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Targeted ^{13}C – ^{13}C Distance Measurements in a Microcrystalline Protein via J-Decoupled Rotational Resonance Width Measurements

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Abstract

Rotational resonance width (R^2W) magic-angle spinning (MAS) NMR experiments are performed to measure ^{13}C – ^{13}C distances in the hydrophobic core of the microcrystalline model protein G_{B1} . Such inter-residue distances are of particular value in NMR structure determinations. The experiments are done at a Larmor frequency of 750 MHz ^1H where the contribution of ^{13}C chemical shift anisotropy (CSA) to the R^2 transfer mechanism is significant. To minimize line broadening in the 2D spectra, we employ a combination of even/odd isotopic labeling with $[1,3-^{13}\text{C}]$ glycerol, and J-decoupling in the indirect dimension. This results in high-precision distance measurements between aromatic side chains of three tyrosine residues and distant methyl groups in the hydrophobic core of the protein. Even in the absence of information on the relative orientation of the shift tensors, we obtain relatively high precision data, which can be further improved by additional constraints on the tensor orientations.

Keywords

biophysics; NMR spectroscopy; proteins; protein structures; solid-state structures

1. Introduction

The accelerating development of biomolecular magic-angle spinning (MAS) NMR spectroscopy is allowing the study of increasingly complex systems, including medically and biologically important targets such as amyloid fibrils and membrane peptides and proteins.^[1–5] In particular, MAS NMR is used to perform structural studies on systems for which it is not possible to obtain analogous information with solution NMR or diffraction techniques. MAS-NMR-based structure determination relies on a variety of methods, among which the measurement of distances via dipolar interactions is the most common. In the

recent past ^{13}C – ^{13}C distance measurements relied on methods such as proton-driven spin diffusion (PDSF) and dipolar-assisted rotational recoupling (DARR; also known as RAD mixing).^[6–8] Although such techniques allow the measurement of a large number of contacts, the absence of a rigorous theory to quantify the experimental results often limits the achievable precision of the resulting structure.^[9] Furthermore, these spin diffusion based techniques function less well at the high spinning frequencies and magnetic fields that are increasingly common in solid-state experiments.

In contrast to techniques such as DARR/RAD and PDSF, experiments based on the rotational resonance (R^2) effect have previously achieved both selectivity and high precision in distance measurements. In the conventional R^2 experiment, the dipolar interaction between two ^{13}C sites is selectively recoupled when the corresponding chemical-shift difference between the two sites of interest is matched to an integer multiple of the sample spinning frequency.^[10–12] Thus, R^2 experiments can selectively recouple distant sites even in the presence of more strongly coupled neighboring spins, circumventing one of the limitations of broadband, first-order recoupling methods, namely, dipolar truncation. When combined with high-power ^1H decoupling, selective recoupling of two ^{13}C sites is described by a relatively simple theoretical model comprised of two spins, leading to reliable simulations of the experimental results and the precise determination of distance constraints. Subsequently, R^2 -based distance measurements have been used in a variety of implementations and for various structural studies, ranging from amyloid peptides to substrate–receptor interactions.^[13–19]

Initially R^2 experiments were performed by measuring and simulating the transfer of magnetization as a function of mixing time,^[10, 12] but a recent alternative implementation examines the polarization transfer by varying the MAS frequency in a constant-time manner (i.e., keeping the mixing time constant while varying the MAS rate in a series of experiments).^[20–23] This so-called R^2 -width (R^2W) experiment allows a more reliable and straightforward interpretation of the measurements, since it efficiently deconvolves contributions from other multispin effects, including relaxation, from the selected dipolar interactions.

Although R^2 -based experiments have provided valuable information, they have typically been limited to either spin pair labeled systems or short-length peptides, with the exception of recent work by Ladizhansky and co-workers.^[24] Furthermore, R^2 -related experiments have not been performed at high magnetic field strengths ($> 500\text{ MHz } ^1\text{H}$ frequency), where the role of chemical shift anisotropy (CSA) becomes increasingly important. As noted in our original papers,^[10, 12] the R^2 mechanism is sensitive not only to the internuclear distance, but also to the magnitude of the shift tensor elements and the relative orientation of the two shielding tensors.^[15, 22] In the case of measuring intra-residue distances with R^2 experiments, the relative CSA orientations are often known a priori and can be included in simulations to improve the accuracy and precision of resulting distance measurements. However, as illustrated in Figure 1, the relative CSA orientations for inter-residue contacts in a protein are generally unknown a priori. As R^2 transfer is sensitive to these orientations (see also Figure 1 b), this uncertainty poses a potential challenge for accurately interpreting long-range, structurally valuable distance measurements.

