# 2D NMR Spectroscopy of Refolding *RNase Sa* using Polarization Transfer from Hyperpolarized Water

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**Abstract**

Polarization transfer from hyperpolarized water through proton exchange is used to enhance the NMR signals of amide protons of the *Ribonuclease Sa* protein. Spectra of the refolding protein are measured within 6 s after dilution of the denaturant urea, at urea-dependent folding rates adjusted in the range of 0.3 – 0.8 $s^{-1}$. Peak patterns including a mixture of folded and unfolded protein at different ratios are observed. The changes in the observed signals indicate that each spectrum accesses a different point in the partial completion of the folding. A comparison to simulated 2D NMR spectra suggests a lower polarization transfer efficiency from water when the protein folds slowly, which may result from the molecular motions in the unfolded protein and the absence of long-range contacts. The ability to acquire 2D NMR spectra under different refolding conditions may open a new avenue for residue specific characterization of the folding process.

**Keywords**

Hyperpolarization, Dynamic Nuclear Polarization, Protein Folding, 2D NMR

## Introduction

Current models describing protein folding emphasize the possibility of folding through multiple routes [1]. The determination of the presence or absence of intermediate structures can provide a key to understand protein folding mechanisms. NMR presents important advantages for the characterization of protein structure, including the ability to work in solution [2]. Towards the protein folding problem, ideally, intermediate structures would be characterized at atomic resolution, on an experimental time scale that is shorter than the lifetime of the corresponding structures. NMR structural information on folding intermediates can be obtained by quenching the process of the folding before signal acquisition. Hu et al. have demonstrated the solid-state 2D NMR measurement of a short-lived transient intermediate, trapped by rapid freezing of a protein during the folding [3]. In hydrogen exchange pulse labeling experiments, $^{1}H$ is introduced to initially unfolded protein in $D_2O$ at a defined time during folding. Information on intermediate structures can be obtained from a read-out of the resulting $^{1}H$ distribution in the folded protein by 2D NMR [4,5]. Folding intermediates may alternatively be characterized by accelerating the NMR data acquisition itself. Instead of 2D NMR, the measurement of a series of 1D $^{1}H$ NMR spectra can be completed within the timescale of the folding, leading to the identification of transient intermediates [6]. However, 1D NMR also reduces the amount of information obtained from each spectrum. The real-time measurement of multi-dimensional spectra can be achieved using pulse sequences with rapid data acquisition, either using rapid sequential scans [7–9] or spatial encoding [10,11]. Using sequential scans in SOFAST-HMQC, for example, the structural changes of a protein that occur on the timescale of several minutes can be monitored [9].

Direct NMR measurements at a faster time scale can be achieved through hyperpolarization of nuclear spins. With an orders of magnitude enhancement in NMR sensitivity, hyperpolarization methods allow the measurement of signals in one, or a small number of scans. Using chemically induced dynamic nuclear polarization (CIDNP), the signals of solvent-exposed aromatic residues can be enhanced to monitor changes in the solvent accessibility as a protein refolds [12]. Dissolution dynamic nuclear polarization (D-DNP) can be used to hyperpolarize $^{13}C$ spins of polypeptides directly [13]. The folding of the protein can then be followed in real-time over several seconds [14]. Additional information on the dynamics of partially folded states is available from spin relaxation that may be measured on the same time scale [15]. Alternatively, hyperpolarization can be introduced to proteins

through proton exchange from hyperpolarized water [16,17]. Different observed exchange rates provide information on solvent exposure. Transferred polarization from water results in signal enhancements of amide protons on proteins, allowing the fast measurement of 2D NMR spectra of intrinsically disordered proteins [18] and folded protein [19] within several seconds. We have recently used hyperpolarized water to facilitate the measurement of 2D NMR spectra of *Ribonuclease Sa* (*RNase Sa*) in the folded state, as well as during the folding [20]. The refolding of the protein was initiated by the dilution of denaturant upon admixing the hyperpolarized water. Spectra measured under folded and under re-folding conditions both showed a peak pattern corresponding to the folded protein, indicating that the folding is almost complete within the experimental time. However, a difference in measured signal enhancement profiles between folded and refolding conditions was observed, which was attributed to the different dynamic motions and molecular contacts during the folding.

Here, we demonstrate the measurement of 2D NMR spectra with polarization transfer from hyperpolarized water to the protein at partial completion of folding. A variable concentration of the denaturant urea permits the tuning of the folding rate of *RNase Sa*. The effect of denaturant on the folding rates of this protein has been previously characterized using fluorescence spectroscopy [21]. Multiple folding rate constants were found, which suggests that folding intermediates may exist. In general, after a rapid dilution of a denaturant to below the denaturation mid-point, the equilibrium distribution of protein conformations shifts towards favoring the folded form. The folding rate depends on the final concentration of denaturant, whereby a higher concentration results in slower folding.

## Materials and Methods

*Sample Preparation*

*RNase Sa* was expressed recombinantly in *E. coli* transfected with pEH 100 plasmid following the protocol described [22]. The protein was purified using cation exchange chromatography, followed by a size exclusion chromatography step for polishing. Samples were lyophilized. Samples of folded or unfolded *RNase Sa* were prepared by dissolving the protein in $D_2O$ buffer (200 mM sodium phosphate, pH 7.0), or in the same $D_2O$ buffer containing 8 M urea-$d_4$. The nominal protein concentration was 2.4 mM, and exact concentrations were determined using UV-Vis spectrophotometry (molar extinction

coefficient of ε = 11,701). For chemical shift calibration, 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; Cambridge Isotope Laboratories, Andover, MA) was included in these samples. For water hyperpolarization, a stock solution of $H_2O$/DMSO-$d_6$ (v/v 1:1) mixture containing 15 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPOL; Sigma-Aldrich, St. Louis, MO) was prepared.

