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Dipolar dephasing for structure determination in a paramagnetic environment

Rubin Dasgupta, Karthick B.S.S. Gupta, Derek Elam1, Marcellus Ubbink, Huub J.M. de Groot *

Leiden University, Leiden Institute of Chemistry, Gorlaeus Laboratories, Einsteinweg, 55 2333 CC, Leiden, the Netherlands

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A B S T R A C T
We demonstrate the efficacy of the REDOR-type sequences in determining dipolar coupling strength in a paramagnetic environment. Utilizing paramagnetic effects of enhanced relaxation rates and rapid electronic fluctuations in Cu(II)-(DL-Ala)2.H2O, the dipolar coupling for the methyl C-H that is 4.20 Å (methyl carbon) away from the Cu2+ ion, was estimated to be 8.8 ± 0.6 kHz. This coupling is scaled by a factor of ~0.3 in comparison to the rigid limit value of ~32 kHz, in line with partial averaging of the dipolar interaction by rotational motion of the methyl group. Limited variation in the scaling factor of the dipolar coupling strength at different temperatures is observed. The C-H internuclear distance derived from the size of the dipolar coupling is similar to that observed in the crystal structure. The errors in the dipolar coupling strength observed in the REDOR-type experiments are similar to those reported for diamagnetic systems. Increase in resolution due to the Fermi contact shifts, coupled with MAS frequencies of 30–35 kHz allowed to estimate the hyperfine coupling strengths for protons and carbons from the temperature dependence of the chemical shift and obtain a high resolution 1H–1H spin diffusion spectrum. This study shows the utility of REDOR-type sequences in obtaining reliable structural and dynamical information from a paramagnetic complex. We believe that this can help in studying the active site of paramagnetic metalloproteins at high resolution.

1. Introduction

Paramagnetic solid-state NMR has undergone a revolution in the past decade due to the development of high spinning frequency probes and tailored pulse sequences. Studying the ligands that directly coordinate the paramagnetic metal ions with NMR can provide insight into the structure and dynamics of the system [1–4]. Among the various interactions, dipolar interactions are attractive for characterizing fast cooperative dynamics that can be measured using rotational-echo, double resonance (REDOR) type solid state NMR experiments including shifted-REDOR for probing anisotropic motions [5–7].

In this study we show that rapid paramagnetic fluctuations favorably affect REDOR and shifted REDOR experiments therefore, those can be performed with high sensitivity and resolution. We find that internuclear dipolar couplings can be determined with high accuracy that is comparable to what has been achieved for diamagnetic systems. We have selected Cu(II)-(DL-Ala)2.H2O (Fig. 1) as a model system to test the utility of the REDOR type sequences and to probe the C-H dipolar coupling of the methyl group in the alanine ligand, which is directly coordinating the paramagnetic metal center [8–11]. Recently, well-resolved heteronuclear dipolar correlation spectroscopy was reported for Cu(II)-(DL-Ala)2.H2O [10,12–14]. The possibility to sustain internuclear dipolar transfer in the immediate vicinity of paramagnetic ions can be attributed to rapid paramagnetic fluctuations that average the anisotropic and traceless electron-nuclear dipolar interactions while leaving the isotropic contact shifts unaffected. Here, the J-coupling between the copper ions determines the fluctuation frequencies for averaging dipolar couplings, which are traceless without isotropic component and is intrinsic to a one-dimensional chain. This results in the motional narrowing from spin waves running along the chain [15]. This opens up a window of opportunity, since essential advantages of paramagnetism, such as rapid acquisition and increased spectral dispersion can be combined with the power of dipolar correlation spectroscopy for resolving static or dynamic structure quantitatively. To demonstrate this, we implement a REDOR method for Cu(II)-(DL-Ala)2.H2O by adapting the REDOR refocusing period to the fast rotation needed for signal detection in paramagnetic systems with their enhanced relaxation that limits the acquisition time. The Cu(II)-(DL-Ala)2.H2O form 1-dimensional chains in which the Cu2+...
ions are weakly coupled \[11\]. The antiferromagnetic coupling \( J = 2 \frac{C_0}{21 \text{ cm}} \) translates into a characteristic frequency of \(~70\ \text{GHz}\) for the electronic spin fluctuations \[11\]. The properties of enhanced relaxation and rapid averaging of electron-nuclear dipolar interactions by paramagnetic fluctuations that are much faster than the frequency scale of the heteronuclear dipolar coupling of \(~10\ \text{kHz}\) or less, allow to perform heteronuclear dipolar recoupling experiments with fast MAS in a very short time, much shorter than for diamagnetic species. In addition, we show how \(^1\text{H}–^1\text{H}\) spin diffusion spectra and the temperature dependence of the chemical shift for carbon and protons where \(CH_3\) and \(C_\alpha\) are in red, \(CH_2\) and carbonyl carbon \(CO\) are in blue and \(H_\alpha\) and \(C_\beta\) are in green. Solid lines are the fit to equation (9). Spinning side bands in the panels showing spectra are marked with asterisks. The assignments in panel (a) to (e) of \(^1\text{H}\) and \(^{13}\text{C}\) responses are shown in italics and blue color.

