

Determination of Internuclear Distances in Uniformly Labeled Molecules by Rotational-Resonance Solid-State NMR

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Abstract: Rotational-resonance magic-angle spinning NMR experiments are frequently used to measure dipolar couplings and to determine internuclear distances. So far most measurements were performed on samples containing isolated spin pairs. Thus, extensive structure elucidation, for example in biomolecules, requires the preparation of a whole set of doubly labeled samples. Here, we describe the analysis of the rotational-resonance polarization-exchange curves obtained from a single, uniformly labeled sample. It is shown experimentally that, at a magnetic field of 14.09 T, the rotational-resonance conditions in uniformly ^{13}C -labeled threonine are sufficiently narrow to permit the measurement of five distances between the four carbon spins with an accuracy of better than 10%. The polarization-exchange curves are analyzed using a modified two-spin model consisting of the two active spins. The modified model includes an additional offset in the final polarization, which comes from the coupling to the additional, passive, spins. The validity of this approach is experimentally verified for uniformly ^{13}C -labeled threonine. The broader applicability of such a model is demonstrated by numerical simulations which quantify the errors as a function of the most relevant parameters in the spin system.

Introduction

High-resolution solid-state NMR is a useful tool to study both micro- and noncrystalline materials. To achieve the spectral resolution required for measuring specific distance constraints, the application of magic-angle spinning (MAS) is usually mandatory. However, the improved spectral resolution comes at the expense of losing the information contained in the anisotropic interactions. One example of such an averaged anisotropic interaction is the dipolar-coupling tensor, which is proportional to the inverse cube of the distance between two nuclei. To reintroduce the anisotropic dipolar interaction into MAS NMR spectra, dipolar recoupling methods are usually applied.^{1,2} In this Article, we concentrate on homonuclear recoupling methods for the determination of distances in uniformly labeled solids.

Recoupling methods may be classified into broad-banded and selective methods. In broad-banded methods, all the spins are recoupled simultaneously. The polarization-transfer dynamics in such multispin experiments contain, possibly among other contributions, the information about all dipolar couplings in the system. It is, however, difficult to extract this information quantitatively³ and the contribution of the small couplings tends to be negligible. Selective methods ideally recouple only two spins at a time and determine accurately a single internuclear distance per experiment. The pair of "active" spins is selected by its spectral properties, for example, the isotropic chemical-shift difference.

The rotational-resonance experiment (RR)^{4–8} is a robust and widely applied method for selective homonuclear recoupling. It requires the MAS frequency to match an integer submultiple of the isotropic chemical-shift difference between the two selected, active spins, i.e., $n\omega_r = |\Omega_1^{\text{iso}} - \Omega_2^{\text{iso}}|$. Despite this inherent selectivity of rotational-resonance recoupling,⁶ most practical applications have been to systems where relatively isolated spin pairs have been introduced chemically by selective labeling. This leads to particularly simple polarization-exchange dynamics. The drawback, however, is that producing the selectively labeled compounds is both expensive and time-consuming.

In rotational-resonance experiments, weak dipolar couplings are characterized through the measurement of polarization exchange between the two recoupled spins. These polarization-exchange curves are analyzed in terms of the internuclear distance and the zero-quantum relaxation time with other parameters such as chemical-shielding tensors playing a minor role at the $n = 1$ rotational resonance condition. This approach is known to work well in selectively labeled systems with isolated spin pairs because there are no additional dipolar couplings to "passive" spins which could influence the polarization-exchange curves.

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In this paper we demonstrate that, under certain circumstances, the polarization exchange at the rotational-resonance condition in uniformly labeled systems can be modeled by a modified two-spin system. In the presence of the additional passive spins the difference polarization evolves towards a finite value, while it evolves toward zero in an isolated two-spin system. Similar effects were observed in systems with inhomogeneously broadened lines.⁹ The modified long-time behavior of the difference polarization can be included phenomenologically into the analysis of the polarization-exchange curves. Such a simple modified two-spin model of rotational-resonance polarization exchange gives accurate results if the isotropic chemical-shift differences between the passive and active spins are far enough from any rotational-resonance conditions.

