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^1H -Detected REDOR with Fast Magic-Angle Spinning of a Deuterated Protein

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Abstract

Rotational echo double resonance (REDOR) is a highly successful method for heteronuclear distance determination in biological solid-state NMR, and ^1H detection methods have emerged in recent years as a powerful approach to improving sensitivity and resolution for small sample quantities by utilizing fast magic-angle spinning (>30 kHz) and deuteration strategies. In theory, involving ^1H as one of the spins for measuring REDOR effects can greatly increase the distance measurement range, but few experiments of this type have been reported. Here we introduce a pulse sequence that combines frequency selective REDOR (FSR) with ^1H detection. We demonstrate this method with applications to samples of uniformly ^{13}C , ^{15}N , ^2H -labeled alanine and uniformly ^{13}C , ^2H , ^{15}N -labeled GB1 protein, back-exchanged with 30% H_2O and 70% D_2O , employing a variety of frequency-selective ^{13}C pulses to highlight unique spectral features. The resulting, robust REDOR effects provide (1) tools for resonance assignment, (2) restraints of secondary structure, (3) probes of tertiary structure, and (4) approaches to determine the preferred orientation of aromatic rings in the protein core.

TOC image

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Supporting Information

Supporting Information includes two figures. Fig. S1 demonstrates the disadvantage of applying REDOR π -pulse trains on ^1H channel. Fig. S2 shows plots and fits of ^1H T_2 of six residues in GB1 protein.

Introduction

Magic-angle spinning (MAS) solid-state NMR (SSNMR) spectroscopy is a powerful technique for elucidating the structures of insoluble proteins, especially amyloid fibrils and membrane proteins, and small molecule ligand-bound biological complexes^{1–7}. Three-dimensional (3D) structure determination of proteins generally relies upon large numbers of semi-quantitative distances and is greatly enhanced by the availability of quantitative distance restraint measurements. Rotational echo double resonance (REDOR) is a well-known SSNMR method for measuring heteronuclear distances^{8–9}, and it has been used extensively for studies of small molecules, proteins and bimolecular complexes^{10–12}. Analogous TEDOR approaches have been developed¹³ and applied to the determination of high-resolution protein structures^{14–15}. The measurable distance depends on the gyromagnetic ratio (γ) of the nuclei. Since ^1H has the highest gyromagnetic ratio of any stable isotope, the longest theoretical distance range for REDOR would be achievable with ^1H - ^{19}F pairs, as demonstrated by Hong and co-workers¹⁶ utilizing low- γ nucleus detection, followed by ^1H - ^{31}P and ^1H - ^{13}C .

Utilizing ^1H as the detected nucleus in such REDOR cases potentially adds another advantage of increased sensitivity. Significant technological advances in the field of SSNMR over the last decade have enabled the routine use of ^1H -detected experiments for biomolecular structure determination^{17–20}. The two main factors that have advanced ^1H detection are (ultra)-fast magic angle spinning (MAS), ranging from 30 to >110 kHz²¹ and complete or partial deuteration of the protein^{21–22}. The ^1H line width is inversely proportional to the spinning rate, and so even fully protonated samples yield sub-ppm line widths at 30 to 40 kHz MAS^{17,23}. Deuteration and partial back-exchange with H_2O further reduce the average ^1H - ^1H dipolar couplings and in combination with fast MAS rates enhance the resolution of the ^1H -detected spectrum and transverse relaxation times (T_2)^{24–25}. State-of-the-art approaches now can often yield ~ 0.1 ppm or better amide ^1H linewidths^{18,20–22}.

Previous ^1H -X REDOR studies have emphasized spectral filtering based on coupling magnitude^{26–28}. The initial implementation, called medium and long distance heteronuclear correlation (MELODI-HETCOR), focused on suppressing the one-bond ^1H - ^{13}C correlation through dephasing of ^1H magnetization by REDOR π pulses on ^{13}C , and detecting medium- and long-range ^1H - ^{13}C correlations in a HETCOR spectrum²⁶. This method was applied at moderate MAS rates (6 to 10 kHz), requiring a frequency-switched Lee-Goldburg (FSLG)²⁹

sequence to suppress the ^1H - ^1H homonuclear couplings. An additional crucial requirement for this experiment is a partially ^{13}C -labeled protein.

Here we introduce a modified pulse sequence that produces a ^{15}N - ^1H correlation spectrum in which the peak intensities depend on selective REDOR dephasing from ^{13}C . Optimal resolution is obtained in a deuterated protein with fast MAS, negating the need for multiple pulse decoupling approaches for removing ^1H - ^1H homonuclear couplings. Further, due to the increased ^1H T_2 relaxation times, the ^1H magnetization can be dephased for a longer time (on the order of 15–20 ms), in principle enabling longer ^1H -X distances to be detected with this pulse sequence (up to 15–20 Å). But since the coupling magnitudes are so large (>1 kHz) for many structurally informative distances (*vide infra*), even REDOR applied for ~1 ms is more than sufficient to reveal a variety of unique effects that depend on the details of protein structure. Band-selectivity of the REDOR dephasing³⁰ is achieved with frequency-selective soft π -pulses applied to the ^{13}C nuclei. In addition to backbone (C α and C') selective versions for spectral filtering, we also evaluate cases in which the π -pulses are applied to the methyl and aromatic regions. These results provide insights into resonance assignment, secondary and tertiary structure, and preferred sidechain orientations.

Experimental Methods

Preparation of uniformly ^{13}C , ^{15}N , ^2H -labeled Ala

$^{13}\text{C}_3$, D_4 , ^{15}N labeled L-Ala was purchased from Cambridge Isotope Laboratories (Andover, MA), exchanged into 99.9% D_2O , lyophilized and then re-crystallized by slow evaporation of a saturated (~250 mg/mL) solution at room temperature. The crystals were crushed to make a fine powder sample and 4 mg was packed in a 1.6 mm FastMAS rotor (Revolution NMR, LLC, Fort Collins, CO).

