

# Measurement of Internuclear Distances in Solid-State NMR by a Background-Filtered REDOR Experiment

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**A background-filtered version of the rotational-echo double resonance (REDOR) experiment is demonstrated. The experiment combines a traditional REDOR pulse sequence with a double-cross-polarization (DCP) sequence to select only those signals coming from spin pairs of interest. The relatively inefficient DCP sequence, which transfers polarization from <sup>1</sup>H to <sup>15</sup>N and subsequently to <sup>13</sup>C, is improved by the use of adiabatic passages through the (−1) sideband of the Hartmann–Hahn matching condition. The result is an efficient 2D-REDOR pulse sequence that does not require a reference experiment for removal of background signals. The data produced by the experiment are ideally suited to analysis by newly developed dipolar transform methods, such as the REDOR transform. The relevant features of the experiment are demonstrated on simple labeled amino acids. Relative efficiencies of several other potential filtering methods are also compared.** © 2000 Academic Press

**Key Words:** solid-state NMR; rotational-echo double resonance (REDOR); double-cross-polarization (DCP); adiabatic passage; internuclear distance; REDOR transform; regularization.

## INTRODUCTION

Measurement of internuclear distances by solid-state NMR has evolved into its own relatively extensive area of research, as demonstrated in several recent reviews of the field (1–4). The NMR techniques utilized for this task are designed to measure dipolar couplings, which are related in a simple manner to the inverse third power of the internuclear distance (5). The majority of the successful applications to date involve the reintroduction and measurement of heteronuclear dipolar coupling under the line-narrowing conditions of magic-angle spinning (6, 7), often using the popular rotational-echo double-resonance (REDOR) methodology (8–10). A number of diverse applications of REDOR distance determinations to studies of biological and chemical systems have appeared recently (11–21). The <sup>13</sup>C–<sup>15</sup>N spin pair has been the subject of much of this attention, since the possibility of measuring distances of 3–5 Å can make the determination of secondary

structure in peptides and other biomolecules a very feasible goal (18, 19). The REDOR experiment functions by using RF pulses to interfere with the MAS averaging of the heteronuclear dipolar coupling. The experiment can be run in either a one-dimensional or a full two-dimensional mode. In the latter experiment the indirectly detected dimension evolves solely under the influence of a reintroduced dipolar Hamiltonian (9, 10).

The signal observed in the dipolar dimension during a 2D-REDOR experiment can be processed using a Fourier transform, yielding a broad powder pattern from which the dipolar coupling (*D*) can be measured (9). Alternatively, the signal can often be fit in the time domain to determine the internuclear distance (9, 10, 22), even if only a few points are available. Analysis of the REDOR signal and extraction of *D* can be improved by designing processing techniques to extract a pure dipolar spectrum from the oscillatory (but nonsinusoidal) components of the REDOR decay. The introduction of these types of processing techniques, such as the REDOR transform and related methods (23, 24), has extended the capability of REDOR by facilitating measurements of multiple dipolar couplings. The REDOR transform has been used to analyze multiple dipolar frequencies in several recent studies (25–27). A further benefit of the REDOR transform is that REDOR data for spin pairs can now be examined in an easily interpretable two-dimensional mode in which the isotropic chemical shift is correlated with the dipolar coupling magnitude via a single cross-peak (25). However, these techniques have led to the more challenging experimental requirement of collecting as many 2D-REDOR data points as possible in order to accurately map out the dipolar spectrum in the second dimension. Due to the rapidly decaying nature of the REDOR signal, this requirement greatly strains the sensitivity of NMR instrumentation. The analysis of the signal is complicated by the need to treat it as a true time-domain signal that often requires apodization and time-domain baseline correction.

The REDOR experiment has several advantages that have led to its success in heteronuclear coupling measurement. The sequence relies on a train of easily calibrated  $\pi$  pulses and has a simple design that can be implemented on most spectrome-

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ters. Phase cycling schemes can correct for pulse timing and amplitude errors (10, 28). The most commonly used sequences are fairly tolerant to small variations in MAS spinning speed (10, 29), and the radiofrequency power levels needed during the sequence can be tolerated by many commercially manufactured MAS probes. The initial stage of a REDOR experiment often involves a cross-polarization (CP) step from a third, more abundant nucleus (usually  $^1\text{H}$ ), so that a triple-resonance REDOR experiment can be performed with the same enhanced sensitivity found in CP-MAS experiments (30). However, one disadvantage of REDOR is its need for a separate reference experiment to account for the presence of uncoupled background nuclei with degenerate chemical shifts, which are not participating in intentionally labeled spin pairs and therefore are not experiencing dipolar dephasing (8–10). An individual reference experiment needs to be acquired for every point in the dipolar dimension, doubling the required experimental time. Subtraction of the REDOR signal ( $S$ ) from the reference signal ( $S_0$ ) yields the difference spectrum, which is then divided by the reference signal to account for  $T_2$  relaxation. The resulting data set is used for the internuclear distance analysis. However, even the 1% natural abundance background of  $^{13}\text{C}$  can pose a problem to this approach, for example, in the increasingly common case of a labeled spin pair in the presence of a much larger carbonaceous molecule.