In this paper we will show experimental rotational-resonance measurements on fully labeled L-threonine that will be analyzed using the modified two-spin model. In addition, synthetic data generated by numerical simulations of model three-spin systems will show in which range of parameters such a model gives accurate results.

Materials and Methods

NMR Experiments. All experiments were performed on uniformly ¹³C and ¹⁵N labeled L-threonine (Cambridge Isotope Laboratories) and diluted in natural-abundance material at a ratio of 1:10 to minimize intermolecular dipolar contacts. Subsequently the sample was recrystallized from a hot aqueous solution. Narrow resonances (full width at half-height 0.15 ppm) observed in the CP-MAS spectra indicated a uniform crystal form throughout the sample. NMR experiments were recorded at a static magnetic field of 14.09 T using a Bruker Avance 600 spectrometer. All data were acquired with a Bruker 2.5 mm o.d. triple-resonance CP-MAS probe. The spinning frequency was stabilized to ± 5 Hz. Rotational-resonance experiments were performed following adiabatic cross polarization from ¹H to ¹³C.¹⁰ Afterward, the polarization of one of the resonances was selectively inverted using a DANTE pulse train.^{11,12} The DANTE inversion was empirically optimized for each exchange curve and typically consisted of a train of between 7 and 10 pulses separated by intervals of up to 100 μ s with an effective 180° pulse of 25 μ s. After a variable mixing time, the polarization was converted to single-quantum coherence by a hard 90° pulse and detected. During the mixing time and data acquisition, TPPM¹³ decoupling was applied using a 10° phase angle and a pulse length of 5 μ s. Each point on the polarization-exchange curve was the result of the summation of 128 transients. An example of a typical spectrum of L-threonine used to determine the difference polarization used for the calculation of the experimental exchange curves is shown in Figure 1 together with its assignment. The data were processed and integrated in Felix 97.0 (Accelrys, CA).

Simulations. Numerical Liouville-space simulations of a hypothetical three-spin system were performed using the GAMMA spin-simulation environment¹⁴ extended by a block-diagonalization package to speed up the diagonalization of the Liouvillian matrixes. The input to these simulations included both the size and the orientation of the chemical-shift anisotropy tensors (CSA), the dipolar-coupling tensors, and the isotropic *J*-couplings (see Table 1). The relaxation was implemented

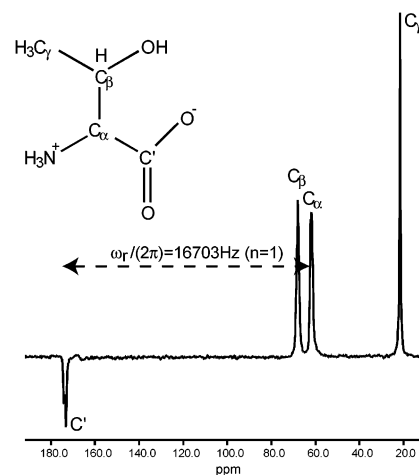


Figure 1. Carbon CP-MAS spectrum of L-threonine ($\omega_r/(2\pi) = 16703$ Hz). The spectrum shown here represents the first time point ($\tau_m = 0$ ms) in a rotational-resonance exchange curve between C' and C_α with the C' selectively inverted through the application of a DANTE pulse train. The broadening and fine structure of the C' and C_α resonances is partially due to matching of the rotational-resonance condition.

Table 1. Simulation Parameters

	spin 2	spin 1	spin 3
$\delta/(2\pi)$	1542 Hz	6083 Hz	9250 Hz
η	0.2432	0.2466	0.8919
(α, β, γ)	(160°, 137°, 82°)	(0°, 0°, 0°)	(42°, 109°, 87°)
$\Omega_{\text{iso}}/(2\pi)$	variable	0	variable
$d_{ij}/(2\pi)$	variable	2248 Hz (1.54 Å)	
(α, β, γ)	(0°, 143°, 166°)	(0°, 148°, 80°)	
J_{ij}	0 Hz	50 Hz	

as an uncorrelated random field fluctuation along the *z*-direction, with an identical rate constant for every spin ($k_z = 100$ s⁻¹). In an isolated two-spin system, this leads to a zero-quantum relaxation time of $T_{2\text{ZQ}} = 5$ ms.