Preparation of uniformly ^{13}C , ^{15}N , ^2H -labeled GB1

Uniformly ^{13}C , ^{15}N , ^2H labeled (U-CDN) GB1 protein was expressed in *E. coli* cells and purified according to Ref.³¹. The purified protein was dialyzed into 30:70 H_2O : D_2O buffer, concentrated to ~25 mg/mL and precipitated with 2-methyl-2,4-pentandiol (MPD) and isopropanol (IPA) solution according to Ref.³². Microcrystals were ultracentrifuged and ~4 mg packed into a 1.6 mm FastMAS rotor (Revolution NMR, LLC, Fort Collins, CO).

^1H -Detected frequency selective REDOR (FSR) pulse sequence

The pulse sequence (Fig. 1) starts with adiabatic cross-polarization (CP)³³ from ^1H to ^{15}N , followed by evolution in t_1 with low power TPPM ^1H decoupling^{34–35}. After evolution, the ^{15}N magnetization is stored along the z-axis in a constant time manner, and MISSISSIPPI solvent suppression is applied³⁶, followed by ^{15}N -to- ^1H CP. The FSR³⁰ period starts with in-phase ^1H magnetization, which evolves under REDOR by applying two π -pulses per rotor period on the dephasing channel (^{13}C in this case). The ^{13}C - ^{13}C dipolar interactions present in a fully ^{13}C labeled protein will be reintroduced by the application of the π -pulse trains on ^{13}C and hence will lead to some additional signal decay. We also attempted versions with one π pulse per rotor period on each channel (Fig. S1) or all the π pulses on ^1H ; both resulted in less satisfactory performance due to ^1H - ^1H recoupling effects.

A simultaneous hard π -pulse on the ^1H channel and a soft frequency selective π -pulse on the dephasing channel are applied in the middle of the REDOR period (after the first train of REDOR π -pulses, with the hard π -pulse centered on the soft π -pulse), with extra rotor periods before and after the REDOR to ensure an in-phase echo for detection with minimal baseline distortion. The soft pulse on ^{13}C will also lead to partial removal of the recoupled ^{13}C - ^{13}C interaction. The S_0 experiment is performed with no REDOR π -pulse train on the dephasing channel, and the S experiment is performed with all the REDOR π -pulses. The ^1H magnetization is then detected in the direct dimension with WALTZ decoupling³⁷ applied to both ^{15}N and ^{13}C .

Solid State NMR (SSNMR) Spectroscopy

Experiments on U-CDN Ala were carried out at 11.7 T (500 MHz ^1H frequency) on a Varian (Walnut Creek, CA and Loveland, CO) VNMRS spectrometer with a 1.6 mm HCDN FastMAS probe. Experiments on U-CDN GB1 (30% H_2O back exchanged) were carried out at 17.6 T (750 MHz ^1H frequency) on Varian VNMRS spectrometer with a 1.6 mm HFX Y FastMAS probe configured in HFCN mode. On both the instruments, the spinning was controlled at 33333 ± 10 Hz by a Varian MAS controller. The variable temperature gas was maintained at 0 °C resulting in an approximate sample temperature of 15–20 °C. ^{13}C chemical shifts were referenced externally with adamantane at 40.48 ppm for the methylene signal³⁸.

^1H detected FSR on U-CDN Ala—The $\pi/2$ pulse widths were 1.4 μs for ^1H , 3.6 μs for ^{15}N , 1.4 μs for ^{13}C . The contact time for ^1H -to- ^{15}N CP transfer and ^{15}N -to- ^1H CP transfer was 1.6 ms. The indirect dimension was not digitized in this experiment (since there is only one ^{15}N frequency). The soft (Gaussian) π pulse was applied for 600 μs Gaussian pulse with Gaussian shape and a maximum nutation frequency of ~ 5.2 kHz power on the ^{13}C channel. The REDOR π -pulse widths were 2.8 μs . The ^{13}C channel transmitter frequency was set to three different values for exciting different ^{13}C atoms in Ala, ^{13}Ca (50 ppm), ^{13}CO (175 ppm) and $^{13}\text{CH}_3$ (20 ppm). The dephasing time was varied from 0 to 1.2 ms. 64 scans were collected for each experiment.

^1H site-specific T_2 measurements on U-CDN GB1—The ^{15}N - ^1H 2D experiment with a ^1H refocusing π -pulse before detection was used to measure the ^1H T_2 for each residue. The echo time was varied in 18 steps from 60 μs to 21.6 ms. The contact time for ^1H -to- ^{15}N CP transfer was 900 μs and ^{15}N -to- ^1H CP transfer was 360 μs . The indirect ^{15}N dimension was digitized to 23 ms and the ^1H acquisition time was set to 20 ms. ~ 10 kHz of decoupling power through TPPM decoupling was applied on ^1H during indirect evolution on ^{15}N .

^1H detected FSR on U-CDN GB1—The contact time for ^1H -to- ^{15}N CP transfer was set to 900 μs and ^{15}N -to- ^1H CP transfer was set to 360 μs . The indirect ^{15}N dimension was digitized to 23 ms. TPPM decoupling (~ 10 kHz, 50° phase, 50 μs pulse width) was applied on ^1H during indirect evolution on ^{15}N . The S_0 experiment was performed with a refocusing hard π -pulse on ^1H and a soft Gaussian π pulse (600 μs duration) on the ^{13}C channel. The S experiment was performed with additional REDOR π -pulse (4 μs pulse width) trains on

the ^{13}C channel. Four versions of the experiment were performed, each with the ^{13}C channel transmitter frequency set to a particular region: ^{13}Ca (50 ppm), ^{13}CO (175 ppm) and $^{13}\text{CH}_3$ (20 ppm) and aromatics (130 ppm). The bandwidth of the soft pulse was ~ 15 ppm. ^{15}N - ^1H 2D S_0 and S spectra for each transmitter frequency were collected with REDOR times ranging from 240 μs to 1.2 ms.

