Communication

Exceeding the limit of dynamics studies on biomolecules using high spin-lock field strengths with a cryogenically cooled probehead

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\textbf{A B S T R A C T}

Internal motions in the microsecond timescale have been proposed to play an active part in a protein’s biological function. Nuclear magnetic resonance (NMR) relaxation dispersion is a robust method sensitive to this timescale with atomic resolution. However, due to technical limitations, the observation of motions faster than \(\sim40\) \(\mu\)s for \(^{15}\)N nuclei was not possible. We show that with a cryogenically cooled NMR probehead, a high spin-lock field strength can be generated that is able to detect motions as fast as 25 \(\mu\)s. We apply this high spin-lock field strength in an NMR experiment used for characterizing dynamical processes. An on-resonance rotating-frame transverse relaxation experiment was implemented that allows for the detection of a 25 \(\mu\)s process from a dispersion curve, and transverse relaxation rates were compared at low and high spin-lock field strengths showing that at high field strengths contributions from chemical exchange with lifetimes up to 25 \(\mu\)s can be removed. Due to the increase in sensitivity towards fast motion, relaxation dispersion for a residue that undergoes smaller chemical shift variations due to dynamics was identified. This technique reduces the previously inaccessible window between the correlation time and the relaxation dispersion window that covers four orders of magnitude by a factor of 2.

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1. Introduction

Internal motions in proteins are tightly related to their particular function. These motions occur in a broad range of timescales from ps to seconds and slower. NMR has been used to measure the kinetics [1-4] of such changes and the structural interconversions [5,6] associated with this except for the kinetic window between the globular rotational correlation time and the fastest kinetics observed with relaxation dispersion (around 40 \(\mu\)s for \(^{15}\)N nuclei). This window, depending on the correlation time of the studied protein, spans approximately four orders of magnitude. Yet, motion in this timescale has recently been proposed to play a role in molecular recognition [1,5,7]. It could be accessed only by lowering the temperature dramatically thus slowing the kinetics down that they became detectable by relaxation dispersion [1,8]. Therefore, we set out to expand the accessible kinetic range of relaxation dispersion as much as technically feasible.

Relaxation dispersion experiments are sensitive to motions that occur on the micro- to millisecond timescale provided that the isotropic chemical shift is modulated by this motion with a sufficient amplitude [3]. The essence of this method lies in the possibility of removing the effect of the modulated isotropic shifts from the induced motion by the application of refocusing or spin-lock pulses with a certain average frequency of rotation (\(\gamma B_1\)) [9].

In particular, rotating-frame transverse relaxation experiments using spin-lock pulses have been ideally suited to probe chemical exchange originating from motions within the microsecond timescale [9-11]. However, due to technical limitations on useable field strengths of spin-lock pulses, a time resolution for the observation of motions is restrained to processes slower than \(\sim40\) \(\mu\)s (\(^{15}\)N nuclei) corresponding to (\(\gamma B_1\))^\(-1\) [12]. We have been able to surpass this limit by utilizing a cryogenically cooled NMR probehead (cryo-probehead) [13] which is a tool commonly used in many NMR facilities. We demonstrate that with a cryo-probehead high spin-lock field strengths for \(^{15}\)N nuclei can be safely applied and used to extend the time resolution to 25 \(\mu\)s and for the observation of motions that induce smaller chemical shift variances potentially allowing observation of faster motions in proteins and accurate characterization of parameters that define the dynamic process.

2. Results

2.1. Measurement of spin-lock fields on a cryo-probehead

We investigated the limit of a cryo-probehead (Bruker QCI) by measuring the highest spin-lock field strength that can be applied
without endangering the intactness of the probehead. Off-resonance continuous wave decoupling during the acquisition time \( (t_{2,\text{max}} = 122.1 \text{ ms}) \) in a \(^{15}\text{N}, ^{1}\text{H}\)-HSQC was performed on a 600 MHz spectrometer [12]. We started to measure the spin-lock field strength by applying the recommended specification for a cryo-probehead (approximately 2 kHz, corresponding to a minimum life time of 80 \( \mu \text{s} \)); black) and increased the spin-lock field strength as shown in Fig. 1. The integrity of the cryo-probehead was secured by ensuring that the coil’s reserve power did not drop below 5%, and that the temperature of the coil did not change during the application of the spin-lock pulse. In this way, we could achieve a spin-lock field strength of 6.4 kHz (Fig. 1; blue) probably because of the cooling of the coil. This exceeds also the recommended specification for a room-temperature probehead which is approximately 4 kHz (Fig. 1; red) and limited to this value since the coil is not cooled [14]. The accurate extraction of the kinetics of the chemical exchange process from relaxation dispersion measurements also relies on the signal to noise of the NMR measurement. This is lower by a factor of 2–3 in a room-temperature probe. Thus, for optimal sensitivity we have to compare the 6.4 kHz with the so far specified value of 2 kHz, constituting a 3.2-fold increase of the field strength.

