Protein NMR Spectroscopy

DOI: 10.1002/anie.201103944

Solid-State NMR Measurements of Asymmetric Dipolar Couplings Provide Insight into Protein Side-Chain Motion**

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Understanding conformational flexibility is of critical importance for understanding protein function, folding, and interactions with other proteins and ligands. NMR spectroscopy is an important tool for such investigations in solution[1] and increasingly also in the solid state[2] since it allows site-resolved studies of dynamic processes. An experimental characterization of all motional modes of a protein is a great challenge and simplified models are necessary. In NMR studies of dynamics, motional amplitudes are generally expressed in terms of a single order parameter,[3] discarding the details of the motion, such as the motional asymmetry. Herein, we show a significant extension of this description, by detecting asymmetric motion of side chains in a protein in the solid state.

Dipolar couplings are particularly powerful probes of local molecular dynamics in the solid state. In the absence of motion, the tensor describing the dipolar interaction between two nuclei is a traceless axially symmetric second-rank tensor. It can be characterized by a single parameter, namely its anisotropy $\delta_{\mathrm{D,rigid}}$ which depends only on the internuclear distance and isotope type of the nuclei involved (for the definition see the Supporting Information). In the presence of "fast" motional processes, in other words, processes with a correlation time shorter than approximately $1/\delta_{\mathrm{D,rigid}}$, (typically 10–100 μs), the dipolar coupling tensor becomes partially averaged. In the case of a motional process with threefold (C_3) or higher symmetry, for example, an isotropic motion within a cone, the averaged tensor remains axially symmetric and is fully characterized by the effective anisotropy δ_{D} which has a reduced value compared to $\delta_{\mathrm{D,rigid}}$. In this case, the motional amplitude can be expressed by a single order parameter[4] $S = \delta_{\mathrm{D}}/\delta_{\mathrm{D,rigid}}$. However, in the case of a general fast motion, the characterization solely by S is incomplete because the averaged dipolar tensor is no longer axially symmetric[5] and one additional tensor parameter, the asymmetry η_{D}, is needed for a complete description (for the definition, see the Supporting Information). The asymmetry η_{D} varies between zero (symmetric tensor) and one.[5a]

In solution-state NMR spectroscopy, dipolar couplings can be measured as residual couplings (RDCs) in anisotropic media. The evaluation of motional amplitudes from RDCs is challenging because RDCs also depend on the (a priori unknown) degree of molecular alignment and the orientation of a given vector relative to the alignment frame and usually data from different alignment media must be combined.[6] The situation is much simplified in solid-state magic-angle-spinning (MAS) NMR, where overall molecular tumbling is absent, allowing the direct measurement of dipolar couplings that depend only on the interatomic distance and dynamics. For the case of one-bond dipolar couplings (C–H, N–H, or C–N) the rigid-limit dipolar coupling tensor is known from the bond lengths. Thus, measurements of the dipolar coupling tensor provide direct access to the amplitude and axial symmetry of the motion sampled by the bond vector. However, owing to the limited precision and accuracy of the currently available experimental data, dynamically averaged dipolar coupling tensors have, so far, always been analyzed in terms of a single order parameter S. Herein, we demonstrate the first direct measurement of asymmetric dipolar coupling tensors in MAS NMR, providing a more detailed picture of motional amplitudes. We exemplify the measurement of asymmetric dipolar couplings by studying side-chain motions in the protein ubiquitin, using a combination of appropriate sample labeling with sensitive and precise NMR measurement techniques. We find that the asymmetry η_{D} of the dipolar coupling tensor of several methyl C–H moieties deviates indeed significantly from zero and provides useful information about the details of the motional processes.