To obtain the apparent distances and relaxation times for the active spin pair from the exchange curves obtained by the numerically exact three- or four-spin simulations or from experiments on fully labeled samples, the data were fitted by the model of a single homonuclear spin pair at rotational resonance⁵ which includes the following parameters: the chemical-shift anisotropy of both spins, the dipolar coupling, a phenomenological zero-quantum relaxation-rate constant, and the initial difference polarization. In addition to these standard parameters, our modified model includes the value of the final difference polarization. Under the influence of $T_{2\text{ZQ}}$ -relaxation, the difference polarization approaches this final difference polarization for $t \rightarrow \infty$.

Nonlinear least-squares fits of the data were carried out using the MINUIT routines.¹⁵ The dipolar-coupling constant, $T_{2\text{ZQ}}$, the initial difference polarization, and the final difference polarization were free parameters for the fit. The remaining parameters were kept fixed. The isotropic chemical shifts of L-threonine were measured in a spectrum recorded at an MAS frequency of 22.5 kHz as follows: C' 173.0 ppm; C_α 62.4 ppm; C_β 68.0 ppm; C_γ 21.6 ppm. The magnitude and the orientation of the chemical-shielding tensors were taken from James et al.¹⁶

Results and Discussion

Six experimental polarization-transfer curves for uniformly ¹³C and ¹⁵N labeled threonine are shown in Figure 2. The

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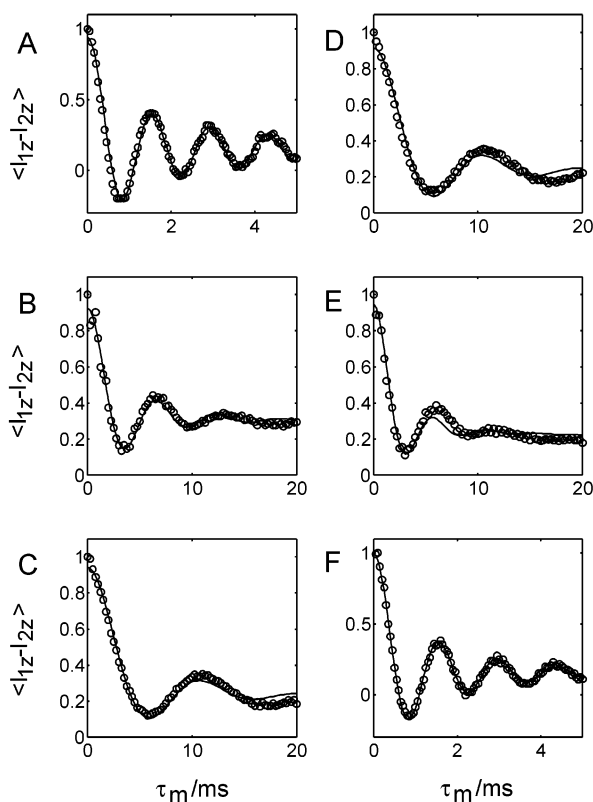


Figure 2. Polarization-exchange curves for rotational-resonance measurements in L-threonine as a function of the mixing time τ_m . The measured data for the transfer from the C' resonance to C_α (A), C_β (B), and C_γ (C), as well as between C_γ and C' (D), C_α (E), and C_β (F) are shown as open circles. The solid lines show the best fit by the modified two-spin model. The resulting fit parameters are given in Table 2.