Here we demonstrate the successful application of R^2W distance measurements to the b1 immunoglobulin-binding domain of streptococcal protein G (G_{B1}) at high magnetic field. Even in the absence of prior knowledge of the relative CSA orientations, we obtain meaningful inter-residue distance measurements within the protein hydrophobic core between aromatic and aliphatic residues (Figure 2). We explore the limitations related to increased CSA effects, and, by including estimates of the relative intermolecular CSA orientations, we are able to significantly improve the resolution and accuracy of specific distance fits. We also examine specific details of the data fitting and compare several measured distances to previously published X-ray diffraction data. Furthermore, with alternate ^{13}C labeling, through the use of $[1,3-^{13}C]$ glycerol as the ^{13}C -labeled carbon source,^[25–27] and a modified version of R^2W that yields J-decoupling in the indirect dimension, we further improve the resolution. Thus, we show that accurate R^2W distance measurements on proteins at high magnetic field strengths are feasible and should be a valuable tool for high-resolution structure determination.

Methods

Sample Preparation—The T2Q mutant of G_{B1} ^[33] (plasmid courtesy of A. M. Gronenborn, University of Pittsburgh, PA) was employed. Protein production and purification were performed according to previous studies.^[34, 35] Briefly, *E. coli* BL21 (DE3) cells were transformed and grown in M9 minimal media containing $^{15}NH_4Cl$ (1 g L^{-1}) and $[1,3-^{13}C]$ glycerol (2 g L^{-1} ; Cambridge Isotope Laboratories, Andover, MA) and $NaH^{12}CO_3$ as the sole nitrogen and carbon sources. Purified, concentrated protein was exchanged from phosphate-buffered saline solution to phosphate buffer (50 mM, pH 5.5) by diluting the protein with the desired buffer and concentrating it with Amicon Ultra-15 devices (Pierce Biotechnology, Rockford, IL) three consecutive times. Microcrystals were then prepared by concentrating the protein to about 25 mg mL^{-1} and precipitation with three additions of 2-methyl-2,4-pentanediol (MPD) and isopropyl alcohol (2/1 v/v) according to a previously published protocol.^[34] The resulting precipitate was previously reported to consist of trigonal microcrystals.^[36] These were centrifuged into a 2.5 mm MAS rotor (Bruker BioSpin Corp., Billerica, MA) and sealed with epoxy to maintain the sample hydration level throughout the experiments.

NMR Measurements—To minimize the experimental time and also to maximize the estimated number of measured distances, previous investigations^[21, 22] employed a pulse sequence in which only the $^{13}C=O$ groups are selected as the pre-existing ^{13}C polarization. As shown in Figure 3 a, this is accomplished through a selective z-filter, which employs selective Gaussian pulses that are on-resonance with the $^{13}C=O$ groups.

Evolution of only these selected sites, which allows a relatively small bandwidth in the indirect dimension, is followed by a R^2 mixing period of a fixed duration. Rather than varying this R^2 mixing time, multiple 2D spectra are obtained over a range of MAS rates. Here we employ a modification of this approach that uses Gaussian refocusing pulses during the evolution period to select aromatic sites and at the same time achieve J-decoupling in the indirect dimension,^[37–39] yielding the pulse sequence illustrated in Figure 3 b. This simple modification, involving both a selective Gaussian p pulse and nonselective p pulse,

refocuses both the chemical shift anisotropy as well as the scalar coupling between the aromatic and aliphatic carbon atoms, and thereby improves the resolution of the spectrum in the indirect dimension. Note that the chemical shift anisotropy of the sites selected by the Gaussian pulse remains unaffected during the R^2 mixing period.

NMR measurements were performed by using a custom-built console (courtesy of Dr. David Ruben) with a 17.6 T magnet (748.9 MHz ^1H frequency). A Bruker three-channel HCN MAS probe with a 2.5 mm rotor/stator (Bruker BioSpin Corp., Billerica, MA) was used at $\omega_r/2\pi = 9\text{--}13$ kHz, and the sample cooled with -10°C gas. The t_1 evolution period was rotor-synchronized at each MAS rate, with an indirect dimension dwell time equal to twice the rotor period and typically 100 t_1 increments. The selective refocusing pulse was placed near the middle of the desired frequency range and optimized to selectively refocus the desired signals. Refocusing of the $^{13}\text{C}=\text{O}$ and Tyr $\text{C}\zeta$ resonances was accomplished with a 220 ms Gaussian pulse. The recycle delay was 2.5 s, with 83 kHz two-pulse phase modulation (TPPM).^[40] ^1H decoupling was used during acquisition and evolution periods, and 100 kHz TPPM during the 30 ms R^2 mixing period.