In order to obtain the necessary accuracy and precision in the measurement of ^{1}H–^{13}C dipolar coupling tensors, we extend a recently developed experimental approach with greatly improved accuracy.[7] In brief, our approach consists of 1) the selective introduction of isolated ^{1}H–^{13}C spin pairs in an otherwise perdeuterated protein, using specifically protonated precursors, and 2) a REDOR recoupling technique in combination with sensitive proton detection. REDOR has a built-in normalization,[8] such that the recoupling data are expressed in a manner that is independent of the peak intensity and the coherence loss during the recoupling period and can be fitted using only δ_{D} (or S) and η_{D} as free parameters.[7]

We prepared two samples of perdeuterated ubiquitin carrying ^{1}H–^{13}C spin pairs on a single methyl group of either Ile (δ1) or Val (γ1 or γ2) and Leu (δ1 or δ2) residues and we

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[**] This work was supported financially by the Swiss National Science Foundation and by ETH Zürich. We thank Isabel Ayala and Carlos Amero for assistance in protein labeling and the Institut de Biologie Structurale in Grenoble for access to the PSB/IBS isotope labeling platform.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201103944.

studied side-chain dynamics as probed by the methyl C–H dipolar coupling tensor. The labeling follows established protocols[9] (see the Supporting Information for details). The low ^{1}H density in such samples largely eliminates ^{1}H–^{1}H couplings and couplings from the ^{13}C spins to remote ^{1}H spins, thus excluding one source of potential systematic errors in dipolar coupling measurements. Furthermore, the low proton density makes it possible to acquire high-resolution proton-detected correlation spectra with high sensitivity.[10] In combination with fast magic-angle-spinning, coherences in such samples are long-lived[11] leading to a further increase in sensitivity and thus precise dipolar coupling measurements. The improved measurement precision, the suppression of systematic errors and the inherent normalization of REDOR recoupling curves are crucial in the detection of dipolar tensor asymmetry, which is manifest as small changes in REDOR curves, as shown in Figure 1.

Figure 2a shows experimental REDOR curves for a number of representative methyl groups in ubiquitin, measured using the pulse sequence of Figure S1 (see the Supporting Information). The full set of experimental recoupling curves is shown in Figure S2, and representative two-dimensional spectra are shown in Figure S3. The results of a two-parameter fit (anisotropy δ_D and asymmetry η_D, red curves in Figure 2a) are shown in Figure 3 and listed in Table S1. Reduced-chi-square (χ^2_{red}) surfaces are shown in Figure 2b.

In comparing the different methyl groups in ubiquitin, a large variation in the fitted anisotropies δ_D is observed, ranging from 4.9 to 11.8 kHz, indicating that site-to-site

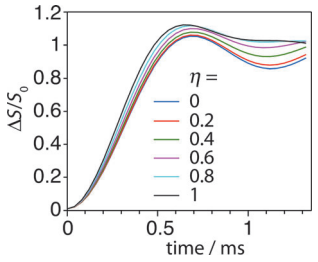

Figure 1. Simulated REDOR recoupling curves, assuming $\delta_D = 7$ kHz and different values of η as indicated. Simulation methods are described in the Supporting Information.

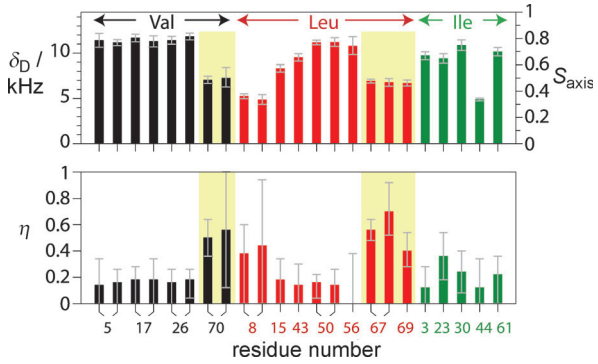

Figure 3. Dipolar tensor parameters (top: anisotropy, bottom: asymmetry) for methyl groups in ubiquitin. Two data points per residue denote methyl groups at positions γ1/γ2 (Val) or δ1/δ2 (Leu). Numerical values are reported in Table S1 in the Supporting Information. Side chains with large tensor asymmetries are highlighted.

