Simultaneous Multiple Distance Measurements in Peptides via Solid-State NMR

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A knowledge of atomic-level structure, including interatomic connectivities, is the link between the observed function of materials and the understanding that enables rational development of novel compounds. Although design paradigms based upon structure−function relationships have long been practiced in the fields of chemistry and materials science, the detailed understanding that enables rational manipulation and development of biologically-active compounds remains elusive. This weakness can mainly be associated with the inherent complexity of biological compounds: their size and sensitivity to experimental conditions makes a detailed accounting of atomic arrangements an experimental and computational challenge. Overcoming these obstacles increases in importance as researchers uncover more and more specific molecules responsible for normal and abnormal cellular behavior.

The traditional methods of structure determination for biomolecules include crystallography and solution-state NMR.[1] These methods provide information on atomic location with accuracies of 0.5−2.0 Å. The primary drawbacks of crystallographic methods include difficulties in obtaining high-quality crystals, expense and time associated with the crystallization process, and difficulties in resolving whether the structure of the crystalline forms is representative of the *in vivo* conformation.[2,3] High-resolution, multidimensional, solution-state NMR techniques[4] represent an attractive alternative, as they can be applied in an aqueous environment. However, analysis of the various mutual correlations between nuclei from the indirect dipole−dipole coupling (studied via the nuclear Overhauser effect, NOE) is time consuming, and provides only a rough measurement of internuclear distances.[1]

Recently, a number of solid-state NMR techniques have been developed that allow the measurement of weak, *direct* dipolar interactions in disordered materials. One of these methods, rotational-echo double-resonance (REDOR), has shown promise for the rapid elucidation of bond distances with an accuracy of 0.1 Å or better[5−7] in peptides and small proteins. Measurements of biologically relevant $^{13}C-^{15}N$ distances up to 6.3 Å have been reported using this technique.[8] As opposed to crystallography, solid-state NMR has the advantage that it can provide high-resolution structural information from disordered (polycrystalline or amorphous) materials. In contrast to solution-state NMR, solid-state NMR relies upon the direct dipolar coupling, which scales as the inverse cube of the internuclear separation, and allows the measurement of longer distances with a higher degree of accuracy and precision. Additionally, due to resolution concerns, solution-state NMR has strict size limitations on the molecules that can be studied.

A limitation with REDOR as it is currently practiced is the slow nature of the information gathering—usually only one distance at a time is measured between specifically labeled spins. Measurement of a number of distances, for instance at an active binding site in a molecule, is then both time consuming and expensive. Garbow and Gullion[9] have shown that these burdens can be reduced by the measurement of REDOR signals from chemically shifted spins. However, as the size of a molecule increases, this strategy (in conjunction with selective labeling) will become limited.

In this paper, we describe the first application of a new analysis method for solid-state NMR, the REDOR transform,[10,11] to the study of biologically-relevant molecules. Theoretically, this purely analytic transform can resolve multiple distances by producing a pure dipolar spectrum representing the different distances of all labeled heteronuclear spins (i.e., ^{13}C) that dephase an observed set of nuclei (i.e., ^{15}N). This immediately makes the use of non-chemically shifted nuclei and/or aggressive labeling schemes more amenable to REDOR analysis. Therefore, the results presented herein represent an important step toward the development of reliable methods for biochemical and materials structure determinations.

The REDOR experiment was performed on a Chemagnetics CMX300 spectrometer equipped with a 5-mm triple-resonance probe. We sampled 28 S and S_0 signals, where S_0 is the echo signal collected in the absence of ^{13}C dephasing pulses, and S is the echo signal collected with dephasing pulses. The sample used in this study was a 1:1 physical mixture of two cyclic peptides, identical except in their labeling. Both peptides were Cys-Asn-Thr-Leu-Lys-*Gly-Asp-Cys-Gly, bound to mBHA resin via the terminal Gly. This particular peptide has been shown to bind to the glycoprotein IIb/IIIa and therefore act as a competitive inhibitor against binding with fibrinogen and resulting platelet aggregation.[12] The peptides were synthesized via FMOC chemistry and cyclized via a disulfide bond (Multiple Peptide Systems, San Diego, CA). One peptide was labeled with ^{15}N,1-^{13}C Gly; the other was labeled with ^{15}N,2-^{13}C Gly (Isotec, Miamisburg, OH). No further dilution of the peptides was necessary, as they were naturally diluted by the presence of the synthesis resin, thus eliminating intermolecular dipolar couplings. The synthesis resin (Advanced ChemTech, Louisville, KY) had 0.1 mequiv of populated mBHA surface sites/g of polystyrene resin. From the peptide molecular weight of 890 g/mol, this gives us 0.09 g of peptide/1 g of resin. Approximately 85 mg of the peptide-resin sample, and therefore ∼7 mg of peptide, was packed into a rotor.