In the 1D (or “single-shot”) version of the REDOR experiment, overlapping sites in such a system can complicate the interpretation of individual REDOR dephasing points by requiring additional correction factors (for example, the schemes used in Refs. 15 and 16). In a 2D-REDOR experiment, this problem is avoided at the expense of additional measurement time, but contributes a constant offset to the signal that must be corrected. In addition, the signal in this indirectly detected dimension is still divided by the reference spectrum to account for transverse relaxation. In the presence of experimental noise, the division process can reduce the fidelity of the signal, especially at longer times. A final drawback to the difference spectrum situation is that the relatively small dipolar-dephasing signal is easily overwhelmed by any strong background signal, resulting in a misuse of the dynamic range of the NMR receiver.

Awareness of many of these issues prompted the development of the transferred-echo double resonance (TEDOR) experiment (31, 32), in which a polarization transfer step is used to avoid the contribution of uncoupled spins to the REDOR-dephased echo. The background signal is effectively phase cycled away so that only the nuclei involved in the polarization transfer (i.e., the intentionally labeled spins) contribute significantly to the signal. No reference experiment is needed for TEDOR, but the polarization transfer efficiency shows a dependence on  $D$  that complicates the measurement, so that an initial estimate of  $D$  is needed to analyze the data correctly (32). There have been relatively few applications of TEDOR for distance measurement despite the apparent need for such an experiment. When TEDOR is used, it is usually employed in

conjunction with REDOR distance measurements (20, 21). More frequently, TEDOR has been used as a heteronuclear correlation technique or as a selective filter for specific spin systems (17, 33–36). One likely reason for its lack of use in distance determination is the relatively low experimental transfer efficiency of 20–30%, even though the TEDOR transfer step theoretically yields an efficiency of 52% (32). The initial estimate of  $D$  required to process TEDOR data also favors the use of REDOR for distance determinations.

The purpose of the present work is to describe and demonstrate a refined version of the REDOR experiment that combines a filtering stage with a robust, traditional version of the REDOR pulse sequence, a possibility first suggested by Schaefer and co-workers (37). This experiment has all of the benefits of TEDOR, without any of the aforementioned drawbacks. A double-cross-polarization (DCP) filter is used instead of the TEDOR transfer. DCP was first utilized prior to the development of REDOR as a means of studying  $^{13}\text{C}$ – $^{15}\text{N}$  connectivity (38). The DCP experiment has been primarily used for determinations of  $^{13}\text{C}$ – $^{15}\text{N}$  bond concentrations in labeled biological systems (39, 40). We note in passing that distance measurements using DCP have been achieved using moment analysis (40) and can also be obtained using transient dipolar oscillations (41–43), but the analysis of the data is often complicated. The shape of the DCP signal is easily distorted by small deviations from a Hartmann–Hahn sideband match. Transform techniques would be more difficult to apply to the DCP signal, which asymptotically approaches one after long time periods (24). However, it is very useful as an initial filter step when combined with a robust REDOR sequence. The low throughput of a flat-profile DCP sequence is improved by the use of adiabatic-passage Hartmann–Hahn cross-polarization (APHH-CP) during both contact time periods (44–46), which greatly reduces the transient dipolar oscillations and recouples crystallites of all orientations. This avoids any improper weighting of the REDOR powder pattern (as is the case, for example, in a combined TEDOR-REDOR experiment).

In the new experiment, which we call DCP-REDOR, the DCP filter will obviate the need for a reference experiment. The DCP-REDOR sequence is found to be sensitive and produces an indirectly detected signal that can be analyzed using the existing REDOR transform and regularization methods. The lack of the reference experiment saves time and eases the analysis of 2D-REDOR data by avoiding arbitrary time-domain baseline correction and noise-induced distortion at longer evolution times. The discussion here will be limited to the 2D version of REDOR measurement on the  $^{13}\text{C}$ – $^{15}\text{N}$  spin pair in triple resonance experiments (also involving  $^1\text{H}$  nuclei). Applications to single-shot REDOR experiments and other nuclei are also possible. As a significant amount of time and money are often spent labeling and preparing samples for REDOR experiments, it seems that a filter for the spin labels may be used to avoid the aforementioned drawbacks of traditional REDOR methods.

## PULSE SEQUENCE DESIGN

For the purposes of efficient signal analysis using known methods, we seek an experiment that will produce a well-understood signal in its indirectly detected dipolar dimension. Specifically, the signal should be modeled by the following equation as a function of time ( $t$ ):

$$S_{\text{REDOR}}(D, t) = \frac{\sqrt{2} \pi}{4} J_{1/4}(\sqrt{2} Dt) J_{-1/4}(\sqrt{2} Dt), \quad [1]$$

where the  $J$  represent Bessel functions of the first kind (23). This is the signal produced in most of the unfiltered REDOR experiments to date (8–10). Note that this is the inverted REDOR signal and is obtained from these experiments by computing either the normalized value  $1 - (S_0 - S)/S_0$  or the ratio  $S/S_0$ . Multiple dipolar couplings can be extracted directly from a signal of this type by applying the REDOR transform or Tikhonov regularization procedures, usually after Blackman–Harris apodization and zero-filling (23, 24).

