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Microsecond Protein Dynamics from Combined Bloch-McConnell and Near-Rotary-Resonance $R_{1\rho}$ Relaxation-Dispersion MAS NMR

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Abstract

Studying protein dynamics on microsecond-to-millisecond (μ s-ms) time scales can provide important insight into protein function. In magic-angle-spinning (MAS) NMR, μ s dynamics can be visualized by $R_{1\rho}$ rotating-frame relaxation dispersion experiments in different regimes of radio-frequency field strengths: at low RF field strength, isotropic-chemical-shift fluctuation leads to "Bloch-McConnell-type" relaxation dispersion, while when the RF field approaches rotary resonance conditions bond angle fluctuations manifest as increased $R_{1\rho}$ rate constants ("Near-Rotary-Resonance Relaxation Dispersion", NERRD). Here we explore the joint analysis of both regimes to gain comprehensive insight into motion in terms of geometric amplitudes, chemicalshift changes, populations and exchange kinetics. We use a numerical simulation procedure to illustrate these effects and the potential of extracting exchange parameters, and apply the methodology to the study of a previously described conformational exchange process in microcrystalline ubiquitin.

Keywords

Conformational exchange; NERRD; numerical spin simulations; crystalline protein dynamics; ubiquitin

1 Introduction

Understanding the mechanisms of protein function requires the characterization of their three-dimensional structures and interactions with their environment. Three-dimensional structures from X-ray diffraction, NMR or EM are highly valuable for understanding biomolecular function, but they are necessarily only part of the story, because they focus on the highly populated "ground states" of these molecules, while transient, low-populated states may be crucial for functional processes. Understanding the conformational space that a protein samples is, thus, an important step for elucidating mechanisms. Nuclear Magnetic Resonance (NMR) spectroscopy is ideally suited to probe the dynamics of proteins with a

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resolution of individual atoms over time scales ranging from picoseconds to seconds and beyond. Many biological processes on the molecular level, such as enzymatic catalysis or folding, occur on time scales of micro-to-milliseconds (μ s-ms), highlighting the particular interest in experimental methods able to quantitatively probe μ s-ms motions.

In solution-state NMR spectroscopy, powerful techniques have been introduced that enable the detailed study of μ s-ms dynamics. On the "slow end", i.e., in the range from tens of ms to seconds, Exchange Spectroscopy (EXSY)[1] and Chemical-Exchange Saturation Transfer (CEST)[2,3] experiments allow the study of exchanging states by their difference in isotropic chemical shift. On the faster side of this window, relaxation-dispersion (RD) experiments have been used with great success in solution-state protein NMR.[4–8] Relaxation dispersion experiments consist of measuring transverse relaxation rate constants, either $R_{1\rho}$, i.e., the relaxation in the presence of a spin-lock radio-frequency (RF) field, or R_2 , i.e., the relaxation decay during a delay with free spin evolution and refocusing pulses. These relaxation rate constants are measured as a function of effective spin-lock RF field, ν_1 , (in $R_{1\rho}$ RD experiments), or as a function of the repetition rate at which refocusing pulses are applied, ν_{CPMG} , in so called Carr-Purcell-Meiboom-Gill (CPMG)[9,10] RD approaches. The time-scale to which these two types of RD approaches are sensitive is related to the effective RF field strength. Typically, RD approaches cover the time-scale from several μ s to hundreds of ms. The frontier towards faster kinetics have been expanded to a few μ s recently by the application of RF field strengths of increasingly high power, [11,12] but, as described below, only part of the parameters can be fitted accurately in this "fast-exchange regime". On the slow side, CPMG RD experiments[13] and low-power $R_{1\rho}$ RD[14–16] allow studying processes occurring up to several hundred ms.

All these experiments, EXSY, CEST, CPMG RD and $R_{1\rho}$ RD, are rooted in fluctuations of the isotropic chemical shift (CS) as the molecule undergoes transitions between different conformations. In EXSY and CEST, the isotropic chemical shifts of the different states are directly seen in the frequency domain. RD approaches exploit the fact that fluctuations of the isotropic CS lead to line broadening which is manifest as enhanced transverse relaxation. The key feature of RD is that the effect of the exchange on the line broadening can be quenched if the effective RF field is sufficiently high, i.e., if the applied spin-lock power (for $R_{1\rho}$) is high, or if the refocusing pulses are applied sufficiently frequently (for CPMG).[4–6] RD profiles, i.e., $R_{1\rho}=f(\nu_1)$ or $R_2=f(\nu_{\text{CPMG}})$, can be fully described in the framework of the Bloch-McConnell formalism,[17] a classical description of spin evolution. In this manuscript we refer to this isotropic-CS-based RD effect as "Bloch-McConnell RD" (BMRD). The Bloch-McConnell formalism or analytical solutions thereof[5,13,18,19] is generally used for fitting RD profiles. Ideally, such RD fits provide: (i) the populations p_i of the involved states, in the simplest case of two states p_A , p_B , (ii) the isotropic CS difference between these states, ω for each spin and (iii) the exchange kinetic rate constant, k_{ex} .

