J|A|C|S
COMMUNICATIONS

Published on Web 05/20/2004

Diluting Abundant Spins by Isotope Edited Radio Frequency Field Assisted Diffusion

Corey R. Morcombe,[†] Vadim Gaponenko,[‡] R. Andrew Byrd,[‡] and Kurt W. Zilm*[,†]

Department of Chemistry, Yale University, P.O. Box 208107, New Haven, Connecticut 06520-8107, and the Structural Biophysics Laboratory, National Cancer Institute, Frederick, Maryland 21702

Received April 10, 2004; E-mail: kurt.zilm@yale.edu

The development of solid-state NMR (ssNMR) methods for determining protein structures has been motivated by problems associated with protein fibrils, membrane proteins, and difficult-to-crystallize or insoluble proteins. In the past few years a great deal of progress has been made toward realizing generally applicable ssNMR protocols for structure determination of large molecules. Nearly complete sequential and side-chain resonance assignments for ^{13}C- and ^{15}N-enriched proteins have been reported,[1−3] and procedures developed for obtaining highly resolved ^{1}H ssNMR spectra of proteins.[4,5] Especially notable are methods that utilize ^{1}H−^{1}H or ^{15}N−^{15}N spin exchange to identify long-range internuclear contacts.[6,7]

When uniform ^{13}C enrichment is used, diffusion via one-bond couplings dilutes and relays the magnetization[8] so rapidly that long-range transfer (>3.5 Å) is difficult to observe.[9] Castellini et al. developed an elegant chemical solution[10] to this problem by using [1,3-^{13}C]- or [2-^{13}C]-enriched glycerol as the carbon source. This produces protein with very few one-bond ^{13}C−^{13}C couplings, facilitating the observation of long-range contacts. Using this approach they determined the first complete structure of a protein by magic angle spinning (MAS) ssNMR.[10]

In this communication we introduce an alternate strategy to directed enrichment of ^{13}C. Uniform deuteration and exchange in normal water is used to produce a dilute ^{1}H pool.[6] We then recouple only those ^{13}C near a ^{1}H, making them appear dilute as well. The technique uses the pulse sequence diagrammed in Figure 1, essentially a modified proton-driven spin diffusion (PSD) experiment. ^{13}C magnetization is labeled by chemical shift evolution during t_1, flipped up along the static magnetic field, and undergoes spin exchange during τ_m. Readout of the ^{13}C magnetization with a $\pi/2$ pulse generates a two-dimensional (2D) data set which yields a 2D ^{13}C−^{13}C correlation spectrum.

Away from rotational resonance the two principal factors governing the exchange rate in a ^{13}C PSD experiment[11] are the square of the ^{13}C−^{13}C dipolar coupling and the degree of spectral overlap between the spins involved. Under slow MAS, ^{13}C spin diffusion is facilitated by fluctuations in the ^{1}H−^{1}H spin bath and the ^{13}C−^{1}H dipolar coupling, but at high MAS rates (ω_R > 20 kHz) such spin exchange slows dramatically. One solution we have found is to apply a ^{1}H radio frequency (RF) decoupling field with an amplitude ω_{1H} set close to ω_R or $2\omega_R$. The ^{13}C−^{1}H dipolar interaction is then recoupled, and the ^{13}C resonances dramatically broaden. The increase in line width and partial recoupling of the ^{1}H−^{1}H dipolar interactions then restores ^{13}C spin diffusion at high MAS rates. Since this is basically a PSD experiment aided by an interfering RF field, we call it *R*F *a*ssisted *d*iffusion, or RAD, and have used it for many years in making ^{13}C resonance assignments for uniformly ^{13}C-enriched proteins.[12] A very similar experiment has been independently developed by Takegoshi et al., but with the principal aim of enhancing rotational resonance recoupling,[13] and is better described as a dipolar assisted rotational resonance (DARR) experiment.