Data Processing

The ^1H -detected FSR 1D spectra collected on Ala were processed in Mnova NMR software (MESTRELAB 2014), with polynomial baseline correction. 2D spectra were processed using NMRPipe³⁹ with back linear prediction and polynomial baseline correction applied to the direct dimension. Lorentzian-to-Gaussian apodization, phase-shifted sine bells and zero filling were applied to both dimensions.

Microsoft Excel for Mac 2011 (version 14.7.1) software was used to plot and fit the ^1H T_2 values assuming a single exponential decay. Error analysis was performed by fitting the natural log of the intensities with the Excel 'LINEST' function to extract the standard error associated with the slope of the linear fit (slope is equivalent to $1/T_2$). The standard error was then converted to standard deviation and propagated to determine the standard deviation associated with T_2 of each residue.

Results and Discussion

REDOR effects on residual amino protons in U-CDN Ala

We initially tested the pulse sequence with U-CDN Ala, a small model compound with $\sim 0.1\%$ residual amino group protons from the crystallization. The low protonation level greatly reduces the average ^1H - ^1H dipolar couplings, resulting in line widths of 0.5 ppm or less at 33.333 kHz MAS rate. Thus, we obtained site-specific ^{13}C -dephased $^1\text{H}_\text{N}$ spectra (Fig. 2), allowing us to evaluate the specificity of the REDOR effect with selective pulses. The ^{13}Ca is an average of ~ 2.2 Å from the amino ^1H (assuming three-site hopping in the fast limit), the shortest ^{13}C - ^1H distance available in this sample, and so with the ^{13}Ca frequency selective pulse, the $^1\text{H}_\text{N}$ signal shows almost complete dephasing ($S/S_0 = 0.7$) already at 360 μs (Fig. 2a). The $^{13}\text{CH}_3$ and the ^{13}CO are each effectively ~ 2.8 Å from the amino (taking motionally averaging into account), and in these cases a slightly smaller dephasing ($S/S_0 = 0.5$) is observed when the REDOR π pulses are on the $^{13}\text{CH}_3$ and ^{13}CO region (Fig. 2b,c).

^1H - ^{15}N correlation spectrum of GB1 at 30% protonation level

The ^1H line width in fully protonated and 100% back-exchanged GB1 protein at 40 kHz MAS was reported as ~ 1 ppm¹⁷ and at least 0.2 ppm²⁴ respectively, with partial overlap especially in central region of the spectrum (115-126 ppm ^{15}N and 8.0-9.5 ppm ^1H), as well as in the Thr residues which were systematically broader due to the high level of sidechain protonation. Akbey et al.²² demonstrated that the optimal combination of ^1H resolution and sensitivity at ~ 30 kHz MAS is in the range of 30% to 40% H_2O back-exchange. We therefore utilized a 30% protonation level for the U-CDN GB1 sample, resulting in a ^{15}N - ^1H spectrum (Fig. 3) that is almost entirely resolved. The ^1H line widths are typically 0.1 ppm

(60 to 80 Hz at 750 MHz ^1H frequency); roughly 20 Hz of this line width is contributed from the magnetic field inhomogeneity (based on the ^{13}C line width in adamantane of ~ 5 Hz). Even the central region is mostly resolved, with only a few partially overlapped peaks (like K31/D36 and K4/A23/A34). The fractional back-exchange has an especially large benefit for Thr residues, and the individual amine protons of the Asn/Gln side chains are resolved.

As expected, based on the overall improvements in the resolution of the ^1H - ^{15}N spectrum, the T_2 values were also greatly improved relative to 100% back-exchanged sample studied previously²⁴. We measured the bulk $^1\text{H}_\text{N}$ T_2 of GB1 to be around 13 ms (measured with a Hahn echo directly before acquisition, with ^{15}N -filtering). We also measured site-specific T_2 values with a series of 2D ^1H - ^{15}N correlation spectra with incremented Hahn echo times prior to detection (Fig. S2). In this manner, we were able to determine site-specific T_2 values for 43 backbone amide sites, which range from 3 to 83 ms (Fig. 4). Even the fastest relaxing sites (e.g., T25, Y45) had more than sufficient echo lifetimes to enable application of the REDOR experiment directly on the ^1H transverse magnetization. In the following sections we demonstrate the magnitude of these REDOR effects and the utility for assignment and conformational analysis.

$^1\text{H}\{^{13}\text{C}\}$ REDOR effects in GB1 spectra

Dephasing by ^{13}Ca emphasizes backbone correlations—Three versions of the ^{13}C -dephased, ^1H -detected REDOR experiment are illustrated in Fig. 5, for different soft pulse bandwidths and mixing times, presented as difference spectra (S_0 - S). First we examined the ^{13}Ca -dephased version, which results in strong REDOR effects at only 240 (Fig. 5a) to 360 μs (Fig. 5b), as expected due to the short $^1\text{H}_\text{N}$ to ^{13}Ca distance (~ 2.1 - 2.2 Å) and corresponding dipolar coupling of ~ 3 kHz. All the residues show significant REDOR effects, with roughly a third (18) exhibiting $>50\%$ dephasing ($S/S_0 > 0.5$) at 240 μs , and most of the remaining (32 additional) sites reaching this threshold by 360 μs . These spectra are effective for providing a filtered spectrum including only the backbone, since the sidechain amine sites show significant dephasing only at 600 μs (Fig. 5c). For example, the N37-H61 ($S/S_0 = 0.3$), N37-H62 ($S/S_0 = 0.5$) and W43-He ($S/S_0 = 0.3$) signals appear with significant intensity. In general, helical Asn/Gln residues have shorter distances from the sidechain amines to the backbone ^{13}Ca atoms (3.2-4.2 Å distance between amine ^1H and intraresidue Ca or $i\pm 1$ residue Ca) than the sidechain amine of those residues in β -strands (4-5.5 Å distance between amine ^1H and intraresidue ^{13}Ca). In GB1, Q2 and N8 are on β -strands, whereas Q32, N35 and N37 are on the helix. In particular, the intraresidue distances to Ca are 3.2 Å for Q32-He1, 3.4 Å for N37-H61, and 4.2 Å for N35-H62. In addition to the short intraresidue distances, helical Asn/Gln residues can have relatively short interresidue Ca distances; both D36 (4.2 Å) and A34 (4.3 Å) Ca sites contribute to the N37H1 dephasing, which is the strongest among all the sidechain signals here.