Notwithstanding the success that BMRD NMR has, its fundamental reliance on isotropic-CS fluctuation limits its range of applications. In particular, as the chemical shift is only an indirect reporter of structure, no direct information about the structural/geometrical differences between the involved states can be retrieved from BMRD data.[20] Furthermore, an important fundamental limitation of BMRD is the fact that in the fast exchange regime

($k_{ex} \gg \omega$), ω and p_B are entangled, and only the product $\phi_{ex} = p_A p_B \omega^2$ can be retrieved from fits of RD data.[21–23]

Biomolecular magic-angle-spinning (MAS) solid-state NMR has only recently reached a stage where RD can be measured quantitatively. High MAS frequencies combined with deuteration have been important ingredients to suppressing so-called dipolar dephasing (which leads to signal decay even in the absence of dynamics), thereby allowing to access "clean" relaxation rate constants that report on dynamics.[24–27] Several very recent applications highlight the potential of RD NMR in solids.[28–37] Unlike in solution, where nanosecond-scale random molecular tumbling averages out all anisotropic interactions (dipolar couplings and chemical-shift anisotropies (CSA)), in MAS NMR these interactions are averaged only in a first-order average-Hamiltonian treatment by the periodic modulation (sample rotation). Interference of spin rotation (applied RF fields) with this spatial modulation of the anisotropic interactions can reintroduce the interactions, as long as the ratio of ω_1 and ω_{MAS} matches certain conditions, i.e., so-called rotary-resonance conditions. The fact that these interactions are not averaged by stochastic tumbling, but by a constant sample rotation has important consequences in the context of RD experiments. The $R_{1\rho}$ spin relaxation induced by fluctuations of the heteronuclear (I-S) dipolar interaction and the S-spin CSA, can be written as follows:[38,39]

$$\begin{aligned}
 R_{1\rho}^{DD, CSA} = & \frac{1}{8} d_{IS}^2 \left\{ \frac{2}{3} J(\omega_1 - 2\omega_{MAS}) + \frac{4}{3} J(\omega_1 - \omega_{MAS}) + \frac{4}{3} J(\omega_1 + \omega_{MAS}) \right. \\
 & + \frac{2}{3} J(\omega_1 + 2\omega_{MAS}) + J(\omega_I - \omega_S) + 3J(\omega_S) \\
 & \left. + 6J(\omega_I) + 6J(\omega_I + \omega_S) \right\} \\
 & + \frac{\omega_S^2 \Delta \sigma^2}{18} \left\{ \frac{2}{3} J(\omega_1 - 2\omega_{MAS}) + \frac{4}{3} J(\omega_1 - \omega_{MAS}) \right. \\
 & \left. + \frac{4}{3} J(\omega_e + \omega_{MAS}) + \frac{2}{3} J(\omega_1 + 2\omega_{MAS}) + 3J(\omega_S) \right\}
 \end{aligned} \tag{1}$$

Importantly, $R_{1\rho}$ depends on the spectral density function at frequencies corresponding to sums and differences of the effective RF field strength (ω_1) and MAS frequency (ω_{MAS}). As an important consequence of this dependence on the spectral density function at sums and differences of ω_1 and ω_{MAS} , in the presence of μs motion, an increase of $R_{1\rho}$ is observed as the RF field strength and the MAS frequency become similar. This effect, described by Redfield theory[39,40] and shown in several studies[26,33,36–38] was dubbed "NEar-Rotary Resonance Relaxation Dispersion" (NERRD) recently.[32] We use this term throughout this manuscript. Non-flat NERRD profiles arise specifically when motions occur in the μs range,[26,31,38,41] and non-flat NERRD profiles are a straightforward sign of μs reorientational dynamics.