Table 2. Parameters Obtained from Analysis of Polarization-Exchange Curves in U- ^{13}C -L-Threonin^a

sites ^e		ω_r	$\frac{d_{12}}{2\pi\text{-Hz}}$	$r_{12}/\text{\AA}^b$		T_{220}/ms^b		init. pop.	final pop.
		$2\pi\text{-Hz}$	fitted	fitted	X-ray ^c	fitted	est. ^d		
A	$C'-C_\alpha$	16703	2011	1.556(4)	1.54	10(8)	2.1	0.94(4)	0.16(1)
B	$C'-C_\beta$	15854	501	2.47(3)	2.55	4(1)	1.7	0.93(5)	0.29(2)
C	$C'-C_\gamma$	22831	300	2.93(3)	3.09	5(1)	2.9	0.94(4)	0.19(2)
D	$C_\gamma-C'$	22831	309	2.91(4)	3.09	5(1)	2.9	0.91(4)	0.19(2)
E	$C_\gamma-C_\alpha$	6128	569	2.37(4)	2.55	2(1)	2.7	0.95(5)	0.19(2)
F	$C_\gamma-C_\beta$	6977	2022	1.554(6)	1.52	4(2)	2.1	1.00(5)	0.17(1)

^a Parameters obtained after fitting the data shown in Figure 2 to theoretical polarization-exchange curves for a homonuclear two-spin system as described in the text. ^b Values in parentheses represent the error (1 standard deviation) calculated during the fitting of the experimental data. ^c Values obtained from the crystal structure of L-threonine.²¹ ^d T_{220} calculated from the single quantum line widths, assuming $1/T_{220} = 1/(2\pi(\Delta\nu_{1/2}^2 + \Delta\nu_{1/2}^2))$.^{22,23} ^e The first site listed is the one whose resonance line was inverted in the experiments.

experimental data were fitted by the model as described in the previous section. The fits yield quite good agreement with the measured data with some systematic deviations. The parameters obtained from the analysis of these six rotational-resonance experiments are given in Table 2. The experimental data were normalized by setting the magnitude of the initial experimental data point to 1. The internuclear distances obtained from these fits compare favorably with the distances obtained from crystal-structure data (see Table 2), with a maximum deviation of 0.18 Å (<10%). The good agreement between the fitted distances and the distances obtained from X-ray diffraction data suggest

that under the given experimental conditions ($B_0 = 14.09$ T) the selectivity of the rotational-resonance condition is sufficiently narrow to permit an analysis of rotational-resonance data from uniformly labeled threonine in terms of a simple modified two-spin model.

To assess if this approach works for spin systems other than threonine and in order to estimate the magnitude of the systematic errors that may be incurred in the analysis, polarization-exchange curves were simulated in Liouville-space for different three-spin systems with two active spins (spins 1 and 2) and one passive spin (spin 3). The passive spin is dipolar coupled to one of the active spins (spin 1) (see schematic in Figure 3). For the simulations used in Figure 3A the dipolar coupling to the passive spin (d_{13}) was chosen such as to represent a one-bond C–C coupling and the chemical-shift difference ($\Delta\Omega_{13}$) corresponds to the one between C' and C_α in an amino acid at 14.09 T. The detailed parameters used in these simulations are summarized in Table 1. The parameters of the two active spins, i.e., the dipolar-coupling constant d_{12} and the isotropic chemical-shift difference $\Delta\Omega_{12}$ were varied in an attempt to mimic the range of parameters found in organic molecules and peptides. The active spin pair was always kept at the $n = 1$ rotational-resonance condition by setting $\omega_r = |\Delta\Omega_{12}|$. The resulting simulated polarization-transfer curves were analyzed in the same manner as the experimental data and the parameters of the fits compared with the input values of the simulation. The values of the parameters were chosen for the simulations shown in Figure 3A such that they do not match directly to the experimentally measured threonine spin system to demonstrate the generality of the observations. However, additional simulations (given as Supporting Information) using a three-spin approximation of the threonine four-spin system show the same qualitative results and confirm that the general features of this analysis do not depend on the precise values of the spin-system parameters.