*Hyperpolarized NMR*

For the measurement of an enhanced spectrum of the folded protein, a total of 100 µL of the water sample was hyperpolarized in a HyperSense DNP polarizer (Oxford Instruments, Abingdon, U.K.). Hyperpolarization occurred by irradiating with microwaves (100 mW power, 94.005 GHz frequency) at a temperature of 1.4 K. After 20 min, the sample was dissolved by 4 mL of dissolution solvent (200 mM sodium phosphate in $D_2O$, pH 7.0) preheated until reaching a vapor pressure of 8 bar. The dissolved sample was automatically injected into a 5 mm NMR tube, which was pre-installed in a 9.4 T NMR magnet (Bruker Biospin, Billerica, MA). In the NMR tube, a volume of 100 µL of folded protein solution was preloaded and mixed with hyperpolarized water. Sample injection [23] was performed with nitrogen gas at 1772 kPa, against 1034 kPa back pressure during $t_{inj}$ = 408 ms. The NMR measurement was automatically initiated after a delay time, $t_{stab}$ = 500 ms.

For measuring spectra under refolding conditions, 100 µL of unfolded protein solution was preloaded in the NMR tube. $D_2O$ buffer (200 mM sodium phosphate, pH 5 – 6) containing 1 – 4 M urea-$d_4$ was used as a dissolution solvent. After mixing with the unfolded protein in 8 M urea, the final concentration of urea ranged from 1.4 to 3.3 M. The dilution of urea initiated the refolding process. It is noted that adding urea in the dissolution solvent causes a pH change in the final sample due to the hydrolysis of urea during the heating process, which results in the formation of ammonia. Therefore, the final sample pH of 7.0 was reached by lowering the pH of the dissolution buffer to 5.0.

2D NMR spectra enhanced through hyperpolarized water were measured following the previously described procedure [20], with minor modifications (Figure S1). In the first scan of the NMR experiment, a hard pulse with small flip angle (~0.01°) was applied to determine the initial polarization of water protons. The signal from this scan was compared to the water signal that was measured after the decay of the hyperpolarization. Subsequently, a [$^{15}$N,$^{1}$H]-HMQC pulse sequence with echo/anti-echo gradient selection was applied to obtain a 2D NMR spectrum of the protein. Selective excitation

and inversion of the amide protons were achieved with PC90 [24] (6.26 ms, 120° flip angle) and RSNOB [25] (1.9 ms, 180° flip angle) pulses centered at 8.5 ppm and covering a bandwidth of ±1.5 ppm to avoid excitation of the hyperpolarized water and urea signals. Pulsed-field gradients were used for coherence selection and to suppress unwanted signals such as from urea, which would saturate the receiver. A total of 40 × 3632 data points were acquired, with acquisition times of $t_{1,max}$ = 16 ms and $t_{2,max}$ = 90 ms for the $^{15}$N and $^{1}$H dimensions, respectively. The center frequency of $^{15}$N was set at 118 ppm, and the corresponding spectral width was 30 ppm. WALTZ-16 decoupling was applied on the $^{15}$N channel with $\gamma B_1$ = 0.7 kHz during the acquisition. The total acquisition time for the experiment was 6 s. The start of folding, $t$ = 0 s, was estimated to occur at half of the injection time ($t_{inj}$), thereby the start and end of the experiment were at $t$ = 0.7 s and $t$ = 6.7 s, respectively. After the DNP experiment, the final concentrations of water and urea were determined by comparing the signals of water protons and urea carbon with those of reference samples containing pure water and 8 M urea. All DNP NMR spectra were acquired using a triple-resonance inverse detection probe (TXI; Bruker Biospin), at a temperature of 304 K. This temperature was equal to the temperature of the dissolved sample previously determined using a thermocouple. All DNP NMR data were processed to yield magnitude spectra using Topspin software (Bruker Biospin), and then imported into the CARA software package [26] for peak integration. The final sample conditions and experimental parameters are summarized in Table S1.

*Reference spectra*

For the comparison with DNP spectra, reference spectra of samples under equilibrium conditions were measured without hyperpolarization. A total of 5 samples of 0.5 mM $^{15}$N-labeled *RNase Sa* with 0, 1.9, 2.4, 3.1 and 8 M of urea were prepared in 90 % $H_2O$ and 10 % $D_2O$ buffer (200 mM sodium phosphate, pH 7.0). The urea concentration in each sample was determined from the corresponding $^{13}$C signal. [$^{15}$N,$^{1}$H]-HSQC spectra were acquired with 512 × 2048 data points and acquisition times of 140 ms and 136.5 ms for $^{15}$N and $^{1}$H, respectively. The center frequency of $^{15}$N was at 118 ppm, and the spectral width was 36 ppm. A relaxation delay of 1 s, and 8 scans per increment were used. These spectra were measured on a 500 MHz NMR spectrometer equipped with a triple resonance TCI cryoprobe with a z-gradient (Bruker Biospin), at 304 K.