To obtain a spectrum of the dipolar couplings present in this peptide system, a REDOR transform is applied to the dephasing data obtained by calculating S/S_0 as a function of total dephasing time. The transformed spectrum, presented in Figure 1, reveals two strong peaks corresponding to frequencies of 200 ± 54 and 900 ± 54 Hz. As detailed in the figure caption, resolution is limited by the sampling rate combined with the number of data points sampled.

The results agree well with X-ray crystallographic data from pure glycine, where the ^{15}N,1-^{13}C crystallographic distance is

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(1) MacArthur, M. W.; Driscoll, P. C.; Thornton, J. M. *Trends Biotechnol.* **1994**, *12*, 149−153.
(2) Shaanan, B.; Gronenborn, A. M.; Cogen, G. H.; Gilliland, G. L.; Veerapandian, B.; Davies, D. R.; Clore, G. M. *Science* **1992**, *257*, 961−964.
(3) Clore, G. M.; Gronenborn, A. M. *J. Mol. Biol.* **1991**, *221*, 47−53.
(4) Clore, G. M.; Gronenborn, A. M. *Protein Sci.* **1994**, *3*, 372−390.
(5) Garbow, J. R.; McWherter, C. A. *J. Am. Chem. Soc.* **1993**, *115*, 238−244.
(6) Gullion, T.; Schaefer, J. *J. Magn. Reson.* **1989**, *81*, 196−200.
(7) Gullion, T.; Schaefer, J. *Adv. Magn. Reson.* **1989**, *13*, 57−83.
(8) Hing, A. W.; Tjandra, N.; Cottam, P. F.; Schaefer, J.; Ho, C. *Biochemistry* **1994**, *33*, 8651−8661.
(9) Garbow, J. R.; Gullion, T. *J. Magn. Reson.* **1991**, *95*, 442−445.
(10) Mueller, K. T. *J. Magn. Res., Ser. A* **1995**, *113*, 81−93.
(11) Mueller, K. T.; Jarvie, T. P.; Aurentz, D. J.; Roberts, B. W. *Chem. Phys. Lett.* **1995**, *242*, 535−542.
(12) O'Neil, K. T.; Hoess, R. H.; Jackson, S. A.; Ramachandran, N. S.; Mousa, S. A.; DeGrado, W. F. *Proteins: Struct., Funct., Genet.* **1992**, *14*, 509−515.

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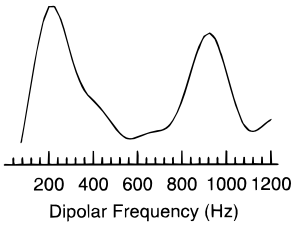

Figure 1. A REDOR transform of data from a 1:1 physical mixture of two cyclic peptides yields a spectrum of dipolar couplings present in the sample. The ^{15}N-detected REDOR S/S_0 data were baseline corrected and smoothed with a three-point Blackman−Harris apodization function prior to transformation. The resonance frequencies for the nuclei were as follows: ^{1}H 299.991 MHz; ^{15}N 30.401 MHz; ^{13}C 75.442 MHz. The ^{13}C π pulse width was 8.8 μs; the ^{15}N π pulse width was 12.2 μs, and the initial ^{1}H $\pi/2$ pulse width was 3.0 μs. Proton decoupling of 90 kHz was used during the REDOR dephasing period and 70 kHz decoupling power was applied during the acquisition period. Data were collected once every four rotor cycles while spinning at 6000 Hz using XY-4 phase cycling, providing an approximate frequency band width of 1500 Hz.[11] 4096 run averages were collected for every S and S_0 point with a recycle delay of 1 s. A total of 28 S and 28 S_0 points were collected.

