

$^2\text{H}\{^{19}\text{F}\}$ REDOR for distance measurements in biological solids using a double resonance spectrometer

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Abstract

A new approach for distance measurements in biological solids employing $^2\text{H}\{^{19}\text{F}\}$ rotational echo double resonance was developed and validated on $^2\text{H},^{19}\text{F}$ -D-alanine and an imidazopyridine based inhibitor of the gastric H^+/K^+ -ATPase. The ^2H - ^{19}F double resonance experiments presented here were performed without ^1H decoupling using a double resonance NMR spectrometer. In this way, it was possible to benefit from the relatively longer distance range of fluorine without the need of specialized fluorine equipment. A distance of $2.5 \pm 0.3 \text{ \AA}$ was measured in the alanine derivative, indicating a *gauche* conformation of the two labels. In the case of the imidazopyridine compound a lower distance limit of 5.2 \AA was determined and is in agreement with an extended conformation of the inhibitor. Several REDOR variants were compared, and their advantages and limitations discussed. Composite fluorine dephasing pulses were found to enhance the frequency bandwidth significantly, and to reduce the dependence of the performance of the experiment on the exact choice of the transmitter frequency.

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1. Introduction

Rotational echo double resonance (REDOR) is a well established technique to obtain accurate distances in biological solids [1]. Typically, stable isotopes of nuclei abundant in these materials, such as ^{13}C , ^{15}N or ^2H , are used to avoid structural modification. Their low sensitivity and small range of dipolar interactions, however, encouraged the use of the high- γ nucleus ^{19}F as an alternative label with greatly enhanced sensitivity and range of measurable distances. Despite the structural modification it presents, it can be successfully employed in numerous biological applications [2–17]. In particular, pharmaceutical compounds (drugs) often contain fluorine, and ^{19}F -NMR is well suited to study the conformation of such compounds and ligand binding sites of proteins [2,13,16]. The use of fluorine, though, is

limited by demanding hardware requirements imposed by the proximity of the ^{19}F and ^1H resonance frequencies. As high power proton decoupling is usually required, REDOR experiments involving ^{19}F can be performed only with specialized triple resonance equipment. Deuterium, however, can be observed without the necessity of ^1H decoupling as the result of the absence of directly bonded protons and the comparatively weak dipolar couplings to protons. Since the resonance frequency of ^{19}F is close to ^1H , it is often possible to use the ^1H channel for ^{19}F . ^2H - ^{19}F double resonance experiments thus giving an opportunity to benefit from the long distance range of fluorine using conventional double resonance (H-X) hardware.

In this study we discuss the feasibility and possible limitations of this approach. A particular concern was to achieve maximum reduction of the signal when the ^2H - ^{19}F dipolar coupling is recoupled, while maintaining the signal as long as possible during the dephasing period. This is important when drawing quantitative conclusions of the REDOR dephasing and when addressing

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long internuclear distances. Different derivatives of the REDOR experiment were compared to address this aim.

REDOR experiments employing the spin-1 nucleus ^2H have been successfully demonstrated in the past, mostly using ^2H as the dephasing spin [4,18–31], but also as the observed spin [32]. Recently, $^2\text{H}\{^{19}\text{F}\}$ REDOR has been successfully applied to a biological system. However, highly specialized NMR equipment allowing ^1H decoupling was used [33]. Whereas peculiarities of spin-1 have to be taken into account in the analysis of REDOR dephasing when the signal is dephased by ^2H , the dephasing of a spin-1 by a spin-1/2 can be treated in the same manner as for systems with two spin-1/2 [32].

Two compounds were studied here, α - ^2H - β - ^{19}F -D-alanine (“DF-Ala”) and an imidazo pyridine derivative (“ d_3 -TM2FPIP”) (Fig. 1), which is a potent inhibitor of the gastric H^+/K^+ ATPase. They comprise different strengths of dipolar couplings and sizes of quadrupolar interactions, and thus allowed investigation of different difficulties inherent in ^2H solid state NMR such as the large spectral range and often short T_2 relaxation of deuterons.

Studies of the inhibitor of the gastric H^+/K^+ -ATPase also address important pharmacological questions. This membrane protein plays an important role in the pH control of mammalian gastro-entestinal digestion by electro-neutral active transport of protons. The protein, a 114 kDa relative of the Na^+/K^+ - and Ca^{2+} P-type ATPases, is involved in a number of diseases such as gastric ulceration or atrophic gastritis, and the development of inhibitors to moderate its function is of great pharmaceutical interest and need. In particular, structural information on the conformation of the inhibitor is desirable to form a rationale basis for drug design [11,34–37]. To this aim, an *internuclear* distance within the inhibitor was investigated here, revealing constraints on its conformation.

The ^2H - ^{19}F distance in DF-Ala depends only on a single torsion angle θ , limiting the possible distances to a

much narrower range than in the case of the inhibitor (Fig. 1). However, knowing the ^2H - ^{19}F distance in TM2FPIP substantially reduces the number of possible conformations and therefore gives an important insight into the structure of this drug. Earlier studies on similar inhibitors indicated an extended conformation, which would give rise to a ^2H - ^{19}F distance of ~ 6 – 7 Å [11]. Both compounds present an opportunity to verify the proposed NMR strategy by comparing results with the expected distance ranges.