## Results and Discussion

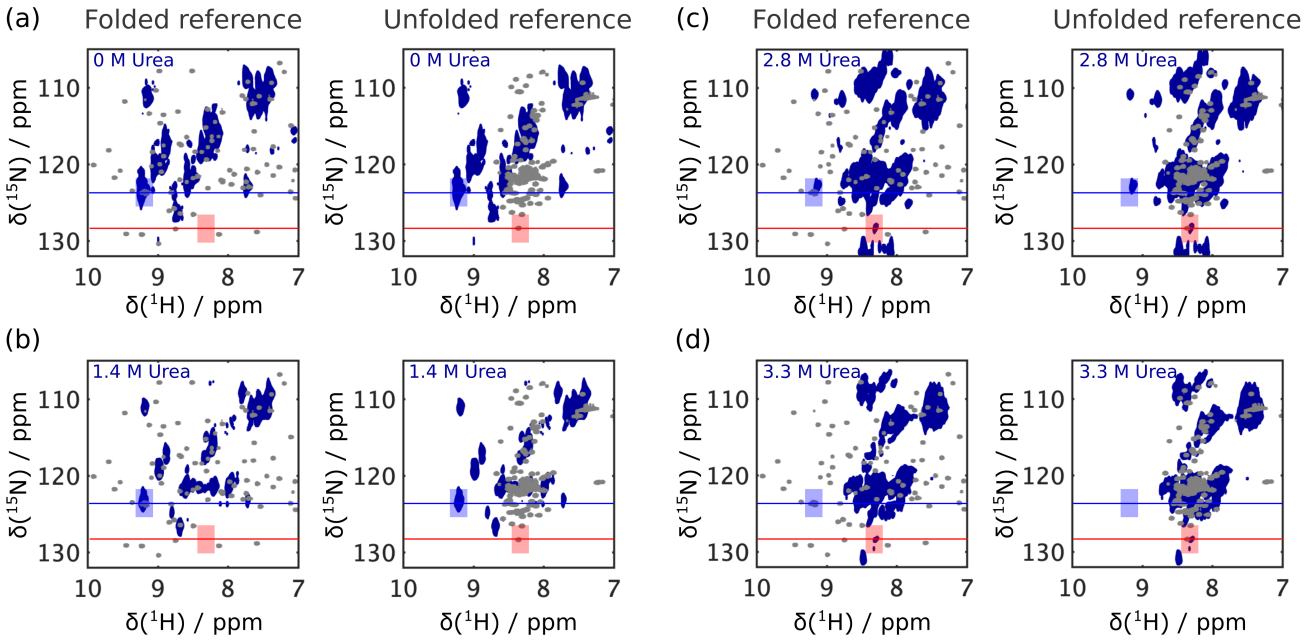


**Figure 1.** Enhanced [$^{15}$N,$^{1}$H]-HMQC spectra of (a) folded and (b-d) refolding *RNase Sa*, measured after the addition of hyperpolarized water (blue, shown in duplicate in the left and right panels). Non-hyperpolarized reference spectra from samples of folded (left panels) and unfolded *RNase Sa* (right panels) in 90 % protonated buffer are shown in gray. The identity of the reference spectra is indicated with lettering "Folded reference" and "Unfolded reference" above the panels. The spectra of refolding protein were measured at different urea concentrations of (b) 1.4 M, (c) 2.8 M, and (d) 3.3 M. In the total of four DNP experiments, the averaged protein concentration and final $^{1}$H content were 0.4 ± 0.1 mM and 6 ± 1 % in $D_2O$ buffer. The spectra are shown as the magnitude of the complex valued signals. The lines and boxes colored blue (δ($^{15}$N) = 123 ppm) and red (δ($^{15}$N) = 128 ppm) indicate the positions of 1D slices, which are shown in Figure 2.

Figure 1 shows a series of 2D [$^{15}$N,$^{1}$H]-HMQC spectra of *RNase Sa* measured after dilution with hyperpolarized water. The folding rate of the protein was modulated at different concentrations of urea. For the spectrum shown in Figure 1a, the protein maintained its native state before and after the addition of hyperpolarized water. The spectra shown in Figures 1b – d were measured after dilution of initially unfolded *RNase Sa* with a larger volume of the dissolution buffer containing hyperpolarized water and urea. In all cases, during the measurement time of 6 s, water hyperpolarization continuously transferred to the exchangeable protons of the protein, resulting in signal enhancements of protein resonances. The transferred polarization results in direct signal enhancements of amide protons, and

can further spread within the protein through spin diffusion mediated by the nuclear Overhauser effect (NOE) [17]. Here, with a $T_1$ relaxation time of water in HDO buffer of 4.5 ± 0.2 s, the acquisition of NMR spectra is completed before the decay of the hyperpolarized water signal.

In the spectra under refolding conditions, different patterns of enhanced peaks are visible depending on the final urea concentration. Most of the observed signals coincide with peaks in the reference spectra of either folded or unfolded protein (gray in Figure 1b – d). At a higher final urea concentration, fewer signals of folded protein and a larger number of signals of unfolded protein are observed. This difference is because the folding occurs at a slower rate when the urea concentration is higher. Specifically, when the spectrum at the low concentration of 1.4 M of urea is compared with the reference spectrum of the folded protein (Figures 1b), the overall pattern of signals is similar to that of the folded protein, but new signals appear near 8.2 ppm / 123 ppm. These signals correspond to unfolded residues. In the spectrum at the higher concentration of 2.8 M urea, the overall pattern is closer to that of the unfolded protein, except for two peaks at 9.2 ppm, which belong to the folded protein (Figure 1c). Finally, the spectrum at 3.3 M urea shows a peak pattern corresponding to unfolded protein (Figure 1d).

