REDOR-Determined Distances from Heterospins to Clusters of ^{13}C Labels

Jacob Schaefer

Department of Chemistry, Washington University, St. Louis, Missouri 63130

The use of rotational-echo double resonance NMR to measure distances from an observed tightly coupled cluster of ^{13}C spins to a distant ^{15}N, ^{31}P, or ^{19}F is practical if all homonuclear ^{13}C–^{13}C dipolar interactions are suppressed by multiple-pulse decoupling during heteronuclear dipolar evolution. This scheme is first calibrated by experiments performed on multiply labeled alanines and then applied in the measurement of ^{19}F–^{13}C distances in *p*-trifluoromethylphenyl [1,2-^{13}C$_2$]acetate. © 1999 Academic Press

It is well known that the information content of REDOR experiments could be improved if more than just the two spins of an isolated pair were involved. Clusters of ^{13}C labels coupled to some heterospin are of particular interest. Unfortunately, the use of rotational-echo double resonance (REDOR) to measure distances from an *observed* tightly coupled cluster of two or more ^{13}C labels (in a uniformly labeled residue of a protein, for example) to a distant ^{15}N, ^{31}P, or ^{19}F is compromised by homonuclear dephasing of the ^{13}C echo train. Long-range distance determinations by REDOR require that rotational echo trains persist for times of the order of 30 ms (*1*). Even though the 5- to 10-kHz magic-angle spinning speeds most commonly used in solid-state NMR experiments reduce the homonuclear dephasing effects of the 2-kHz dipolar coupling between directly bonded ^{13}C's, spinning at these rates does not completely remove them. In addition, spinning has no effect on ^{13}C–^{13}C scalar couplings, which are typically 50–100 Hz for directly bonded carbons and 10 Hz for carbons separated by two bonds (*2*). The result is that ^{13}C echo trains for coupled carbons are attenuated and lose coherence after about 15 ms (Fig. 1, top).

One way to improve the integrity of the echo train is to spin much faster, on the order of, say, 30 kHz. Fast spinning quenches homonuclear dipolar (but not scalar) coupling. This is a promising approach that is being pursued in several laboratories (*3, 4*), despite the fact that the volumes of current high-speed rotors limit the sample size to about 10 mg. A second approach is to create multiple-quantum coherence whose time evolution is independent of ^{13}C–^{13}C coupling within the cluster (*5*). However, this method gives up infor-

mation (all ^{13}C spins within the cluster experience the same average heteronuclear dipolar coupling) and suffers from reduced sensitivity when the clusters have more than two carbons. A third approach to maintain the integrity of a rotational echo train for a cluster of ^{13}C spins is to suppress residual dipolar ^{13}C–^{13}C interactions by multiple-pulse decoupling (Fig. 1, bottom). In this communication, we show that it is possible to observe heteronuclear REDOR dephasing for a ^{13}C cluster while using multiple-pulse decoupling (MPD) to suppress homonuclear interactions.

The pulse sequence for multiple-pulse decoupled REDOR (MPDR) had 5-μs ^{13}C $\pi/2$ pulses on the rotor periods (four pulses in six T_r) and a pair of X-spin π pulses within each rotor period. The X-spin π pulse lengths were 10 μs for ^{15}N and 5 μs for ^{19}F. The complete MPDR pulse sequence is illustrated in Fig. 2. Standard MREV-8 phase cycling (*6*) was used for MPD, and XY-8 phase cycling (*7*) for REDOR. Data acquisition began after multiples of 48 rotor periods so that each phase-cycling scheme was performed an even number of times before and after the central π pulse for optimum refocusing.

Results of MPDR experiments on a directly bonded ^{13}C–^{13}C pair in alanine and in *p*-trifluoromethylphenyl acetate are shown in Fig. 3. If the ^{13}C carrier frequency is chosen so that none of the isotropic peaks are on resonance (or off resonance by a multiple of the spinning frequency), REDOR-difference and full-echo spectra remain in phase after 48, 96, and 144 rotor cycles with spinning at 6.250 kHz. The cycle time of the MPD is $6T_r$, which corresponds to about 1-kHz decoupling. This is apparently sufficient to suppress dipolar couplings only partially averaged by spinning.