The full $R_{1\rho}$ rate constant comprises, in addition to the part induced by CSA and dipolar-coupling fluctuations, a contribution due to isotropic CS fluctuations, often dubbed the "exchange contribution".

$$R_{1\rho} = R_{1\rho}^{DD, CSA} + R_{1\rho}^{ex} \quad (2)$$

Analytical equations for $R_{1\rho}^{ex}$ can be found elsewhere.[5]

On the experimental level, the BMRD and NERRD measurements are identical, differing only in the RF field strength. While BMRD probes the quenching of the exchange-contribution to $R_{1\rho}$ in the low-RF regime (typically $\lesssim 15$ kHz), NERRD uses RF fields close to the MAS frequency (tens of kHz) to sense fluctuations of internuclear angles, and possibly distances, although distances of directly bonded nuclei are constant, i.e., for ^{15}N experiments only the HN bond angle fluctuation matters.

In this study, we explore the potential of the *joint* analysis of BMRD and NERRD data. We explore the possibility to obtain more insight into μs motions than from either BMRD or NERRD alone; particularly, we use numerical simulations to show that the combined fit can help to disentangle populations and CS-differences, which would be difficult to separate from BMRD alone. We measured a comprehensive $R_{1\rho}$ RD data set on deuterated, partially back-exchanged ubiquitin and provide insight into a previously studied exchange phenomenon involving a peptide-plane flip. Even though $R_{1\rho}$ RD data on wild-type ubiquitin in this particular crystal form have been presented before,[26,32] the present data set is more complete, covering 23 different RF field strengths.

2 Results and Discussion

2.1 Numerical simulations: possibilities of MAS RD NMR to provide structural insight into dynamics

We first used *in silico* data to investigate the potential benefit of fitting BMRD and NERRD data jointly to gain insight into μs motions. At this stage it is noteworthy that there has been some debate in the literature whether Redfield relaxation theory, which underlies equation 1, is generally valid in solid-state NMR. In the derivation of Redfield theory it is assumed that the motion that induces spin relaxation is fast compared to the spin relaxation rate constants, [39,40] an assumption which is generally valid in solution due to molecular tumbling, but not necessarily in solids. Another complication in MAS NMR arises from the fact that spin relaxation in solids is always multi-exponential.[39,42,43] Consequently, mono-exponential fits of spin relaxation curves are not physically meaningful, raising additional questions about the best way of treating relaxation data.

Comparisons of Redfield-theory relaxation rate constants with relaxation decays obtained by numerical simulations of a spin system undergoing dynamics have indeed indicated that there are some deviations,[34,44] particularly in the μs range. It has been suggested that in this regime one shall resort to numerical simulations instead of Redfield treatments.[34] However, as pointed out by A. Krushelnitsky and K. Saalwächter later, in analyzing the numerical simulations the authors have treated the non-exponential character differently in numerical simulations and Redfield-type calculations, which explains the deviations of

numerical and analytical treatments.[45] Therefore, it appears that analytical Redfield calculations of the type shown in equation 1 are valid, and can be used similarly as numerical spin simulations, as long as the non-exponential character of the spin relaxation is properly accounted for.[28] The obvious advantage of analytical calculations is their ease and computational efficiency.

As the computationally more efficient Redfield-type calculations can be rigorously used for standard $R_{1\rho}$ data analyses, the advantages of numerical simulations are limited to three aspects. (i) During the first few milliseconds of $R_{1\rho}$ coherence decay, oscillations of magnetization occur, which are not described by Redfield theory, but which may be captured by simulations. The safest choice, however, is to avoid sampling the very beginning of $R_{1\rho}$ experimentally.[28] (ii) The analytical equations describe the initial decay of magnetization only, and they require, therefore, that the experimental data are fitted in a way to retrieve the initial decay, which to good approximation may be achieved by bi-exponential fitting.[41] If data are fitted in a mono-exponential manner, the resulting decay constants are best compared to numerical simulations of the decay, which can be done with a similar sampling grid as the one used experimentally to probe the decay. (iii) Arguably the most important advantage of numerical simulations over analytical treatments at this point is that they are more versatile than available Redfield-type relaxation equations. In particular, new experiments are emerging in which the continuous-wave spin-lock is replaced by composite-pulse sequences. McDermott and co-workers proposed such a relaxation measurement to measure $R_{1\rho}$ that avoids CSA-induced coherent spin evolution, which is not of interest for dynamics studies.[46] When analyzing such complex experiments quantitatively, the only viable route currently is the use of numerical spin simulations.

In this work, we used exclusively numerical spin simulations, rather than Redfield-type equations. We note, however, that for the simple continuous-wave spin-lock experiments treated herein, analytical treatments would be equally valid and computationally more efficient.

The spin simulations, used herein and described before by us[26,39,47] and similarly also by others,[38,46,48] simulate the evolution of ^{15}N coherence in a two-spin ^1H - ^{15}N spin system that undergoes explicit exchange between two states. These states generally differ in their H-N bond orientation (i.e., dipole coupling and CSA tensors) and isotropic CS (see Methods for details).