Extent of methyl- ^{13}C dephasing depends on secondary structure—The second version of the experiment was performed with methyl-specific dephasing. It is notable that even for small methyl-bearing residues (Ala, Thr and Val), the $^1\text{H}_\text{N}$ and ^{13}C (methyl) are separated by at least three bonds and therefore the internuclear distance depends on the

backbone ϕ angle (for all cases) and additionally on the χ_1 angle for Thr and Val. This is notable and unlike the ^{15}N - $^{13}\text{C}\beta$ distance in Ala, for example, which is ~ 2.5 Å and invariant to dihedral angles, or the ^{15}N - $^{13}\text{C}\gamma$ distances in Thr and Val which depend only on χ_1 . As a result of the $^1\text{H}_\text{N}$ - ^{13}C (methyl) conformation dependence, at 240 μs REDOR time (Fig. 5d) the methyl-bearing helical residues (A23/A34, A24, T25, A26 and V29) appear with greatest intensity ($S/S_0 > 0.2$), along with some loop (V21 and T49) and residues neighboring methyls (F30). No β -strand residues show up at this short REDOR time; thus the effect in this case begins to provide a restraint on secondary structure. At increasing mixing time (Fig. 5e) the magnitude of the REDOR effect increases ($S/S_0 > 0.5$) and additional methyl-bearing residues (I6, T16, T18, A20, V39, A48 and T55) show up prominently. In addition there are sites that begin to report on longer-range interactions, such as K13 and Q32. After 960 μs of dephasing (Fig. 5f), almost all the backbone amide ^1H start showing strong modulation in intensity ($S/S_0 > 0.5$). Thus the most informative mixing times for the methyl-dephased version of the experiment are in the range 240 to 600 μs .

Aromatic dephasing reveals tertiary contacts—Performing the aromatic-selective version selects for ^{13}C sites that are at least (for the $^{13}\text{C}\gamma$) four bonds from the intraresidue $^1\text{H}_\text{N}$ and often (for the remainder of the ring) closer to the amides of neighboring or long-range residues. This observation is immediately apparent in Fig. 5g, since GB1 has six aromatic residues (Y3, F30, Y33, W43, Y45 and F52), yet roughly 15 peaks show similar intensities at short REDOR time, and none of the aromatic residues show strong attenuation in intensity of their own backbone amide ^1H . In fact, the two strongest REDOR effects are observed to W43- H_ϵ (which has two aromatic ^{13}C sites, $^{13}\text{C}\delta_1$ and $^{13}\text{C}\delta_2$, within ~ 2.2 Å bonds of the H_ϵ) and, surprisingly, D46. (We will discuss this observation further in the following section). Within 600 μs of aromatic REDOR ^{13}C dephasing (Fig. 5h), the backbone amide $^1\text{H}_\text{N}$ signals of the aromatic residues appear, and Y3, W43, Y45 and F52 exhibit $S/S_0 > 0.4$. Few other non-aromatic amino acids like V21/E27, N37 (sidechain) also show substantial REDOR effects at this time point, and the magnitude continues to increase as expected at 840 μs (Fig. 5i).

Quantitation of REDOR effects and structural interpretation

In order to investigate the more nuanced site-specific details of the REDOR effects observed above, we examined the intensity changes (S/S_0) of the ^{15}N - ^1H correlations by residue number at 600 μs for the methyl and aromatic versions of the experiment (Fig. 6). The plot of the methyl-dephased data (Fig. 6a) illustrates the extent of backbone and sidechain $^1\text{H}_\text{N}$ sites that have been dephased. To examine the detailed variations in REDOR effects and their relationships to the structural elements, we modeled the $^1\text{H}_\text{N}$ positions using WHATIF web server (Add Protons to the Structure)⁴⁰ and the high-resolution crystal structure (PDB 2QMT). Then we considered the distances from $^1\text{H}_\text{N}$ to methyl ^{13}C sites. Almost all backbone $^1\text{H}_\text{N}$ sites are within 5 Å of some methyl groups, and so the average S/S_0 value is ~ 0.4 , but some sites show significantly larger effects ($S/S_0 > 0.6$). For example, helical Ala residues exhibit nearly the shortest possible intraresidue H_N -C(methyl) distance, which is 2.5 Å because a ϕ angle of -60° results in an eclipsed H_N -C β (H_N -N-C α -C β dihedral of $\sim 0^\circ$). Therefore A23 and A34 exhibit a REDOR effect of > 0.6 at 600 μs , and even larger

effects are observed for A24 and A26, which have additional interresidue HN-C(methyl) distances of < 3.5 Å. Specifically, the A24-H_N to A23-C β distance is 3.3 Å and the A26-H_N to A20-C β distance is 3.2 Å (Fig. 7a).