The observed MPDR values of $\Delta S/S_0$ (96 T_r) for the carboxyl carbons of [1-^{13}C, ^{15}N]alanine, [1,2-^{13}C$_2$, ^{15}N]alanine, and [1,2,3-^{13}C$_3$, ^{15}N]alanine are 0.59, 0.48, and 0.35, respectively. The 48% dephasing in the 96-T_r ^{13}C$_2$-alanine spectra of Fig. 3 represents about 70% of that calculated in three-spin simulations (*8*) (assuming δ-function pulses) explicitly including MPD. This limit is not related to the expected scaling (*6*) of the ^{13}C–X dipolar coupling which is taken into account in the simulation, and is not exceeded by increasing the dephasing time. Standard isolated two-spin REDOR experiments yield close to 100% of the calculated dephasing ($\Delta S/S_0 \approx 1$ for 96

AP
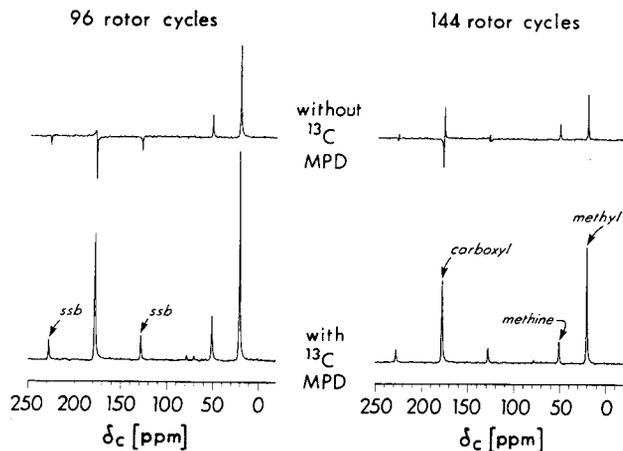
FIG. 1. 125-MHz ^{13}C NMR spectra of L-[^{13}C$_3$,^{15}N]alanine (5%, recrystallized with 95% natural-abundance alanine) obtained after 96 rotor cycles (left) and 144 rotor cycles (right) of evolution with (bottom) and without (top) ^{13}C multiple-pulse decoupling using the pulse sequence of Fig. 2 but with the X-spin dephasing pulses omitted.

T_r). We attribute the shortfall in MPDR to (i) the difficulty in compensating for the imperfections in $\pi/2$ pulses; (ii) the possibility that some ^{13}C spins may not be in the MPD toggling frame, but rather along the static magnetic field during dipolar evolution, but not during detection; and (iii) destructive interference in coherent averaging by the combination of magic-angle spinning and imperfect MPD. With respect to the latter, the difference in cycle times for MPD and spinning in the experiments of Fig. 3 is only a factor of 6, and the combination of averaging techniques, although greatly extending the useful lifetime of the ^{13}C rotational echo train, is limited. The decrease in intensity of the full-echo spectrum of ^{13}C$_3$-labeled alanine for 144 T_r compared to 96 T_r (Fig. 1, bottom) is about double that observed in the standard Hahn-echo spectra of natural-abundance ^{13}C alanine. Spinning faster should improve this situation by reducing the cycle times of both magic-angle and MPD averaging.