Figure 1 shows simulated $R_{1\rho}$ RD curves for different scenarios, involving isotropic CS fluctuations, bond-angle fluctuations or both. As predicted from eq. 1, RD effects (i.e., a dependence of $R_{1\rho}$ on the RF-field strength) are found in the low-RF regime (BMRD) if the exchange involves isotropic CS fluctuations, and in the high-RF regime (where ω_1 approaches ω_{MAS}) if the exchange involves bond angle fluctuations. The effects are additive (see an example in Figure 1B), as expected from eq. 2. The "width" of the RD "peaks" depends on the rate of the exchange process. On the low-field side (BMRD) it requires a higher RF-field strength to quench the exchange contribution when the exchange process occurs at a higher rate (compare, e.g. the two left or the two right panels in the lower row of Figure 1A). Similarly, for a "pure NERRD effect" the tail of the $R_{1\rho}$ peaks extends further

away from the rotary-resonance condition when the process occurs at 18000 s^{-1} than at 2000 s^{-1} (top row in Figure 1A).

We next revisited the ability of BMRD profiles to retrieve exchange parameters, k_{ex} , ν and p_{B} . We simulated BMRD profiles of a two-site exchange process and a range of exchange time constants. After adding random noise at different levels to simulated BMRD curves, we subjected the resulting RD profiles to a grid-search procedure against a grid of simulations with systematically varied exchange parameters, probing whether the initial parameters of exchange can be retrieved. Figure 2 shows the fitted chemical-shift differences (ν) and minor-state population (p_{B}) for a representative exchange rate constant ($k_{\text{ex}}=18000\text{ s}^{-1}$) and four different noise levels. Only at very low experimental noise, well below levels we obtain experimentally, the majority of fits retrieves ν and p_{B} accurately (Figure 2A). When realistic error bars are assumed, the fitted ν and p_{B} are highly entangled, reflecting the well-known difficulty of BMRD approaches in the fast exchange regime, reported already decades ago.[21]

Figure 3 investigates how the addition of NERRD data improves the outcome of the fit, which now comprises a fourth parameter, the bond-vector fluctuation angle θ_{D} , in addition to k_{ex} , ν and p_{B} . Three different scenarios of conformational exchange were considered, involving either only isotropic CS fluctuations ($\theta_{\text{D}}=0$, left column), only angular fluctuations ($\nu=0$, right column) or both (middle column). When only low-RF-field RD data points are fitted (i.e., BMRD, second row in Figure 3) the fitted parameters ν and p_{B} are poorly defined and, trivially, the fluctuation angle θ_{D} cannot be obtained. Similarly, when fitting only data obtained near the rotary resonance condition ($\omega_1=20\text{--}33\text{ kHz}$ at $\omega_{\text{MAS}}=35\text{ kHz}$, third row in Figure 3), isotropic CS differences are ill-defined, but the fluctuation angle θ_{D} can be confidently retrieved. Interestingly, population p_{B} and angle θ_{D} appear much less correlated in NERRD fits than are p_{B} and ν in BMRD fits (compare Figure 3D and I). When data from the entire range of RF fields are used in a joint fit, the dynamic parameters can be retrieved with high precision (first row in Figure 3 and Figure S1)

2.2 Insight into conformational dynamics in ubiquitin

Having shown the potential of combined BMRD and NERRD fits, we measured ^{15}N $R_{1\rho}$ RD profiles in microcrystalline ubiquitin at 23 RF field strengths from 2.25 kHz to 33 kHz, at a MAS frequency of 35 kHz. In order to suppress the effect that dipolar interactions to remote ^1H spins would have on the ^{15}N relaxation parameters, we used a highly deuterated sample, in which all non-exchangeable hydrogens were replaced by ^2H and the exchangeable sites (such as amides) were re-protonated to ca. 30% (see Methods section). When measuring the relaxation-dispersion profile, we explicitly avoided RF field strengths close to the $n=1/2$ rotary-resonance condition, i.e., around 17.5 kHz. At this condition the homo-nuclear ^{15}N - ^{15}N dipolar coupling contributes to the signal decay, and the quantitative analysis would require more complex models that are not within the scope of this study. The effects are discussed in Figure S4.

Ubiquitin is a convenient model system, because its μs dynamics has been characterized extensively by solution-NMR, solid-state NMR and MD simulations.[25,32,49–53] The

exchange process involves a flip of the peptide plane of D52/G53, along with reorientation of the side chain of E24, which is H-bonded in one of these two states, but not in the other state. In light of the nature of this process, one would expect that the largest RD effects are centered around residues G53 and E24.