The spectra of refolding protein (Figure 1b – d) can further be compared to the reference spectra measured at different urea concentrations under equilibrium. All of these reference spectra, which were measured with a urea concentration of up to 3.1 M, show a peak pattern corresponding to the folded protein (Figure S2). They indicate that the protein at equilibrium is folded in its native form under these conditions. On the other hand, the DNP spectra show peak patterns corresponding to a mixture of folded and unfolded proteins, with different ratios depending on the urea concentrations. This comparison results in the conclusion that the DNP experiment occurs under non-equilibrium conditions, and provides the capability to measure transiently observable NMR spectra.

The peak patterns of the spectra of refolding protein in the current experiments are different from those in previously described experiments incorporating polarization transfer from hyperpolarized water. The previously recorded spectra showed a peak pattern of the folded protein both under folded and under refolding conditions (Figure 1 of ref [20]). With the refolding initiated at a urea concentration of 0.8 M, a folding rate on the order of 1.1 $s^{-1}$ [21] resulted in completion of the folding. In contrast, in the present experiments, the refolding rates are varied between 0.3 – 0.8 $s^{-1}$, which was

achieved by adjusting the urea concentration in the range of 1.4 – 3.3 M [21]. The resulting spectra show peak patterns depending on the urea concentrations, corresponding to partially completed folding.

The fractions of folded and unfolded proteins in each spectrum can be estimated based on the known folding rate of the protein. The NMR experiment extends over a time between 0.7 s and 6.7 s after dilution of urea. Based on the folding rates mentioned above, the calculated fraction of the folded protein at the start of the experiment would be 43 % at 1.4 M urea, and 18 % at 3.3 M urea. At the end of the experiment, these fractions would be in the range of 99 – 85 %.

Comparing the spectrum of folded protein (blue in Figure 1a) to the corresponding previous measurement (Figure 1a of ref [20]), it is evident that the signals of several peaks are absent in the current spectrum. This difference is in part because of line broadening at faster proton exchange rates in the present experiments, which were measured at a higher pH. Due to this effect, the peaks showing fast exchange rates such as G66, V2, and G61 are not observable in both DNP and the reference spectra measured here. Some additional signals, which are seen in the reference spectrum, are not observed in the present DNP-NMR spectrum. These include signals from the residues T56, V57, and I58. As these peaks previously showed relatively weaker signals, the disappearance of these signals may result from a 1.6 times lower signal-to-noise ratio under the present experimental conditions.

Although most of the observed peaks in the DNP spectra appear in the same positions as peaks in the reference spectra of either folded or unfolded protein, some additional peaks are seen in the spectra at the intermediate urea concentrations of 2.8 M and 3.3 M (Figure 1c and d). For example, the peak at 8.7 ppm / 112 ppm is present neither in the reference spectra of folded nor unfolded protein. On the other hand, some observed peaks are overlapped with peaks of either folded or unfolded residues, but the appearance and disappearance of these peaks as a function of the urea concentration do not match what would be expected from the overall peak pattern changes in Figure 1b – d. An additional peak at 8.9 ppm / 114 ppm in Figure 1c is close to signals from folded protein, but the urea concentration dependence of this peak intensity is different from that of a folded residue. All of the above described additional peaks may result from the existence of a transient intermediate, or from the interaction between urea and the unfolded fraction of the protein. Both of these effects would be uniquely observable under non-equilibrium conditions in the hyperpolarized experiments.

The changes in peak intensities of folded and unfolded protein as a function of urea concentration are most clearly visible by comparing the peaks obtained from 1D slices of the DNP spectra (Figure 2). The intensity of the peak at 9.2 ppm / 123 ppm, which belongs to the residue S31 in the folded protein, becomes smaller when the urea concentration is increased (Figure 2a). Under the same conditions, the peak at 8.3 ppm / 128 ppm, corresponding to the unfolded protein, appears (Figure 2b). The opposite trend of the signal changes between folded and unfolded peaks is consistent with the observation of peak patterns as a function of the urea concentration.

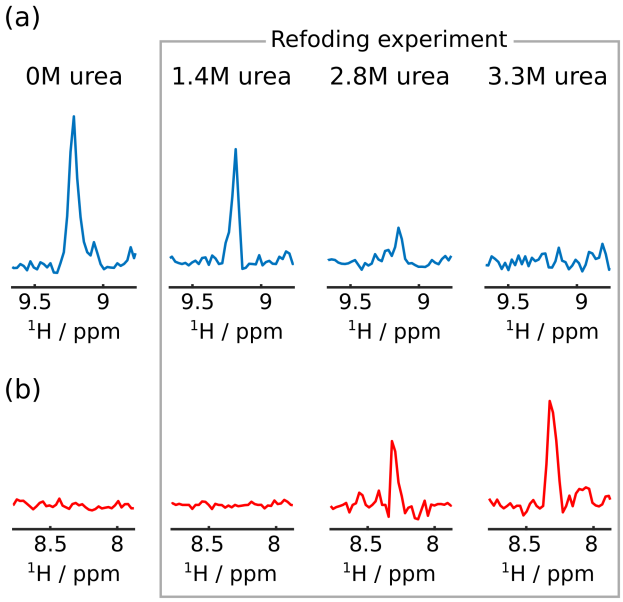


**Figure 2.** 1D slices at (a) $\delta(^{15}N)$ = 123 ppm for the folded residue and (b) $\delta(^{15}N)$ = 128 ppm for the unfolded residue. The exact positions of 1D slices are marked in Figure 1 with boxes and lines. Each 1D slice was normalized by the initial enhancements of water and protein concentrations.