This signal is not produced by versions of the TEDOR experiment used to date (22). Instead, the signal is given by

$$\begin{aligned} S_{\text{TEDOR}} &= \frac{[J_0(\Omega_-)]^2}{2} - \frac{[J_0(\Omega_+)]^2}{2} \\ &- \sum_{k=1}^{\infty} \frac{1}{16k^2 - 1} [J_k(\Omega_-)]^2 \\ &+ \sum_{k=1}^{\infty} \frac{1}{16k^2 - 1} [J_k(\Omega_+)]^2, \quad [2] \end{aligned}$$

where the frequency and time parameters are contained in

$$\Omega_{\pm} = \sqrt{2} D(t_{\text{prep}} \pm t_{\text{evol}}). \quad [3]$$

The form of this signal prevents use of the REDOR transform method. However, as shown below, a signal like that of Eq. [1] can also be produced by a special form of the TEDOR experiment, removing the requirement for an *a priori* estimate of  $D$ . We refer to this as a “symmetric” TEDOR sequence, because both the preparation time ( $t_{\text{prep}}$ ) and the evolution time ( $t_{\text{evol}}$ ) are simultaneously stepped so that  $t_{\text{prep}} = t_{\text{evol}}$  for each point in the dipolar dimension. Despite the useful form of its signal, this experiment will in fact prove to be unsuitable. We briefly derive its signal to illustrate the problem. Equation [2] can be reduced by use of the following identity (47) with  $a = \frac{1}{4}$ , as was done for the REDOR signal in Ref. (23):

$$\begin{aligned} \sum_{k=1}^{\infty} \frac{1}{k^2 - a^2} [J_k(z)]^2 &= \frac{1}{2a^2} [J_0(z)]^2 \\ &- \frac{\pi}{2a} \csc(a\pi) J_a(z) J_{-a}(z). \quad [4] \end{aligned}$$

Upon simplification, this yields

$$S_{\text{TEDOR}} = \frac{\sqrt{2} \pi}{4} [J_{1/4}(\Omega_-) J_{-1/4}(\Omega_-) - J_{1/4}(\Omega_+) J_{-1/4}(\Omega_+)]. \quad [5]$$

This signal reduces for the symmetric TEDOR case ( $t_{\text{prep}} = t_{\text{evol}}$ ) to a simpler form, by setting the value of  $\Omega_+$  equal to  $\sqrt{2} Dt$  (where  $t$  is now  $t_{\text{prep}} + t_{\text{evol}}$ ) and  $\Omega_-$  equal to zero. Use of the limit:

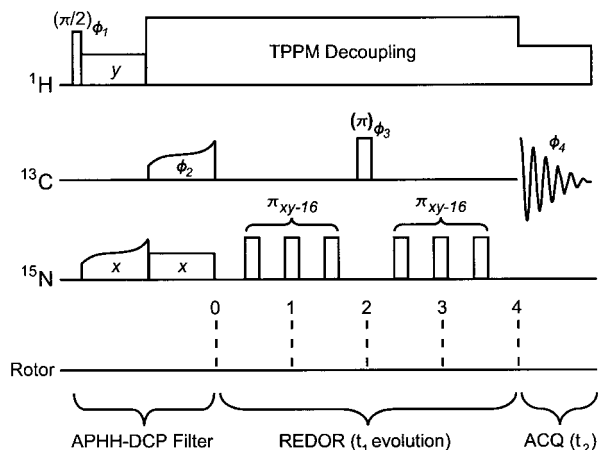
$$\lim_{z \rightarrow 0} J_{1/4}(z) J_{-1/4}(z) = \frac{4}{\sqrt{2} \pi} \quad [6]$$

then yields a simplified form similar to Eq. [1],

$$S_{\text{sym-TEDOR}}(D, t) = 1 - \frac{\sqrt{2} \pi}{4} J_{1/4}(\sqrt{2} Dt) J_{-1/4}(\sqrt{2} Dt), \quad [7]$$

where  $t$  now represents the combined evolution time (i.e.,  $t_{\text{prep}} + t_{\text{evol}}$ ). Unfortunately, instead of decaying to zero like Eq. [1], the symmetric TEDOR signal of Eq. [7] now theoretically grows to 1. In practice, relatively rapid  $T_2$  relaxation causes the signal to return to zero and distorts the spectral analysis. A reference experiment may be able to correct for the relaxation effect. However, the low efficiency of TEDOR is still an issue and would pose a problem in more challenging applications. In our experience, the distorted signals acquired in symmetric-TEDOR experiments are impractical to process because of this effect, even on simple model compounds.

A superior experiment can be constructed by treating the filter and distance measurement stages of the experiment separately. A DCP experiment that employs a  $^1\text{H} \rightarrow ^{15}\text{N} \rightarrow ^{13}\text{C}$  transfer is useful as a filter, because signals not arising from the  $^{13}\text{C}$ – $^{15}\text{N}$  cross-polarization step can be phase cycled away via spin-temperature inversion (48). As we have noted, the recently developed APHH method (44–46) can improve the efficiency of the DCP filter to the extent that it is a viable approach to filtration. Most importantly, its removal of transient dipolar oscillations observed between spin pairs greatly reduces any weighting of the powder pattern caused by the filter, so that the REDOR transform and regularization techniques are still valid. Combining a simple DCP filter stage with a REDOR pulse sequence yields the 2D DCP-REDOR pulse