2. Materials and methods

2.1. Materials

The fluorine and deuterium substituted analogue α - ^2H - β - ^{19}F -D-alanine (DF-Ala) was a kind gift by Merck (Rahway, NJ). The cationic inhibitor of the gastric H^+/K^+ -ATPase, 1-trideuteromethyl-2,3-dimethyl,8-(p-fluorophenylmethoxy)-imidazo[1,2- α]pyridinium iodide (d_3 -TM2FPIP), was synthesized as described [11]. Neither compound was diluted and both were measured at room temperature. About 20 mg of DF-Ala and 6 mg of d_3 -TM2FPIP were filled in the central part of a 6 mm NMR rotor for measurement.

2.2. NMR experiments

All NMR experiments were performed on a two-channel Chemagnetics CMX400 spectrometer operating at 61.078 MHz ^2H resonance frequency and 374.340 MHz ^{19}F resonance frequency. A double resonance 6 mm magic angle spinning (MAS) probe was used, with the proton channel tuned to the fluorine resonance frequency. The high frequency amplifier was tuned to 374.340 MHz, and a broadband amplifier was employed for the low frequency channel. The ^{19}F 90° pulse length was 3.0 μs , the ^2H 90° pulse was 4.4 μs . All spectra were obtained under 5 kHz magic angle sample spinning and with a sweep width of 500 kHz. For each REDOR data set a dephased and non-dephased FID with 8192 points were acquired. 32 scans for DF-Ala and 640 scans for d_3 -TM2FPIP were averaged for each FID if not otherwise stated. The scans for one REDOR set were alternately acquired, to assure the same conditions for the dephased and non dephased spectrum. Due to slow T_1 relaxation, long recycle times of 200 s in the case of DF-Ala, and 30 s in the case of d_3 -TM2FPIP were chosen.

2.3. Data evaluation

The signal intensities in the REDOR curves were determined through integration of the individual sideband intensities and their successive summation.

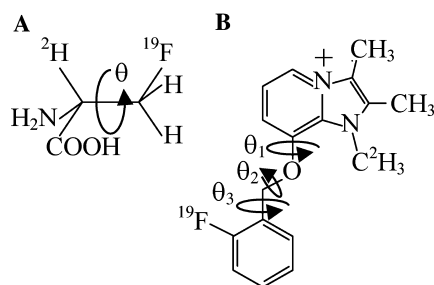


Fig. 1. Two compounds were used to evaluate the $^2\text{H}\{^{19}\text{F}\}$ REDOR approach in this study. The alanine derivative DF-Ala (A) possesses a ^2H - ^{19}F distance in the range of 2.4 and 3.2 Å depending on the torsion angle θ . The ^2H - ^{19}F distance in the inhibitor of the gastric H^+/K^+ -ATPase, TM2FPIP (B), is determined by several degrees of freedom (θ_1 , θ_2 , θ_3), and similar inhibitors have been shown to adopt an extended conformation [11].

The errors given for the signal intensities and REDOR ratios $\Delta S/S_0$ were obtained from the signal-to-noise ratio, taking into account the summation of the signal intensities and error propagation.

2.4. Simulations

The dipolar interaction between the fluorine and the three methyl deuterons of d_3 -TM2FPIP was calculated as a function of the distance between the centre of the deuteron triad and the fluorine position and their relative orientation. An average ^2H - ^{19}F dipolar tensor was computed assuming fast methyl rotation, employing the Simpson rule for integration of the tensor components [38]. Since the asymmetry parameter η of the resulting average dipolar tensor was found to be negligible ($|\eta| \leq 0.001$) in the relevant distance range of ~ 5 – 6 Å, the averaged coupling constant could be related to an effective distance.

To assess the influence of proton decoupling, simulations of the REDOR experiment were performed with the SIMPSON program [39]. The simulated pulse sequence consisted of a central ^2H 180° -pulse, and 180° dephasing pulses every half rotor cycle. Dephasing times up to 128 rotor cycles assuming 5 kHz MAS spinning frequency were computed. For each point of the REDOR calculated curve, 144 orientations with respect to the spinning axis and 18 angles around the spinning axis were averaged.

3. Theory

The rotational echo double resonance (REDOR) experiment was introduced by Gullion and Schaefer [1] to allow the determination of heteronuclear dipolar couplings under magic angle spinning (MAS) conditions. This is achieved by preventing the dipolar coupling from being averaged by MAS through the application of rotor synchronized rf pulses. To measure the dipolar coupling, the signal S , subjected to dephasing during time NT_R , and the signal S_0 subject only to relaxation for the same time, are measured for a series of dephasing times NT_R . The dipolar coupling constant:

$$D = -\frac{\mu_0}{4\pi} \frac{\gamma_I \gamma_S \hbar}{2\pi r^3} \quad (1)$$

then provides a direct measure of the internuclear distance r .

Various pulse sequences have been developed to optimize the performance in different situations. In the case discussed here, where the observed nucleus is of spin-1, the different nature of spin-1 must be considered as well. Use of a 90° -pulse in place of 180° -pulse on the observe channel to form a quadrupolar echo is not necessary, as the quadrupolar interaction is refocused by MAS.