The relative deviation of the distances extracted with our two-spin model from the “true” distance, $(r_{12}^{\text{fit}} - r_{12})/r_{12}$ is plotted in Figure 3A as a function of the distance r_{12} and the isotropic chemical-shift difference, $\Delta\Omega_{12}$. As expected, accurate results (indicated in green) for the internuclear distance r_{12} were obtained if the passive spin is far from rotational resonance, while the largest deviations (indicated in red) occur when the passive spin is also close to a rotational-resonance condition, i.e., $n\omega_r \approx |\Delta\Omega_{13}|$. The width of these unfavorable recoupling conditions is of the size of the dipolar coupling to the passive spin (d_{13}) and becomes smaller for higher-order rotational-resonance conditions. In addition, the J -couplings to the passive spins can contribute to the deviations in the fitted distance. For Figure 3A, we have chosen the worst case scenario with the passive coupling being about $d_{13}/(2\pi) = 2.25$ kHz, corresponding to a one-bond carbon–carbon coupling. The errors in Figure 3A, therefore, correspond to the errors in the value of the small coupling, measured in the presence of a one-bond coupling. It is noteworthy that the relative size of the unfavorable regions with large errors will be reduced at higher B_0 -fields as the dipolar coupling is independent of the static magnetic field. In the regions with large errors, the fitted distances are shorter than the theoretical ones because the oscillation frequency increases as the passive spin (spin 3) approaches a rotational-resonance condition. For some parameter values good agreement for the