For Thr and Val residues, the H_N-C(methyl) distances range from 2.6 to 3.0 Å for eclipsed or gauche conformations. For example, T49 (despite being present in a turn), has a H_N-C γ 2 methyl distance of 3 Å, as well as an interresidue T49H_N-A48C β distance of 3.3 Å, leading to leading to a strong REDOR effect of ~ 0.65 at 600 μ s. V29, within the helix, has $S/S_0 > 0.6$, arising from the H_N-C γ 1 distance of 2.9 Å (the H_N-C γ 2 distance is 4 Å). T25 is another helical residue that demonstrates a large $S/S_0 (> 0.7)$, in this case due to the short interresidue distance of 3.0 Å from T25-H_N to A24-C β , which is significantly shorter than the T25H_N-C γ 2 intraresidue distance of 4 Å (Fig. 7b). Thus the strongest methyl-¹³C-dephased REDOR effects (S/S_0 between 0.6 and 0.8 at ~ 600 μ s) arise from Ala residues in helical conformations or from some Thr/Val residues with favorable conformations. A number of other methyl-bearing residues or neighbors of methyl-bearing residues show REDOR effects are between 0.4-0.6.

Dynamics plays an important role in determining the REDOR effects shown by methyl groups. The C3 axes of the methyl groups can undergo libration, thereby attenuating the ¹H_N-¹³C dipolar couplings. The methyl bearing residues whose side chains are extended in the solvent and not buried within the protein core will show such librational motions. One particular example in GB1 protein consists of V21 and V29. V21 is in a turn, with the ¹H_N-C γ 1 distance as 2.6 Å and ¹H_N-C γ 2 distance as 3 Å. Hence, V21 should show larger REDOR effect than V29. But as mentioned in Shi *et al.*³², the side chain of V21 is more mobilized than that of V29. This should explain the similar REDOR effects shown by V21 and V29.

Fig 6b plots the S/S_0 intensity versus the residue number for the backbone ¹H_N and sidechain amino group ¹H for aromatic residues at 600 μ s. The strong ¹³C-¹³C dipolar interactions present in a fully ¹³C labeled protein results in an average S/S_0 of 0.2. The residue showing the biggest REDOR effect ($S/S_0 \sim 0.6$) at 600 μ s is surprisingly not an aromatic residue, but a neighboring one, D46, which we attribute to the Y45 aromatic ring being trans relative to its intraresidue ¹H_N (distances > 4.3 Å) but in much closer proximity to D46H_N; the distances from D46H_N to Y45C δ 2, Y45C γ and Y45C δ 1 are 3 Å, 3.3 Å and 4.1 Å respectively (Fig. 7c). Hence, the REDOR effect shown by Y45 is ~ 0.45 . F52 shows the strongest intraresidue REDOR effects among the aromatic residues ($S/S_0 \sim 0.5$), given a gauche conformation and F52H_N-C δ 1 distance of 3 Å, as well as other aromatic carbons within 4.5 Å of the amide. The F52 aromatic ring is also responsible for dephasing of the E27H_N which is ~ 4.3 Å from the F52C ϵ (Fig. 7d); the E27 REDOR effect of 0.4 is second strongest among the non-aromatic residues. Y3 and W43 are the two other aromatic residues that have intraresidue REDOR effects > 0.4 . Both Y3 and W43 aromatic rings are in a gauche conformation with respect to the H_N (Fig 7e). Y3 ¹H_N-C γ distance is 3.4 Å. W43 ¹H_N-C δ 1 distance is 3.5 Å. A surprising observation among the sidechain amines is N37 δ 1, which shows a REDOR effect of ~ 0.4 , due to its orientation relative to the Y33 aromatic ring (Fig. 7f); the N37H δ 1 to Y33C δ distance is 3.3 Å.

To summarize, for aromatic residues with gauche conformations, the H_N REDOR effects are between 0.4 and 0.6 at 600 μ s. This is observed for Y3, W43 and F52 in GB1. Substantially weaker intraresidue REDOR effects are observed for trans conformations (Y33 and Y45), and in these cases the neighboring (*i+1*) residues can show larger effects, as we observed for A34 and D46. In some cases, long-range effects are detected involving the sidechain protons, such as in the case of N37.

We note that for the aromatic version of the experiment utilized here, the soft pulse was centered among the aromatic signals at ~ 130 ppm, with a bandwidth of ~ 15 -16 ppm, thus exciting the region from ~ 122 to ~ 138 ppm. This region encompasses the majority of aromatic frequencies, but with some notable exceptions. For example, we might expect some attenuation of REDOR effects for Tyr C ϵ sites with upfield shifts (~ 120 ppm), and clearly the Tyr C ζ at ~ 160 ppm is outside of the range noted here. For Trp, the C δ atoms were inverted by the soft pulses here, but C γ (110 ppm) was outside of the bandwidth and other carbons would be near the edge of the soft pulse (C ϵ at 120 ppm or 140 ppm and C ζ at 120 ppm). Finally the Phe C γ atom is near the edge at 140 ppm. It is apparent that the effects are sensitive to this range of offset, and given this large range of possible excitable frequencies, further selectivity could be achieved with soft pulses centered at ~ 160 ppm or ~ 110 ppm in order to detect backbone correlations in a residue-specific manner. For example, narrower bandwidth pulses could be used to detect the W43C ζ long-range distances, such as the K31H $_N$ (Fig 7e). There are many additional opportunities for more selectivity and given the long T_2 values observed, detection of at least 10 Å distances may be feasible.

Conclusions

The 1H -detected FSR pulse sequence introduced here is a broadly applicable technique for measuring long-range distances in SSNMR and leverages the high sensitivity of 1H detection in combination with fast MAS. The REDOR difference spectra are collected as 2D ^{15}N - 1H planes, so majority of the residues in the protein can be site-specifically resolved. Involving 1H as one of the nuclei in the dipolar coupled spin pair also enhances the distance measurement range. No multiple pulse sequences are required for effective 1H - 1H dipolar decoupling, given the fast MAS rates and deuteration. Another advantage of deuteration is enhancement of echo lifetimes of the detected nucleus (1H), thereby increasing potential distance measurement range.