In fact, a 90° -pulse does not yield the full dephasing, and the 180° -pulse implemented in the original REDOR sequence to refocus isotropic chemical shifts might be used with ^2H as well, or omitted completely.

The dephased signal $\Delta S/S_0 = (S_0 - S)/S_0$ differs depending on whether the dephasing nucleus is spin-1/2 or spin-1 [32]. However, in the case where the observed nucleus is spin-1 and the dephasing nucleus possesses spin-1/2, the situation is the same as for a two spin-system, and the reduction of the signal due to the recoupled dipolar interaction is given by [40,41]:

$$\frac{\Delta S}{S_0} = 1 - \frac{1}{4\pi} \int_0^{2\pi} d\alpha \times \int_0^\pi \sin \beta d\beta \cos(NT_R D 2\sqrt{2} \sin 2\beta \sin \alpha). \quad (2)$$

Fitting “REDOR-curves” of $\Delta S/S_0$ as a function of the dephasing time NT_R , the internuclear distance r can be determined for the case of dilute spin pairs, or small spin networks with known geometry. In situations where the observed spin is dephased by a group of spins without known spatial arrangement, the distribution of dipolar couplings can still be characterized with the aid of an expansion of $\Delta S/S_0$ in powers of NT_R [42]:

$$\frac{\Delta S}{S_0} = \sum_{n=2,4,6,\dots}^{\infty} A_n (NT_R)^n. \quad (3)$$

Then, the coefficient

$$A_2 = \frac{16}{15} \sum_k D_k^2 \quad (4)$$

is independent of the relative orientations of the internuclear vectors and proportional to the second moment of the dipolar coupling. The REDOR transform [43] to obtain a distribution of dipolar couplings was not used here, as this conversion is only applicable to a distribution of isolated spin pairs with different couplings.

4. Results

4.1. Comparison of different REDOR sequences

A first assessment of the feasibility of the proposed approach for $^2\text{H}\{^{19}\text{F}\}$ REDOR measurements is shown on DF-Ala in Fig. 2. The close ^2H - ^{19}F distance in this compound gives rise to a large dipolar coupling and rapid dephasing. Using an optimized standard REDOR sequence (Fig. 3D), the dephased signal S (right column in Fig. 2) is completely lost after ~ 3 ms. The dephasing is uniform among the sidebands within the error limits of the experiment.

The second compound, d_3 -TM2FPIP, was used to compare different REDOR sequences in more detail. All experiments employed $xy8$ phase cycling [44] and composite pulses [45] for the fluorine 180° -pulses, which will

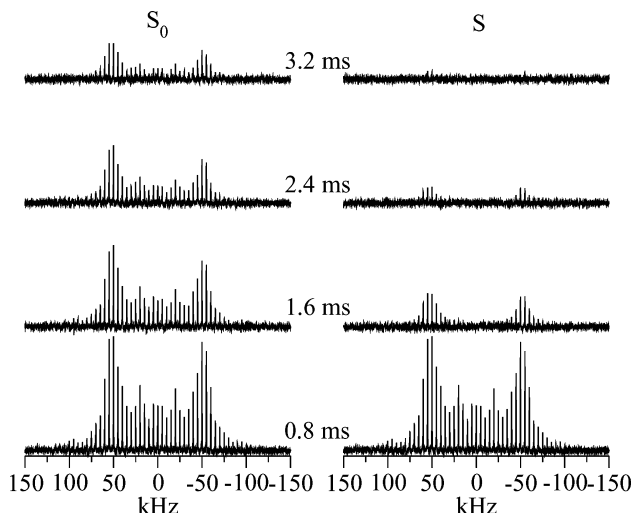


Fig. 2. ^2H spectra of DF-Ala after 0.8, 1.6, 2.4, and 3.2 ms without ^{19}F dephasing (left column) and with ^{19}F dephasing pulses (right column). The spectra were obtained using the sequence in Fig. 3D, employing $xy8$ phase cycling and ^{19}F composite pulses.