Even in the presence of the same ^{13}C–X dipolar coupling, the observed MPDR dephasing is not the same for different types of carbons, although the calculated dephasing is the same. In general, carbons with sizeable chemical shift anisotropies show less dephasing than those with small shift tensors, particularly for weak coupling after only 48 rotor cycles. Because these variations appear to be related to the response of the ^{13}C spins to imperfect MPD, REDOR dephasing remains proportional to the heteronuclear dipolar coupling for a *fixed* dipolar evolution time. We have therefore adopted an empirical scaling procedure to determine internuclear distances from MPDR dephasing, which we illustrate for the carboxyl carbons of directly bonded ^{13}C–^{13}C pairs. The calculated MPDR dephasing for the 185-Hz (9), two-bond ^{13}C–^{15}N dipolar coupling in L-[1,2-^{13}C$_2$, ^{15}N]alanine after 96 T_r is $\Delta S/S_0 = 0.74$, and the observed dephasing for the carboxyl carbon is $\Delta S/S_0 = 0.48$ (Fig. 3, left; Fig. 4, open circle). The ratio of the two is 0.65, which we take as the scaling factor for all calculated values of $\Delta S/S_0$ for this carbon in the 96-T_r experiment. A scaling factor of 0.67 is generated for the 48-T_r experiment by comparison of the corresponding calculated and experimental dephasing for the carboxyl carbon. The resulting scaled versions of the predicted $\Delta S/S_0$ (Fig. 4, solid lines) can now be used to determine the dipolar coupling between a carboxyl carbon in a ^{13}C–^{13}C pair and any X spin in an analytical sample.

As a test of this method, the ^{13}C–^{19}F coupling between the CF$_3$ group and the carboxyl carbon in the *p*-trifluoromethylphenyl acetate of Fig. 3 (right) is measured in Fig. 4 (experimental $\Delta S/S_0$ shown by solid symbols) as 75 Hz (7.1 Å) after 48 rotor cycles and 70 Hz (7.3 Å) after 96 rotor cycles. These values are in agreement with the calculated, conformation-independent, pseudo two-bond distance of 7.2 Å. This is the distance from the carboxyl carbon to the center of the triangle defined by the three fluorines. This distance is long enough that the orientation of the CF$_3$ cone relative to the internuclear vector connecting the acetate oxygen and carboxyl carbon is not important (10), but short enough that the dephasing behavior of the CF$_3$ and that of a single fluorine are indistinguishable. The ^{13}C–^{19}F coupling between the CF$_3$ group
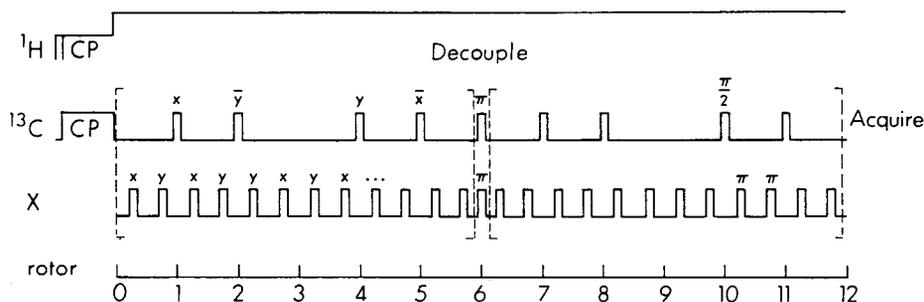
FIG. 2. Pulse sequence for multiple-pulse decoupled REDOR. Phase cycling of the ^{13}C $\pi/2$ pulses is (x, $-y$, y, $-x$, $-x$, $-y$, y, x) and that of the X-spin π pulses, (x, y, x, y, y, x, y, x). The former is repeated $2n$ times ($n = 1, 2, 3, \ldots$) in $24nT_r$ before and after the central ^{13}C π pulse (this pulse refocuses isotropic chemical shifts), while the latter is repeated $6n$ times before and after the central X π pulse (this pulse prevents refocusing of ^{13}C–X dipolar dephasing). Maximum REDOR dephasing occurs if the spacing between X-spin pulses is $T_r/2$.
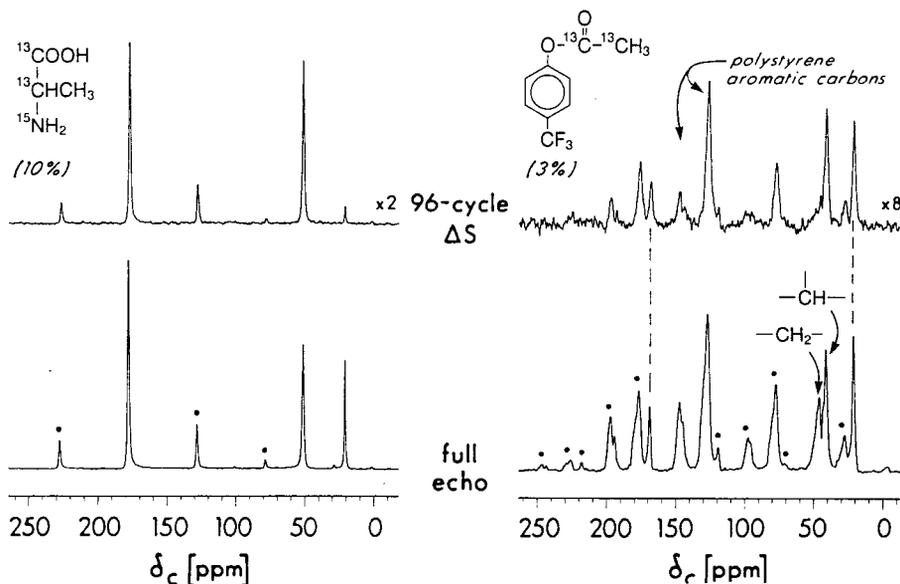
FIG. 3. 125-MHz multiple-pulse decoupled REDOR ^{13}C NMR spectra of L-[1,2-^{13}C$_2$,^{15}N]alanine (left, 10%, recrystallized with 90% natural-abundance alanine) and p-trifluoromethylphenyl [1,2-^{13}C$_2$]acetate (right, 3% by weight, homogeneously dispersed in glassy polystyrene) obtained after 96 rotor cycles of dipolar evolution with magic-angle spinning at 6.250 kHz. The spacing between the ^{15}N (left) and ^{19}F (right) dephasing pulses was T_r/2. The REDOR-difference spectra are at the top of the figure and the full-echo spectra at the bottom. Solid circles indicate spinning sidebands; dashed lines connect peaks associated with the labeled acetate carbons. The sizeable dephasing of the natural-abundance ^{13}C methine-carbon peak suggests a preferred location for the CF$_3$ group in the polystyrene glass.