Although many residues both for folded and unfolded proteins are observed in the spectra, not all are individually identifiable due to signal overlap. Including the peaks selected in Figure 2, a total of 4 peaks from folded and 2 peaks from unfolded proteins were identified for peak integration, to observe intensity changes as a function of urea concentration. The positions of the integrated peaks are marked in Figure S3.

The signal intensities of all the selected peaks as a function of urea concentration are plotted in Figure 3. However, these intensities are not directly proportional to the fraction of protein in each state because the magnitude of polarization transfer from water contributes to the observed signal intensity. As polarization is transferred from water to amide protons of proteins through proton exchange and the NOE, observed signals can be correlated with the solvent accessibility and the local correlation time of the receiving spins on a protein [17,19,20].

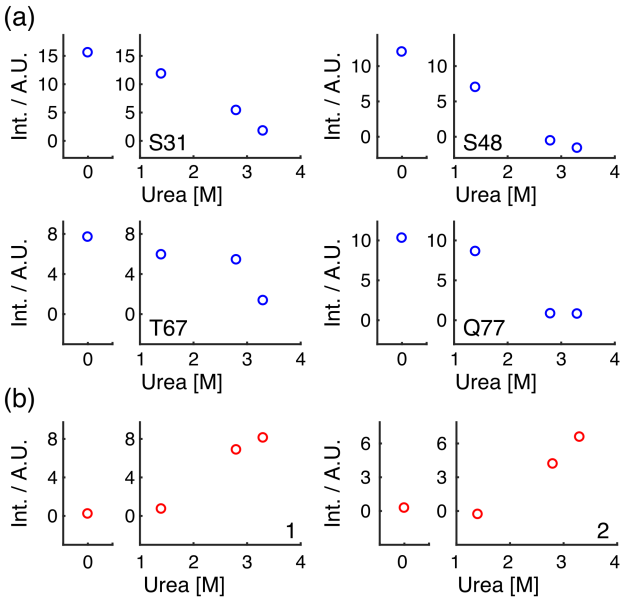


**Figure 3.** Intensities as a function of urea concentration for (a) residues belonging to folded protein and (b) peaks corresponding to unfolded protein. The intensities were normalized by the initial water enhancements and the concentrations of the protein. The data points at 0 M Urea were measured with initially folded protein. For the other data points, the protein was initially unfolded.

The effects of the change in fractions of folded and unfolded proteins during the measurement time on the NMR spectra were investigated by simulating 2D NMR spectra under changing concentrations and signal amplitude due to decay of the original water hyperpolarization (Figure S4-5 and Table S2). Because the changes in concentration are slow compared to the acquisition of a single scan, these simulations assumed that concentrations remained constant in each scan. Thereby, no lineshape effects are considered in the acquired $^{1}$H dimension. However, the changing concentrations from scan to scan affect the indirectly acquired $^{15}$N dimension. For signals of the folded protein, the increase in the

fraction of the folded form as time progresses results in a larger amplitude of the data points corresponding to longer indirect time evolution. The opposite occurs for signals of the unfolded protein (Figure S4 a and d). As a result, the signal of the folded protein after Fourier transform is not in-phase (Figure S4b). This effect becomes larger as the refolding rate is increased. On the other hand, an increased folding rate merely causes a broader linewidth of the signals from unfolded protein. Because it is not possible to correct the phase for peaks of both folded and unfolded protein at the same time, magnitude spectra may be shown (Figure S4c,f and Figure S5).

When the urea concentration is increased from 1 to 4 M, the simulated intensity of the folded peak decreases, while that of the unfolded peak increases (Figure S6), similar to the intensity changes observed from the experiments. Nevertheless, the relative intensity of the simulated signal for the folded peak at a high urea concentration is larger than that of the D-DNP experiments. For example, the simulated intensity at 3.3 M urea is more than half compared to that at 1 M urea, but the intensity of residue S31 at 3.3 M urea obtained from the experiment is close to zero (Figures S6 and 3a).

The comparison between simulations and experiments suggests that the polarization transfer from water to protein is less efficient when the protein folds slowly. This observation is opposite to what may be expected based on the higher solvent accessibility of an unfolded protein, compared to a folded protein. However, the observation is in line with our previous work [20], where we also observed less polarization transfer for the protein undergoing refolding compared to the folded state. It is also in agreement with the results from Szekely et al. [27], who observed a similar phenomenon under the equilibrium condition where both folded and unfolded states coexist. In addition to other relaxation effects not considered in the simulation, suggested explanations for this phenomenon include differences in molecular motions between folded and unfolded proteins, the existence of long-range cross-relaxations, and the possible presence of structural water in the folded form.

By varying the final denaturant concentration, the folding rate for *RNase Sa* in this work was tuned in the range of 0.3 – 1.1 $s^{-1}$. These folding rates are compatible with the acquisition of 2D SOFAST-[$^{15}$N,$^{1}$H] HMQC spectra with a number of increments sufficient for resolving amide groups of the protein. Future improvements may include the application of spectroscopic techniques for resolution enhancement, such as non-uniform sampling [28,29].

Folding rate constants of similar order are typical for proteins that undergo processes such as the formation of disulfide bonds or trans/cis interconversion of prolines [30,31]. These proteins can be interesting targets for attempting to identify intermediate structures. Future developments of the experimental setup may aim at changing the temperature of the solvent after dissolution but prior to injection and mixing through contact with a heated or cooled heat exchanger. This would allow a change of the folding rate by a factor depending on the activation energy, thus further broadening the range of folding processes that are observable.