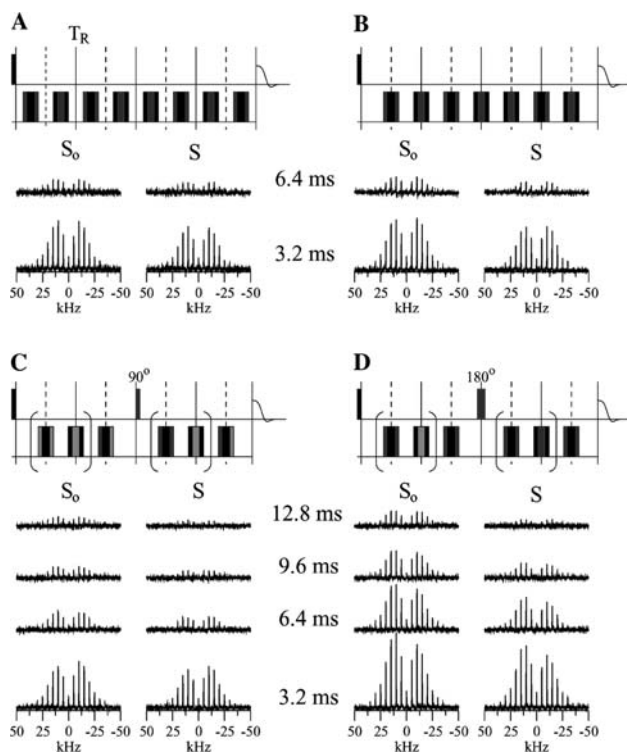


Fig. 3. Four REDOR experiments were compared on the gastric H^+/K^+ -ATPase inhibitor TM2FPIP: no ^2H refocusing pulse and ^{19}F dephasing pulses at a $1/2$ and $3/4$ of each rotor cycle T_R (A), no ^2H refocusing pulse and ^{19}F dephasing pulses every rotor cycle (B), a 90° ^2H refocusing pulse and ^{19}F dephasing pulses every $1/2$ rotor cycle (C), a 180° ^2H refocusing pulse and ^{19}F dephasing pulses every $1/2$ rotor cycle (D). All spectra were obtained using $xy8$ phase cycling and ^{19}F composite pulses. Non-dephased (left columns) and dephased spectra (right columns) are shown for dephasing times as noted in the figure.

be discussed below. However, different implementations of the pulses on the observe channel were tried to optimize the signal amplitude as well as dephasing at long

dephasing times. In the experiments shown in Figs. 3A and B, the observe refocusing pulse was omitted completely, since the reason for it in refocusing the isotropic chemical shift should be a minor issue with ^2H . It is possible, as demonstrated in [32], to place the dephasing pulses at $1/4 T_R$ and $3/4 T_R$ to permit stroboscopic detection of the signal every half rotor cycle (sequence Fig. 3A). Figs. 3C and D compare the use of a 90° and 180° refocusing pulse to form a quadrupolar echo or Hahn echo. The most significant difference in these four experiments is a different decay of the overall signal S_0 , demonstrated in Fig. 4. Using a 180° ^2H pulse for refocusing yields the least loss in S_0 signal, while omitting this pulse causes the largest loss in intensity. Decay times from exponential fits (Fig. 4) also reflect this tendency. Signal decay times of 3.12 ± 0.23 , 2.89 ± 0.08 , 4.52 ± 1.01 , and 6.04 ± 0.47 ms were found for sequences depicted in Figs. 3A–D, respectively.

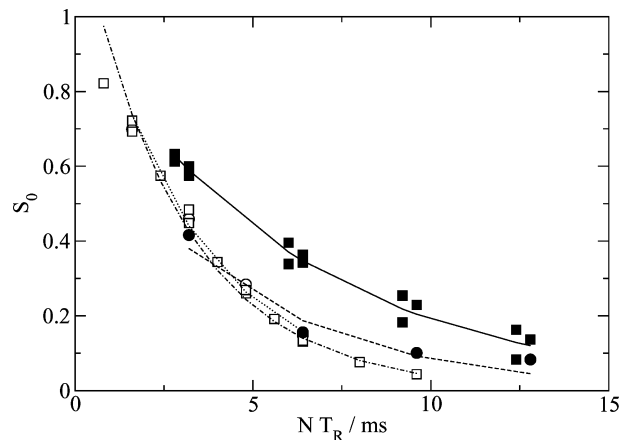


Fig. 4. Non-dephased, normalized signal S_0 (symbols) and exponential fits (lines) to the data of the REDOR experiments of Fig. 3. (A) \circ ; (B) \square ; (C) \bullet ; (D) \blacksquare .

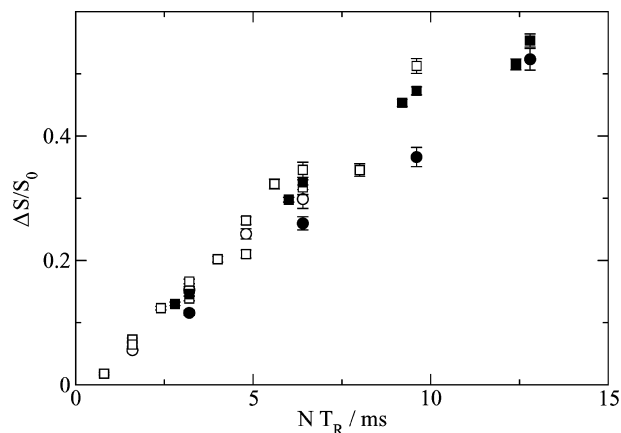


Fig. 5. REDOR curves, showing the reduced REDOR signal $\Delta S/S_0$ as a function of the dephasing time NT_R , of the experiments on TM2FPIP discussed in Fig. 3. (A) \circ ; (B) \square ; (C) \bullet ; (D) \blacksquare .

The REDOR curves for all four sequences are depicted in Fig. 5. Though the general intensity decreases at different rates, the dephasing $\Delta S/S_0$ caused by the dipolar coupling does not show large sequence-specific differences. The dephasing curves of the experiments using no or a 180° refocusing pulse (Figs. 3A, B, and D) behave similarly, while the dephasing of the experiment employing a quadrupolar echo (Fig. 3C) is lower than for the three previous examples.

