as in solution-state NMR experiments.[14] 2) If bond angle fluctuations are involved upon conformational exchange (thus reorienting the dipolar coupling and CSA interactions), the situation gets more complex. $R_{ip}$ rate constants in this case are overall increased, because the fluctuating anisotropic interactions induce relaxation.[15] Of particular note for the present discussion is the fact that the increase of $R_{ip}$ rates is particularly pronounced when the spin-lock field strength ($v_{l}$) approaches the sample rotation frequency ($v_{MASS}$), that is, close to the so-called rotary resonance conditions.[8]

Importantly, as shown in Figure 1 and Figure S1 and reported previously,[4, 5] conformational exchange broadens these rotary resonance recoupling conditions, and the $R_{ip}$ relaxation rate in the vicinity of the rotary resonance conditions depends on the kinetics of the exchange, the relative populations of the involved states, and the jump angle between the different conformations. Taken together, these simulations demonstrate that MAS ssNMR $R_{ip}$ RD experiments may provide rich information about conformational exchange, and that they may report not only on chemical shift fluctuations between the states (as is the case in solution-state NMR measurements), but also on the bond angles by which a short-lived “excited” state differs from the predominant “ground-state” conformation.

Exploiting this potential of MAS ssNMR $R_{ip}$ RD experiments to quantitatively analyze conformational exchange in proteins has so far been hampered by the fact that $R_{ip}$ decay rates in ssNMR not only contain a dynamics-related component, but also have substantial contributions from coherent decay mechanisms (i.e. dipolar dephasing). Dipolar dephasing contributes to $R_{ip}$ decay particularly in multispin systems, such as proteins with numerous proton spins. Extracting the dynamics-related part of $R_{ip}$ decay rates thus requires suppressing this dipolar-dephasing part. As shown recently, at high MAS frequencies of roughly $40$ kHz or higher, and spin-lock fields above $15$ kHz this dipolar dephasing is significantly reduced and appears negligible even in the proton-rich environment of a protein.[7] However, the requirement for high spin-lock fields eliminates the possibility of observing $R_{ip}$ RD in the window below $5$–$10$ kHz spin-lock field, where RD profiles are particularly sensitive to isotropic CS fluctuations.

We circumvent these limitations here by employing high degrees of sample deuteration, which strongly reduces the dipolar-dephasing contribution to $^{15}$N $R_{ip}$ rates.[9] In highly deuterated samples, that is, samples that are fully deuterated at non-exchangeable sites, and in which the exchangeable (amide) sites are (partly) reprotonated, the proton dipolar coupling network is strongly diluted, allowing for high-resolution highly sensitive proton-detected ssNMR spectra, and long $^{15}$N coherence life times.[8, 9] Figure 2a shows representative examples of residue-wise $R_{ip}$ RD profiles obtained on a sample of deuterated microcrystalline ubiquitin that has been reprotonated at exchangeable sites to $50\%$.

$^{15}$N $R_{ip}$ data have been measured at a MAS frequency of $39.5$ kHz, and spin-lock rf field strengths of $2$–$15$ kHz, that is, far from the $n=1$ rotary resonance condition (which is at $39.5$ kHz). The reported rate constants are on-resonance $R_{ip}$ that is, the $R_{i}$ contribution in the tilted rotating frame has been eliminated (using standard formulae,[14] see the Supporting Information).

For the vast majority of residues, for example, residues Ile3, Leu15, Lys33, and Gly47 in Figure 2a, we find that the RD profiles are flat over the entire range of rf field strengths. This finding confirms that coherent dephasing is indeed efficiently suppressed by deuteration and fast MAS; it further suggests that nonflat $R_{ip}$ RD profiles can safely be ascribed to motional processes. Indeed, for a few residues we observe nonflat $R_{ip}$ RD curves, that is, increased relaxation rate constants at low spin-lock rf fields, as expected for the Bloch–McConnell regime of exchange. The residues with such nonflat $^{15}$N $R_{ip}$ RD profiles are located in a well-defined region of ubiquitin, in the N-terminal part of the helix and a loop that is hydrogen-bonded to this helix (residues Ile23, Lys27, Glu51, Asp52, Arg54, Thr55; see Figure 3 and Figure S5). Conformational exchange in this region of the protein has been reported in numerous studies in solution,[10] and we have recently also reported conformational exchange in this part of the protein in microcrystals from Carr–Purcell–Meiboom–Gill (CPMG) RD and multiple-quantum relaxation experiments.[11]