## Conclusions

In summary, we have measured enhanced 2D NMR spectra of *RNase Sa* using polarization transfer from water hyperpolarized by dissolution DNP. After initiating the folding of the protein by dilution of urea to different final concentrations, transient NMR spectra were observed at different folding rates. Differences in the signals stemming from folded or unfolded protein observed in these spectra correlated to the progress of folding under each condition. A comparison between simulated and experimental data further indicated that the polarization transfer rate to the protein is reduced when the protein folding occurs at a slow rate. This observation may be attributed to the molecular dynamics in the unfolded protein. Observing the polarization transfer from hyperpolarized water combined with the ability to tune the urea-dependent folding rate allows accessing structural and molecular properties of the protein at different points of completion of folding. This capability opens a new avenue for the residue specific characterization of protein folding through real-time NMR.

## Acknowledgments

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**Supporting Information**

*NMR Pulse Sequence*

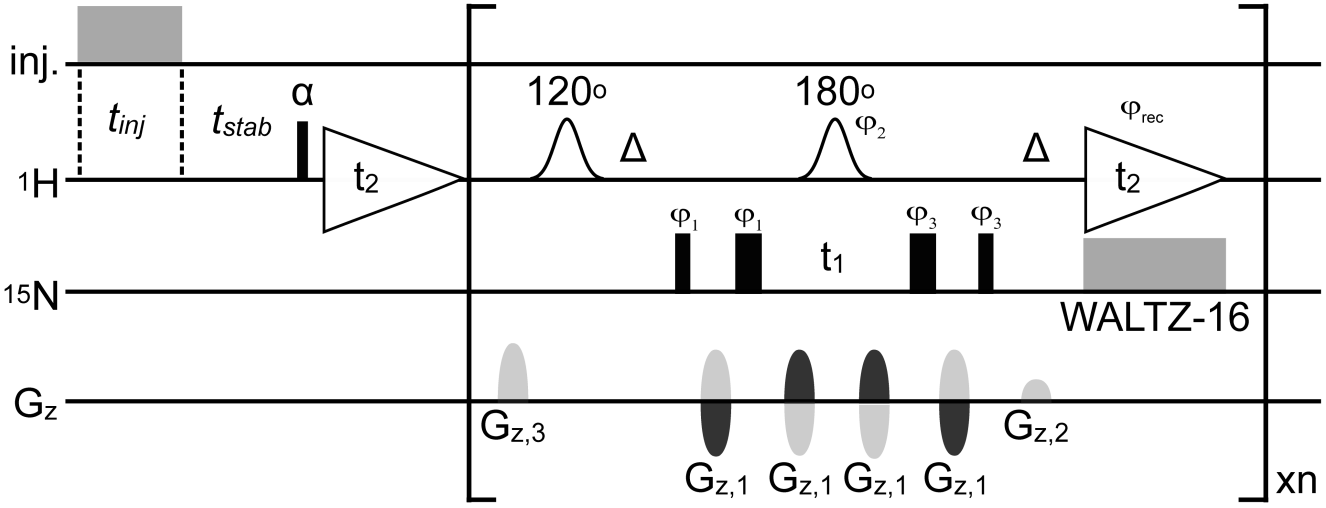


**Figure S1.** [$^{15}$N,$^1$H]-HMQC pulse sequence. Shapes represent 120° and 180° selective pulses. Narrow and wide black bars indicate 90° and 180° hard pulses, unless denoted as flip angle α = 0.01°. The sample injection was carried out during injection time, $t_{inj}$ = 408 ms and the acquisition was started after the stabilization time, $t_{stab}$ = 500 ms. Pulsed-field gradients were applied for 1 ms with $G_{z,1}$ = 54.5 G·cm$^{-1}$, $G_{z,2}$ = 22.1 G·cm$^{-1}$ and $G_{z,3}$ = 68.1 G·cm$^{-1}$. Echo and antiecho data sets were recorded with opposite sign of $G_{z,1}$. The phase cycle was $\varphi_1$ = x,-x, $\varphi_2$ = 2x,2(-x), $\varphi_3$ = 4x,4(-x), and $\varphi_{rec}$ = 2(x,-x),2(-x,x).

*Sample Conditions and Reference Spectra*

**Table S1.** Summary of the final sample conditions and experimental parameters from the DNP experiments. The signal enhancement of water, ε, was determined by comparing the peak integral from 1D $^{1}$H spectrum acquired in the first scan of the DNP experiment with the integral from that measured after the decay of hyperpolarization.

| Urea [M] | Final pH | Protein Conc. [mM] | ε | $H_2O$ [M] | $T_{1,water}$ [s] |
|---|---|---|---|---|---|
| 0.0 | 7.0 | 0.34 | 443 | 4.44 | 4.8 |
| 1.4 | 6.9 | 0.36 | 443 | 2.78 | 4.4 |
| 2.8 | 6.9 | 0.60 | 336 | 3.33 | 4.4 |
| 3.3 | 7.0 | 0.44 | 258 | 3.33 | 4.5 |

