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Capturing Excited States in the Fast-Intermediate Exchange Time-Limit in Biological Systems Using ^1H NMR Spectroscopy.

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Abstract

Changes in molecular structure are essential for the function of biomolecules. Characterization of these structural fluctuations can illuminate alternative states, correlating their structure to function. NMR Relaxation Dispersion (RD) is currently the only method for detecting these alternative, high-energy states. In this study, we present a versatile ^1H $R_{1\rho}$ RD experiment that extends the exchange timescales at least 3x beyond the rate limits of $^{13}\text{C}/^{15}\text{N}$ $R_{1\rho}$ and 10x for CPMG-experiments and adds easily accessible probes, thus allowing a general description of biologically important excited states. We can extract chemical shifts for structural characterization of excited states and elucidate complex excited states. With this experiment we increase the toolbox for characterization of biomolecular dynamics and overcome many previous limitations, allowing for a more widespread application/characterization of biologically relevant excited states for nucleic acids and proteins.

Over the past two decades, nuclear magnetic resonance (NMR) has opened the way to understanding how molecular dynamics can explain biological function. Methodological developments, along with new labeling schemes, have revealed that molecules can undergo conformational changes and access discreet, low-populated excited states (ES). It has been shown that inter-conversion between ground (GS) and excited states are essential for biomolecular function, such as molecular recognition, ligand binding, enzyme catalysis or protein folding.¹⁻⁶ These structural rearrangements on the micro- to millisecond timescale are invisible to conventional structural methods and can therefore only be studied using NMR relaxation dispersion (RD) experiments.⁴ Most commonly studied nuclei have so far been ^{13}C or ^{15}N , whose relaxation dispersion is usually measured using either CPMG- or $R_{1\rho}$ -based techniques. The latter measure the relaxation rate $R_{1\rho}$ as a function of the radiofrequency field to quantify the exchange contribution allowing the description of thermodynamic and kinetic parameters of the ES (populations, pES, and exchange rates, k_{EX}) as well as the chemical shift differences $\Delta\omega$ that are directly linked to the structural properties of the ES. In contrast to CPMG methods, measuring $R_{1\rho}$ allows convenient detection of more than one ES and determination of the associated chemical shift differences including the sign.

Figure 1. Building blocks of A) RNA (A: Adenine, U: Uracil), B) protein (numbering according to IUPAC⁹). $^1J_{\text{CH}}$ couplings^{10,11} of the ^1H investigated (orange) are indicated in blue and C) ^1H - ^{13}C HSQC of the aromatic region of RNA with assignments for adenines and guanines indicated, inset shows secondary structure of the 25 nucleotides RNA construct. RD profiles of highlighted nucleotides in Fig. 3.

Thus, NMR ^{13}C or ^{15}N $R_{1\rho}$ RD pulse sequences, measuring relaxation rates in the rotating frame as a function of spinlock (SL) power and SL offset, are advantageous. Thanks to technical improvements allowing cryoprobes to handle higher SL powers,¹² $R_{1\rho}$ RD has been able to extend the accessible exchange timescale over CPMG techniques, but is still limited due to the small gyromagnetic ratio and the recommended r.f. limits of cryoprobes¹³ (~100 ms SL at 4 kHz for ^{13}C and 3 kHz for ^{15}N , Bruker). ^1H , with its large magnetic moment, is an ideal nucleus to probe and measuring ^1H $R_{1\rho}$ RD increases the independent number of probes. Importantly a wider range of exchange rates can be covered (with SL up to 20 kHz (Bruker) exchange rates up to 50 kHz can be covered), allowing us to characterize conformational exchange faster than 10 kHz accurately, for the first time in RNA. ^1H $R_{1\rho}$ measurements allow us to detect chemical shift differences between GS and ES. These ^1H chemical shifts are very sensitive to structural changes and many studies have been dedicated to correlating them to secondary and tertiary structures.¹⁴⁻²⁰ Difficulties of using ^1H $R_{1\rho}$ can be caused by cross-relaxation mechanisms originating from dipolar interactions with other protons that can complicate the relaxation behavior,²¹ therefore relatively few studies have been dedicated to it.²¹⁻²⁵ Addressing these issues and taking advantage of the benefits, we propose a versatile ^1H $R_{1\rho}$ RD experiment that probes conformational dynamics at ^1H positions connected to ^{13}C and ^{15}N in nucleic acids and proteins to elucidate ES in the fast-intermediate exchange range. We present results obtained for different types of ^1H spin systems, specifically H5, H1' and H8 in RNA and for H $\square$ positions in proteins (Fig. 1A & B), without the need for selective ^{13}C or ^2H labeling.²⁶ The pulse sequence (Fig. 2) is built on ideas from Lundström,²⁷ Weininger,²⁵ Eichmüller²¹ and Hansen.²⁸