This method can be used with backbone ^{13}C dephasing for purposes of spectral filtering, and the methyl- and aromatic-selective versions give insights into the secondary and tertiary structure of the protein. Specifically, the dephasing by the methyl bearing residues is stronger in general for helical residues, particularly Ala, and Thr or Val residues helical/loop regions can also yield strong REDOR effects ($S/S_0 > 0.6$) at 600 μ s dephasing time. The aromatic-specific version gives some initial insights into tertiary structure, since aromatic rings can be significantly closer to neighboring or long-range amides than intraresidue amides (e.g., Y33, Y45). This information can also restrain the side chain orientation of the aromatic ring with respect to its own backbone amide proton, based on the relative REDOR magnitude.

We envision that this approach can be extended to incorporate ^{31}P or ^{19}F dephasing in the case of phospholipids or fluorinated drugs bound to proteins. Notably, the detection range for $^1\text{H}\{^{19}\text{F}\}$ REDOR effects with $>10\text{ ms } ^1\text{H } T_2$ values would be well in excess of $15\text{ \AA}$. We also anticipate that the large REDOR effects observed here could facilitate 3D TEDOR studies¹³ on sparsely ^{13}C -isotopically labeled samples¹⁵. Sparse labeling in this case would be necessary in order to minimize signal loss due to recoupling of ^{13}C - ^{13}C interactions from directly attached pairs. Using these approaches, ^1H -detected FSR has excellent potential for improving biomolecular SSNMR-based structure determination with enhanced sensitivity, resolution and distance measurement range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Petkova AT, Ishii Y, Balbach JJ, Antzutkin ON, Leapman RD, Delaglio F, Tycko R. A Structural Model for Alzheimer's β -Amyloid Fibrils Based on Experimental Constraints from Solid-State NMR. *Proc Natl Acad Sci U S A*. 2002; 99:16742–16747. [PubMed: 12481027]
2. Schutz AK, Soragni A, Hornemann S, Aguzzi A, Ernst M, Bockmann A, Meier BH. The Amyloid-Congo Red Interface at Atomic Resolution. *Angew Chem Int Ed Engl*. 2011; 50:5956–5960. [PubMed: 21591034]
3. Lu JX, Qiang W, Yau WM, Schwieters CD, Meredith SC, Tycko R. Molecular Structure of β -Amyloid Fibrils in Alzheimer's Disease Brain Tissue. *Cell*. 2013; 154:1257–1268. [PubMed: 24034249]
4. Prade E, Bittner HJ, Sarkar R, Lopez Del Amo JM, Althoff-Ospelt G, Multhaup G, Hildebrand PW, Reif B. Structural Mechanism of the Interaction of Alzheimer Disease A β Fibrils with the Non-Steroidal Anti-Inflammatory Drug (NSAID) Sulindac Sulfide. *J Biol Chem*. 2015; 290:28737–28745. [PubMed: 26416887]
5. Tuttle MD, Comellas G, Nieuwkoop AJ, Covell DJ, Berthold DA, Kloepper KD, Courtney JM, Kim JK, Barclay AM, Kendall A, et al. Solid-State NMR Structure of a Pathogenic Fibril of Full-Length Human α -Synuclein. *Nat Struct Mol Biol*. 2016; 23:409–415. [PubMed: 27018801]
6. Walti MA, Ravotti F, Arai H, Glabe CG, Wall JS, Bockmann A, Guntert P, Meier BH, Riek R. Atomic-Resolution Structure of a Disease-Relevant A β (1–42) Amyloid Fibril. *Proc Natl Acad Sci U S A*. 2016; 113:E4976–E4984. [PubMed: 27469165]
7. Colvin MT, Silvers R, Ni QZ, Can TV, Sergeyev I, Rosay M, Donovan KJ, Michael B, Wall J, Linse S, et al. Atomic Resolution Structure of Monomorphous A β 42 Amyloid Fibrils. *J Am Chem Soc*. 2016; 138:9663–9674. [PubMed: 27355699]
8. Gullion T, Schaefer J. Rotational-Echo Double-Resonance NMR. *J Magn Reson*. 1989; 81:196–200.
9. Gullion T, Schaefer J. Detection of Weak Heteronuclear Dipolar Coupling by Rotational-Echo Double-Resonance Nuclear-Magnetic-Resonance. *Adv Magn Reson*. 1989; 13:57–83.
10. Pan Y, Gullion T, Schaefer J. Determination of C-N Internuclear Distances by Rotational-Echo Double-Resonance NMR of Solids. *J Magn Reson*. 1990; 90:330–340.
11. Strojek W, Eckert H. Medium-Range Order in Sodium Phosphate Glasses: a Quantitative Rotational Echo Double Resonance Solid State NMR Study. *Phys Chem Chem Phys*. 2006; 8:2276–2285. [PubMed: 16688310]