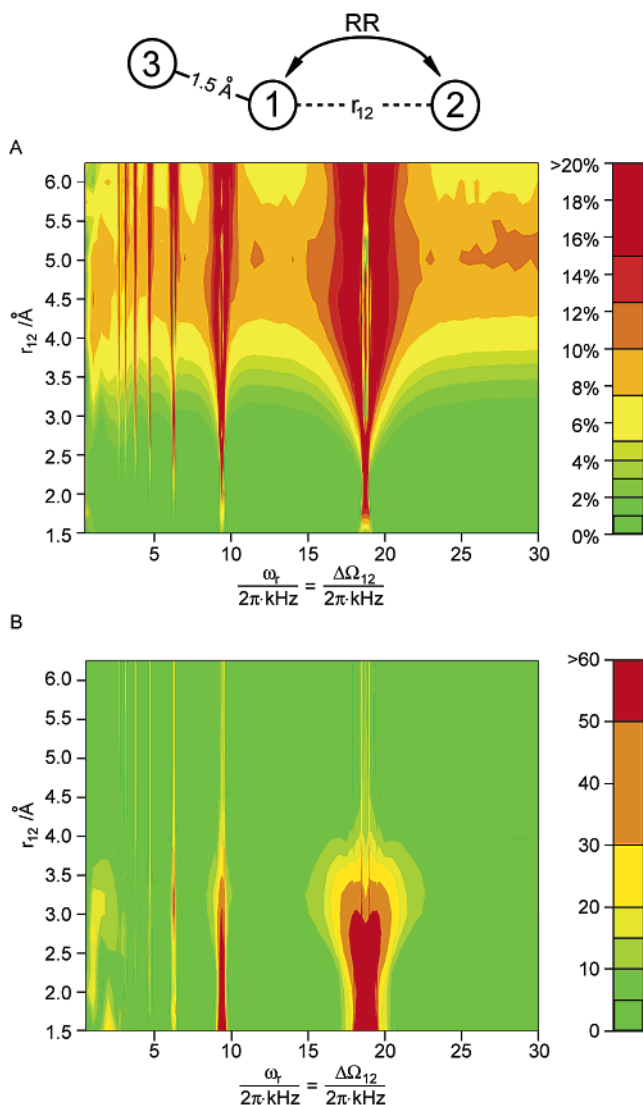


Figure 3. Contour plot of $(r_{12}^{\text{fit}} - r_{12})/r_{12}$, i.e., the relative difference of the fitted distance r_{12}^{fit} from the two-spin model and the theoretical distance r_{12} (A). Simulated polarization-transfer curves for a three-spin system were calculated and fitted by the modified two-spin model. A χ^2 contour plot of the fits is shown in (B). Green regions indicate that the exchange curve can be fitted well by a two-spin model. Both plots are shown as a function of the isotropic chemical-shift difference ($\omega_r = |\Delta\Omega_{12}| = |\Omega_1^{\text{iso}} - \Omega_2^{\text{iso}}|$) of the two active spins (spins 1 and 2) and the distance r_{12} between the two active spins. The chemical-shift difference between the active spins and, therefore, the MAS frequency was incremented in steps of 500 Hz, with additional sampling points at and near the $n = 1$ to $n = 7$ rotational-resonance conditions with the passive spin. The distance r_{12} was incremented in steps of 0.25 Å. The chemical-shift difference to the passive spin, $\Delta\Omega_{13}/(2\pi)$, was set to 18750 Hz, a typical difference between C' and C_α in amino acids at a magnetic field of 14 T. The mixing time was increased as a function of the distance according to $\tau_m = 5 \text{ ms } (r_{12}/1.5 \text{ \AA})^3$ to a maximum of 100 ms, to account for the decreasing oscillation frequency of the transfer process. The differences obtained are negative in most areas, i.e., the fit leads to an underestimation of the true distance.

distance was observed even when there was poor agreement between the fit and the simulated spectra, corresponding to a high value of χ^2 . A contour plot of χ^2 as a function of the chemical-shift difference, $\Delta\Omega_{12}$, is given Figure 3B. Poor fits with high χ^2 (areas indicated in red) are again obtained when a resonance condition of an active/passive spin pair is approached. For larger distances, the values of χ^2 decrease, because polarization-exchange curves without large oscillations can be fitted quite well even in the proximity of a rotational-resonance

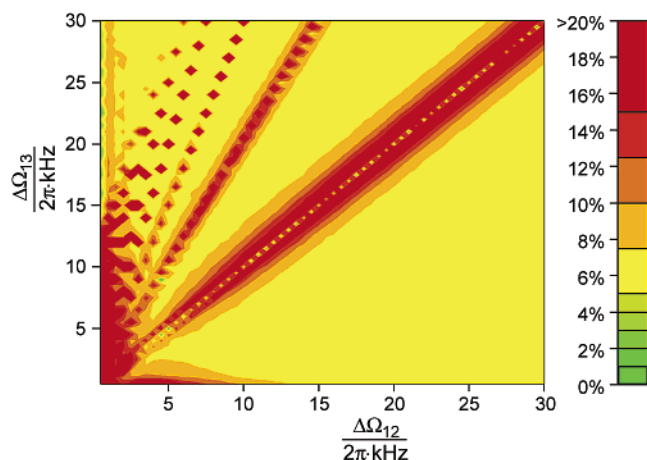


Figure 4. Contour plot of $(r_{12}^{\text{fit}} - r_{12})/r_{12}$, i.e., the relative difference of the fitted distance r_{12}^{fit} from the theoretical distance r_{12} as a function of the isotropic chemical-shift differences between the two active spins ($\Delta\Omega_{12}$) and the active/passive spin pair ($\Delta\Omega_{13}$). The chemical-differences were incremented in steps of 500 Hz. Because the stepsize is larger than the width of the higher order rotational-resonance conditions, they do not manifest themselves in all slices, leading to gaps in the red bands. Clear discrepancies (shown in red) are observed when the spinning frequency ($\omega_r = |\Delta\Omega_{12}|$) is also close to a rotational-resonance condition of the active/passive spin pair ($n\omega_r \approx |\Delta\Omega_{13}|$). The dipolar-coupling constants were set to $d_{12}/(2\pi) = 118 \text{ Hz}$ ($r_{12} = 4 \text{ \AA}$) and $d_{13}/(2\pi) = 2.248 \text{ kHz}$ ($r_{13} = 1.5 \text{ \AA}$), respectively.

condition with a passive spin. Thus, from Figure 3 we conclude that internuclear distances up to 4.5 Å can be measured, in the presence of a one-bond coupling, with an error of less than 10% for spin pairs with favorable chemical-shift values (areas indicated in green/yellow in Figure 3).