1.49 Å. In the peptide, the distance measured via the REDOR transform is 1.50 ± 0.03 Å. The ^{15}N,2-^{13}C crystallographic distance is 2.47 Å, and the measured distance in the peptide is 2.48 ± 0.25 Å. For the measurement of nontrivial distances (3 or more bonds between atoms), smaller dipolar couplings are present, and therefore a smaller spectral width than was necessary for the 900-Hz coupling is sufficient. Smaller sweep widths at reasonable spinning speeds make improved phase cycling via XY-8 or XY-16 possible,[13] and result in a finer distance resolution for a given number of S/S_0 data points collected. REDOR transformation of simulated data with small band widths and a large number of S/S_0 data points indicate that 0.1-Å resolution for 5−6-Å distances is possible. In practice, however, effects such as relaxation and long-term spectrometer stability may interfere with the precision of measurements at long distances. The trade-off gained when using this technique is the ability to measure dispersions in distances present within a given spin-pair, and to effectively eliminate the need to correct for the dephasing from naturally abundant spins.

Figure 2 presents the two-dimensional NMR analysis of the same data set. This processing takes advantage of the inherent two-dimensional nature of the dipolar-dephasing experiments. In this analysis, one dimension is the Fourier transform of the accumulated ^{15}N echo signal, giving a chemical shift spectrum of the observed nucleus. The other dimension is a REDOR transform of time-domain dephasing amplitudes, yielding the dipolar coupling frequencies correlated to each resolved chemical shift.

As this example indicates, the accurate, simultaneous determination of multiple distances in complex biomolecules is readily obtained using a REDOR transform. The extension of this technique to the simultaneous measurement of more than two distances in peptides and to simultaneous distance measurements in other molecules of biological and materials science importance is now possible. Additionally, this transform is not limited exclusively to the REDOR experiment, as a number of other NMR experiments have the same functional form for time evolution as the REDOR signal.

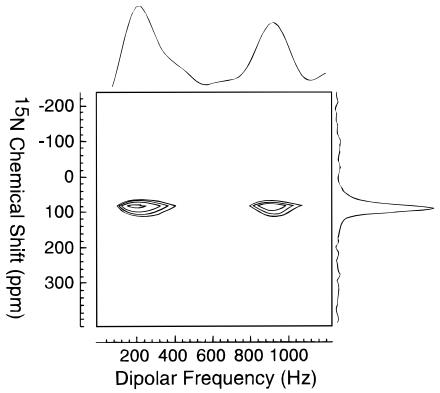

Figure 2. Two-dimensional presentation of the REDOR data shows the correlation between the chemical shift of the observed nucleus and the heteronuclear dipolar couplings. Prior to Fourier transform, the 1024 point ^{15}N FIDs were truncated to 32 points and line broadened to 500 Hz with an exponential function. A REDOR transform was then performed on each of the 32 points in the chemical shift spectra.

Ultimately, the REDOR transform has the potential to aid in the drug discovery process via improved local structural studies of regions of peptides that bind proteins. The technique is not strictly limited by the molecular weight of the protein/ligand complex, or by the availability of highly regular crystals for diffraction. The application of this transform to one- and two-dimensional data processing substantially increases the number of useful REDOR measurements per experiment and, in the case of peptides, could allow the active site to be quickly studied. Additionally, the application of this improved technique to small protein domains is foreseeable. One important and immediate extension of the two-dimensional version of this technique is the measurement of multiple distances from many chemically shifted spins, as a means to ultimately enable the measurement of all distances in broadly labeled material. Such labeling methods would enhance the utility of the technique by lowering the cost of experimental samples through more cost-effective chemical synthesis or perhaps by allowing biological synthesis. To simplify assignment of the resonances in samples with multiple labels, one could envision a three-dimensional version of this experiment that correlates the isotropic chemical shifts of both heteronuclei to the dipolar coupling frequency.

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(13) Gullion, T.; Baker, D. B.; Conradi, M. S. *J. Magn. Reson.* **1990**, *89*, 479−484.