and the methyl carbon is determined similarly, with a scaling factor of 1.00 (48 T_r) and 0.78 (96 T_r) established by the methyl-carbon results (not shown) for both double- and triple-^{13}C labeled alanines. The conformation-dependent experimental distance from the methyl carbon to the CF$_3$ group is 8.1 Å (48 T_r) or 7.7 Å (96 T_r).

We anticipate a broad applicability for MPDR. Even though the dephasing is generally reduced relative to stan-

FIG. 4. Multiple-pulse decoupled REDOR dephasing ($\Delta S/S_0$) for the carboxyl carbon of a ^{13}C–^{13}C directly bonded pair as a function of the heteronuclear dipolar coupling to a spin-$\frac{1}{2}$ X nucleus. The solid lines show the scaled calculated dephasing in the presence of multiple-pulse decoupling (see text). The open and closed symbols are, respectively, experimental results for the carboxyl carbons of the alanine and acetate ^{13}C–^{13}C pairs of Fig. 3, obtained using the pulse sequence of Fig. 2 with magic-angle spinning at 6.250 kHz.

dard REDOR for isolated pairs of spins, the information content of any single experiment is dramatically increased. As long as chemical-shift resolution is available in the ^{13}C NMR spectrum, multiple independent clusters can be used. For example, one could imagine a protein binding site that had both ^{13}C$_6$-histidine and ^{13}C$_4$-threonine so that seven or eight distances could be measured to a ligand containing ^{19}F or ^{31}P. Preliminary results on L-[^{13}C$_6$, ^{15}N$_3$]histidine indicate that MPDR works as well for 6-carbon clusters as for 2- and 3-carbon clusters. However, a dephasing scaling factor must be generated *for each carbon type* by experiment and either used in conjunction with multispin simulations (as in Fig. 4), or with calibration experiments on one or more model compounds containing the *identical* ^{13}C spin cluster to be used in the analytical application but with known hetero-nuclear dipolar couplings. In calibration and analytical experiments, strong and weak couplings are measured together using *fixed* dipolar evolution times by systematic variation of the separation of the REDOR dephasing pulses (*11*). In the event that the natural-abundance ^{13}C background over-whelms the signals of the ^{13}C clusters in the analytical application, one of a variety of homonuclear recoupling schemes (*12–16*) can be used to isolate the signals from ^{13}C labels and generate the spin count needed for S_0.