12. Cegelski L. REDOR NMR for Drug Discovery. *Bioorg Med Chem Lett*. 2013; 23:5767–5775. [PubMed: 24035486]
13. Jaroniec CP, Filip C, Griffin RG. 3D TEDOR NMR Experiments for the Simultaneous Measurement of Multiple Carbon-Nitrogen Distances in Uniformly ^{13}C , ^{15}N -Labeled Solids. *J Am Chem Soc*. 2002; 124:10728–10742. [PubMed: 12207528]
14. Nieuwkoop AJ, Wylie BJ, Franks WT, Shah GJ, Rienstra CM. Atomic Resolution Protein Structure Determination by Three-Dimensional Transferred Echo Double Resonance Solid-State Nuclear Magnetic Resonance Spectroscopy. *J Chem Phys*. 2009; 131:095101. [PubMed: 19739873]
15. Nieuwkoop AJ, Rienstra CM. Supramolecular Protein Structure Determination by Site-Specific Long-Range Intermolecular Solid State NMR Spectroscopy. *J Am Chem Soc*. 2010; 132:7570–7571. [PubMed: 20465251]
16. Wi S, Sinha N, Hong M. Long-Range ^1H - ^{19}F Distance Measurement in Peptides by Solid-State NMR. *J Am Chem Soc*. 2004; 126:12754–12755. [PubMed: 15469252]
17. Zhou DH, Shah G, Cormos M, Mullen C, Sandoz D, Rienstra CM. Proton-Detected Solid-State NMR Spectroscopy of Fully Protonated Proteins at 40 kHz Magic-Angle Spinning. *J Am Chem Soc*. 2007; 129:11791–11801. [PubMed: 17725352]
18. Linser R, Dasari M, Hiller M, Higman V, Fink U, Lopez del Amo JM, Markovic S, Handel L, Kessler B, Schmieder P, et al. Proton-Detected Solid-State NMR Spectroscopy of Fibrillar and Membrane Proteins. *Angew Chem Int Ed Engl*. 2011; 50:4508–4512. [PubMed: 21495136]
19. Zhou DH, Nieuwkoop AJ, Berthold DA, Comellas G, Sperling LJ, Tang M, Shah GJ, Brea EJ, Lemkau LR, Rienstra CM. Solid-State NMR Analysis of Membrane Proteins and Protein Aggregates by Proton Detected Spectroscopy. *J Biomol NMR*. 2012; 54:291–305. [PubMed: 22986689]
20. Stanek J, Andreas LB, Jaudzems K, Cala D, Lalli D, Bertarello A, Schubeis T, Akopjana I, Kotelovica S, Tars K, et al. NMR Spectroscopic Assignment of Backbone and Side-Chain Protons in Fully Protonated Proteins: Microcrystals, Sedimented Assemblies, and Amyloid Fibrils. *Angew Chem Int Ed Engl*. 2016; 55:15504–15509. [PubMed: 27865050]
21. Andreas LB, Le Marchand T, Jaudzems K, Pintacuda G. High-Resolution Proton-Detected NMR of Proteins at Very Fast MAS. *J Magn Reson*. 2015; 253:36–49. [PubMed: 25797003]
22. Akbey U, Lange S, Trent Franks W, Linser R, Rehbein K, Diehl A, van Rossum BJ, Reif B, Oshkinat H. Optimum Levels of Exchangeable Protons in Perdeuterated Proteins for Proton Detection in MAS Solid-State NMR Spectroscopy. *J Biomol NMR*. 2010; 46:67–73. [PubMed: 19701607]
23. Zhou DH, Graesser DT, Franks WT, Rienstra CM. Sensitivity and Resolution in Proton Solid-State NMR at Intermediate Deuteration Levels: Quantitative Linewidth Characterization and Applications to Correlation Spectroscopy. *J Magn Reson*. 2006; 178:297–307. [PubMed: 16289756]
24. Zhou DH, Shea JJ, Nieuwkoop AJ, Franks WT, Wylie BJ, Mullen C, Sandoz D, Rienstra CM. Solid-State Protein-Structure Determination with Proton-Detected Triple-Resonance 3D Magic-Angle-Spinning NMR Spectroscopy. *Angew Chem Int Ed Engl*. 2007; 46:8380–8383. [PubMed: 17907259]
25. Knight MJ, Webber AL, Pell AJ, Guerry P, Barbet-Massin E, Bertini I, Felli IC, Gonnelli L, Pierattelli R, Emsley L, et al. Fast Resonance Assignment and Fold Determination of Human Superoxide Dismutase by High-Resolution Proton-Detected Solid-State MAS NMR Spectroscopy. *Angew Chem Int Ed Engl*. 2011; 50:11697–11701. [PubMed: 21998020]
26. Yao XL, Schmidt-Rohr K, Hong M. Medium- and Long-Distance ^1H - ^{13}C Heteronuclear Correlation NMR in Solids. *J Magn Reson*. 2001; 149:139–143.
27. Yao XL, Hong M. Dipolar Filtered ^1H - ^{13}C Heteronuclear Correlation Spectroscopy for Resonance Assignment of Proteins. *J Biomol NMR*. 2001; 20:263–274. [PubMed: 11519749]
28. Kiihne SR, Creemers AF, de Grip WJ, Bovee-Geurts PH, Lugtenburg J, de Groot HJ. Selective Interface Detection: Mapping Binding Site Contacts in Membrane Proteins by NMR Spectroscopy. *J Am Chem Soc*. 2005; 127:5734–5735. [PubMed: 15839640]
29. Lee M, Goldberg WI. Nuclear-Magnetic-Resonance Line Narrowing by a Rotating Rf Field. *Phys Rev*. 1965; 140:A1261–A1271.