**FIG. 1.** The DCP-REDOR pulse sequence used to obtain background-filtered REDOR signals is illustrated. The DCP stage of the experiment uses two adiabatic (tangent-ramped) pulses for polarization transfer. The XY-16 phase cycle is used for the repetitive  $\pi$  pulse trains. TPPM decoupling was used at all times after the initial CP step. The phase cycle includes spin-temperature inversion to suppress signals not coming through the DCP filter, with additional cycling to eliminate artifacts from the  $^{13}\text{C}$  echo pulse. The phase cycle is as follows: pulse  $\phi_1 = \{16 \times 0, 16 \times 2\}$ ,  $\phi_2 = \{4 \times 3, 4 \times 0, 4 \times 1, 4 \times 2\}$ ,  $\phi_3 = \{0, 1, 2, 3\}$ , and receiver phase  $\phi_4 = \{3, 1, 3, 1, 2, 0, 2, 0, 1, 3, 1, 3, 0, 2, 0, 2, 1, 3, 1, 3, 0, 2, 0, 2, 3, 1, 3, 1, 2, 0, 2, 0\}$ , where 0 is a  $0^\circ$  phase shift, 1 is a  $90^\circ$  phase shift, and so on.

sequence. No reference experiment is needed to account for uncoupled spins. However, because of the lack of a reference experiment, the DCP-REDOR signal is affected by relaxation and takes the form

$$S_{\text{DCP-REDOR}}(D, t) = \frac{\sqrt{2}}{4} \pi J_{1/4}(\sqrt{2} Dt) \times J_{-1/4}(\sqrt{2} Dt) \exp(-t/T_2). \quad [8]$$

In the resulting dipolar spectra, the relaxation appears primarily as a broadening of the individual dipolar lines.

## EXPERIMENTAL

The triple resonance DCP-REDOR pulse sequence used here is illustrated in Fig. 1. The DCP filter step involves a sequential transfer from  $^1\text{H}$  to  $^{15}\text{N}$  and then from  $^{15}\text{N}$  to  $^{13}\text{C}$ . Both transfers utilize adiabatic sweeps (approximated by a tangential function) over the  $(-1)$  sideband of the Hartmann–Hahn matching profile. The sweeps have a depth ( $\Delta$ ) that is set equal to the spinning frequency (45, 46). A standard REDOR sequence follows the filtration step. This sequence is known to be fairly tolerant to small fluctuations of the spinning speed (29), and its design also avoids any possibility of unintended homonuclear recoupling. The pulses on the  $^{15}\text{N}$  channel make use of the XY-16 phase cycle to compensate for pulse width errors, resonance-offset effects, and phase-shifting errors (28). Proton

decoupling during the DCP-REDOR stage and the acquisition period utilizes the TPPM sequence (49), although Lee–Goldburg decoupling could also be efficiently employed during the DCP period (46). TPPM decoupling has been shown to be useful in conjunction with REDOR dipolar evolution periods (50). The decoupler is maintained at a high power (approximately three times the  $B_1$  field on the  $^{13}\text{C}$  and  $^{15}\text{N}$  spins) during both the DCP and the REDOR stages to avoid detrimental signal loss from any additional Hartmann–Hahn contact (51), which would result in broadened peaks in the dipolar spectrum.

Experiments were performed on an 11.7-T Varian/Chemagnetics Infinity spectrometer operating at irradiation frequencies of 499.62, 125.63, and 50.63 MHz for  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$ , respectively. A Chemagnetics triple resonance 5-mm MAS probe (Model MPRB-500377) was used, and the spinning speed was stabilized to  $8000 \pm 1$  Hz by a Chemagnetics spin rate controller. During the DCP filter stage, the  $^{13}\text{C}$  spin-lock pulse and the first  $^{15}\text{N}$  spin-lock pulse were both tangentially ramped from 32 to 40 kHz across the  $(-1)$  sideband matching condition. For the REDOR stage of the sequence, all  $\pi$  pulses were 10 to 12.5  $\mu\text{s}$  in duration corresponding to nutation frequencies of 40 to 50 kHz. The TPPM decoupling power was set to 65 kHz during acquisition and increased to 100 kHz for the stages requiring a Hartmann–Hahn mismatch. All TPPM phase shifts were  $15^\circ$ , and the phase alternation time was set to an experimentally optimized value slightly shorter than the width of a  $\pi$  pulse (49). A recycle delay of 5 s was used for all experiments discussed here.

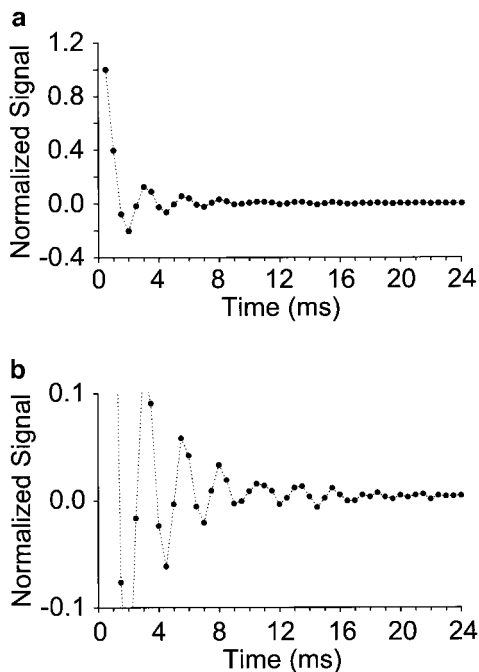
Two test samples were employed in this work. The first was  $2\text{-}^{13}\text{C}$ ,  $^{15}\text{N}$ -glycine (Cambridge Isotope Labs, Inc.) diluted to a level of 10% (w/w) in natural abundance glycine by recrystallization from water. A second glycine test sample consisted of  $1\text{-}^{13}\text{C}$ ,  $^{15}\text{N}$ -glycine (Isotec, Inc.), also diluted to 10% in unlabeled glycine.