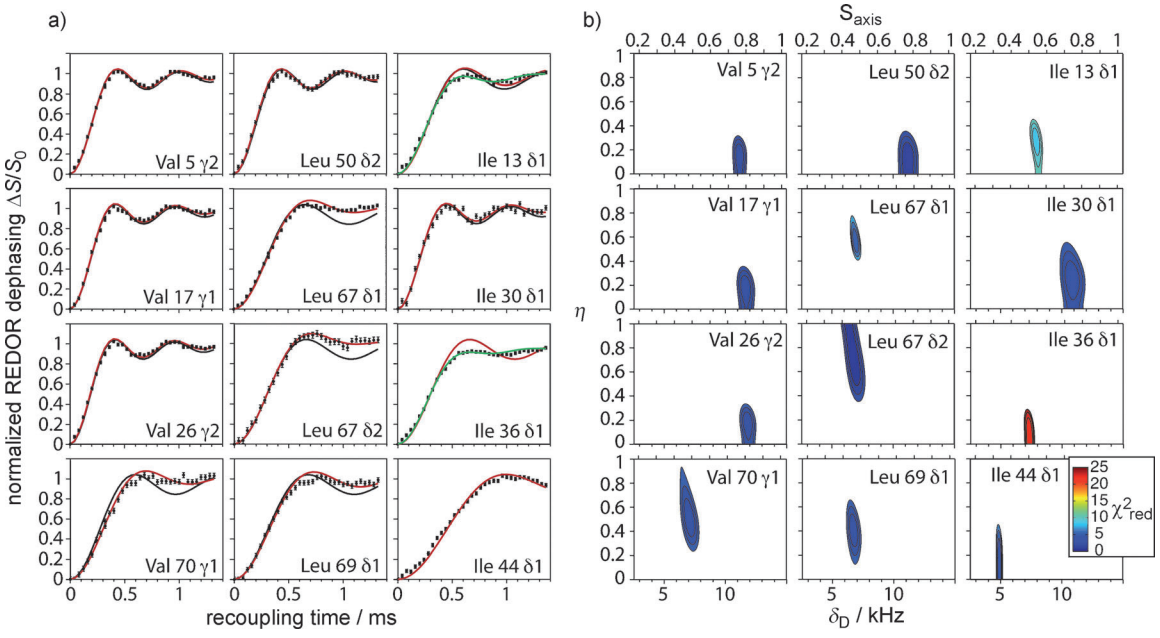

Figure 2. a) REDOR recoupling curves for methyl ^{1}H–^{13}C sites in crystalline ubiquitin. Each data point was obtained from 2D recoupling and reference spectra (recording time 95 min per spectrum). Experimental points are shown along with error bars, based on twice the standard deviation of the spectral noise. Black curves show fits assuming an axially symmetric dipolar coupling tensor, red curves use asymmetric tensors, and green curves (only Ile13/36) assume a superposition of two general tensors. b) Plots of the reduced chi-square (χ^2_{red}) values for the two-parameter fits of the (red) REDOR curves of Figure 2a. Shown are three contours at the values of χ^2_{red} corresponding to the minimum of χ^2_{red} +1, +2 and +3. Thus, the innermost contour denotes the confidence interval. The full set of χ^2_{red} plots is shown in Figure S4. S_{axis} is defined as $\delta_D/\delta_{D,rigid\ axis} = \delta_D/14.53$ kHz.

variations of side-chain motional amplitudes are large. We also detect significant variation of the asymmetries η_D between different side chains (between 0 and 0.58). Based on the fit of the anisotropy and the asymmetry to the REDOR data, the methyl groups can be classified into three groups: 1) methyl groups that have a low χ^2_{red} value and an asymmetry that is not significantly different from 0, 2) methyl groups that have a low χ^2 value but a value of the asymmetry parameter that is significantly different from 0, and 3) methyl groups that cannot be properly fitted (with correspondingly large χ^2 value) by a single asymmetric dipolar tensor. The majority of the methyl groups in ubiquitin (22 out of 29), such as those in Val5, Val17, Val26, Leu50 (Figure 2) fall into category 1 with an almost symmetric dipolar coupling tensor, that is, an asymmetry below about 0.2. These methyl groups can be described by the conventional symmetric-tensor assumption. A significant asymmetry with values of $\eta \geq 0.4$ (category 2) is observed for the methyl groups of Val70, Leu67, and Leu69 (5 out of 29). For two methyl groups the asymmetric dipolar coupling model does not result in satisfactory fits (category 3, δ1 of Ile13 and Ile36), pointing to slow motional processes. Thus, for several sites the traditional symmetric-tensor model clearly does not apply, and the reported asymmetric tensors provide further insight into the motion of these side chains.