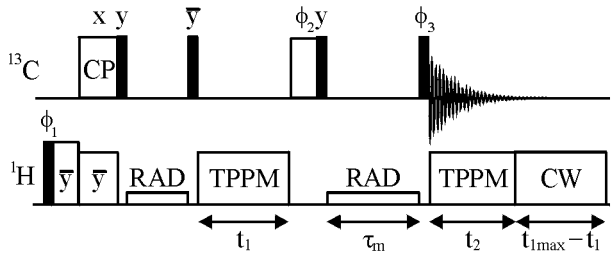

Figure 1. RAD mixing pulse sequence. Solid bars $\pi/2$ pulses (^{1}H 2.0 μs, ^{13}C 2.8 μs). Spin lock 10 μs prior to CP with $\omega_{1C}/2\pi$ = 80 kHz, $\omega_{1H}/2\pi$ = 100 kHz, τ_{CP} = 2 ms, $\omega_R/2\pi$ = 20 kHz. RAD applied with $\omega_{1H} = \omega_R$. RADCP period 50 ms, τ_m 200 ms. ^{13}C spin lock of 104 μs applied at the end of t_1. Decoupling interval $t_{1max}-t_1$ maintains constant RF duty cycle. Phases: $\phi_1 = x,x,x,x,-x,-x,-x,-x$; $\phi_2 = x,-x,x,-x,x,-x,x,-x$; $\phi_3 = x,y,-x,-y,x,y,-x,-y$; receiver = $x,y,-x,-y,-x,-y,x,y$. ϕ_2 advanced for TPPI quadrature detection in t_1. ^{1}H carrier set on resonance with water, ^{13}C carrier set to ~80 ppm.

The pulse sequence diagrammed in Figure 1 creates ^{13}C magnetization by cross polarization (CP) at the $\omega_{1H} = \omega_{1C} + \omega_R$ matching condition and then applies 50 ms of RAD to even out nonuniform CP enhancements (RADCP). After t_1 evolution a component of the transverse magnetization is selected by spin locking, flipped up along *z*, and RAD is applied for τ_m to establish ^{13}C−^{13}C correlations. When applied to a perprotio protein, setting τ_m to 12.5 ms provides very clean ^{13}C spin exchange spectra symmetric about the diagonal, dominated by one-bond correlations, with cross-peak intensities averaging 10−20% of the diagonal. We have recently used this technique to determine the ^{13}C side-chain assignments for human ubiquitin,[3] and the spectra observed are quite similar to the RFDR[9] ^{13}C−^{13}C map shown to the left of Figure 2.

In a perprotio protein sample nearly every ^{13}C has a directly attached ^{1}H, and when RAD is applied, all ^{13}C participate in spin diffusion. Extensive deuteration and equilibration in normal water results in a very different situation. Applying RAD mixing to the ^{1}Hs now only broadens the ^{13}C centers close to sites with exchangeable ^{1}Hs. The line widths for the deuterated sites remain narrow, and spin exchange is halted even through one-bond dipolar couplings. Pairs of ^{13}C spins separated by several bonds, but close to an exchangeable ^{1}H, can have faster mutual exchange rates, since the increase in spectral overlap provided by RAD mixing can more than compensate for the smaller dipolar coupling. The combination of using a dilute ^{1}H pool and RAD mixing to effect restricted spin diffusion among a subset of uniformly enriched ^{13}C nuclei provides many of the advantages of directed enrichment, while preserving

[†] Department of Chemistry, Yale University
[‡] Structural Biophysics Laboratory, National Cancer Institute