## RESULTS AND DISCUSSION

### Initial Demonstration of DCP-REDOR

The DCP-REDOR pulse sequence can be easily demonstrated on isotopically labeled glycine samples. The results of a DCP-REDOR experiment on the  $2\text{-}^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled glycine sample are shown in Fig. 2. A total of 48 points were acquired every 4 rotor cycles (0.5 ms) for a total of 192 rotor cycles and a spectral width in the dipolar dimension of 2 kHz. The initial DCP transfer required a 10-ms contact time period for the  $^{15}\text{N}$  to  $^{13}\text{C}$  polarization transfer. The complete experiment (without a reference) required approximately 15 h of acquisition time. A useful signal could be acquired in far less time; however, our goal in this case is to demonstrate the possibility of observing long-time oscillations in the dipolar signal. In the case of glycine, oscillations can be seen as far out as 164 rotor cycles ( $\sim 20$  ms), leading to relatively high resolution in the dipolar dimension. The DCP filter effectively removes any contribu-



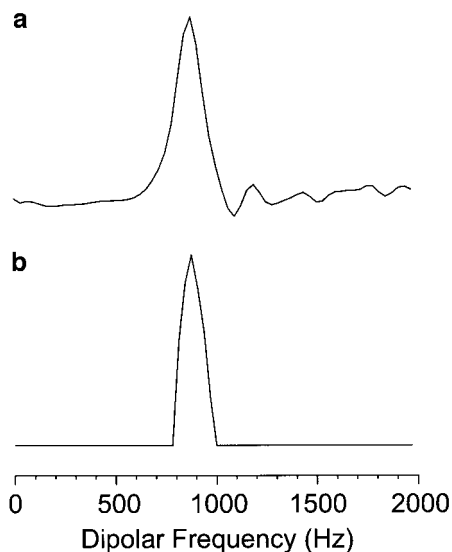


**FIG. 2.** (a) Forty-eight points of DCP-REDOR data obtained from the  $2\text{-}^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled glycine sample. (b) Expanded plot of DCP-REDOR data showing the smaller dipolar oscillations observed at longer evolution times. The dashed line is a guide to the eye.

tion from uncoupled  $^{13}\text{C}$  spins to the dipolar signal, which decays to zero in the time domain.

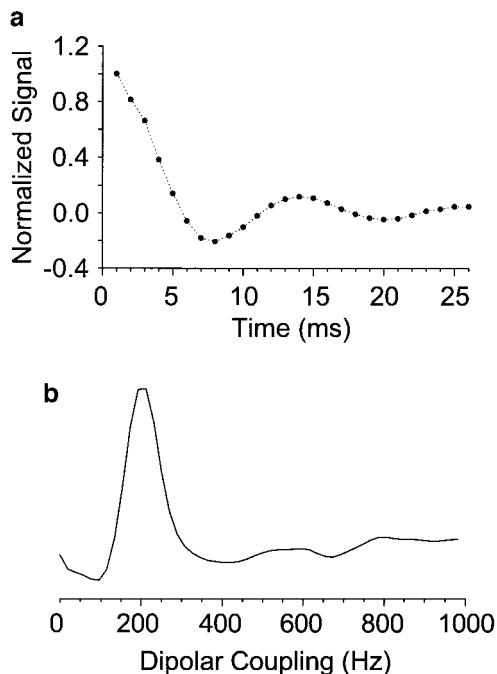
The dipolar couplings can be extracted directly from this signal by applying the REDOR transform after Blackman–Harris apodization and zero-filling to 64 points. The results are shown in Fig. 3a, where the peak at  $\sim 880$  Hz corresponds to an internuclear distance of  $\sim 1.50$  Å. This is close to the  $1.47$ -Å distance known from the crystallographic structure of glycine (53). The difference between the two values is consistent with the known vibrational elongation of distance measurements in NMR (54, 55). Application of the closely related Tikhonov regularization procedure to this data set results in the improved spectrum of Fig. 3b, where the high frequency noise evident in Fig. 3a has been effectively removed (24). The surprising quality of this spectrum is most likely caused by the positivity constraints of the Tikhonov procedure, combined with its optimal handling of Gaussian experimental noise. As we have noted, the  $T_2$  relaxation that occurs during the dipolar evolution contributes only to the linewidth of the peak in the dipolar spectrum. These spectra illustrate that this effect does not appear to significantly reduce the dipolar resolution of the spectra.

One potential limitation of the DCP-REDOR sequence is the lengthy mixing periods required to measure weaker couplings. In order to address this issue, we present a second demonstration of the DCP-REDOR pulse sequence in the form of a measurement of a weaker dipolar coupling constant, corre-



**FIG. 3.** (a) The REDOR transform and (b) Tikhonov regularization of the data presented in Fig. 2, after 3-point, 67-dB Blackman–Harris apodization and zero-filling to 64 points. The measured coupling of 880 Hz corresponds to a  $\sim 1.50$ -Å internuclear distance.

sponding to an internuclear distance of approximately  $2.50$  Å in the  $1\text{-}^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled glycine sample. A DCP-REDOR experiment on this sample resulted in 26 points of data acquired every eight rotational periods (Fig. 4a). This corre-



**FIG. 4.** (a) Twenty-six points of DCP-REDOR data obtained from the  $1\text{-}^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled glycine sample. (b) REDOR transform of the data set in (a), after apodization and zero-filling to 52 points. The measured coupling of 200 Hz indicates a internuclear distance of  $\sim 2.50$  Å.