Data Analysis and Simulation: Spectra were processed and analyzed by using the NMRPipe^[41] and Sparky^[42] software packages. The MAS rate-dependent polarization transfers were determined and analyzed with minor modifications to methods described previously.^[22] Peak integrations were performed with the Gaussian peak-fitting routine in Sparky. Cross-peaks were normalized relative to the diagonal peaks in reference 2D spectra with 0 ms mixing. The resulting MAS rate-dependent polarization profiles were analyzed with an analytical fitting program that relies on a description of the polarization transfer via multipole-multimode Floquet theory (MMFT).^[22, 43] In this description of the spin dynamics, the ZQ relaxation employed to describe other-spin effects has three distinct contributions, which were kept equal in these simulations (as this was previously found to produce reliable and accurate distances in a model peptide with known distances). The significant contribution from CSA at the magnetic field applied in our measurements makes it necessary to consider both the orientation and magnitude of the CSA of the recoupled sites.^[10] The CSA magnitudes were based on literature values (Table 1), and orientations relative to the molecular frame were based on known CSA orientations in standard compounds.^[44] For certain simulations (see below), the relative CSA orientations were determined and visualized with the SIMMOL software package,^[29] as illustrated in Figure 2 b. Given the trigonal nature of the microcrystals,^[36] we used the trigonal X-ray structure by Gallagher et al.^[32] as a reference point, except where specified otherwise. The isotopic labeling scheme applied here results in nonuniform ^{13}C labeling.^[27, 45] Based on published degrees of labeling, we assumed complete labeling of all sites, except for the Thr $\text{C}\gamma_2$ and Asp $\text{C}\beta$ sites (see Supporting Information). These sites are incompletely ^{13}C labeled, and the peak volumes were normalized accordingly for the data analysis.

2. Results

2.1. Identification and Selection of Contacts

Distances involving aromatic residues in the hydrophobic core of proteins are among the most valuable restraints for NMR structure determinations.^[48] Accordingly, a

broadband ^{13}C – ^{13}C 2D experiment with 300 ms DARR mixing period was used to identify possible long-distance contacts in the hydrophobic core of G_{B1} involving the three resolved Tyr $\text{C}\zeta$ sites (Y3, Y33, and Y45) and adjacent ^{13}C atoms. Subsections of the broadband spectrum are shown in Figure 4, along with resonance assignments based on (and consistent with) analogous data from Franks et al.^[48] Several long-distance contacts between the aromatic Tyr $\text{C}\zeta$ sites and aliphatic sites (CH_2 and CH_3 groups) are identified.

2.2. R^2W Measurements

Particularly interesting contacts were subsequently examined with R^2W measurements to obtain more accurate distance estimates. Figure 5 shows representative 2D panels from the pseudo-3D R^2W experiment (with 30 ms mixing time), showing polarization transfer from the Tyr $\text{C}\zeta$ atoms to distant methyl groups (a–c), and $\text{C}\beta$ (CH_2) sites (d–f). The latter were included to provide reference data in the form of intra-residue Tyr $\text{C}\zeta$ – $\text{C}\beta$ cross-peaks, which have a fixed distance and a restricted range of relative CSA orientations (see below). The dependence of the observed cross-peak intensity on the MAS frequency $\omega_r/2\pi$ is plotted in Figure 6 as R^2 polarization transfer profiles (triangles indicate experimental data). The experimental profiles were simulated with an MMFT-based analytical simulation program,^[22, 43] by using the estimated CSA tensor elements (Table 1). Since the relative CSA orientations are not (always) known beforehand, we explored the range of distances consistent with all possible orientations by analyzing specific CSA alignments analogous to those shown in Figure 1 and discussed previously.^[22] These reflect relative CSA alignments that result in either the shortest distance or the longest distance that optimally fits a particular polarization profile, thereby delineating the full range of distances consistent with the unknown relative CSA orientations.^[15, 22] The resulting simulated curves are included as solid and dashed lines in Figure 6. A good fit to the data can be obtained for all CSA orientations, but with different corresponding ^{13}C – ^{13}C distances (see Table S6 in the Supporting Information for further details). This results in a CSA-related (and thus field-dependent) systematic uncertainty of $\pm 0.5 \text{ \AA}$, in addition to the random error in the measurements. Table 2 lists the fit distances along with the overall uncertainty resulting from this approach, assuming that the relative CSA orientations are completely unknown. The zero-quantum relaxation rates ($1/T_{\text{ZQ}} = 10\text{--}70 \text{ Hz}$) are similar to values for comparable distances in a model peptide.^[22] These relaxation parameters, along with more detailed simulation results, are listed in Tables S6 and S7 in the Supporting Information.