4.2. Composite pulses

Composite pulses have been successfully employed to increase the bandwidth in REDOR experiments [18]. Therefore the use of both composite pulses replacing the ^2H refocusing pulse or the ^{19}F dephasing pulse were evaluated. Refocusing ^2H with the aid of $90^\circ_x-180^\circ_y-90^\circ_x$ or $90^\circ_x-90^\circ_y-90^\circ_x$ composite pulses produced an increase in the decay of the overall signal, and was thus found to be inferior compared to a regular 180° -pulse (data not shown). However, dephasing ^{19}F with $90^\circ_x-180^\circ_y-90^\circ_x$ composite pulses in place of 180° pulses, greatly reduced the dependence of dephasing on the ^{19}F transmitter offset, and thus improved the applicability of the experiment. Fig. 6 compares the reduction of the ^2H signal of DF-Ala caused by dephasing with either simple 180° ^{19}F pulses or composite pulses for 0.8 ms, using the pulse scheme depicted in Fig. 3A. Applying composite pulses yields complete dephasing over a ^{19}F transmitter frequency range of ~ 120 kHz, whereas with simple 180° pulses the transmitter has to be set within a range of 20 kHz in order to achieve full dephasing. Fluorine composite pulses were therefore used throughout the remainder of this study.

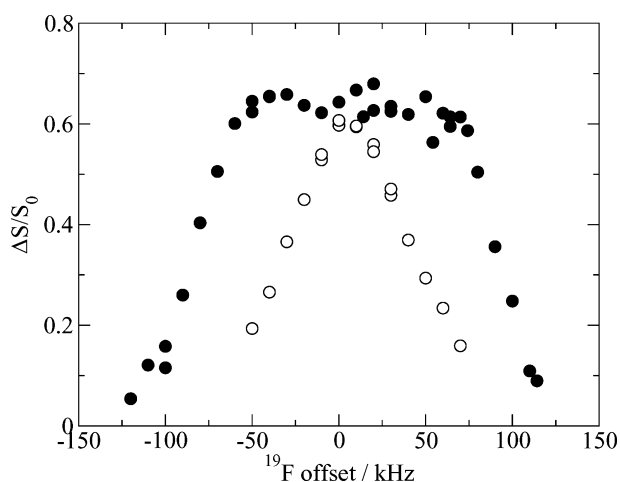


Fig. 6. Signal dephasing $\Delta S/S_0$ of DF-Ala after a dephasing time of 0.8 ms as a function of the ^{19}F transmitter offset. Using $90^\circ_x-180^\circ_y-90^\circ_x$ composite pulses (●) instead of simple 180° (○) for ^{19}F dephasing, greatly enhances the frequency range over which complete dephasing is achieved.

4.3. Distance determination in DF-Ala

Although no crystal structure of DF-Ala is available, only a limited range of $^2\text{H}-^{19}\text{F}$ distances, confined by the $^2\text{H}-\text{C}-\text{C}-^{19}\text{F}$ torsion angle θ (Fig. 1), is possible. The distance obtained from the REDOR experiment is thus expected to fall within the range of 2.4 and 3.2 Å.

Fig. 7 displays the REDOR curves obtained with DF-Ala with different versions of the experiments. The stroboscopically observed experiment yielded slightly higher dephasing, otherwise all experiments show similar dephasing behaviour within errors. To estimate the $^2\text{H}-^{19}\text{F}$ distance in DF-Ala, the experimental REDOR curve was compared with simulated curves. The theoretical curves were calculated with SIMPSON [39] for a two spin $^2\text{H}-^{19}\text{F}$ system. It was found that the relative orientation of the involved interaction tensors, as well as their size, had no significant impact on the simulated REDOR curves, as long as the total intensity of the signal is considered. A considerable deviation from the 2-spin situation is expected because the compound was not diluted with unlabelled material, which is difficult because the fluorine substitution results in a change in the physico-chemical properties of the chemical. However, the initial behaviour is represented fairly well by the 2-spin simulations. The REDOR curves deviate from the 2-spin calculations only in the lack of a fitting oscillatory behaviour and a lower plateau level $\Delta S/S_0 < 1$ for long dephasing times. It therefore seemed justified to use the 2-spin approximation for a distance estimate. A distance of 2.5 ± 0.3 Å was found to be in agreement with the REDOR measurements this way, which translates into a torsion angle θ in the range 0° to 90° . From this we can conclude that the molecule assumes a *gauche*, rather than a *trans* conformation. As the systematic error due to the approximation by a

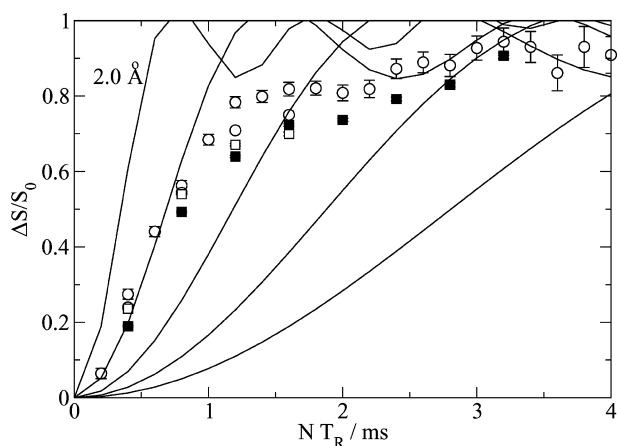


Fig. 7. REDOR curves, showing the reduced REDOR signal $\Delta S/S_0$ as a function of the dephasing time NT_R , of the experiments on DF-Ala using sequences discussed in Fig. 3. (A) ○; (B) □ and (D) ■. For comparison, calculated curves for a single $^2\text{H}-^{19}\text{F}$ spin pair at inter-nuclear distances of 2.0, 2.5, 3.0, and 3.5 Å are drawn with solid lines.