sponds to a sampling interval of 2 ms with the given spinning rate of 8 kHz. In this case, a 25-ms contact time period was needed to transfer polarization from  $^{15}\text{N}$  to  $^{13}\text{C}$  via the second APHH-CP stage. The REDOR transform of this data set is shown in Fig. 4b. The single peak at 200 Hz indicates a distance of  $\sim 2.50 \text{ \AA}$  and corresponds well to the 2.48- $\text{\AA}$  distance determined by X-ray analysis (53). The experiment shown here was run much more rapidly than that discussed above, requiring only an hour to complete. We also note that the longer  $T_2$  of the glycine carbonyl site, from which these data were obtained, makes this distance a favorable case for REDOR measurements. Nevertheless, this serves to demonstrate the potential limits of DCP-REDOR measurements, as further acquisition of time-domain data was limited in our case by spectrometer and probe hardware.

Both of the time-domain data sets shown here exhibit an interesting effect that is due to the nature of the DCP-REDOR experiment. At shorter times, the normalized signal intensity in Figs. 2 and 4 dips excessively below zero compared to the model decay of Eq. [1]. We attribute this effect to incomplete transfer from all crystallite orientations by the DCP filter. This has been noted and discussed previously (45) and is caused by the initial jump in RF amplitude when the adiabatic sweep pulse is turned on. The problem might be avoided by performing the adiabatic sweep upwards from zero; however, an inevitable loss of signal would occur as multiple sidebands are matched. The spectra illustrated here do not seem to be appreciably affected by this issue. We therefore continue to use an adiabatic sweep over a single sideband for the remainder of this work and recommend its use in general.

### *The Reference Experiment and Signal Processing Implications*

The REDOR reference signal ( $S_0$ ) has two functions. The first is to account for uncoupled background spins (via their subtraction in the difference spectrum). The  $S_0$  signal is also intended to remove the effects of  $T_2$  relaxation by subsequent division of the signal. The first function of the reference signal is no longer required in the DCP-REDOR experiment. However, it is less clear whether the reference experiment is needed for the removal of  $T_2$  relaxation effects in DCP-REDOR and what its effect would be if used. We have already noted that  $T_2$  relaxation will appear as a line broadening in the dipolar spectrum. Here we examine additional effects of dividing an experimental REDOR (or DCP-REDOR) signal by its reference. It will be shown that the result is a tradeoff between increased noise in signals that have been divided by their reference and increased linewidth in signals that have not. To delineate this tradeoff more clearly, an error analysis of the REDOR and DCP-REDOR experiments is required.

The divided REDOR signal is obtained from the  $S_0$  reference signal and the  $S$  (dipolar-recoupled) signal,

$$S_{\text{REDOR}} = 1 - \frac{S_0 - S}{S_0} \equiv \frac{S}{S_0}. \quad [9]$$

We will assume that the noise distribution for  $S_0$  and  $S$  is the same. This is a valid assumption since the level of random noise entering the experiment should not depend upon which experiment is conducted. It is also assumed that the errors in  $S_0$  and  $S$  are uncorrelated, since the two experiments are performed separately. If the noise or error present in a given signal is  $\sigma$ , then the rules of error propagation yield the following noise level for the divided REDOR signal (56):

$$\sigma_{\text{div}} = \sqrt{(S_{\text{REDOR}})^2 \left( \frac{\sigma_S^2}{S^2} + \frac{\sigma_{S_0}^2}{S_0^2} \right)}. \quad [10]$$

Again, we neglect cross-correlation terms (which should be zero). Since the amount of noise does not depend on the experiment, such that  $\sigma_S = \sigma_{S_0} \equiv \sigma$ , the expression above becomes

$$\sigma_{\text{div}} = \sqrt{\left( \frac{S}{S_0} \right)^2 \left( \frac{\sigma^2}{S^2} + \frac{\sigma^2}{S_0^2} \right)}. \quad [11]$$

Rearranging, we have

$$\sigma_{\text{div}} = \sqrt{\frac{\sigma^2}{S_0^2} \left( 1 + \frac{S^2}{S_0^2} \right)}. \quad [12]$$

This expression determines the effect of experimental noise in the individual  $S$  and  $S_0$  signals on the noise level of the final divided signal.

The theoretical effect of the reference signal is to remove relaxation effects at all times. Experimental noise will affect this process, especially at longer times, where the REDOR signal appears as  $S/S_0 \approx 0$ . Equation [12] then becomes

$$\sigma_{\text{div}} \approx \frac{\sigma}{S_0}. \quad [13]$$

The meaning of Eq. [13] is made clear if the  $S_0$  signal is modeled by an exponential decay, which is the form it usually takes in REDOR experiments:

$$S_0 = \exp\left(\frac{-t}{T_2}\right). \quad [14]$$

Equation [13] then becomes

$$\sigma_{\text{div}} \approx \sigma \cdot \exp\left(\frac{t}{T_2}\right). \quad [15]$$

From Eq. [15], the behavior of the noise level in the divided signal is immediately apparent. As the dephasing time  $t$  is increased, the noise becomes exponentially more significant. Also, as  $T_2$  becomes shorter, the uncertainty again grows larger. Thus, as  $S_0$  approaches zero, the uncertainty or random noise in the signal becomes quite large. This consideration often precludes the acquisition of important REDOR data points at longer dephasing times.