2. Experimental procedures

2.1. Sample preparation and solid-state NMR experiments

\[^{11}\text{Cu}-(\text{DL-Ala})_2\cdot\text{H}_2\text{O}\] complex was synthesized as reported earlier \[10\]. All chemicals including the uniformly labelled \(^{13}\text{C}\)-\text{c}-alanine were obtained from Sigma Aldrich (USA).

Solid-state NMR experiments were performed with AV-I (17.6 T) 750 MHz and AV-HDIII (22.31 T) 950 MHz spectrometers equipped with a 2.5 mm and 1.3 mm MAS probe, respectively, at spinning frequencies ranging from 30 to 62.5 kHz 1D proton and carbon spectra were recorded with a rotor synchronized Hahn-echo sequence (Fig. S4a). The high-power \(\pi/2\) pulses were 2 \(\mu\)s and 2.5 \(\mu\)s for \(^1\text{H}\) and \(^{13}\text{C}\), respectively. A dead time of 4.5 \(\mu\)s was subtracted from the refocusing period in the Hahn-echo pulse sequence. A comparison of spectra obtained with single 90° pulse (Fig. S1) with rotor synchronized Hahn-echo over single 90° pulse is elimination of probe artifacts as observed from Fig. S1a and Fig. 2a. This prompted us to use rotor-synchronized Hahn-echo over single 90° pulse sequence to obtain 1D spectra for both carbon and proton for further study.

The 1D and 2D dipolar-INEPT sequence was adopted from
Wickramasinghe et al. (2006) with a π/2 pulse length similar to the rotor synchronized Hahn-echo experiment \[13\]. Temperature dependent experiments were performed at temperatures between 247 K and 318 K. For the 2D $^1$H–$^1$H spin diffusion (Fig. S4b) experiment the mixing time was varied from 1 ms to 5 ms with a total acquisition time of 530 μs and a delay of 30 ms between scans. 200 slices of data were acquired in the t1 dimension in a total experiment time of <5 min. REDOR and shifted-REDOR data were collected by direct excitation of $^{13}$C (Figs. S4c – S4d) \[5, 6, 16–18\]. The π/2 pulse lengths were 2 μs and 2.2 μs for $^1$H and $^{13}$C respectively. Temperature calibration was done using samarium tin oxide and lead nitrate \[19\].

2.2. REDOR and shifted-REDOR data analysis

The REDOR and shifted-REDOR curves were fitted with SIMPSON 4.2.1 \[22, 23\]. The geometry of Cu(II)-(DL-Ala)$_2$H$_2$O was adopted from Calvo et al. (1991) and Zhang et al. (2005) to generate the spin system parameters, dipolar coupling and Euler angles using SIMMOL-VMD \[8, 21\]. Data analysis was performed with TOPSPIN 4.0.5 (Bruker) and Igor pro 6.37.