2-spin system dominates statistical errors, no error analysis based on statistical deviations from the fit was attempted.

4.4. Distance estimates in TM2FPIP

To estimate the ^2H – ^{19}F distance, the experimental REDOR curves (with the exception of the quadrupolar echo type REDOR, Fig. 3C) were compared with 2-spin simulations for various distances in a similar fashion as for DF-Ala (see Fig. 8). The agreement between the considered 2-spin model and the experimental data, however, was significantly less in the case of TM2FPIP (Fig. 8) than for DF-Ala (Fig. 7). For this reason, a more general approach for fitting the REDOR curve was pursued by using an expansion of $\Delta S/S_0$ in powers of $(NT_R)^n$, up to $n = 6$ [Eq. (3)]. The coefficients determined in this way were $A_2 = 0.127 \times 10^5 \text{ s}^{-2}$, $A_4 = -0.120 \times 10^9 \text{ s}^{-4}$, $A_6 = 0.386 \times 10^{12} \text{ s}^{-6}$. As outlined in the theory section, the coefficient A_2 is also linked to the second moment of the dipolar coupling [42], giving $M_2 = 3\pi^2/4 A_2 = 0.942 \times 10^5 \text{ s}^{-2}$. With the help of the expansion fit, an estimate for the lower limit of ^2H – ^{19}F distances can also be derived. On the assumption that the initial behaviour of the curve is dominated by the quadratic term of the expansion in NT_R , the dephasing can be either explained as due to a single spin with a dipolar coupling D_1 with $A_2 = (D_1)^2$, or as due to many spins with dipolar couplings $D < D_1$ such that the sum of their squares yields A_2 . Thus, the dipole couplings in a multi-spin situation can not exceed $(A_2)^{-1/2}$. As higher terms also contribute to the initial REDOR curve, $(A_2)^{-1/2}$ only approximates an upper

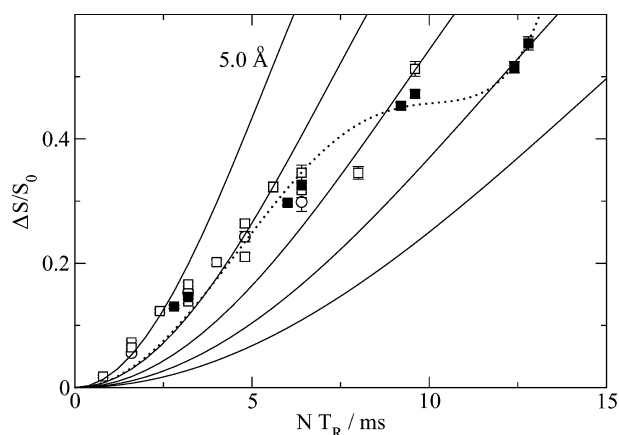


Fig. 8. REDOR curves, showing the reduced REDOR signal $\Delta S/S_0$ as a function of the dephasing time NT_R , of the experiments on TM2FPIP discussed in Fig. 3. (A) \circ , (B) \square and (D) \blacksquare . Calculated curves for a single ^2H – ^{19}F spin pair at internuclear distances of 5.0, 5.5, 6.0, 6.5, and 7.0 Å are drawn with solid lines. In addition, the REDOR curves are also fitted (dotted line) to the expansion in powers of NT_R [Eq. (3)]. The initial part of the expansion fit is compatible with a distance of 5.4 Å.

limit for the dipolar coupling, from which an estimate for a lower limit for the internuclear distance can be derived. The lower distance limit obtained from the expansion fit is found to be 5.4 Å.

Due to the uniaxial rotation of the deuterated methyl group in the inhibitor compound, the observed dipolar coupling reflects a motionally averaged value. The lower distance limit of 5.4 Å thus refers to a distance which deviates from the distance between the fluorine nucleus and the centre of the deuteron triad because of the averaging process. This deviation depends on the relative orientation of the methyl axis and the fluorine–methyl vector. A stronger than expected coupling is observed compared to a distance between the triad centre and the fluorine if the fluorine is located in an equatorial position, and a weaker coupling is observed if the fluorine is located along the methyl rotation axis. With the aid of numerical calculations of the averaged dipolar interaction tensor, a fluorine–triad centre distance of 5.9 Å for the equatorial fluorine position and of 5.2 Å for the axial fluorine position were found to correspond to the upper limit for the dipolar coupling that had been determined. Hence, the lower limit for the distance between the fluorine and the deuteron triad centre, taking into account the averaging, is 5.2 Å.