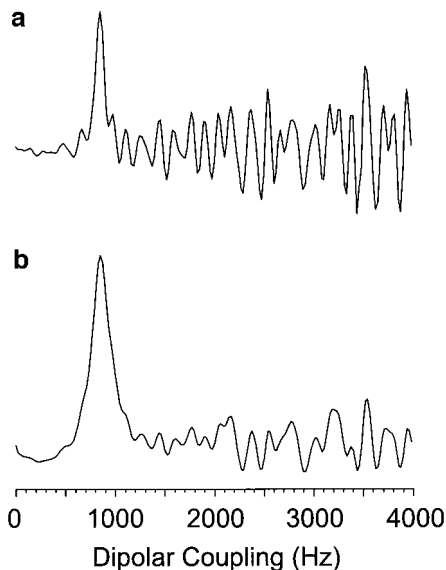
It is also interesting to examine the short-time behavior of the noise propagation in the divided REDOR signal. Here  $S/S_0 \approx 1$  and Eq. [12] becomes

$$\sigma_{\text{div}} = \sqrt{2} \sigma. \quad [16]$$

Thus, even at shorter dephasing times, the noise level in the divided REDOR signal is enhanced by a factor of  $\sqrt{2}$ . To summarize, at all time points during the REDOR experiment the division by  $S_0$  results in the possibility of more random uncertainty being introduced into the time-domain signal. The divided signal becomes especially sensitive to noise during the latter part of the signal (as the dephasing time is increased) or when  $T_2$  is small.

The situation is different for the DCP-REDOR experiment. Since it does not require a reference, the uncertainty is independent of both the dephasing time and  $T_2$ . The experimental data sets shown to this point (for the glycine samples) do not suffer from this enhancement of the noise level. If a reference was used in the DCP-REDOR experiment, which is possible by simply deleting the REDOR dephasing pulses and repeating the experiment, then the above analysis would again apply. This allows for a convenient demonstration of these effects using DCP-REDOR, which also clarifies the role of the reference in a REDOR experiment. Since the reference is required for dipolar processing of REDOR, this demonstration cannot be made without the DCP filter.

A DCP-REDOR experiment was performed on the  $2\text{-}^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled glycine sample, under conditions similar to those used to produce the data of Fig. 2. However, in this case, a total of 96 points in the dipolar dimension were sampled every two rotor periods. This oversampling of the dipolar signal was done to more clearly illustrate the predictions of Eq. [15]. A second DCP-REDOR experiment was performed as a reference, with the  $\pi$  pulses on the  $^{15}\text{N}$  channel turned off. This yielded a smooth, exponential decay. Both experiments were also run over a shorter interval than was used above; each required 5 h to complete. The REDOR-transformed result of the DCP-REDOR experiment divided by its reference is shown in Fig. 5a. (Note that the Tikhonov regularization procedure would in fact suppress this noise and distort the analysis). In Fig. 5b, the transform of the DCP-REDOR signal alone (with no division) is illustrated. From a comparison of these two data sets, it is clear that the division of data using the reference experiment



**FIG. 5.** (a) REDOR transform of an oversampled DCP-REDOR data set after division by a separate DCP-REDOR reference experiment. (b) REDOR transform of the same data set, but without division by the reference experiment. The tradeoff between additional noise and narrowing of the peak (at 900 Hz) is clearly shown. The spectra each consist of a total of 192 data points.

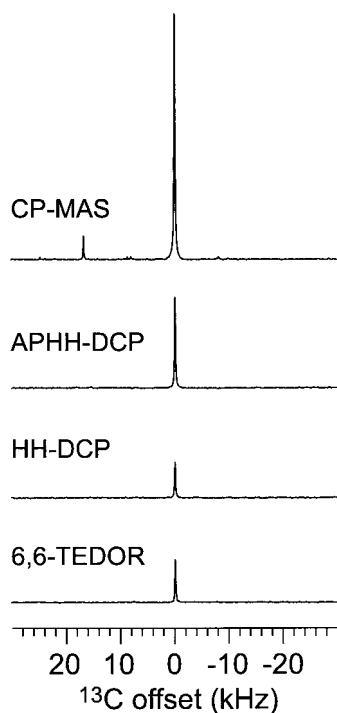
narrows the peak in the dipolar spectrum, but at the expense of increased noise.

Division by the reference signal is necessary for single-shot REDOR measurements, but in the full 2D approach to REDOR, the effect of the division is to introduce more random uncertainty into the measurement. The effect is especially severe in its amplification of the noise at the end of the time-domain signal. A partial avoidance of this problem would require the user to arbitrarily truncate the signal. This could be an undesirable penalty in samples with weaker dipolar coupling and shorter  $T_2$  relaxation times, as in many biologically relevant samples. In practice, we have found that this uncertainty, coupled with the additional time required for a DCP-REDOR reference experiment, is in general not worth the line-narrowing effect. However, in samples with multiple dipolar couplings of similar magnitude, division by the reference signal could prove to be worthwhile.

The ultimate solution to this issue may take the form of a constant-time version of the REDOR experiment (9, 10), such as the XDM sequence (43). This would avoid the effect of relaxation in its entirety. We note, however, that a constant-time experiment suitable for the transform analysis is not yet available. Also, the issue of reintroduced heteronuclear  $J$ -coupling could further complicate the matter.