30. Jaroniec CP, Tounge BA, Herzfeld J, Griffin RG. Frequency Selective Heteronuclear Dipolar Recoupling in Rotating Solids: Accurate ^{13}C - ^{15}N Distance Measurements in Uniformly ^{13}C , ^{15}N -Labeled Peptides. *J Am Chem Soc.* 2001; 123:3507–3519. [PubMed: 11472123]
31. Franks WT, Zhou DH, Wylie BJ, Money BG, Graesser DT, Frericks HL, Sahota G, Rienstra CM. Magic-Angle Spinning Solid-State NMR Spectroscopy of the $\beta 1$ Immunoglobulin Binding Domain of Protein G (GB1): ^{15}N and ^{13}C chemical shift assignments and conformational analysis. *J Am Chem Soc.* 2005; 127:12291–12305. [PubMed: 16131207]
32. Shi X, Rienstra CM. Site-Specific Internal Motions in GB1 Protein Microcrystals Revealed by 3D ^2H - ^{13}C - ^{13}C Solid-State NMR Spectroscopy. *J Am Chem Soc.* 2016; 138:4105–4119. [PubMed: 26849428]
33. Metz G, Wu XL, Smith SO. Ramped-Amplitude Cross-Polarization in Magic-Angle-Spinning NMR. *J Magn Reson Ser A.* 1994; 110:219–227.
34. Bennett AE, Rienstra CM, Auger M, Lakshmi KV, Griffin RG. Heteronuclear Decoupling in Rotating Solids. *J Chem Phys.* 1995; 103:6951–6958.
35. Kotecha M, Wickramasinghe NP, Ishii Y. Efficient Low-Power Heteronuclear Decoupling in ^{13}C High-Resolution Solid-State NMR Under Fast Magic Angle Spinning. *Magn Reson Chem.* 2007; 45:S221–S230. [PubMed: 18157841]
36. Zhou DH, Rienstra CM. High-Performance Solvent Suppression for Proton Detected Solid-State NMR. *J Magn Reson.* 2008; 192:167–172. [PubMed: 18276175]
37. Shaka AJ, Keeler J, Freeman R. Evaluation of a New Broad-Band Decoupling Sequence - Waltz-16. *J Magn Reson.* 1983; 53:313–340.
38. Morcombe CR, Zilm KW. Chemical Shift Referencing in MAS Solid-State NMR. *J Magn Reson.* 2003; 162:479–486. [PubMed: 12810033]
39. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a Multidimensional Spectral Processing System Based on UNIX Pipes. *J Biomol NMR.* 1995; 6:277–293. [PubMed: 8520220]
40. Hekkelman ML, Te Beek TA, Pettifer SR, Thorne D, Attwood TK, Vriend G. WIWS: a Protein Structure Bioinformatics Web Service Collection. *Nucleic Acids Res.* 2010; 38:W719–23. [PubMed: 20501602]

Pulse sequence for ^1H -detected frequency selective REDOR spectroscopy. The ^1H magnetization is transferred to ^{15}N for indirect evolution and then transferred back to ^1H for detection. A REDOR period is added before detection to dephase the ^1H magnetization with nearby ^{13}C atoms (more details in text). The phase cycles were as follows: $\phi 1 = x, x, -x, -x$; $\phi 2 = x, x, x, x, -x, -x, -x, -x$; $\phi 3 = y, -y$; $\phi 4 = y, y, -x, -x$; $\phi 5 = y, y, x, x, x, y, y, -x, -x, y, y, y, -x, -x, -y, -y, -x, -x, -x, -y, -y, x, x, y, y, y, x, x$; $\phi 6 = x, y, x, y, -x, -y, -x, -y$; $\phi 7 = x, -x, -y, y, x, -x, -y, y, -x, x, y, -y, -x, x, y, -y$

Fig. 2.

Application of ^1H detected FSR pulse sequence on uniformly ^2H , ^{13}C , ^{15}N labeled Ala (residual protonation level is 1%). The $^1\text{H}_\text{N}$ 1D spectra are shown when the REDOR π pulse trains are on (a) $^{13}\text{C}_\alpha$ region, (b) ^{13}CO region, and (c) $^{13}\text{CH}_3$ region. S_0 spectrum is in black (collected without REDOR π pulses on ^{13}C channel) and S spectrum is in red (collected with REDOR π pulses on ^{13}C channel)

Fig. 3. ^{15}N - ^1H ^2D spectrum of uniformly ^{13}C , ^{15}N , ^2H labeled GB1 protein, back-exchanged with 30% H_2O . The high level of deuteration of GB1 allows optimal resolution in ^1H dimension.

Fig. 4. Site-specific T_2 measurements of backbone amide ^1H in uniformly ^2H , ^{13}C , ^{15}N labeled GB1 protein, back exchanged to 30% amide ^1H . The transverse relaxation time varies from 3 ms (T25) to 83 ms (D40). The residues that are not observable or overlapped in a hNH 2D spectrum are not shown here. The error bars represent standard deviation calculated using linear regression model (described in text in details)

Fig. 5.

^{13}C dephased ^{15}N - ^1H REDOR difference spectra (S_0 - S) collected on uniformly ^2H , ^{13}C , ^{15}N labeled GB1 protein, back exchanged with 30% $^1\text{H}_\text{N}$. (a-c) Spectra with $^{13}\text{C}\alpha$ dephasing at 240 μs , 360 μs and 600 μs respectively. (d-f) Spectra with $^{13}\text{C}(\text{methyl})$ dephasing at 240 μs , 600 μs and 960 μs . (g-i) $^{13}\text{C}(\text{aromatic})$ dephasing at 240 μs , 600 μs and 840 μs .

Fig. 6. Plot of S/S_0 intensity vs backbone amide (left) and sidechain (right) amino protons in uniformly labeled ^2H , ^{13}C , ^{15}N labeled GB1 protein, back-exchanged with 30% amide protons. (a) shows the plot for $^{13}\text{CH}_3$ dephasing for 600 μs , and (b) shows the plot for aromatic ^{13}C dephasing for 600 μs . The residues showing maximum dephasing have been labeled. Red asterisks indicate that the corresponding residues have overlapped peaks in the ^{15}N - ^1H spectrum.

Fig. 7.
 (a-f): GB1 structure illustrating origins of REDOR effects for the methyl bearing and aromatic residues. (a-b) shows a few methyl bearing residues Ala (a) and Thr,Val (b), (c) shows Y45 and D46, (d) shows F52 and E27, (e) shows Y3, K31 and W43, (f) shows Y33 and N37. The black dotted lines indicate the distances between ^1HN and C(methyl) (a-b) and C(aromatics) (c-f). Figure f shows distances to sidechain amino 1H of N37. All the figures have been rendered in VMD.