2.3. Structure-Based CSA Orientations

The analysis above assumed no a priori knowledge of the relative CSA orientations. Fortunately, we can often integrate known structural information and well-established CSA orientations from model compounds into the data fitting. In particular, we examined the intra-residue distance between Tyr $\text{C}\zeta$ and $\text{C}\beta$, which is constrained by the geometry of the Tyr ring. While the relative orientation of their CSAs does vary with the side-chain torsion angle χ_2 , which in general is not known, the accessible range of relative CSA angles is much reduced due to the restricted geometry. In simulations, this residual variability yields a much smaller range of distances. The two Tyr $\text{C}\zeta$ – $\text{C}\beta$ distances that were measured show very similar R^2W experimental profiles for both (Figure 6 a and e), consistent with expected identical distances. Figure 7 shows contour plots of the root mean square deviation (RMSD)

between the experimental and simulated profiles for the Tyr-3 C ζ –C β data, which reflect a range of ^{13}C – ^{13}C distances and ZQ relaxation times (T_{ZQ}). Let us first examine the hypothetical situation in which we would be unsure of the relative CSA orientations. Figure 7 a and b show contour plots for two distinct CSA orientations that are analogous to the CSA orientations illustrated in Figure 1. For each CSA alignment we obtain a different distance that is equally consistent with the experimental data. A χ^2 error analysis was used to estimate the standard deviation due to the random error in each of these fits : the contour levels marked 0.05 approximately correspond to the standard deviation in the distance and relaxation. Between panels a and b of Figure 7, with optimal fits of 5.5 ± 0.4 Å and 4.4 ± 0.4 Å, the results imply an overall range of 5.0 ± 1.0 Å that would be consistent with the experimental results. The last-named value is significantly larger than the expected distance of about 4.25 Å. However, for this case we can define the relative CSA alignments on the basis of the geometric restrictions of the aromatic ring and the side-chain torsion angles, as found in a reference X-ray crystal structure^[32] (Figure 2 b). Panel c of Figure 7 shows the RMSD contour plot for a simulation that uses those CSA orientations. The measured distance is now 4.5 ± 0.4 Å, within 0.3 Å of the actual distance. Clearly, the inclusion of CSA orientation improves both the accuracy and the precision of the measurement. The same is true for the Tyr-45 C ζ –C β measurement (data not shown).

Unfortunately, for distances of structural interest between residues that are distant in the primary sequence, the relative CSA orientations are in general unknown, and determined by the details of protein folding. For the case considered here, the model protein G_{B1}, we can combine the G_{B1} crystal structure,^[32] typical CSA orientations,^[44] and CSA principal values (Table 1) to refine the distance measurements with the correct CSA orientations (Figure 2). The results of such an analysis are tabulated in the final column of Table 2, with additional details in Table S7 of the Supporting Information. Upon elimination of the uncertainty in the CSA orientations, the precision of the measurements improves significantly. However, in contrast to the results with the “reference” Tyr C ζ –C β measurements (see above), we do not appear to observe as large an improvement in the apparent accuracy of the measurements: compared to the simulations without a predefined relative CSA orientation, the agreement with the X-ray structure improves only marginally. Possible causes and implications of this observation are examined in the discussion below.

3. Discussion

The accuracy of R²W methods is well documented,^[20–22] but such measurements have typically been performed on smaller test systems and at lower magnetic fields where the inclusion of the CSA orientations is less essential. It has remained unclear to what extent the method would be practical at high fields and on larger systems. To address these issues we measured several ^{13}C – ^{13}C distances in the model protein G_{B1} with R²W measurements at 750 MHz ^1H frequency. Under these experimental circumstances it was necessary to explicitly include contributions from the CSAs to optimally refine the distance measurements. We have shown above that even in the absence of information on the CSA orientations (assuming an unknown structure for our model protein) we can obtain a reasonable range of distances, with a typical uncertainty of less than 1 Å. More importantly, the resulting distances deviated at most by 0.75 Å from the known crystal structure. The one

exception to this is the Y33–T16 contact, which deviates more. We note here that these residues do not form part of the main hydrophobic core and discuss this distance in greater detail later in the discussion. Our results suggest that our R²W analysis (which does explicitly treat the CSA orientations) provides useful structural information even in the absence of prior knowledge of the secondary and tertiary structure.