10.1021/ja047919t CCC: $27.50

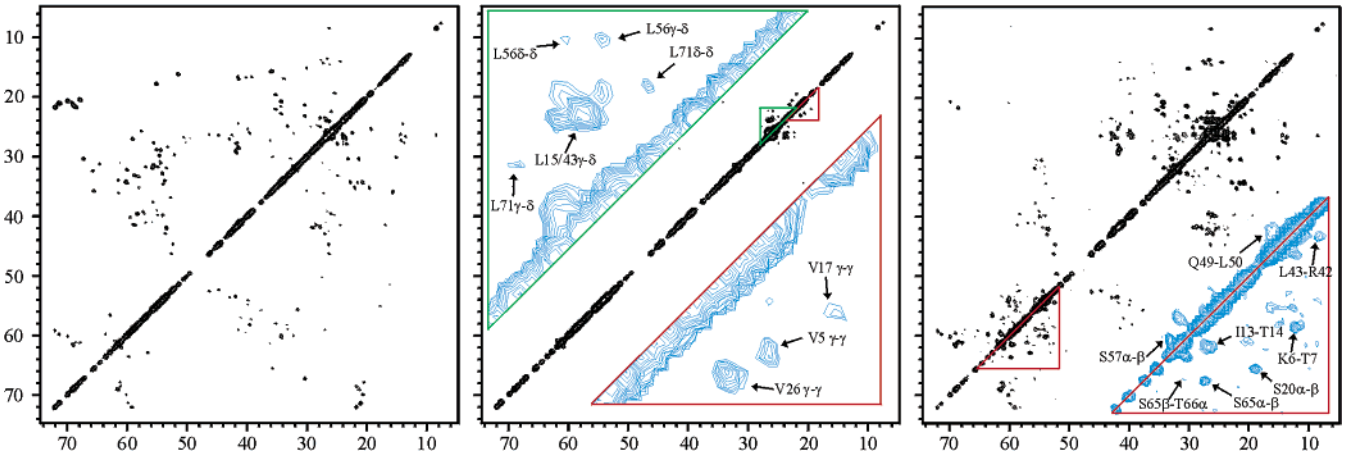

Figure 2. ^{13}C spin exchange spectra for ubiquitin taken on an 800 MHz NMR instrument. For instrumental details and sample preparation see ref 7 and citations therein. RFDR (left), PSD (middle), and iseRAD mixing (right). PSD spectrum obtained using the RAD mixing sequence with the RAD field set to zero amplitude and τ_m set to 50 ms. RFDR spectrum acquired removing the RADCP step, and applying RFDR with XY-8 phase cycling and no ^1H decoupling for 1.6 ms during τ_m. $\omega_R/2\pi = 20$ kHz in all cases. Dwell time in both t_1 and t_2 10 μs, and both acquisition times 15.36 ms. Data zero-filled to 4096 and apodized with a slightly shifted cosine bell function in both t_1 and t_2. 16 scans collected per t_1 increment. Colored insets are expansions, and cross-peaks are between Cα carbons unless otherwise indicated otherwise.

the possibility of observing all one-bond correlations by other techniques. We refer to this method as isotope-edited RAD mixing, or iseRAD.

The effectiveness of this strategy is illustrated in application to a sample of uniformly $^{13}C/^{15}N/^2D$-enriched ubiquitin exchanged in normal water, where the val, leu and ile δ_1 methyls are also perprotio.[14] In this sample, as well as ones with perdeuterated methyls, CP from bound water and the remaining protein ^1Hs is fairly efficient. ^{13}C line widths are essentially identical for perprotio and perdeutero samples, indicating that MAS alone is sufficient for deuterium decoupling.

The spectrum in the left-hand panel was obtained using RFDR. All one-bond correlations are obtained, allowing for complete side-chain assignments. The middle panel displays the PSD spectrum. No cross-peaks appear except for ^{13}C in perprotio methyls. As depicted in the insets, val $C\gamma-C\gamma$, leu $C\delta-C\delta$, and leu $C\delta-C\gamma$ peaks are easily identified.

The final panel depicts the iseRAD spectrum. This is strikingly different from the RFDR spectrum or the RAD spectrum that is obtained on a perprotio sample. A 200 ms RAD mixing time was used, yet the only cross-peaks found involve ^{13}C close to a ^1H. In the 10−40 ppm window the cross-peaks are among the perprotio methyls or to their adjacent carbons. The 40−70 ppm region likewise contains a select subset of cross-peaks. Serines and threonines have exchangeable protons close to the $C\beta$ carbons, and therefore their $C\alpha-C\beta$ cross-peaks are observed as indicated. No other strong $C\alpha-C\beta$ or $C\beta-C\gamma$ cross-peaks are seen. More interesting are the additional long range $^{13}C-^{13}C$ contacts that are cleanly established. The inset in the iseRAD spectrum identifies five such peaks, all sequential $C\alpha_i-C\alpha_{i+1}$ or $C\beta_i-C\alpha_{i+1}$ peaks, corresponding to a range of distances from 3.79 to 4.43 Å. Even though these ^{13}C nuclei have many intervening ^{13}C centers and stronger $^{13}C-^{13}C$ dipolar couplings to other spins, the long-range cross-peaks are more intense due to the selective iseRAD recoupling. Many CO_i-CO_{i+1} cross-peaks are also obtained, and at longer τ_m observation of additional long-range correlations is possible. The absence of one-bond $^{13}C-^{13}C$ cross-peaks, and the elimination of spin exchange past the $C\beta$ carbon centers significantly simplifies the spectrum. This saves magnetization for long-range mixing, and keeps the 2D map simple, facilitiating unambiguous assignment of the long range $^{13}C-^{13}C$ contacts. Such correlations cannot be observed using perprotio samples as the $^{13}C-^{13}C$ map is severely congested by two-bond and relayed transfer at sufficiently long τ_m.