Figure 2. 2D ^1H $R_{1\rho}$ RD pulse sequence for characterizing chemical exchange in proteins and nucleic acids. Filled (open) rectangles correspond to 90° (180°) hard pulses. Pulses are applied along x unless specified. Phase cycle is $\phi_1=(x,-x)$ and $\phi_{\text{rec}}=(x,-x)$ for data acquisition in phase-sensitive mode. Delay τ is set to $1/4J_{CH}$. Purge gradients (0, 2, 3, 4) and refocusing gradients (1) are applied for a duration of $650\text{ }\mu\text{s}$ and $600\text{ }\mu\text{s}$, respectively, with the strength (G/cm):

$g_0=42$, $g_1=11$, $g_2=35$, $g_3=80$, $g_4=20.1$. During the pulse sequence, anti-phase magnetization (created at point a) is aligned by adiabatic ramps along the ^1H spinlock axis and brought back along the z direction after the relaxation period T_{EX} (point b). Overlapping signals are resolved through the second dimension where anti-phase ^{13}C magnetization evolves (point c). Magnetization is returned to in-phase ^1H for detection (point d). Experiments are performed for incremented T_{EX} and at different spinlock powers. Detailed explanation and the pulse programs, for both Bruker and Varian spectrometers, are available in SI.

For probing different ^1H , the delay τ ($=1/4J_{\text{CH}}$), as well as the adiabatic decoupling on the ^{13}C channel during t_1 for the removal of ^{13}C homonuclear couplings of the attached carbon, have to be modified (Table S1, SI). Protons at ^{15}N positions can be probed by interchanging the ^{13}C and ^{15}N channels. Potential artefacts that could arise from pulse sequence elements (such as water suppression and efficiency of the adiabatic ramps flanking the SL pulse) and/or from interfering mechanisms during the spinlock (such as ^1H - ^1H Hartmann-Hahn transfers, rotating-frame Overhauser (ROE) cross-relaxation and J-coupling evolution) have been carefully investigated and have been proved to be insignificant or manageable when measuring ^1H $R_{1\rho}$ with the pulse sequence proposed in Figure 2 and within the proposed range of SL strengths (Fig. S5-10 & Table S2), as shown by well-behaved mono-exponential relaxation decays.

To demonstrate the scope of the developed pulse sequence, we investigated ES in an RNA as well as a protein sample. ^1H $R_{1\rho}$ RD experiments were recorded on uniformly ^{13}C and ^{15}N labelled samples of the 25 nucleotide tip of H44 of the ribosome and the N-terminal lobe of the regulatory domain of the protein calcium dependent protein kinase 3 from *P. falciparum*. (sample preparation and assignments in SI).

Dispersion profiles were recorded for spinlock strengths ranging from 0.8 kHz to 16.8 kHz. For each spinlock strength, peak intensities were measured as a function of T_{EX} durations (SI for fitting procedures) and mono-exponential decays were obtained (Fig. 3C & Fig. S11-17), indicating that no additional relaxation mechanism is observable during the ^1H spinlock. On-resonance 2D $R_{1\rho}$ RD experiments on RNA ^1H were performed and compared to ^{13}C $R_{1\rho}$ data, recorded with a selective 1D pulse sequence²⁸ (Fig. 3 & Table S2). R_2+R_{EX} as a function of the effective field is presented except in cases where the R_1 contribution cannot be determined or ignored. There, $R_{1\rho}$ as a function of the effective field is shown. The results for different

nucleotides are shown in Figure 3. Flat profiles, with $^1\text{H}R_{1\rho} = 29.19 \pm 0.04$ Hz and $^{13}\text{C}R_2 = 29.39 \pm 0.13$ Hz, as expected for the less dynamic RNA G21 base, were observed in both nuclei RD curves, proving the reliability of the NMR pulse sequence developed (Fig. 2, Table S4) (RD curves were fitted to a model excluding chemical exchange as confirmed by F-test).

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A8C8

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Figure 3. On-resonance C8 (left) and 2D H8 (right) RD profiles for the RNA bases (highlighted in Fig. 1) A) G21, B) G16 and C) A8 (inset: monoexponential decays at 1.2 and 14 kHz SL strengths). Solid lines correspond to best fits to an exchange-free (A) and a two-state fast exchange model (B and C). Details in SI.