In order to obtain quantitative insight into the exchange process occurring in this part of the protein, we have fitted an exchange model to the $R_{ip}$ RD profiles of these residues using the Bloch–McConnell formalism. The application of this formalism is strictly speaking not correct in MAS ssNMR if bond reorientation is involved (cf. Figure 1). However, it appears justified here by the fact that the RD curves of Figure 2 were collected in a range of rf fields well outside the rotary-resonance conditions; bond angle fluctuations have only very small impact on $R_{ip}$ RD in this regime, especially when changes in the bond vector orientations are small (cf. Figure 1). That bond angle fluctuations are indeed rather small is justified a posteriori with independent measurements (see below). We fitted a two-state exchange model to the $R_{ip}$-derived RD profiles of residues 23, 27, 51, 52, 54, and 55 (Figure 2 and Figure S6). Because of their close spatial proximity, we assumed that these residues are involved in the same exchange process. Consequently, the exchange rate constant, $k_{ex} = k_{AB} + k_{BA}$ and minor-state population, $p_{m}$ were assumed to be identical for all these residues, and only the
chemical-shift differences between the major and minor states, \( \Delta \delta \), which are sensitive to changes in the local environment, were assumed to be specific to individual residues. Solid lines in Figure 2a show best-fit curves of this fit. The fit yields an exchange rate constant \( k_{\text{ex}} = 8600 \pm 1700 \text{ s}^{-1} \) and a minor-state population \( p_B = 3.1 \pm 1.2\% \). Residue-wise chemical-shift differences \( \Delta \delta \) are in the range of 2–5 ppm (all values are reported in Table S1). In order to investigate the reliability of these results, which were obtained from a single R\(_{1p}\) RD measurement, we explored the inclusion of additional, independent data sets: a first possibility, often used in solution-state NMR, would be the measurement of R\(_{1p}\) RD at additional static magnetic field strengths (requiring, however, access to another spectrometer equipped with a fast-MAS probe). As an alternative, we use here a combined fit with CPMG RD data, obtained previously under similar conditions of fast MAS and deuteration.[11] CPMG RD is sensitive to exchange processes on \( \mu \text{s}–\mu \text{ms} \) timescales, making a combined fit with R\(_{1p}\) possible. Such an analysis of the present R\(_{1p}\) RD data with CPMG data, also obtained at a magnetic field strength of 14.1 T, yields values of \( \Delta \delta \) that are very similar to those obtained from the above fit of only R\(_{1p}\) RD data (see Table S1). The obtained exchange rate \( k_{\text{ex}} = 2900 \pm 140 \text{ s}^{-1} \) and population \( p_B = 9.3 \pm 0.6\% \). Although these values slightly differ from the values obtained from fitting only a single R\(_{1p}\) RD data set (where \( k_{\text{ex}} = 8600 \pm 1700 \text{ s}^{-1}, p_B = 3.1 \pm 1.2\% \) ) it is noteworthy that the fit curves of the combined R\(_{1p}\)/CPMG fit, shown as orange lines in Figure 2a, are almost indistinguishable from the fits of R\(_{1p}\) data only, which shows that the present data are in excellent agreement with independent CPMG data. The differences of the fitted parameters point to the well-known fact that it is difficult to disentangle all fit parameters from a single measurement (here, a single \( B_0 \) field strength).

The R\(_{1p}\) RD experiment has advantages over the previously proposed CPMG RD experiment:[11] In solids, even at...
fast MAS and high degrees of deuteration, measured R_1 rates contain a substantial amount of dipolar dephasing, in contrast to R_m (see Figure S10). This has two important consequences: 1) Due to the more rapid coherence decay, sensitivity in the CPMG experiment is significantly lower than in the R_m experiment. 2) The remaining coherent contributions to the effective R_1 rates in a CPMG experiment are almost, but not entirely independent of the CPMG frequency. Thus, observed variations of the effective R_1 in the CPMG experiment may to some extent be artificial. We note that the timescales probed by R_m and CPMG RD experiments overlap but are not exactly identical, such that the approaches should be considered as complementary.

In solution, the same part of ubiquitin undergoes conformational exchange, but the process is much faster than observed here. Even at a temperature 20 K lower than what was used here, the exchange occurs much faster than here (k_ex ≈ 12 500–25 000 s⁻¹). At 298 K, a similar temperature to that used here, the R_1 RD profile in solution for example, for Ile23 is flat (to within 0.5 s⁻¹), which is in stark contrast to the pronounced dispersion of R_m seen in crystals in this study. This finding unequivocally establishes that the motional process in microcrystals is slower than in solution. Intramolecular contacts are at the origin of this slowdown, acting as additional energy barriers for motion in the crystal.

Having thus established that R_m RD experiments at high MAS frequencies can provide information about the thermodynamics and kinetics of exchange, as well as site-specific chemical-shift values of the minor states, akin to the situation in solution-state NMR experiments, we investigated whether additional structural information about the minor state may be obtained. As Figure 1 shows, ¹⁵N R_m RD profiles in the vicinity of the rotary resonance condition (ν₁ ≈ ν_MAS) are sensitive to the angle by which the NH bond (i.e. the dipolar coupling and ¹⁵N CSA) is altered upon the conformational transition. For technical reasons we refrained from performing such measurements at high MAS frequencies, because the required high spin-lock field strengths (ν₁ ≈ ν_MAS) would challenge the integrity of hardware and sample. Instead, we performed experiments at lower MAS frequency (20 kHz), where the rotary-resonance condition is met at lower rf field. To ensure that coherent contributions to the ¹⁵N R_m decay remain negligible even at slower MAS, we employed a higher degree of deuteration (20% reprotonation of amide sites instead of 50%); the larger volume of the sample rotor that can be used at lower MAS frequency compensates for the resulting sensitivity loss. Figure 2b shows representative examples of ¹⁵N R_m RD profiles obtained at spin-lock field strengths of 12–19 kHz. Many of the residues, such as Ile3, Leu15, Lys33, and Gly47 in Figure 2b, show flat RD profiles over the entire range of sampled spin-lock field strengths, or only show an increase when the spin-lock field strength is within 2–3 kHz of the n = 1 rotary resonance condition. Interestingly, the obtained plateau values are similar to the values obtained at fast MAS, implying that coherent contributions to R_m are indeed efficiently suppressed, even at the lower MAS frequency. An increase of R_m when approaching the rotary resonance condition (ν₁ = ν_MAS) can be understood from partial recoupling of dipolar coupling and CSA inter-