Figure 4 shows the experimental RD profiles for selected residues with non-flat RD profiles. The residues neighboring the flipping peptide plane, D52 and R54, have indeed very strong NERRD effects, in agreement with the nature of the process, involving G53 peptide plane flip. The amide H-N sites of E24 and N25 are not observable in MAS spectra and HN(G53) is strongly overlapped and weak, possibly due to the line broadening effects induced by the exchange event, excluding these residues from quantitative analysis. The HN of I23, which is H-bonded to the carbonyl of R54 displays much lower NERRD effects than D52 and R54, but a larger BMRD effect, as one may expect from the fact that it is not itself involved in the peptide plane flip and that even subtle changes in H-bonding can lead to significant ^{15}N chemical-shift changes. Residues further away from this "epicenter" of the conformational exchange process have gradually smaller RD effects, both on the BMRD and the NERRD sides.

We have fitted a two-state model with four parameters pertaining to the exchange process (θ_D , k_{ex} , ν and p_B) to these $R_{1\rho}$ RD profiles. We have performed these fits either in a residue-wise manner, or in a global fit, where a common (k_{ex} , p_B) parameter set was assumed. The determined values for the residue-wise fit is shown in the panels in Figure 4. Exchange rate constants of ca. 2000 s^{-1} are found for the majority of HN sites in this cluster; some sites have higher k_{ex} , up to 20000 s^{-1} . The largest θ_D values are found for residues D52 and R54, adjacent to the flipping peptide plane, and are of the order of 20° . This value is in reasonable agreement with the difference in the orientations observed in crystal structures of the two exchanging state, which is ca. 30° , as found by comparing crystal structures of ubiquitin in these two states.[26] Residue-wise chemical-shift differences are largest for residues E52, D52, R54, T22 and I23, up to 6 ppm. A joint fit with $k_{\text{ex}}=2000\text{ s}^{-1}$ and $p_B=2\%$ is shown in Figure S6, and shows comparable values of ν and θ_D .

3 Conclusions

We have reported here how $R_{1\rho}$ RD profiles over a wide range of RF fields can be used to quantitatively describe μs conformational exchange processes in proteins, exploiting isotropic (chemical shift) and anisotropic (bond vector orientation) interactions. This study focused on ^{15}N in proteins, but the approach can readily be extended to other moieties. Numerical simulations reveal that the inclusion of NERRD data into RD analyses can provide more information than the use of BMRD data alone, in particular in the fast exchange regime ($k_{\text{ex}} \gtrsim 5000\text{--}10000\text{ s}^{-1}$), where BMRD data fail to disentangle the exchange parameters. An extensive set of new experimental RD profiles on ubiquitin determined in this study are in good agreement with the previously proposed mechanism of peptide-plane flip.

A remarkable finding of this study is the fact that in almost all cases observed experimentally the NERRD profile is larger than the BMRD profile. This observation

suggest that μ s motions are more robustly determined through NERRD effects than from BMRD profiles. Experimentally, however, NERRD measurements have their own challenges. First, the high RF field strengths required may lead to sample heating. In our case, using a 1.6 mm probe and RF fields of 33 kHz for ca. 50 ms at most, the RF-induced heating is below 2° in the presence of the highest ^{15}N RF field strengths employed here (data not shown). The high RF field strengths may also challenge the hardware. We find, however, that this is not a limitation for MAS frequencies of the order of 40-50 kHz, as in our hands ^{15}N RF fields up to 45 kHz can be handled for ca. 50 ms for measurements lasting days without any effect on pulse lengths or sensitivity (Bruker 1.3 mm probe; data not shown).

A more fundamental challenge related to NERRD measurements may arise from the recoupling of anisotropic interactions that lead to a fast oscillatory behavior (cf. Figure S3 and Krushelnitsky *et al.*[28]). As a consequence of this rapid oscillation (typically < 2 ms), which is not informative of dynamics,[28] the residual spin magnetization is reduced, and therefore the signal-to-noise ratio is reduced. This behavior is indeed reflected in the increased error bars of $R_{1\rho}$ data close to the rotary resonance condition in our experimental data (Figure 4). McDermott and co-workers have recently proposed schemes to suppress this CSA recoupling.[46] The use of such composite spin-lock schemes shall increase signal-to-noise in NERRD measurements. Fitting such data, however, requires a numerical approach, as no analytical solutions are available. The analysis scheme that we used here, based on numerical spin simulations, can readily incorporate such complex pulse sequences.