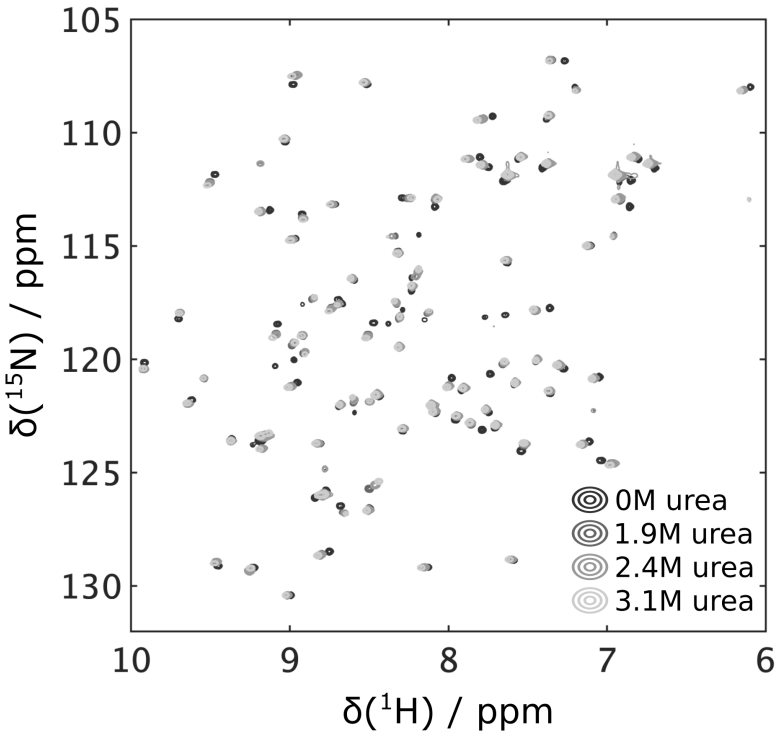


**Figure S2.** Overlaid reference [$^{15}$N,$^{1}$H]-HSQC spectra of *RNase Sa* measured under equilibrium without hyperpolarization. Solutions of 0.5 mM $^{15}$N-labeled *RNase Sa* were prepared under conditions close to those used in the DNP experiments but in 90 % protonated solvent.

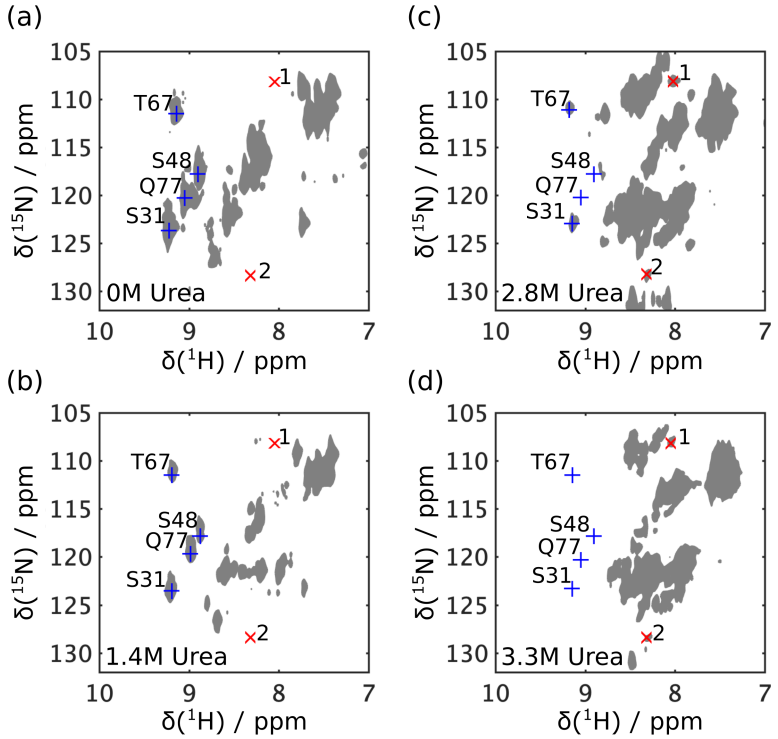


**Figure S3.** DNP [$^{15}$N,$^{1}$H]-HMQC spectra of *RNase Sa*, shown in Figure 1, with marked positions for peak integration. Peaks corresponded to the residues of the folded protein are denoted as blue +, while those of the unfolded protein are indicated as red ×. Peaks from folded protein are identified by the residue number and the type, and for the unfolded protein with unknown assignments are numbered.

*Simulations of Spectra*

As the protein folding progresses during the acquisition of 2D NMR spectra, the fractions of folded and unfolded proteins change depending on the folding rate under the experimental condition. Effects of this change on the resulting 2D spectra were examined by performing a simulation using Matlab (Mathworks, Natick, MA). In this simulation, the fraction of folded and unfolded protein was calculated at the beginning of each scan for the indirect dimension of the 2D spectrum. Folded and unfolded resonances were assumed to be located at frequency offsets ($^{1}$H / $^{15}$N) = (-200 Hz / -200 Hz) and (200 Hz / 200 Hz), respectively. The polarization transfer from water was assumed to be uniform, with water signal decaying with a time constant $T_1$ = 4.5 s. Because the acquisition time was short compared to the folding time constant, exchange effects occurring during each signal acquisition were not considered. The simulation was carried out using the same spectral width, acquisition time, and number of data points as in the experiments. $T_2$ relaxation times of the amide proton and the amide $^{15}$N, active during the acquisition period $t_2$ and the indirect evolution period $t_1$ were calculated based on average molecular parameters and used in the simulation. As a reference, a 2D spectrum was simulated under a condition, where the fractions of folded and unfolded proteins were maintained as a constant during the measurement time. In processing, no window function or zero filling was applied.