\[
D_{avg} = S^*D, \tag{1}
\]

with $D$ the dipolar coupling strength in the rigid limit. The scaling factor is

\[
S = (3\cos^2\theta - 1) / 2, \tag{2}
\]

where $\theta$ is the angle between the methyl C-H and the rotational axis, which in this case is the bond between C$_3$ – C$_4$. For a tetrahedral geometry of the methyl group $\theta = 109.5^\circ$. This gives a theoretical scaling factor of $S = 0.333$ (Fig. S5) and $D_{avg} = 10.69$ kHz for the C-H bond in Cu(II)-(DL-Ala)$_2$H$_2$O in the rotating methyl group. For the analysis of the data, the scaled $D_{avg} = 10.69$ kHz was changed to fit the experimental data. The average dipolar coupling strength was optimized to fit the REDOR curve using the fitting algorithm of SIMPSON. The homonuclear dipolar coupling between the three protons of the methyl group was also scaled by the theoretical value of 0.333 to give a $D_{avg} = 9.76$ kHz \[26\]. The codes for fitting and to obtain the spin-system can be downloaded from zenodo.org (https://doi.org/10.5281/zenodo.4599603).

2.3. Theory of temperature dependence of Fermi contact shifted resonances

The chemical shift of the paramagnetic system is temperature dependent and the observed chemical shift is given by \[29\].

\[
\delta_{bs} = \delta_{dia} + \delta_{FCS} + \delta_{PCS}, \tag{3}
\]

where $\delta_{dia}$ is the diamagnetic chemical shift and $\delta_{FCS}, \delta_{PCS}$ are the contributions from the Fermi contact shift (FCS) and pseudo-contact shift (PCS) respectively \[29, 30\]. For the nuclei of coordinating ligands FCS >> PCS and eq. (1) can be written as

\[
\delta_{bs} \approx \delta_{dia} + \delta_{FCS}. \tag{4}
\]

The contribution from the FCS is given by \[30\].

\[
\delta_{FCS} = \frac{\Delta S}{\hbar \gamma_B B_0} \tag{5}
\]

with $A = \frac{3}{2} \hbar \gamma_B \mu_0 \delta_\rho$, the isotropic hyperfine coupling that is proportional to the spin density $\rho$ on a nucleus. The $S_i$ is the expectation value of the $z$ magnetization, $\hbar$ is the reduced Planck constant, $\gamma_B$ is the gyromagnetic ratio of the nucleus in question, and $B_0$ is the external magnetic field.

Since the $S_i$ is related to the magnetic susceptibility according to \[30, 31\].
The magnetic susceptibility is given by [32].

\[
\chi = \frac{S(S+1)\mu_B^2}{3k_B T}.
\]

Substituting eq. (8) in eq. (7), eq. (4) can be written as

\[
\delta_{\mu} \approx \frac{S(S+1)\mu_B^2}{3k_B T} \delta \mu
\]

for the temperature dependent chemical shift, which was used to determine hyperfine coupling constant by fitting to the data in Fig. 3.

3. Results and discussion

3.1. NMR characterization of Cu(II)-(DL-Ala)₂H₂O

There are several advantages when working with Cu(II) paramagnetic systems. Since Cu(II) has small susceptibility the Fermi-contact interaction is stronger than the paramagnetic relaxation enhancement (PRE) [29]. This results in a better resolution because of the increase in the spectral dispersion without adversely affecting the linewidth. Additionally, reduction of experimental time due to fast longitudinal relaxation compared to diamagnetic systems can be achieved. In contrast, with other metal ions such as Tb(III) the susceptibility anisotropy is large and may lead to resonances broadened beyond detection due to increase in PRE-effects [29].

The 1D spectra of \(^1H\) and \(^13C\) at different spinning frequencies and magnetic fields. The resolution is enhanced by the paramagnetic shifts at very fast MAS (≥ 20 kHz) [12-14,33-35].

The 1D \(^1H\) rotor-synchronized Hahn-echo spectra (Fig. 2a and b) show three prominent resonances attributed to \(CH_3\) at 8 ppm and \(CH_2\) at 32 ppm and \(CH_2\) at 21 ppm. We observed that the intensity of \(CH_3\) is 4 times that of \(H_2\) and \(CH_2\), while \(H_2\) and \(CH_2\) show similar intensities. The carbon spectrum shows three prominent resonances for \(C_1\) at –280 ppm, \(CO\) at –200 ppm and \(C_1\) at 180 ppm and two minor resonances for \(C_2\) at –258 ppm and \(C_2\) at 88 ppm (Fig. 2c and d and Table S1) [10,13,35].