The drawback of the fit approach proposed here, however, is its fairly high computational cost, as it requires simulating and fitting a grid of $R_{1\rho}$ decays in five dimensions (k_{ex} , p_{B} , ν , θ_{D} , ν_1). The general idea proposed here, namely the joint fit of BMRD and NERRD data is not bound to the implementation with numerical spin simulations. As long as the multi-exponential nature of the decay is properly taken into account when fitting the experimental data,[28] a Redfield-theory-based numerical analysis approach, combined with a Bloch-McConnell-style treatment is equally applicable to analyzing BMRD and NERRD data. For standard continuous-wave $R_{1\rho}$ experiments, the analysis via such analytical equations is clearly beneficial, as it comes at much lower computational costs, and thus allows a more precise fit that is not bound to the potentially coarse grid of simulations. Implementations of joint BMRD and NERRD fits are on the way in our laboratory.

While the focus of this study is to show the principles and potential of such a joint fit, rather than the application, we have provided also a new data set on ubiquitin, which is more exhaustive than previously presented ones. It provides a comprehensive view of Bloch-McConnell and NERRD effects throughout the structural part that undergoes conformational exchange.

The present study highlights the detail at which conformational dynamics can be probed to date with MAS NMR RD measurements, providing insight into geometric details of the motion which are difficult to obtain with solution-state NMR methods. This study demonstrated the BMRD and NERRD fit with a fairly rigid crystalline protein, arguably an "easy" target. Recent applications show that this approach can also be applied to non-crystalline samples, such as amyloid fibers.[34,37] We have data on large protein

assemblies, with subunit sizes that exceed ubiquitin by 5-6 fold, and interestingly we obtain similar quality data. Relaxation data on GB1 in the context of a 300 kDa complex show that quantitative analyses of $R_{1\rho}$ data are possible even for more challenging case where sensitivity is limited. Furthermore, data on sites other than backbone- ^{15}N on such large systems, soon to be published, indicate that the approach can be applied to a variety of systems. We believe that the measurement and analysis scheme that we propose here, which involves measurement of $R_{1\rho}$ over a wide range of RF field strengths will likely become a routine tool in such studies.

4 Materials and Methods

Numerical simulations and fit procedures

Simulations of spin coherence evolution—All numerical simulations of the evolution of ^{15}N in-phase coherence were performed using a code written using the GAMMA simulation library.[54] which can be obtained from the authors or downloaded at <https://github.com/BandaIslands/NERRD>.—below A composite Liouville space is constructed from two ^1H - ^{15}N spin pairs, representing the two exchanging states (A, B), which may differ in the most general case by the ^1H and ^{15}N isotropic chemical shift and CSA tensors, as well as the orientation of the HN dipolar tensor. The populations of the two states and the exchange rate constant is a variable parameter in the simulation. The simulation used standard piecewise constant Hamiltonian evolution, using 100 time steps per rotor period. The spin evolution was sampled during 150 ms, which is similar to experimentally used maximum relaxation delays, and 656 time points were sampled. Powder averaging was performed according to the scheme of Cheng *et al.*,[55] using 100 orientations. The HN dipolar coupling tensor anisotropy was set to 22.49 kHz, corresponding to a bond length of 1.025 Å; the ^{15}N CSA tensor, assumed to be axially symmetric, had an anisotropy of 6600 Hz, corresponding to a $\sigma = \sigma_{zz} - \sigma_{xx}$ of 165 ppm at 14.1 T B_0 field strength; the ^1H CSA tensor was assumed axially symmetric with an anisotropy of 480 Hz, but simulations revealed that its exact tensor parameters have negligible effect. In all cases, the time evolution of N_x coherence was fitted using an in-house-written program in python language. In some cases numerical instabilities arise as a consequence of the initial oscillatory behavior, which in an experimental setting is dampened by the RF-field inhomogeneity.[28] In principle, the RF field inhomogeneity can be taken into account in simulations, but for reasons of computational efficiency such explicit averaging over RF field strengths was omitted here. Examples of time traces are shown in Figure S3.