4.5. Influence of the lack of decoupling on the distance measurements

The major difference of the proposed strategy compared to previous REDOR measurements is the lack of high power proton decoupling, allowing the proton channel to be used for the fluorine dephasing. To estimate the potential influence of the coupling between surrounding protons and the ^2H – ^{19}F spin pair, the

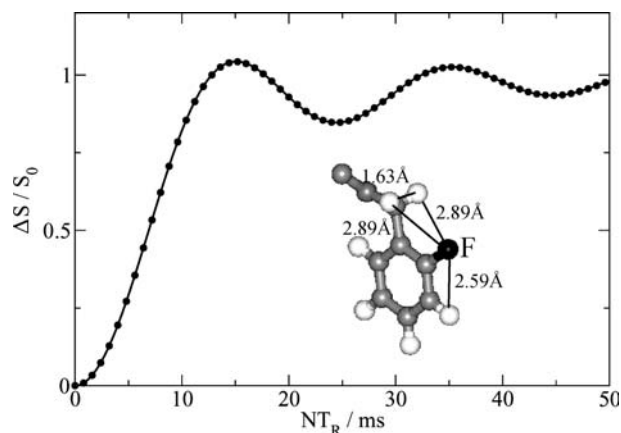


Fig. 9. Simulation of $^2\text{H}\{^{19}\text{F}\}$ REDOR-curves with (symbols) and without (line) ^1H proton couplings. To approximate the local proton environment of the fluorine, three adjacent protons were considered, assuming a molecular conformation which brings the protons closest to the fluorine. The inset shows the considered ^1H – ^{19}F and ^1H – ^1H couplings in the fluorinated phenyl-ring of TM2FPIP.

$^2\text{H}\{^{19}\text{F}\}$ REDOR experiment was simulated in the presence and absence of proton couplings. As couplings between fluorine and proton are significantly stronger than between deuterium and proton, the protons in the vicinity of the fluorine were considered in the simulated spin system. The three closest protons to the fluorine were used to approximate the surrounding proton network (see inset Fig. 9). No significant difference between simulated $^2\text{H}\{^{19}\text{F}\}$ REDOR-curves with and without ^1H couplings was found within the considered dephasing time of ~ 50 ms.

5. Discussion and conclusion

5.1. Pulse sequences

This study presents $^2\text{H}\{^{19}\text{F}\}$ REDOR as a way to benefit from the high gyromagnetic ratio of fluorine in heteronuclear distance measurements without the need for specialized fluorine NMR equipment, which is often compromised by the proximity of proton and fluorine resonance frequencies and high fluorine probe background signals. Observing ^2H without ^1H decoupling raises the question whether the signal can be kept over long enough dephasing times to address large distances. Although the lack of decoupling is not expected to alter the accuracy of obtained distances significantly, since it affects both dephased and non-dephased signals in a similar way, it might decrease the overall signal intensity at long dephasing times. Several versions of the REDOR experiment were compared to elucidate this issue by comparing different ^2H refocusing pulses. It was found that a 180° -pulse gave the strongest signal, and a 90° -pulse or no pulse at all lead to a larger loss of the overall signal. The poorer performance of the 90° refocusing pulse compared to the 180° -pulse might be explained by a larger influence of finite pulse effects in the former experiment. The 90° -pulse refocuses the quadrupolar interaction in addition to the refocusing by MAS, thus the finite pulse width can lead to a loss in signal due to a lack of synchronization of both types of refocusing. As the 180° -pulse does not refocus the quadrupolar interaction, such effects are not present in this version of the experiment. Furthermore, the 180° -pulse does refocus the isotropic chemical shift, whereas a 90° -pulse or no refocusing pulse does not, yielding a higher intensity in the former experiment. Second-order quadrupolar shifts are refocused in different ways as well, and may contribute to the observed differences in performance of the compared sequences.

5.2. Composite pulses

Although composite pulses did not enhance dephasing (Fig. 6), they were shown to improve the bandwidth of the

fluorine dephasing pulses significantly. While accurate positioning of the fluorine transmitter frequency within ~ 20 kHz is crucial to achieve full dephasing without composite pulses, their application renders the experiment independent of the ^{19}F transmitter frequency over a wide range of ~ 120 kHz. The proposed $^2\text{H}\{^{19}\text{F}\}$ REDOR therefore also becomes applicable without compromising the performance in situations where the precise ^{19}F frequency in the sample is not known. As it is often difficult to detect weak fluorine signals with standard probes because of the ^{19}F background from conventionally used fluoropolymers, the strategy presented here benefits especially from broadband composite pulses.

5.3. Distance measurements

Though an exact interpretation of the REDOR data in terms of a unique distance is hampered by the presence of multinuclear dephasing in these systems, it could be shown that sufficiently long dephasing times up to 12 ms are possible. It is thus feasible to address long distances of up to approximately 8 \AA by $^2\text{H}\{^{19}\text{F}\}$ REDOR without ^1H decoupling. Furthermore, in both studied compounds, a distance or distance limit could be obtained. The DF-Ala distance of 2.5 \AA falls within the expected range of possible distances, and a conclusion that a *gauche* conformation of the $^2\text{H}-\text{C}-\text{C}-^{19}\text{F}$ torsion angle could be drawn. A lower $^2\text{H}-^{19}\text{F}$ distance limit of 5.2 \AA was determined in the case of the inhibitor TM2FPIP. In good agreement with earlier measurements on a similar inhibitor of the same family [11], this is compatible with an extended conformation of the ATPase-bound ligand. Although the accuracy of these distance measurements, and the effect of the lack of ^1H -decouplings on the obtained distance, could not be judged according to the present experimental observations, the observed distances are in good agreement with expected distances in both cases. Theoretical estimation of the influence of couplings to surrounding protons by simulations indicate no significant alteration of the observed $^2\text{H}-^{19}\text{F}$ coupling in the absence of decoupling. As the pulse sequence of the proposed strategy does not differ from routine REDOR experiments except for the absence of decoupling, but rather relies on the specific choice of nuclei, the distances obtained are therefore not expected to be influenced by the new approach. The $^2\text{H}\{^{19}\text{F}\}$ REDOR experiment is thus a viable approach for distance measurements.