The observed dipolar tensor parameters δ_D and η_D for a methyl group are the result of several averaging processes. Invariably, at room temperature, the methyl groups are in fast rotation around the local threefold axis with correlation times typically in the picosecond range. This rotation leads to an averaged axially symmetric tensor with an asymmetry $\delta_{D,rigid\,axis} = \delta_{D,rigid}/3 \approx 14.53$ kHz (based on the canonical tetrahedral angle $\theta_{HCC} = 109.47°$ and a C–H bond length of 1.115 Å). This tensor is further affected by motional processes involving the direction of the threefold methyl axis, which result from librational motions, and, more importantly in terms of amplitude, from jumps between discrete rotamer states. Under such fast (< 10–100 μs) rotamer jumps, the observed tensor is the average of the involved orientations, and will, thus, be generally asymmetric, characterized by its asymmetry η and the anisotropy δ_D or the axis order parameter $S_{axis} = \delta_D/\delta_{D,rigid\,axis}$.

Rotamer jumps should, thus, have a measurable impact on tensor anisotropies and asymmetries, and information about rotamer equilibria should be contained in the dipolar tensors. Furthermore, rotamer jumps equally affect the methyl groups γ1 and γ2 attached to a given Val, and the δ1/δ2 methyl groups attached to a given Leu side chain, and the tensors should be identical, provided that librational motions are negligible or similar to both sites. Indeed, we find that the tensor parameters for methyl groups attached to the same side chain always agree within error bars. The significant asymmetry ($\eta \geq 0.4$) observed for some of the Val and Leu methyl groups can be rationalized when one looks into the details of possible rotamer transitions for different side chains.

The simplest situation arises for valine side chains where a single dihedral angle χ1 is relevant. Rotations by 120° around χ1 interconvert *trans*,*gauche*(+), and *gauche*(−) rotamer states[12] (see Figure 4a). Assuming that the methyl axis undergoes only transitions between these three rotameric states and neglecting any other motional processes, it is straightforward to calculate the resulting $^1H–^{13}C$ tensor parameters as a function of the populations of the three states (see Figure 4b,c). The asymmetry in this model is zero for the case of only a single rotamer state being populated (which in this model means no mobility), or if all three rotamer states are equally populated. These two cases are readily distinguished by the tensor anisotropy, which is three times larger for the case of a single rotamer state (see also Figures S5–S8 in the Supporting Information for examples of REDOR curves resulting from different motional models and illustrative examples).

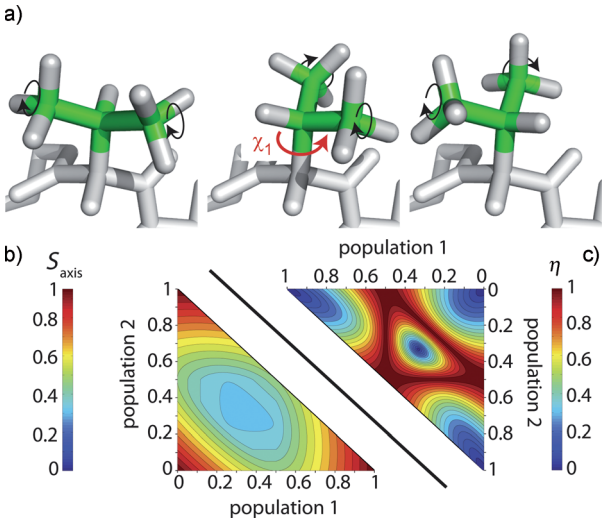

Figure 4. a) Rotamer transitions in the valine side chain. Fast methyl rotations (black arrows) and jumps around χ1 are considered. b) Calculated methyl $^1H–^{13}C$ tensor anisotropy $\delta_D/\delta_{D,rigid\,axis}$ and c) asymmetry η for the three-site jump model of the methyl axis around χ1, as a function of the population levels of the three rotamer states p_1, p_2, and $p_3 = 1-p_1-p_2$. See Figures S4–S6 for more details.