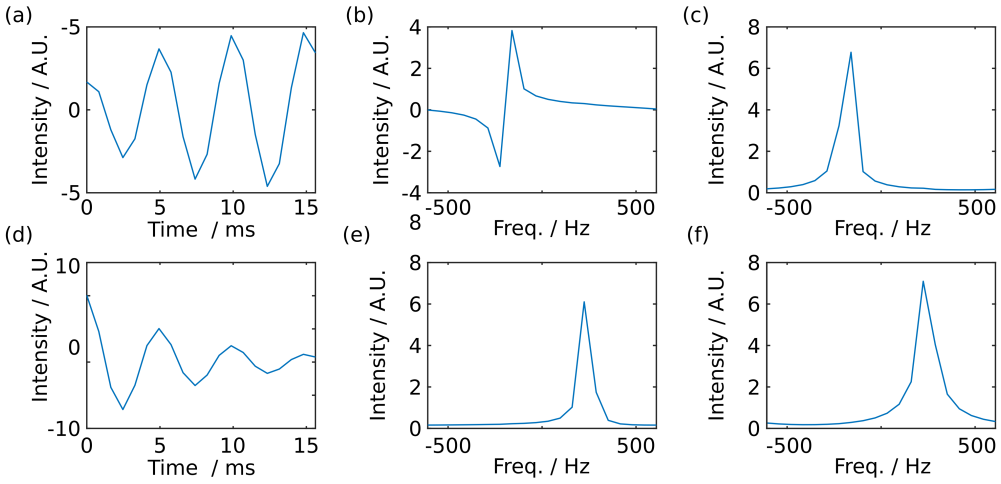


**Figure S4.** Simulated free induction decay (FID) in the indirect dimension and Fourier transform spectra using phase and magnitude modes for the resonance of folded (a – c) and unfolded protein (d – f) under the refolding condition with the rate of 0.4 $s^{-1}$. In this simulation, folded and unfolded resonances were assumed to be located at -200 Hz / -200 Hz and 200 Hz / 200 Hz, respectively.

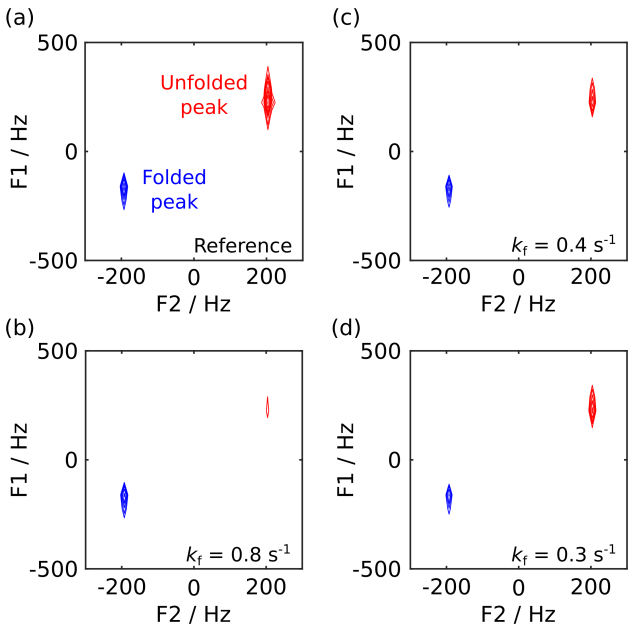


**Figure S5.** Simulated 2D NMR spectra under different conditions. (a) Reference spectrum simulated under the condition that the fractions of folded and unfolded proteins are maintained as a constant during the measurement time. (b – d) Simulated spectra under refolding conditions with different folding rates, $k_f$.

**Table S2.** Expected changes in linewidth and peak intensity under reference and refolding conditions ($k_f$ = 0.8, 0.4, and 0.3 s$^{-1}$), obtained from the simulation of 2D spectra.

| | Ref. | $k_f$ = 0.8 s$^{-1}$ | $k_f$ = 0.4 s$^{-1}$ | $k_f$ = 0.3 s$^{-1}$ |
|---|---|---|---|---|
| $\nu_{1/2}$ (folded, Hz) | 90 | 93 | 96 | 97 |
| $\nu_{1/2}$ (unfolded, Hz) | 90 | 163 | 118 | 106 |
| Rel.int (folded) | 1.0 | 0.9 | 0.7 | 0.6 |
| Rel.int (unfolded) | 1.0 | 0.1 | 0.3 | 0.4 |

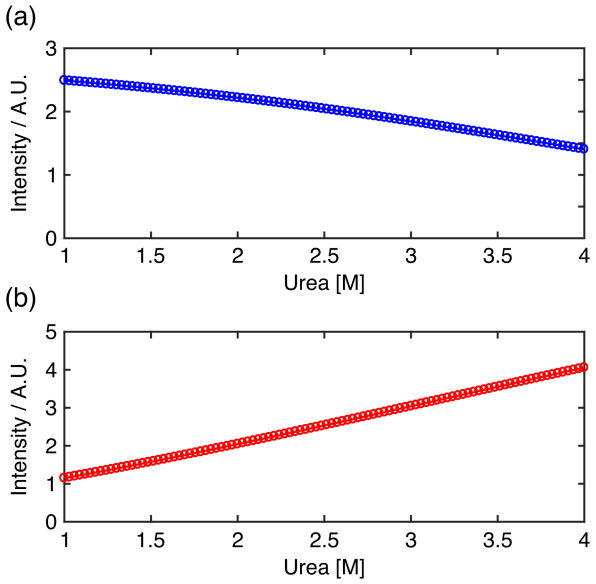


**Figure S6.** Intensities for (a) folded and (b) unfolded peaks as a function of urea concentration obtained from the simulated 2D NMR spectra.