Our measurements of the Tyr C ζ –C β “reference distances” show that the precision and accuracy of the measurements can be improved substantially by limiting the range of CSA orientations. For those particular intra-residue distances, the accessible orientations are limited due to the geometry of the rigid Tyr side chain, and inclusion of this information improves the apparent accuracy by as much as 0.45 Å. The ¹³C–¹³C distances measured for these well-defined sites are within 0.3 Å of the corresponding X-ray crystallographic distances. Our analogous examination of inter-residue contacts employed CSA orientations based on a trigonal X-ray crystallographic structure,^[32] in an attempt to also delineate those relative CSA alignments. A variety of other known X-ray structures of G_{B1} yielded similar CSA orientations and thus similar distance measurements (data not shown). This observation is consistent with previous reports that indicate that the global fold and in particular the hydrophobic core of G_{B1} are consistent among crystal forms.^[32] Note that in the absence of a known structure, one might instead employ a coarse structural model that constitutes an intermediate step in an iterative structure determination. The inclusion of X-ray-structure-based CSA orientations improved the precision of the measurement (reduced the resulting standard deviation). It also resulted in a small improvement in accuracy for all the measurement distances, in terms of correspondence with the X-ray structure. The residual deviation between the X-ray and solid-state (SS) NMR distances is typically within about 0.5 Å, which might well be within the standard deviation of the X-ray data. The correspondence between the SSNMR and X-ray data is plotted in Figure 8, which reveals a seemingly consistent overestimation of the distance.

As indicated above, one notable exception is Y33C ζ –T16C γ 2, both in terms of the extent of deviation from the X-ray distance as well as this deviation reflecting a shorter rather than longer distance. We believe this indicates a deviation of the structure of our microcrystals from the reference (trigonal) single-crystal X-ray structure used in our analysis. GB1 is known to display polymorphic crystallization behavior,^[36] and when one compares the distances examined here between different known X-ray studies the largest variations (by far) are found for the Y33–T16 contact (Table S2 in the Supporting Information). This is not entirely surprising, since these residues are not part of the protein’s hydrophobic core and are both more surface-exposed and mobile. Interestingly, even though these GB1 microcrystals are reported to be trigonal,^[36] we find that the Y33–T16 distance measured in our R²W analysis matches well to an otherwise-similar recent orthorhombic structure (see Figure 8 c).

Let us now focus on the other hydrophobic core distances and their seemingly systematic overestimation. As discussed above, there may be some uncertainty about how representative the X-ray data are for our sample.^[36] However, these are sites that compose the hydrophobic core, and previous studies have indicated that the core structure of G_{B1} is not very sensitive to differences in crystallization.^[32] This is indeed borne out by our

examinations of several crystal structures. Second, it could reflect a larger deviation of the extent of labeling from what we assume, based on previously published work. If the aliphatic “target” sites are labeled significantly less than 100 %, then a straightforward analysis would be expected to overestimate the ^{13}C – ^{13}C distances. In the case of the distance measurement involving T16Cg2 and D47Cb, which appear to be about 30 and about 80% labeled, respectively,^[27, 45] we took the reduced labeling into account by scaling the data prior to analysis. This yielded significantly shorter distances, especially in the former case, which was found to have a 1.5 Å shorter distance than if one assumed 100% labeling. Finally, a systematic underestimation like we observe would also be consistent with the presence of dynamics that partially average the dipolar interaction. These dynamics could include both backbone fluctuations and side-chain motion that average the internuclear distances. Note that aromatic ring oscillations might do this, but that 1808 flips would not. Previous MAS NMR data suggested that among the three Tyr residues of G_{B1}, Tyr33 is relatively mobile.^[34] This is also reflected in increased crystallographic temperature factors in at least some of the known crystal structures (see Supporting Information). Such dynamics would result in a partial averaging of the ^{13}C – ^{13}C dipolar interaction, yielding weaker crosspeak intensities and biasing the results toward overestimating the distances relative to the crystal structure. However, the chosen C ζ sites should be less sensitive to ring flips than other sites along the aromatic ring. Note that inter-residue distances should be more sensitive to the motion than the intra-residue Tyr C ζ –C β pairs. This appears consistent with our observations that the Tyr C ζ –C β distances show the best correspondence with the X-ray data. Further study of different samples with varying degrees of motional averaging by means of temperature-dependent spectra may be beneficial to examine these questions.