There are four valine residues in ubiquitin. Only one of these (Val70) shows significant asymmetry, while the asymmetry for Val5, Val17, and Val26 is not significantly different from zero (see Figure 3 and Table S1 in the Supporting Information). Likewise, the order parameter S_{axis} is about 0.5 for Val70, but significantly higher (close to 0.8) for the others. The small asymmetry and high anisotropy observed for the valine residues 5, 17, and 26 can only be explained if these side chains populate primarily one rotamer state. Calculations show that these three side chains populate primarily one rotamer state (at levels of 80–90%, see Table 1). This 85% population has to be considered as a lower limit, resulting from the assumption that no librational motion is present: if part of the reduction of S_{axis} from 1 to 0.8 is ascribed to librational motion rather than rotamer jumps, an even higher population of the dominant rotamer state is calculated. For Val70 the tensor asymmetry is significantly different from zero, and the calculated populations of the three rotamer states are roughly 60, 25, and 15% (see Table 1); that is, all three rotamer populations deviate significantly from zero within this model.

Table 1: Rotamer jumps for Val/Leu side chains in ubiquitin as derived from dipolar coupling tensors.

Side chains	Population ranges derived from dipolar coupling measurements [%][b]			Populations derived from solution-state NMR measurements [%][c]		
Valines[a]	p_1	p_2	p_3	p_1	p_2	p_3
5 (γ2)	81–88	6–19	0–9	92±3 (g−)	6±2 (t)	2±2 (g+)
17 (γ1)	83–87	6-17	0-8	96±3 (t)	4±3 (g−)	0±2 (g+)
26 (γ2)	82–86	7–16	0–8	100±1 (g−)	0±1 (g+)	0±3 (t)
70 (γ1)	58–63	20–29	9–18	59±10 (t)	36±11 (g−)	5±5 (g+)

Leucines	p_1[b,d]	p_2[b,d]	S_{axis}, expected from p_1/p_2[b]	S_{axis}, experimentally observed [b]
67 (δ1)	68–74	26–32	0.6–0.67	0.46–0.49
67 (δ2)	57–73	27–43	0.51–0.66	0.44–0.47
69 (δ1)	80–83	17–20	0.74-0.77	0.44–0.49

[a] Data for the two equivalent methyl groups on the same side chain are in agreement, and only one is reported. [b] Confidence limits of the populations were derived from confidence intervals of η and S_{axis}. [c] Data as reported in Ref. [14]. t, g+, and g− denote *trans*, *gauche*+, and *gauche*− rotamers, respectively. [d] Populations within the model of Figure 5, derived only from values of η.

We have thus identified a valine side chain (Val70) with significant tensor asymmetry and ascribed this asymmetry to jumps between unequally populated rotamers and estimated the relative populations based on dipolar tensor parameters. It is interesting to compare our findings to data from complementary approaches. Scalar ($^3J_{C'C\gamma}$ and $^3J_{NC\gamma}$) couplings and residual dipolar couplings in liquid-crystalline media can also provide insight into the population levels and identity of side-chain rotamers.[13] While the small size of the scalar couplings (below 3–4 Hz) currently makes direct measurement in the solid state difficult, a solution-state study has used residual dipolar couplings in two alignment media and ^3J scalar couplings to investigate side-chain rotamer jumps.[14] Interestingly, and in agreement with our findings, Val5, Val17, and Val26 were found to populate primarily one single rotamer state in solution, while in Val70 all three rotamer states are populated to similar extents as found here (Table 1).