The data represented in Figure 3 correspond to a special, although practically important, situation because $\Delta\Omega_{13}$ was kept constant at a value typical for a $C'-C_\alpha$ spin pair. The influence of variations in $\Delta\Omega_{13}$ is illustrated in Figure 4 where both $\Delta\Omega_{12}$ and $\Delta\Omega_{13}$ are varied. In these simulations, both internuclear distances r_{12} and r_{13} were fixed at 4 and 1.5 Å, respectively. Again, large discrepancies between the apparent distance obtained in our two-spin analysis and the true distance occur only when the spinning frequency ($\omega_r = |\Delta\Omega_{12}|$) is close to a rotational-resonance condition involving the passive spin ($n\omega_r \approx |\Delta\Omega_{13}|$). Simulations of a selection of four-spin systems (data not shown) gave results similar to those described for a three-spin system.

The chemical-shielding tensor orientations are known to influence the rotational-resonance polarization-exchange curves obtained for an isolated two-spin system. The effects on the fitted distance are, however, usually small at the $n = 1$ rotational-resonance condition and are often neglected.⁴ Additional simulations (data not shown) indicate that the same assumption is justified in the context of the analysis of a three-spin system.

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These simulations on three- and four-spin systems support also the empirical findings in threonine and indicate that similar accuracies can be expected for other compounds. We have observed for threonine (see Table 2) that the $C'-C_\gamma$ distance of 2.9 Å corresponding to a dipolar coupling constant of 309 Hz was measured relatively accurately in the presence of two one-bond $C'-C_\alpha$ coupling of more than 2 kHz. The largest deviation between the X-ray and the NMR distances measured in L-threonine (see Table 2) is found for the spin pair $C_\alpha-C_\gamma$. The measurement of the distance between these two spins represents the determination of a relatively weak two-bond coupling ($d_{12}/(2\pi) \approx 569$ Hz) in the presence of two one-bond couplings of more than 2 kHz. In addition, one of the passive spins (C_β) is separated by only 850 Hz from a rotational-resonance condition with one of the active spins (C_γ). Despite these unfavorable conditions, the distance measured by rotational-resonance NMR and the distance measured by X-ray diffraction differ only by about 7%.

Conclusion

The experimental data and our numerical simulations show that, in many practical cases, it is possible to determine internuclear distances of up to 4.5 Å with an accuracy better than 10% using rotational-resonance measurements in uniformly labeled samples. The experiments were performed on a small molecule. They can be translated directly to biologically interesting compounds like neurotransmitters (e.g., acetylcholine where preliminary measurements have been performed) and hormones whose structure could be determined bound to proteins.¹⁷ We also foresee that the method is applicable to larger

molecules like peptides. Spectral crowding does, by itself, not compromise the power of the analysis presented here as only passive spins with a sizeable coupling need to be taken into account in the analysis.

The analysis introduced here describes a many-spin system as a pair of active spins and a set of passive spins. If the chemical-shift differences between the active and the passive spins are not closer to a rotational-resonance condition than the dipolar coupling constant between the active/passive spin pair, the presence of the passive spins only leads to a finite final polarization approached in the dynamics of the active spin pair and to a moderate systematic error in the internuclear distance. We have quantified the systematic errors for simple model systems and conclude that it is possible to identify critical cases based on the assignment of the resonances in the molecule under study.

The increase in uncertainty in the distance evaluated from the exchange curves in uniformly labeled samples using our modified two-spin model, compared to the one obtained in selectively labeled samples, is often more than offset by the increase in the number of distances which can be measured from a single, uniformly labeled sample. The method presented can be looked at as a homonuclear version of the recently introduced selective heteronuclear recoupling experiments in uniformly labeled molecules (REDOR²⁰).

Supporting Information Available: A table listing additional simulations using a three-spin approximation of the threonine four-spin system. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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