It is also possible that certain limitations inherent to the assumptions and description of the data fitting limit the accuracy of our approach, even though such effects appeared limited in previous applications to more rigid model peptides.^[22] The fitting routine assumes a two-spin model and ignores multispin effects. Our use of even/odd isotopic labeling greatly reduces the number of adjacent labeled sites and can thus limit intramolecular multispin interactions. Multispin interactions from intermolecular contacts could contribute to observed cross-peak intensities and were considered in previous R²W studies of model peptides. However, in this model protein system, we can exclude multispin effects due to overlapping intramolecular as well as intermolecular interactions on the basis of our examination of the known crystal structures (see Supporting Information). Another potential concern involves modeling of the relaxation dynamics, which worked well for a model peptide at lower magnetic fields but may not be applicable here. Our good results for the intra-residue reference distances suggest that the current approach works reasonably well, also in this hydrated model protein. Lastly, the assumed CSA tensors are themselves approximations (based on model compounds). Direct experimental measurements of the CSA magnitudes and relative orientations would therefore be helpful for complete evaluation of these uncertainties.

We should also examine additional potential concerns that need to be addressed in future applications of this technique. If we use standard experimental protocols, the recoupling of all possible carbonyl/aromatic sites to all aliphatic site combinations would require a very

large number of time-consuming 2D experiments. This particular issue led Ladizhansky and coworkers to propose a version of the experiment with reduced selectivity.^[24] Here our intent was to illustrate an alternative approach in which the selective R²W experiment is used to refine data from broadband recoupling experiments, which generally exhibit lower precision.^[48] For optimum efficiency it can actually be useful to initially identify sites in sufficiently close proximity in those broadband recoupling experiments, by using data such as those shown in Figure 4.

For this study we specifically examined the three Tyr residues in G_{B1}, since contacts between aromatic and methylated side chains are essential contacts characteristically found in the hydrophobic core of G_{B1}. However, the MAS NMR signals of aromatic ¹³C sites are often characterized by broad line shapes and low intensities, due to strong ¹³C–¹³C interactions within uniformly labeled aromatic rings,^[10, 49] and in many cases ring motions (e.g., ring flips) interfere with heteronuclear ¹H–¹³C decoupling.^[50, 51] One approach to reducing the ¹³C–¹³C coupling is to use a nonuniform labeling scheme, for instance by using 1,3-¹³C-labeled glycerol during protein expression.^[25, 45] This results in much improved aromatic line shapes, but leaves aromatic side chains with some directly bonded ¹³C atoms. In particular, the targeted Tyr C ζ sites tend to have partially labeled adjacent C ϵ sites in the same molecule (see Table S5 in the Supporting Information).^[26, 27] The use of J-decoupling in the indirect dimension^[37] can partially remove these residual J-couplings, which improves the 2D line shape of the cross peaks and facilitates more reliable peak fitting and integration. This J-decoupling implementation is designed to also perform the spin selection that was done previously by means of a selective Gaussian pulse and z-filter^[21, 22] or selective double cross-polarization (CP) transfers.^[24] We used a Gaussian pulse for J-decoupling, but improved performance might be obtained by the use of alternate selective pulse designs.^[38, 39]

While many of the ¹³C sites of interest have previously been reported to be close to 100% labeled in proteins obtained with [1,3-¹³C]glycerol as ¹³C source, this is not true for all cases. Here we relied on published labeling ratios (see Supporting Information) rather than quantifying the labeling on this particular sample. This appears justified by the fact that many of the sites involved in the actual hydrophobic core (i.e., in contact with Y3 and Y45) were reported to be either fully (Ala C β and Tyr C β) or highly (Asp C β) labeled. For sites of much reduced labeling, such as Thr C γ , experimental quantification becomes more important. We note that this site is not part of the hydrophobic core and is merely included here for completeness. Clearly, for more extensive structural measurements that include a variety of residues, reliable quantification of residue-specific and site-specific degrees of labeling will be essential to attain optimal precision and accuracy. Properly taking the presence of differently labeled isotopomers into account may bring another level of complexity to the analysis of broadband experiments (perhaps more so than for more selective variants like R²W).

4. Conclusions

We have successfully performed and analyzed R²W distance measurements at high magnetic field. The analysis highlights the expected dependence on the magnitude and