For fits of exchange parameters to the experimental RD data (Figure 4) or to the *in-silico* RD profiles (Figures 2 and 3), a grid search procedure was chosen. Simulations of the coherence decay, as described above, were performed for a grid of (θ_D , k_{ex} , ν , p_B , ν_1) values. The grid dimensions ([lowest,highest],number of steps) for these five variables were ([0°,24°],11; [0 s⁻¹,50000 s⁻¹],16; [0 Hz,360 Hz],13; [0%,50%],8; [2.25 kHz,33 kHz],23) for the fits of the experimental data (Figure 4), and ([0°,36°],13; [0 s⁻¹,50000 s⁻¹],21; [0 Hz,600 Hz],16; [0%,50%],8; [1.75 kHz,33 kHz],28) for the fits of the simulated curves (Figures 2 and 3). Each of these simulations was fitted to a mono-exponential decay function to retrieve an apparent $R_{1\rho}$ value. We note here that in principle the decay is multi-exponential, and the

fitted value is thus not strictly reflecting the relaxation rate constant.[39] The rationale behind the approach is that the experimental decay curves were treated analogously (see below), and thus the simulated and experimental decay rate constants are comparable. The fitted rate constants were arranged in a five-dimensional matrix ($\theta_D, k_{ex}, \nu, p_B, \nu_1$). Each experimental RD profile ($R_{1\rho} = f(\nu_1)$) was compared to each of the simulated RD profiles across the grid, leaving as an additional parameter a vertical offset, $R_{1\rho}^0$ applied to all points of a given dispersion curve, which reflects contributions to $R_{1\rho}$ other than from μs motions. A χ^2 value was calculated for each grid point as the sum (over all RF field strengths ν_1) of the squared deviation between the experimental and simulated $R_{1\rho}$ weighted by the square of the experimental uncertainty. From the final four-dimensional matrix of χ^2 values, the lowest 10 values were considered; examples are shown in Figures S5 and S6. The python scripts and GAMMA simulation program have been deposited in Github at <https://github.com/BandaIslands/NERRD>.

Experimental methods

U- 2H , ^{15}N ubiquitin (residues 1-76, no His-tag) was prepared by bacterial overexpression in D_2O -based M9 medium with deuterated glucose and $^{15}NH_4Cl$ as sole carbon and nitrogen sources. Expression was induced by addition of 1 mM IPTG at 37°C and the cells were harvested 4 hours after induction. Cells were disrupted by sonication and the supernatant of the lysate was subjected to Q sepharose chromatography (40 mL column) and a Superdex 75 HiLoad 16/60 prep-grade column (GE). Fractions containing protein were pooled and dialyzed against distilled water, and lyophilized. Crystallization of the protein was performed with 1-methyl-2,4-pentanediol (MPD) in a buffer containing H_2O/D_2O at a ratio of 30:70, using a method described in detail in reference.[32] Protein crystals, obtained after ca. 1-2 weeks at 4°C, were filled into a Agilent 1.6 mm rotor.

1H - ^{15}N correlation experiments with a ^{15}N spin-lock RF field of incremented duration were recorded as described before[47] on a 600 MHz Agilent spectrometer. The cooling gas was set to -1°C, resulting in an actual sample temperature of 298 K at 35 kHz MAS, as determined from the bulk water frequency[56] relative to the frequency of the MPD peak at 4.1 ppm. We determined the amount by which the application of the ^{15}N RF field heats the sample, by measuring one-dimensional 1H spectra, preceded by a ^{15}N RF field of variable duration and amplitude. We find that the highest applied field strength (33 kHz, 60 ms) heats the sample by ca. 2-3°C. When measuring the high-RF-field RD profiles, we accounted for this RF-induced heating by lowering the cooling gas temperature to achieve the desired effected temperature (involving ca. 5° colder cooling gas in our setup). A compensatory ^{15}N RF block was inserted at the beginning of the recycle delay such that the total duration of irradiation, and thus the sample heating, was kept constant, irrespective of the spin-lock duration within the pulse sequence. Details about the measurement of the RF-induced sample heating and the effects of the recycle delay are presented in Figure S7.

The intensity decay over the spin-lock duration was followed by recording a series of 2D spectra for each RF field strength. The maximum spin-lock duration was 150 ms for the lower RF field strengths (up to 20 kHz, 12 data points per RF field strength), and gradually reduced for higher RF field strengths, down to 60 ms (11 data points) for the highest RF

field strength (33 kHz). The recycle delay was increased from 0.8 s for the low RF field strengths to 3 s for the highest RF fields (to avoid sample heating and hardware damage). More than 250 2D spectra were collected overall. Site-specific decay rate constants and error bars were obtained from mono-exponential fits of the $R_{1\rho}$ decays with the nLinLS module in NMRpipe[57] and Monte Carlo analysis.

All experimentally determined $R_{1\rho}$ rate constants (from mono-exponential fits) have been deposited in the BioMagResBank under accession number 27635.

Supplementary Material

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Figure 1.