The use of deuteration to dilute the ^1H pool makes it possible to obtain both resonance assignments and long-range dipolar contacts on the same uniformly $^{13}C/^{15}N$-enriched sample. This concept of using the differential distribution of spin pools in combination with RAD to effect a selective enhancement of homonuclear spin exchange has wide potential applications. The same method should enhance $^{15}N-^{15}N$ spin exchange, and could be used to establish selective $^1H-^1H$ mixing by irradiating attached ^{15}N or ^{13}C nuclei. The simplicity of the iseRAD approach makes it a versatile and robust technique, and we expect it to find widespread use in structural studies of proteins by ssNMR.

Acknowledgment. This work was supported in part by the Wm. M. Keck Foundation and Yale University. C.R.M. gratefully acknowledges the support of a NSERC post-graduate fellowship.

References

(1) Pauli, J.; Baldus, M.; van Rossum, B.; de Groot, H.; Oschkinat, H. *ChemBioChem* **2001**, *2*, 272−281.
(2) Igumenova, T. I.; Wand, A. J.; McDermott, A. E. *J. Am. Chem. Soc.* **2004**, *126*, 5323−5331.
(3) Igumenova, T. I.; McDermott, A. E.; Zilm, K. W.; Martin, R. W.; Paulson, E. K.; Wand, A. J. *J. Am. Chem. Soc.* **2004**, *126*, 6720−6727.
(4) Paulson, E. K.; Morcombe, C. R.; Gaponenko, V.; Dancheck, B.; Byrd, R. A.; Zilm, K. W. *J. Am. Chem. Soc.* **2003**, *125*, 15831−15836.
(5) Chevelkov, V.; van Rossum, B. J.; Castellani, F.; Rehbein, K.; Diehl, A.; Hohwy, M.; Steuernagel, S.; Engelke, F.; Oschkinat, H.; Reif, B. *J. Am. Chem. Soc.* **2003**, *125*, 7788−7789.
(6) Reif, B.; van Rossum, B. J.; Castellani, F.; Rehbein, K.; Diehl, A.; Oschkinat, H. *J. Am. Chem. Soc.* **2003**, *125*, 1488−1489.
(7) Paulson, E. K.; Morcombe, C. R.; Gaponenko, V.; Dancheck, B.; Byrd, R. A.; Zilm, K. W. *J. Am. Chem. Soc.* **2003**, *125*, 14222−14223.
(8) Gehman, J. D.; Paulson, E. K.; Zilm, K. W. *J. Biomol. NMR* **2003**, *27*, 235−259.
(9) Bennett, A. E.; Rienstra, C. M.; Griffiths, J. M.; Zhen, W. G.; Lansbury, P. T.; Griffin, R. G. *J. Chem. Phys.* **1998**, *108*, 9463−9479.
(10) Castellani, F.; van Rossum, B.; Diehl, A.; Schubert, M.; Rehbein, K.; Oschkinat, H. *Nature* **2002**, *420*, 98−102.
(11) Gan, Z. H.; Ernst, R. R. *Chem. Phys. Lett.* **1996**, *253*, 13−19.
(12) Zilm, K. W. Presented at the 40th Experimental NMR Conference, Orlando, FL, 1999.
(13) Takegoshi, K.; Nakamura, S.; Terao, T. *Chem. Phys. Lett.* **2001**, *344*, 631−637.
(14) Goto, N. K.; Gardner, K. H.; Mueller, G. A.; Willis, R. C.; Kay, L. E. *J. Biomol. NMR* **1999**, *13*, 369−374.

JA047919T