#### Comparison of Filtering Methods

The efficiency of the APHH-DCP filtering method used here is demonstrated in Fig. 6. For all of the spectra shown, the initial  $^1\text{H} \rightarrow \text{X}$  transfer always utilized an identical single



**FIG. 6.** A performance comparison is presented of the three filtering methods discussed in the text. The  $^1\text{H} \rightarrow ^{13}\text{C}$  CP-MAS spectrum of the labeled glycine sample is given as a reference and was acquired using a single-CP APHH sequence with the  $^{13}\text{C}$  ramp set for a  $\Delta = 8$  kHz. The APHH-DCP spectrum was obtained using the filter stage shown in Fig. 1 with both  $\Delta$  values set to 8 kHz. A DCP spectrum without a second APHH ramp and a 6,6-TEDOR spectrum are shown for comparison.

APHH ramp of 8 kHz on either the  $^{13}\text{C}$  or  $^{15}\text{N}$  channel. The CP-MAS spectrum obtained in this manner is shown at the top as a reference. The other three sequences act as filters for the  $^{13}\text{C}$ - $^{15}\text{N}$  spin pair in the glycine sample, as can be seen from the suppression of the carbonyl signal. The APHH-DCP stage (the initial part of the sequence given in Fig. 1) shows an efficiency of about 44% of that of the CP-MAS spectrum, after correction for natural abundance effects in the CP-MAS data. The flat-profile (HH-DCP), using an initial  $^1\text{H} \rightarrow ^{15}\text{N}$  APHH transfer, showed a transfer efficiency of just 16%. The use of TEDOR as an initial filter for a REDOR experiment was also examined. In Fig. 6, we show TEDOR data acquired with six preparatory and six dephasing rotor periods, which yields the maximum coherence transfer for this spinning speed (32). This shows an approximate intensity of 20% relative to the other methods. The performance of both the HH-DCP and the 6,6-TEDOR filter is relatively poor, making their use as an initial filter for a REDOR experiment somewhat uneconomical.

The superior performance of the APHH-DCP filter has been attributed to two effects: the efficiency gain of an adiabatic passage over the  $(-1)$  sideband and the additional effect of the ramp broadening the HH match for all crystallites and alleviating many RF inhomogeneity concerns (45, 46). The APHH-DCP filter used here also allows maintenance of an efficient

match with proton decoupling turned on during the second contact time period, thus preserving overall signal intensity. The original DCP experiments were performed with decoupling turned off during this period to broaden the very sensitive  $^{13}\text{C}$ - $^{15}\text{N}$  matching condition (38–40).

#### Experimental Optimization of DCP-REDOR

The combination of DCP and REDOR introduces some complications in the choice of optimum spinning speed for the experiment. In general, a faster spinning speed allows for a large adiabatic sweep depth ( $\Delta$ ) and higher DCP efficiency (45, 46). The REDOR stage of the experiment shown in Fig. 1 can also benefit from faster MAS speeds. Theoretical simulations have shown that the presence of a significant chemical shift anisotropy (CSA) on the  $^{15}\text{N}$  spin (or the spin receiving the dephasing pulses) affects the performance of the type of REDOR experiment discussed here. In particular, the XY-type phase cycles perform best at spinning speeds  $\omega_r$  that exceed the approximate relation (57, 58)

$$\omega_r \geq \frac{3}{4} \omega_0 (\sigma_{33} - \sigma_{\text{iso}}), \quad [17]$$

where  $\omega_0$  is the Larmor frequency and the  $\sigma$  values characterize the magnitude of the chemical shift tensor. If this condition is not exceeded, finite pulse widths can lead to distortions of the apparent dipolar frequency in the REDOR spectrum. In practice, we have noticed this effect in many REDOR experiments at higher static field strengths. Fast spinning thus benefits both the REDOR and the DCP phases of this experiment.

However, a speed limit on the REDOR stage of the experiment is also enforced by the presence of finite pulse widths. If these widths exceed  $\sim 10\%$  of the rotor period, additional dipolar frequencies can be produced that would significantly alter the dipolar spectrum (58). It seems that the best balance between these criteria is to set the spinning speed based on the highest achievable RF powers, so that the speed is as high as possible without the  $\pi$  pulse widths consuming more than 10% of the rotor period. We have followed this prescription in the present work. Current power limitations might make this situation more challenging on higher field instruments (i.e., greater than 11.7 T) and when studying nuclei with more significant CSA magnitudes (such as  $^{19}\text{F}$ ). In these cases, the recently developed SFAM pulse sequence can provide an alternative solution (59).

## CONCLUSIONS

A background-filtered version of the REDOR experiment has been successfully demonstrated. This experiment is relatively efficient due to its use of two APHH-CP stages and has a number of advantages that ease the processing of the experimental data. The sequence generates a well-understood



REDOR signal that decays to zero, the analysis of which can be accomplished using a number of complementary processing techniques. The loss of the background signal allows better use of the dynamic range of a spectrometer and simplifies the analysis of complex materials with strong background signals. The sequence is particularly useful for suppressing natural-abundance signals in  $^{13}\text{C}$ -detected REDOR, which also avoids the complicated three-spin problems inherent in  $^{15}\text{N}$ -detected REDOR (19). The signal lost in the DCP filter is partially recovered over time by the lack of a reference experiment, so that the experiment is a viable alternative to REDOR when working with moderate to weak couplings in systems such as bound biomolecules. In cases where a single coupling is observed, further improvement could be realized by combining the DCP selection stage with a synchronous-detected REDOR experiment (9) for simplified, one-dimensional distance measurements. Although we have focused only on 2D experiments, the single-shot REDOR methodology can also benefit from this approach if transverse relaxation can be quantified by a reference experiment.

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