Simulated $R_{1\rho}$ relaxation profiles for different two-site exchange scenarios, computed using in-house written programs based on the GAMMA simulation library and exponential fits of the simulated relaxation decays. In the two-site exchange model, a major and a minor state (populations p_A , $p_B = 1 - p_A$), exchange with a rate constant ($k_{ex} = k_{AB} + k_{BA}$), and they differ by their ^{15}N chemical shift (Δv) and H-N bond-vector angle (θ_D), as stated in the different panels. The panels in (A) consider separately the effects of Δv and θ_D , and a general case with both $\Delta v \neq 0$, $\theta_D \neq 0$ is shown in panel (B). In all simulations, a MAS frequency of 35 kHz was assumed, matching the experimental conditions used in this study.

Figure 2.

The populations and the chemical shift difference cannot be independently disentangled in the fit of two-site exchange. 200 $R_{1\rho}$ profiles were generated using a two-site exchange with values of p_B , k_{ex} , ν and θ_D as indicated above the panels. In the four different panels, different levels of random noise were added to the BMRD profiles. In agreement with the experimental errors shown in Figure 4, the noise added to each $R_{1\rho}$ was proportional to its value. The relative ratio of the noise is increasing from panel A to panel D as 1.0, 2.0, 3.0, 6.0 and panel B matches roughly to the ubiquitin measurements shown in Figure 4. The

noisy data sets were subjected to a grid search procedure, as described in the Materials and Methods section, and the size and color of the symbols denotes the number of fits that result in a given fitted (ν , θ_D) pair. At low experimental noise (panel A), the correct value of parameters ($p_B = 5\%$, $\nu = 240$ Hz) are found by the fit in the majority of cases ($>95\%$). But when the noise increases (panel D), the obtained values for the population p_B and the chemical shift ν become more scattered with a strong correlation between the 2 parameters.

Figure 3.

Fitting RD profiles from both the BMRD and the NERRD regimes improves the fit quality. The panels report the fitted exchange parameters in fits that used either only the low-field part of the RD profile (1.7 to 15 kHz RF field strength; denoted with a half-filled red square), or only the high-field part (20-33 kHz), or the entire range (1.7-33 kHz RF field strength; denoted with a full red symbol). These two-dimensional plots report on selected parameter combinations; all possible projections are shown in Figures S1 and S2. In each case, a series of 200 $R_{1\rho}$ RD profiles was generated with random noise added, akin to Figure 2. The exchange parameters assumed for these simulations are denoted on the top of the figure. In the left-hand column (panels A, D and G), only the chemical shift difference is active ($\theta_D = 16^\circ$), in the right hand one (panels C, F and I), only the fluctuation of the dipolar interaction ($\nu = 0$ Hz) and in the central one, both contribute ($\nu = 240$ Hz, $\theta_D = 16^\circ$). The 3 rows depict how accurately the various parameters can be determined, depending whether the full range of r.f. spin-lock field strength (1.7 to 33 kHz) is used (A, B, C) or only the low-field (1.7 to 15 kHz) (D, E, F) or only the high-field (20 to 33 kHz). The angular fluctuation of the dipolar tensor can be more accurately determined than the chemical shift difference (compare panel A and C). As expected, ν or θ_D cannot be evaluated if the range of rf fields where these interaction are active has not been sampled: see panel G for ν and panel F for θ_D .

Figure 4.

Experimental ^{15}N relaxation dispersion data obtained for selected residues on perdeuterated Ubiquitin at 298K. Data were recorded on a 600 MHz Agilent-Varian spectrometer at a MAS frequency of 35 kHz. The plotted on-resonance $R_{1\rho}$ (in red) were corrected from the chemical shift offset using the R_1 value measured independently. Data were processed using nmr-Pipe and the relaxation rates and experimental errors were obtained using nLinLS and associated software. The only residues that exhibit significant relaxation dispersion are clustered in two stretches in the protein (21 to 27 and 51 to 58) as shown on the Ubiquitin model. For comparison with the simulated data (solid blue line), a vertical offset of $R_{1\rho}$ accounting for other sources of relaxation than the two-site exchange was included and the best solution was chosen on the basis of the χ^2 value by systematic search. The best parameter set (populations p_B , exchange rate k_{ex} , chemical shift difference ω and angular fluctuation) is displayed for each residue. For each residue, several parameter sets corresponding to similar χ^2 value can be found, and the 10 best solutions for each residue are shown in Figure S5 (individual fits) and Figure S6 (joint fit of all 11 residues of the cluster). All data have been deposited in the BioMagResBank (accession number 27635).