The two components were attributed to two orientations of the methyl group as a consequence of crystal packing, where the major component (CH₃/C₁) is closer to the copper ion, while the minor component (CH₂/C₂) is more distant [8,36]. The \(^1H\) signals from the NH₂ group (data not shown) were reported to be at –132.7 and –159.5 ppm [10]. The \(^1H\) from the water molecule are generally broadened beyond detection due to fast exchange dynamics [10,35]. The methyl group of the major component was reported to be undergoing fast rotation which results in a single resonance for the three methyl protons (Fig. 2a and b) [10].

A dipolar-INDEPT experiment was performed to check the quality of the sample and the efficiency of polarization transfer via dipolar interactions, which was previously shown to be superior to cross polarization based transfer for paramagnetic systems [13,37]. The spectrum is similar to the one reported earlier (Fig. 2d), albeit with better resolution and S/N, allowing to identify the splitting of the methyl group response that arises due to crystal packing effects [8,13,36].

The 2D \(^1H\)-\(^1H\) spin diffusion spectra acquired at 22.31 T with a MAS frequency of 60 kHz (Figs. 2e and S3), show that a high resolution homonuclear 2D spectrum can be obtained under paramagnetic conditions with modification of the pulse sequence to consider the paramagnetic effects of increased relaxation and rapid fluctuations. Similar observations were reported for copper cyclam complexes [37].

The temperature dependence of the chemical shift shows strong Curie behavior for CO, C₃, C₁, CH₃ and CH₂ while weak anti-Curie behavior is observed for H₄ (Fig. 2f and Fig. S2a). The hyperfine coupling strengths are summarized in Table S2, obtained after fitting the data with equation (9) (Fig. 2f) [38]. It is interesting to observe that, although the H₄ is close to the copper, the hyperfine coupling constant and hence the spin density on it is less than for H₃. This was also observed in DFT calculations for Cu(II)-(DL-Ala)₂H₂O showing very little electron density localized on the H₄ atom [26,38]. The sign of the hyperfine coupling strength provides the spin polarization of the electron on the nucleus with respect to the external magnetic field. It was observed to be parallel for C₁, CH₃ and CH₂ and anti-parallel for CO, CH₃ and H₄ (Table S2 and Fig. 2f). These observations are in line with the calculations performed on copper alanine complexes and previously reported data (Table S3) [38]. The estimated δH₄ from the fit of the temperature dependences of the chemical shift (Fig. 2f) is summarized in Table S2. They differ from the expected diamagnetic chemical shift of ω-alanine (Table S4). This difference can be attributed to the differences in crystal packing of Cu(II)-(DL-Ala)₂H₂O and ω-alanine (Fig. S6). The NMR chemical shift tensor for small molecules in solid state NMR differs significantly between different crystal forms due to changes in hydrogen bonding, stacking, ring current, C–H–π interactions etc. [39] DFT calculations of different crystal forms of purine derivatives show that the change in the diamagnetic chemical shift can be as high as 6.5 ppm, 20 ppm and 55 ppm for proton, carbon and nitrogen respectively [39].

The full width at half of the maximum (FWHM) of the resonances decreases with increasing temperature (Figs. S2b and S2c). Most likely, this increase in the electronic relaxation rate with temperature reduces the paramagnetic relaxation enhancement.

3.2. Dipolar coupling strength estimation

Dipolar coupling measurements can provide insight into the dynamics of the molecule [5,40]. Here, we have employed REDOR (Fig. S4c) and shifted-REDOR (Fig. S4d) to measure the dipolar coupling between \(^1H\) and \(^13C\) of the methyl group in Cu(II)-(DL-Ala)₂H₂O [5,6]. The choice of these pulse sequences was based on their robustness for amplitude mis-setting, inhomogeneities in the radiofrequency field, carrier offset and CSA [5]. The methyl group was used as a probe because of the presence of intrinsic methyl rotation that partially averages the dipolar coupling and its higher S/N ratio compared to that of other protons (Fig. 2a and b).