orientations of CSA tensors for such high-field measurements, and shows how inclusion of specific CSA information significantly reduces these uncertainties. Thus, high-field studies using other frequency selective experiments that are less sensitive to the CSA than the $n = 2$ R^2 matching performed here (e.g. $n = 1$ R^2W , but also other approaches) could prove beneficial to address this effect. Our results also suggest that the moderate dynamics present in this particular hydrated model protein, which should exceed those present in previously used model peptides,^[22] might affect the observed distances but can be accommodated without significant loss of accuracy. We show how a selective approach to ^{13}C – ^{13}C distance measurements can be complementary to more broadband recoupling methods and can be used for the targeted measurement of distances of particular interest, such as those that define the protein hydrophobic core. These kinds of measurements should aid in the refinement of protein structures, and can also be applied to enzyme binding sites and their interactions with substrates, ligands, or retinals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Relative CSA orientations and rotational resonance width polarization transfer. a) Relative orientations of the ^{13}C chemical shielding tensors for an inter-residue pair of ^{13}C atoms. The relative tensor alignment depends on the spatial distribution and orientation of the residues, even if the individual CSA tensors remain the same. This is illustrated here for a Tyr $\text{C}\zeta$ and two hypothetical Ala $\text{C}\beta$ sites, both at a distance of 4 Å. For site (1) the relative CSA tensor orientations along the internuclear vector are $\Omega_{\text{PM,CZ}} = (0,0,0)$; $\Omega_{\text{PM,CB}} = (0,0,0)$, whereas for site (2) the respective CSA orientations are $(0,90^\circ,0)$; $(0,0,0)$. b) The theoretical R^2W profile for either case, indicating the fraction of polarization transferred from the aromatic ^{13}C to the methyl site as a function of the MAS rate. A mixing time of 30 ms and a typical relaxation parameter of $1/T_{\text{ZQ}} = 50$ Hz were assumed. Other CSA alignments result in transfer profiles intermediate between these two cases.

Figure 2.

a) Illustration of the recoupled sites in the hydrophobic core of protein GB_1 (spheres) and the inter-residue $^{13}\text{C}_\zeta$ - ^{13}C distances (solid lines) that were measured. Note that the Y33-T16 contact (in the back) is not part of the main hydrophobic core pocket. b) The estimated CSA tensor magnitudes and orientations for Tyr-3 C_ζ and its aliphatic contacts (see also Table 1). The lengths of the ellipsoid axes reflect the shielding tensor components (longest axis=most shielded component, δ_{33}). The figures were prepared with UCSF Chimera,^[28] SIMMOL,^[29] geomview,^[30] and rendering program Pixie.^[31] Both panels are based on the X-ray structure from Gallagher et al. (PDB 1PGB).^[32]

Figure 3. Illustration of a) selectively filtered^[21, 22] and b) J-decoupled pulse programs for R^2 -width ^{13}C - ^{13}C distance measurements. Solid black and gray bars indicate 90 and 180° pulses, respectively. Phases are indicated as: $\phi_1 = (x)_8(-x)_8$, $\phi_2 = xy$, $\phi_3 = x$, $\phi_4 = y$, $\phi_5 = (x)_2(y)_2(-x)_2(-y)_2$, $\phi_{\text{acq}} = x(-x)y(-y)(-x)x(-y)y(-x)x(-y)yx(-x)y(-y)$. Additional experimental details are provided in the Methods Section.

Figure 4.

Subsections from a 2D ^{13}C - ^{13}C spectrum with long (300 ms) DARR mixing, highlighting the contacts between three Tyr C_ζ and various aliphatic sites in G_{B1} . a)–d) are from the same experiment, but (c) and (d) have been transposed for convenience of display. Note the asymmetry between the top and bottom halves of the figure, which complicates direct interpretation of relative distances in this particular experiment.

Figure 5.

2D ^{13}C - ^{13}C panels showing MAS-dependent, R^2 -induced polarization transfer from three resolved Tyr C_ζ sites to various aliphatic sites in GB_1 . Experiments were done at 750 MHz (^1H) and MAS rates of a) 13.094, b) 13.177, c) 13.29, d) 10.736, e) 10.803, and f) 10.845 kHz.

Figure 6.

R^2 width profiles of the polarization transfer for different intra- and inter-residue contacts from $Y_3C\zeta$ to a) $Y_3C\beta$, b) $A_{23}C\beta$, c) $A_{26}C\beta$; d) from $Y_{33}C\zeta$ to $T_{16}C\gamma_2$; and from $Y_{45}C\zeta$ to e) $Y_{45}C\beta$, f) $D_{47}C\beta$, g) $A_{23}C\beta$, and h) $A_{26}C\beta$. Normalized intensities of the experimental cross-peaks are indicated as triangles, which include zero intensities from data sets that lacked cross-peaks that were distinguishable from the noise. Solid and dashed lines indicate two representative best-fit solutions that reflect distinct CSA alignments and correspond to two different ^{13}C - ^{13}C distances. Note that these distinct data fits explain the experimental data equally well, and thus a significant systematic uncertainty results (see also Table 2 and the Supporting Information).

Figure 7.