For the residue G16 (Fig. 3B), dispersion was observed for both ^1H and ^{13}C nuclei. Individual fitting of the data resulted in exchange rates of $k_{EX}^{13\text{C}} = 2169 \pm 84$ Hz and $k_{EX}^{1\text{H}} = 1858 \pm 926$ Hz. As the k_{EX} obtained are comparable, a global fit was performed leading to a $k_{EX}^{glob} = 2168 \pm 78$ Hz (fitting in Table S2) and confirmed by F-test, showing that $^1\text{H} R_{1\rho}$ RD measurements complement ^{13}C data, allowing a more precise description of conformational exchange.

In case of the bulged out nucleotide A8, where the most interesting dynamics are expected as it is involved in an A-minor motif, attempts at fitting the ^{13}C $R_{1\rho}$ RD curve failed, indicating that exchange processes are faster than the timescale ^{13}C RD is sensitive to, evading characterization of the ES (Fig. 3C). Conversely, data obtained through ^1H $R_{1\rho}$ RD measurements resulted in a measureable $k_{EX}^{1H} = 16457 \pm 326$ Hz, clearly indicating the relevance of ^1H $R_{1\rho}$ RD experiments for extending accessible timescales. A global analysis allowed fitting both profiles, resulting in $k_{EX}^{glob} = 16208 \pm 423$ Hz and allowing, using ^1H $R_{1\rho}$ RD, to extend timescales of ^{13}C $R_{1\rho}$ RD to characterize ES structures.

To characterize the chemical shift difference and sign for excited states in A8H8, a selective 1D off-resonance version of the pulse sequence was developed. A8H8 displayed a three-state exchange process (Fig. 4) defined by the following excited states parameters: $k_{EX1} = 10535 \pm 1577$ Hz, $p_{ES1} = 0.28 \pm 0.17$ %, $\Delta\omega_1 = -1372 \pm 194$ Hz, $k_{EX2} = 14801 \pm 1149$ Hz, $p_{ES2} = 0.68 \pm 0.19$ % and $\Delta\omega_2 = 1263 \pm 164$ Hz.

Figure 4. Off-resonance ^1H $R_{1\rho}$ RD curves obtained for A8H8 at 1.2 (red), 1.5 (blue) and 2 kHz (green). Solid lines correspond to the three-state Laguerre model fitting and allowed for chemical shift extractions to characterize the ES's.²⁹ Uncertainties are expressed by 1-sigma error bars.

To prove that the method can be adapted to a variety of protons in biomolecules, in fact any single proton attached to any labelled hetero-nucleus, ^1H $R_{1\rho}$ RD experiment was performed on $\text{H}^\square$ in proteins (Fig. S2, S3 & Table S3). As for RNA, prominent $\text{H}^\square$ dispersions were observed for certain residues, indicating conformational exchange in this part of the protein, while residues, which are not involved in any dynamics within the accessible time window can be identified by flat dispersion profiles. (Fig. 5)

Figure 5. ^1H $R_{1\rho}$ RD curves obtained for the protein residues D417, K472, Y437 and I438 (Structure in Fig. S3). RD curves were individually fitted, using the same procedure as for ^1H in RNA (fitted parameters available in SI Table S3). Data points with large error bars are measured far off-resonance.

In conclusion, the new ^1H $R_{1\rho}$ RD methodology, presented here, allows identification of conformational exchange in a variety of biomolecular systems – from proteins over DNA to RNA – with no selective isotopic labelling requirement. This will lead to the first characterizations of excited state structures in the fast-intermediate exchange limit. ^1H $R_{1\rho}$ RD easily extends the accessible chemical exchange timescale to 50 kHz, illuminating faster processes and allows for characterization of single and multiple excited states via extraction of the complete chemical shift information. The method we present here is widely applicable, less time consuming than versions carried out on hetero-nuclei and easy to implement. It has therefore the potential to vastly expand the number of studies of dynamics on biomolecules carried out, shedding a light on the complex interplay between structure and function.

Kommentarad [PL1]: I would prefer single proton attached to ^{13}C . A reviewer might point out that there already are (superior) experiments for ^1H N.

Katja: I understand your point. I still think we should be able to say it here. And one can argue that measuring RD with one pulse sequence ($^{13}\text{C}/^{15}\text{N}$ attached ^1H) will make the rates more comparable...

I would argue that Nikolais experiment, if performed for other tilt-angles than 35° , is more or less equivalent to ours except that it employs sensitivity enhancement. However if you want to keep it this way I am ok with that.

Kommentarad [m2]: We should maybe also add A, B, ... panel labels in this figure.

Katja: Absolutly. Can you adjust the figure?

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