Fig. S4a shows the \(^1H\)-\(^13C\) REDOR curves of the C-H in CH₃ group at 277 K, 287 K and 297 K obtained at a MAS frequency of 30 kHz. The dipolar coupling strengths estimated after the fitting are 8.6 kHz, 10.0 kHz and 9.2 kHz respectively (Table 1 and Fig. 3b). These values correspond with a scaling factor of ~0.3 (Table 1) relative to the rigid limit value of ~32 kHz, consistent with previously reported data for alanine in the diamagnetic crystalline state and similar to the theoretical scaling factor of 0.333 [27]. The accuracy of the paramagnetic measurement is limited...
similar to that reported for the diamagnetic systems [5,6].

The corresponding internuclear $^1$H-$^{13}$C (C$_6$1 and CH$_2$1) distances derived after dividing the dipolar coupling strength with the scaling factor is $\sim$1.0 Å (Table 1) which is very similar to the crystal structure (1.0 Å) [8,26]. This demonstrates the efficacy of the REDOR sequence in obtaining reliable distance information in a paramagnetic system. It is noteworthy that although the paramagnetic shift is temperature dependent the dipolar coupling strength is preserved. This property can be exploited to characterize the dipolar coupling strength between the nuclei in the vicinity of the metal at the active site of the paramagnetic metalloproteins and consequently get insight into the dynamics.

It was recently reported that using a variant of REDOR where the $\pi$ pulses are shifted from its original position, known as shifted-REDOR (Fig. S4d), is able to identify anisotropic motions present in the protein via the asymmetry parameter $\eta$ [5,6]. We investigated the dephasing behavior in the presence of the paramagnetic metal center at a spinning frequency of 30 kHz and at 297 K with the $\pi$ pulse shift ($\tau_{p}$) of 0.685 or 0.580 or 0.440 times $\tau_{p}/2$ (Fig. 3c). The dipolar coupling strength between the methyl C–H nuclei does not change with varying $\tau_{p}$ and it matches with the values obtained from REDOR (Tables 1 and 2). The derived internuclear distance is $\sim$1.0 Å (Table 2), consistent with the crystal structure and similar to REDOR. Hence, shifted-REDOR appears also robust and reliable for obtaining structural information. The asymmetry parameter $\eta$ of 0.1 gave the best fit, which is in line with the lack of anisotropic motion in the methyl group rotation. While there was no anomaly in the implementation of shift-REDOR for Cu(II)-(DL-Ala)$_2$H$_2$O, in shifted-REDOR (Fig. 3c), the oscillatory behavior observed in the REDOR (Fig. 3a) is diminished which can be due to the presence of additional protons (NH$_2$ and Hx). This is consistent with the observations of Schanda et al. (2011), who showed that the presence of additional protons near the observed nuclei reduces the oscillatory nature of the shifted-REDOR for ubiquitin crystals [5,6].

In summary, both REDOR and shifted-REDOR sequences provide reliable estimates of the dipolar coupling strength and consequently information about the structure and methyl rotation.

4. Conclusions

This study shows that high resolution $^1$H based solid-state NMR spectra can be obtained for a paramagnetic system at fast MAS ($\sim$25 kHz). Using the high sensitivity of $^1$H and the high resolution due to the paramagnetic nature of Cu(II), a temperature dependence study of the model compound Cu(II)-(DL-Ala)$_2$H$_2$O could be performed that shows that the shift correlates with the spin delocalization over the ligands. The fast MAS has enabled the implementation of $^{13}$H–H homonuclear spin diffusion, REDOR and shifted-REDOR pulse sequences. Our benchmark is the existing crystal structure. The error in the measured values were similar to that observed for diamagnetic systems and are largely systematic errors from e.g. thermal motions that effectively reduce the dipolar coupling from its rigid limit value. We present a technical step forward by showing that both REDOR and shifted-REDOR are quantitatively reliable in determining the dipolar coupling strength and internuclear distances near the paramagnetic metal center, which paves the way for future application in metalloproteins to probe fast dynamics. In addition, the results contribute to the converging and convincing evidence that paramagnetic solid-state NMR is increasingly accessible and can be interpreted in the same way as the NMR spectra of diamagnetic solids.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ssnmr.2021.101728.

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