Contour plots of the RMSD error in the fits of the R^2W data for the Tyr-3 C_ζ - C_β distances. The plots correspond to a) relative CSA orientations of $\Omega_{PM,CZ} = (0,0,0)$ and $\Omega_{PM,CB} = (0,0,0)$, b) relative CSA orientations of $\Omega_{PM,CZ} = (0,90,0)$ and $\Omega_{PM,CB} = (0,0,0)$, and c) CSA orientations as defined by the Tyr ring geometry and the X-ray structure (see also the Supporting Information). The expected ^{13}C - ^{13}C distance is indicated by a vertical dashed line in each panel.

Figure 8.

Comparison of MAS NMR distance measurements with the equivalent distances in X-ray crystallographic structures. a) Results allowing for the full range of CSA orientations, and b) data obtained with estimated CSA orientations based on the 2 Å-resolution X-ray structure by Gallagher et al.^[32] The apparent outlier (in gray) represents the Y33-T16 contact, which lies outside the hydrophobic core. c) The same analysis for a more recent 1.14 Å-resolution orthorhombic X-ray structure,^[9] for which there is a better match of the Y33-T16 distance. Note that in all cases there is also an uncertainty in the X-ray distances, which is not indicated in the figure.

Table 1

Principal values δ_{ii} of the chemical shift tensors for the aromatic Tyr ^{13}C , Ala and Asp $^{13}\text{C}\beta$, and Thr $\text{C}\gamma$ atoms used in the simulations (in ppm relative to TMS). Where multiple references are listed, the tensor elements were averaged. δ and η are obtained by using the formulas $\delta = \delta_{11} - \delta_{\text{iso}}$ and $\eta = \frac{\delta_{22} - \delta_{33}}{\delta_{11} - \delta_{\text{iso}}}$ when $|\delta_{11} - \delta_{\text{iso}}| > |\delta_{33} - \delta_{\text{iso}}|$, or $\delta = \delta_{33} - \delta_{\text{iso}}$ and $\eta = \frac{\delta_{22} - \delta_{11}}{\delta_{33} - \delta_{\text{iso}}}$ when $|\delta_{11} - \delta_{\text{iso}}| < |\delta_{33} - \delta_{\text{iso}}|$ – where $\delta_{\text{iso}} = (\delta_{11} + \delta_{22} + \delta_{33})/3$.

Site	Isotropic	δ	η	δ_{11}	δ_{22}	δ_{33}	Refs.
Tyr C ζ	156	−91.4	0.97	246	157	65	[46,47]
Tyr C β	37	13.3	0.86	50	36	25	[46,47]
Ala CB	20	12.0	1.00	31	19	7	[47]
Asp C β	37.7	−18.0	0.94	55	38	20	[47]
Thr C γ	20	−16.0	0.31	31	26	4	[47]

Table 2

Overview of measured ^{13}C – ^{13}C distances [Å]. For comparison, a column is included with the distances based on X-ray crystallography on a trigonal single crystal (PDB: 1PGB). The next column shows the measured SSNMR distances assuming a lack of knowledge of the relative CSA orientations (i.e., when the protein tertiary structure is unknown). The final column shows results obtained by using the relative CSA tensor orientations consistent with the 1PGB X-ray structure^[32] (see Table S7 in the Supporting Information). Note the improvement in the precision that reflects elimination of the systematic error due to unknown relative CSA orientations.

^{13}C – ^{13}C contact	X-ray distance [Å]	SSNMR R ² W distances [Å]	
		“Unknown” CSA orientations $\mathcal{Q}_{\text{PM}}$	Estimated CSA orientations $\mathcal{Q}_{\text{PM}}$
Y3C ζ –Y3C β	4.2	4.95±0.95	4.5±0.4
Y3C ζ –A23C β	4.4	4.55±0.95	4.8±0.4
Y3C ζ –A26C β	4.7	5.45±1.0	5.4±0.5
Y33C ζ –T16C γ ^[a]	4.7	3.0±0.9	3.3±0.4
Y45C ζ –A23C β	3.9	4.0±0.9	4.4±0.4
Y45C ζ –A26C β ^[b]	7.2	6.45±1.65	6.8±1.0
Y45C ζ –Y45C β	4.3	5.05±0.95	4.6±0.4
Y45C ζ –D47C β ^[a]	4.1	4.4±0.9	4.6±0.4

^[a] The normalized peak volumes for these contacts were scaled to account for the reduced isotopic labeling of the aliphatic site (ca. 30% for Thr C γ and ca. 80% for Asp C β ; see also Supporting Information),

^[b] This cross-peak was very weak (see also Figure 6 h), and thus a substantially larger uncertainty resulted.