### 2.2. Validation for relaxation dispersion experiments

We then applied this high spin-lock field strength to an on-resonance rotating-frame transverse relaxation \( (R_{1\text{p}}) \) experiment (Fig. 2) on the same cryo-probehead [15]. Since the maximal kinetic rate of an exchange process detectable by the on-resonance \( R_{1\text{p}} \) experiment is proportional to the applied spin-lock field strength, only motions slower than \( \sim 80 \mu \text{s} = (2\pi \times 2 \text{ kHz})^{-1} \) \( (\sim 40 \mu \text{s} = (2\pi \times 4 \text{ kHz})^{-1} \) could be detected using the previously recommended specification of a spin-lock field for a cryo-probehead of 2 kHz (4 kHz on a room-temperature probehead). With the newly achieved high spin-lock field strength of 6.4 kHz, fast motions around 25 \( \mu \text{s} = (2\pi \times 6.4 \text{ kHz})^{-1} \) are accessible with an on-resonance \( R_{1\text{p}} \) experiment. In order to control sample heating from different employed spin-lock field strengths a control scheme was implemented in which the same power was used in all experiments [16]. For each experiment, the length and strength of the employed spin-lock and the maximum power that would be used for the dispersion measurement at the highest spin-lock field were considered and a variable duration temperature control block was applied with the highest power at the end of acquisition for every collected transient. This scheme was effective in controlling the fluctuation in temperature when field strengths were varied as flat dispersion curves were obtained (see Fig. S1 for an example). A recycle delay was chosen that kept the duty cycle at 5%.

Using a \(^{15}\text{N}\)-labeled ubiquitin sample at 277 K, we measured relaxation dispersion for residues Ile13, Thr55, and Val70, which have been previously reported to show dispersion [1,17,18]. We used an on-resonance \( R_{1\text{p}} \) experiment [15] in which the spin-lock field strength was varied from 1 to 6 kHz allowing for residues that undergo chemical exchange to be modulated solely by the utilized spin-lock field strength (Fig. 2). Using a fast exchange model, values for exchange lifetime \( (\tau_{\text{ex}}) \) (chemical shift variance \( (\Phi) \) of 50 ± 9 \( \mu \text{s} \) \( (29 \pm 7 \times 10^{3} \text{ rad}^{2} \text{ s}^{-1}) \), 51 ± 5 \( \mu \text{s} \) \( (53 \pm 7 \times 10^{3} \text{ rad}^{2} \text{ s}^{-1}) \), and 33 ± 5 \( \mu \text{s} \) \( (112 \pm 38 \times 10^{3} \text{ rad}^{2} \text{ s}^{-1}) \) for Ile13, Thr55, and Val70, respectively, were obtained. They are in good agreement with previous studies [1,17,18]. When the previously recommended specification of spin-lock field strength for a cryo-probehead (up to 2 kHz; hashed box in Fig. 2) was used for fitting the dispersion data we could not achieve reliable fitting results due to the fact that the changes in the effective relaxation rate (\( R_{2\text{eff}} \)) of residues are very small \( R_{2\text{eff}}^{\text{Ile13}} = R_{2\text{eff}}^{\text{Thr55}} = 0.26, 0.64, \) and 0.43 s\(^{-1}\) for Ile13, Thr55, and Val70, respectively).

Measuring up to a spin-lock field strength specification for a room-temperature probehead (4 kHz; dashed line in Fig. 2), now performed on a cryo-probehead, gave changes in \( R_{2\text{eff}} \) that were
large enough to provide fits that agree with literature values [1,17]. In order to evaluate the dependency between the quality of the fits and the field strengths, we recorded a dispersion curve of Val70 with a total of 51 spin-lock field strengths up to 6 kHz and conducted a jackknife procedure for the fitting. Eleven field strengths up to 2, 4, and 6 kHz were randomly selected 500 times and then subsequently fit to a fast-exchange model. As stated before, data up to 2 kHz could not provide a reliable fit. The average values and errors from the runs yielded $\Phi$ and $\tau_{ex}$ of $163 \pm 149 \times 10^3$ rad$^2$ s$^{-2}$ and $32 \pm 10 \mu$s $(101 \pm 58 \times 10^3$ rad$^2$ s$^{-2}$ and $36 \pm 10 \mu$s) for 4 (6) kHz. Evidently, the use of higher field strengths gives fits with lower errors.

In addition, we measured relaxation dispersion for residue Gln49, which is involved at the binding interface for ubiquitin associated interactions [19,20] and has not been previously identified when spin-lock field strengths lower than 6 kHz were utilized (Fig. 2) [1,17,18]. Although smaller changes in $R_{2,eff}$ are observed (an order of magnitude smaller than Ile13), it was possible to extract $\tau_{ex}$ and $\Phi$ of $101 \pm 32 \mu$s and $7.1 \pm 1.2 \times 10^3$ rad$^2$ s$^{-2}$, respectively. Comparison of experimental $\Phi_{Gln49}$ with $\Phi_{Ile13}$ yields a ratio of $4.1 \pm 1.7$, which compares well with ratios predicted from the published ubiquitin ensembles [1,5,7] ranging from 3.8 to 1.5 (Table S1).