5.4. Applications to biological samples

The extension of the proposed NMR strategy to membrane proteins such as TM2FPIP bound to its target protein, the gastric H^+/K^+ -ATPase, remains to be investigated. In particular, the fast T_2 relaxation of ^2H due to the presence of motions in such samples could

impose a limitation on the distance range which can be studied by ^2H observed REDOR without ^1H decoupling. It has been demonstrated successfully that ^2H observed REDOR with ^1H decoupling allows studies of weak couplings in biological samples. However, as the quadrupolar interaction is the dominant T_2 relaxation mechanism, and the time scale of molecular motions can be altered by working at low temperature, we expect that the lack of ^1H decoupling has an only minor impact on the general applicability on biological samples.

In the recent application of $^2\text{H}\{^{19}\text{F}\}$ REDOR [33], the importance of an accurate setting of the magic angle in the case of the quadrupolar nucleus ^2H was reported. Deviations from the magic angle contribute to the loss of signal during the dephasing time and were compensated by the use of a 90° pulse in the centre of the dephasing period on the ^2H channel. Similar effects were not encountered in this study, possibly due to the stronger couplings and therefore shorter dephasing times used here.

The signal-to-noise achievable and distance range obtainable with the choice of nuclei presents a concern in extending the proposed strategy to biological systems. When comparing the ^2H observed experiment to REDOR observing nuclei such as ^{15}N , ^{13}C or ^{31}P , which have been used in conjunction with ^{19}F in the past, the gyromagnetic ratio, spin quantum number, spectral width and relaxation times determine the signal to noise of the experiment. The sensitivity ratio of the spin-1 nucleus ^2H and a spin-1/2 nucleus X, with gyromagnetic ratios γ_{D} and γ_{X} , is $r = 8/3(\gamma_{\text{D}}/\gamma_{\text{X}})^{5/2}$ [46]. Accounting for the gyromagnetic ratio and the spin-1 of deuterium thus renders the sensitivity of ^2H almost comparable to ^{13}C ($r = 0.777$), higher than ^{15}N ($r = 7.525$), but lower than ^{31}P (0.236). However, cross polarization, which cannot be used together with the proposed approach, yields an increase in intensity in REDOR applications detecting ^{13}C , ^{15}N , or ^{31}P . When observing the integrated signal, summed over all eventual sidebands, the large spectral width resulting from the quadrupolar interaction of ^2H leads to a higher statistical error of the data points of the REDOR curve. The long deuterium T_1 -relaxation times observed in the present study are the result of the poly-crystalline environment of the compounds investigated. In biological samples, even at low temperatures such as below -100°C , the molecular mobility gives rise to T_1 -relaxation times on the milliseconds time scale, thus significantly shorter than those of this investigation, but also than typical T_1 -relaxation times of ^{15}N , ^{13}C or ^{31}P in these systems. In ^2H -NMR experiments of biological samples fast recycle times of 100 ms–1 s are typically used, giving ^2H an advantage over ^{15}N , ^{13}C or ^{31}P . When considered as a whole, the integral intensity as measured in the ^2H detected REDOR experiment is comparable in sensitivity to ^{15}N .

Provided the same dipolar coupling frequency can be resolved, the distance range of the REDOR experiment

depends only weakly ($\sim (\gamma_{\text{X}})^{1/3}$) on the gyromagnetic ratio γ_{X} of the detected nucleus. On this basis, the distance range in the ^2H -detected REDOR experiment is $\sim 15\%$ larger than the ^{15}N -detected, 15% lower than the ^{13}C -detected and 28% lower than the ^{31}P -detected experiment. The distance range of the new approach suggested here thus depends more on the achievable dephasing times in the absence of decoupling and T_2 -relaxation, than on the choice of ^2H per se.

5.5. Summary

The feasibility of $^2\text{H}\{^{19}\text{F}\}$ REDOR for structural investigations in biological solids employing standard double resonance hardware has been investigated. Among the studied variations of the REDOR experiment, using a single 180° -pulse for refocusing ^2H and a train of 90°_x – 180°_y – 90°_x composite pulses with $xy8$ phase cycling with timings as in the original REDOR experiment, was found to yield the largest overall signal intensity, whilst not compromising the amplitude of dephasing.

Dephasing was observed for recoupling times up to 12 ms, and ^2H – ^{19}F distance information obtained from two compounds, an alanine derivative and an inhibitor of the gastric H^+/K^+ -ATPase, was in good agreement with expectations. The approach presented here has proven to be a simple and viable method of benefiting from the large coupling strengths of fluorine in heteronuclear distance measurements without the need of specialized fluorine equipment.

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