Jumps around two side-chain dihedral angles (χ1, χ2) have to be considered for Leu (Figure 5). Clearly, the complete characterization of such motions based on only the ^1H–^{13}C dipolar coupling tensor of the terminal methyl groups is difficult and in general ambiguous. In general, the measurement of several dipolar coupling tensors along the side chain would be needed. In Leu side chains, however, jumps around χ1 and χ2 are highly correlated, and these side chains

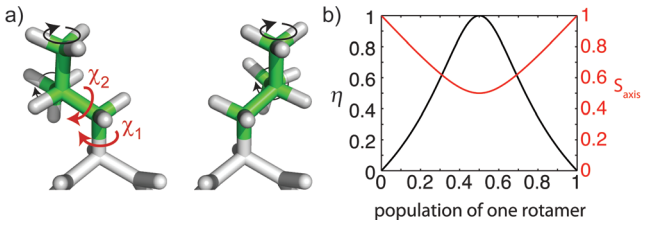

Figure 5. a) Transitions between two rotamer states in Leu side chains, resulting from correlated jumps around χ1 and χ2 b) Resulting dipolar coupling tensor parameters for the methyl group as a function of the population of one of the two states. Numerical expressions are shown in the Supporting Information.

populate primarily two states.[12,15] We have thus attempted to interpret the dipolar coupling parameters for the two Leu side chains that show significant asymmetry (Leu67, Leu69) in the framework of such a simple two-site jump model (see Figure 4). Based on the relations shown in Figure 4b, the observed asymmetry η_D for Leu67 and Leu69 (see Figure 2 and Table S1 in the Supporting Information) points to population levels of roughly 70% and 30%, respectively (see Table 1). However, these populations would result in tensor anisotropies that are higher than those found experimentally. We speculate that the model used here, where only transitions between two rigid conformations are considered, is too simplistic, and that additional librational motions are present that give rise to this observed reduction of the anisotropy. To date, no scalar-coupling-based rotamer populations have been reported in solution state for these residues.

As in Leu, two torsion angles (χ1, χ2) are necessary to describe the side-chain motion in isoleucines as probed by the methyl group δ1. However, several rotameric states corresponding to different combinations of χ1 and χ2 are generally populated to significant amounts;[12,15,16] more experimental parameters would be required to accurately describe the motion, and we refrain from deriving rotamer populations from a single dipolar coupling measured on the δ1 site. The REDOR curves of two side chains (δ1 methyl groups of Ile13 and Ile36, Figures 2) could not be fit satisfactorily by a single dipolar coupling tensor. The minimum values of χ^2_{red} for these sites are 8 and 22, respectively. These REDOR curves can only be described by taking into account an additional process that is slow on the timescale of the dipolar coupling. For such processes, the REDOR curve has to be described by a weighted superposition of several REDOR curves, each characterized by different tensor parameters. We consider the simplest possible model of two exchanging sites, each described by a general dipolar coupling tensor. A fit with such a model leads to a good description of the experimental curves with χ^2_{red} values of 2.6 and 3.1 for Ile13 and Ile36, respectively (green lines in Figure 2a, see Table S1 for fitted values). This improvement is statistically significant, as investigated by an F-test.

In this study we have only used methyl groups residing on the end of the side chain to characterize the motional processes. Accordingly, the availability of only two tensor parameters for each terminal methyl position precludes the detection of more complex modes of motion or distinguish between different models. Our approach can be extended to other moieties in the protein backbone and along the side chains. Access to more coupling tensors will make it possible to characterize the side-chain and backbone mobility at higher precision and with less ambiguity. Measuring the

dipolar coupling tensors at multiple positions in combination with selective or sparse labeling[17] will allow the separation of small-amplitude fluctuations (librations) and rotamer jumps around different dihedral angles and thus provide a comprehensive picture of backbone and side-chain motion. Importantly, a single experiment provides this information simultaneously for many sites, covering a wide range of time scales, as contrasted by studies of motional asymmetry in solution[18], which require several experimental parameters and interpretation within motional models.

In conclusion, we have shown for the first time that MAS solid-state NMR spectroscopy can be used for the accurate determination of dipolar coupling tensors in terms of both the anisotropy and the asymmetry. The hitherto never exploited information about dipole tensor asymmetries provides direct access to the details of dynamics, as exemplified here with the exchange between side-chain rotameric states. Such large-scale motions may be crucial to the function of membrane proteins[2d] and other proteins in the solid state.

Received: June 9, 2011
Published online: September 14, 2011

Keywords: asymmetric dipolar couplings · isotopic labeling · methyl groups · protein dynamics · solid-state NMR spectroscopy

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