2.3. Application to the accurate determination of transverse relaxation rates

In addition to dispersion type experiments, high spin-lock field strengths can be used to quantify transverse relaxation rates ($R_2$) [11,21]. Typically, $R_2$ together with longitudinal relaxation rate ($R_1$) and heteronuclear NOE is used to obtain information of motions faster than the overall tumbling motion of a macromolecule (the Lipari–Szabo order parameter, $S_L$) [22,23]. For this analysis, an $R_2$ experiment which ideally suppresses exchange contributions ($R_{ex}$) to $R_{2,eff}$ is highly desirable. Residual $R_{ex}$ contributions systematically disturb the analysis and the fitting procedure for $R_2$ [24,25].

Thus, the higher spin-lock field strength would provide more accurate $R_{ex}$ free $R_2$ values since motions slower than 25 $\mu$s, as described earlier, can be refocused by using a -6 kHz spin-lock field.

We compared $R_{2,eff}$ values with spin-lock field strengths of 1 and 6 kHz on a $^{15}$N-labeled ubiquitin sample at 277 K. From the 72 potentially observable sites, 46 resonances provided sufficient intensity and frequency separation in either the $^1$H or $^{15}$N dimension to be reliably queried with the employed selective off-resonance experiment (Fig. 3A) [15]. Their differences can be found in the Supplementary Material (Fig. S2). Fig. 3B shows $R_{2,eff}$ as being scaled by $S_{LS}$ [26]. This reveals $R_{2,eff}$ without the impact of motions faster than the overall tumbling time of ubiquitin and isolates the effect of exchange on $R_{2,eff}$. Although this scaling does not account for diffusion anisotropy, ubiquitin has only small rotational anisotropy [25] and therefore, the effect of this motion has nearly a uniform effect for all $R_{2,eff}$. The variances of $R_{2,eff}/S_{LS}^2$ using 6 kHz (0.57 s$^{-2}$) and 1 kHz (0.95 s$^{-2}$) spin-lock field strengths, show a reduction by 40% (Fig. 3B). Also, the average $R_{2,eff}/S_{LS}^2$ is smaller for 6 kHz as compared to 1 kHz as expected from the more efficient removal of exchange contributions.

3. Discussion and conclusion

We have shown that cryogenically cooled NMR probeheads can generate higher spin-lock field strengths than previously specified without endangering the intactness of the probehead. The newly achieved high spin-lock field strength (~6 kHz) makes chemical exchange kinetics down to 25 $\mu$s accessible using an on-resonance $R_{1P}$ experiment. Using $^{15}$N nuclei we could achieve a factor of two improvement in the time resolution. Since sampling of the dispersion curve over larger $\gamma_B$ ranges improves the analysis, motion that induces smaller chemical shift variances can be observed. This revealed dynamics in Gln49 of ubiquitin that were previously not observed.

Adapting the use of a cryo-probe for nuclei with larger gyromagnetic ratios [27–30] could also further enhance the currently set time resolution of 25 $\mu$s, and is our current focus for future development. In principle, off-resonance $R_{1P}$ experiments can be conducted by combining large spin-lock fields and offsets which will yield even greater effective fields and thus provide dynamic information of motions even faster than 25 $\mu$s [10]. For example, an offset of 16.5 kHz provides an effective field of 17.5 kHz using a spin-lock field strength of 6 kHz. With this large effective field, motions slower than 9 $\mu$s could be detected. However, with this large offset, only 12% of exchange induced chemical shift variance contributes to the relaxation dispersion. Thus, the chemical shift variance induced by motions has to be large enough to observe this dynamics using off-resonance $R_{1P}$ experiments. The detection of small chemical shift variations could be improved by the cryo-probe detection making this off-resonance experiment especially attractive for observation of fast timescale motion. Furthermore, multiple quantum double resonance experiments, which was shown to be applicable for the studies of motions up to 50 $\mu$s [18], can be used to explore further insight into dynamics that have smaller conformational amplitudes by combining the large spin-lock fields demonstrated here. We have also shown that the newly introduced high spin-lock field strength allows for better refocusing of exchange processes with lifetimes up to 25 $\mu$s, thereby suppressing chemical exchange processes that are inherent to the
transverse relaxation rate. The high spin-lock field can only be achieved with a cryo-probehead probably because of the cooling of the coil absent in normal temperature probeheads, which in addition provides a 2–3-fold sensitivity gain [31] compared to a room-temperature probehead thus making this extension to faster kinetics even more feasible.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jmr.2012.05.005.

References