EXPERIMENTAL DETAILS

MPDR was performed using a 6-frequency transmission line probe having a 12-mm long, 6-mm inside-diameter

analytical coil and a Chemagnetics/Varian ceramic stator. Powdered samples (typically 100 mg) were contained in thin-wall Chemagnetics/Varian 5-mm outside diameter zirconia rotors. The rotors were spun at 6250 Hz with the speed under active control to within ± 2 Hz. The spectrometer was controlled by a Tecmag pulse programmer. ^{13}C and ^{15}N radiofrequency pulses were produced by 1- and 2-kW American Microwave Technology power amplifiers, respectively. ^{1}H and ^{19}F radiofrequency pulses were generated by 1-kW Creative Electronics tube amplifiers driven by 100-watt American Microwave Technology power amplifiers. A 12-T static magnetic field was provided by an 89-mm bore Magnex superconducting solenoid. Proton-carbon cross-polarization transfers were made in 2 ms at 50 kHz. Proton dipolar decoupling was 100 kHz during data acquisition. Spectra of alanine standards and the *p*-trifluoromethylphenyl acetate analytical sample resulted from the accumulation of 64 and 3072 scans, respectively.

ACKNOWLEDGMENTS

REFERENCES

1. L. M. McDowell, A. Schmidt, E. R. Cohen, D. R. Studelska, and J. Schaefer, *J. Mol. Biol.* **256**, 160–171 (1996).

2. J. B. Stothers, "Carbon-13 NMR Spectroscopy," Academic Press, New York (1972), p. 327; pp. 370–373.

3. A. K. Mehta, B. A. Tounge, S. T. Burns, X. Wu, and K. W. Zilm, 39th Rocky Mountain Conference on Analytical Chemistry, Denver (1997).

4. M. Ernst, A. Verhoeven, and B. H. Meier, *J. Magn. Reson.* **130**, 176–185 (1998).

5. X. Feng, M. Edén, A. Brinkmann, H. Luthman, L. Eriksson, A. Gräslund, O. N. Antzukin, and M. H. Levitt, *J. Am. Chem. Soc.* **119**, 12006–12007 (1997).

6. P. Mansfield, *Phil. Trans. R. Soc. Lond. A* **299**, 479–496 (1981).

7. T. G. Gullion, D. B. Baker, and M. S. Conradi, M. S., *J. Magn. Reson.* **89**, 479–484 (1990).

8. S. M. Holl, G. R. Marshall, D. D. Beusen, K. Kociolek, A. S. Redlinski, M. T. Leplawy, R. A. McKay, S. Vega, and J. Schaefer, *J. Am. Chem. Soc.* **114**, 4830–4833 (1992).

9. T. Gullion and J. Schaefer, *Adv. Magn. Reson.* **13**, 57–83 (1989).

10. J. M. Goetz and J. Schaefer, *J. Magn. Reson.* **127**, 147–154 (1997).

11. A. W. Hing and J. Schaefer, *Biochemistry* **32**, 7593–7604 (1993).

12. D. P. Raleigh, M. H. Levitt, and R. G. Griffin, *Chem. Phys. Lett.* **146**, 71–76 (1988).

13. V. Bork and J. Schaefer, *J. Magn. Reson.* **78**, 348–354 (1988).

14. R. Tycko and G. Dabbagh, *Chem. Phys. Lett.* **173**, 461–465 (1990).

15. T. Gullion and S. Vega, *Chem. Phys. Lett.* **194**, 423–428 (1992).

16. W. Zhu, C. A. Klug, and J. Schaefer, *J. Magn. Reson.* **108**, 121–123 (1994).