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Solid-State NMR Approaches to Internal Dynamics of Proteins: From Picoseconds to Microseconds and Seconds

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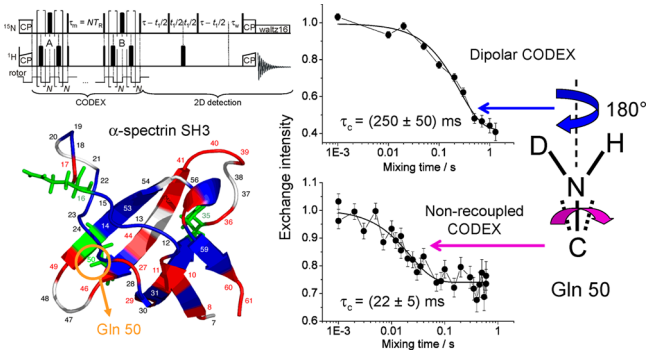

Solid-state nuclear magnetic resonance (NMR) spectroscopy has matured to the point that it is possible to determine the structure of proteins in immobilized states, such as within microcrystals or embedded in membranes. Currently, researchers continue to develop and apply NMR techniques that can deliver site-resolved dynamic information toward the goal of understanding protein function at the atomic scale. As a widely-used, natural approach, researchers have mostly measured longitudinal (T_1) relaxation times, which, like in solution-state NMR, are sensitive to picosecond and nanosecond motions, and motionally averaged dipolar couplings, which provide an integral amplitude of all motions with a correlation time of up to a few microseconds. While overall Brownian tumbling in solution mostly precludes access to slower internal dynamics, dedicated solid-state NMR approaches are now emerging as powerful new options.

In this Account, we give an overview of the classes of solid-state NMR experiments that have expanded the accessible range correlation times from microseconds to many milliseconds. The measurement of relaxation times in the rotating frame, $T_{1\rho}$, now allows researchers to access the microsecond range. Using our recent theoretical work, researchers can now quantitatively analyze this data to distinguish relaxation due to chemical-shift anisotropy (CSA) from that due to dipole—dipole couplings. Off-resonance irradiation allows researchers to extend the frequency range of such experiments. We have built multidimensional analogues of T_2-type or line shape experiments using variants of the dipolar-chemical shift correlation (DIPSHIFT) experiment that are particularly suited to extract intermediate time scale motions in the millisecond range. In addition, we have continuously improved variants of exchange experiments, mostly relying on the recoupling of anisotropic interactions to address ultraslow motions in the ms to s ranges. The NH dipolar coupling offers a useful probe of local dynamics, especially with proton-depleted samples that suppress the adverse effect of strong proton dipolar couplings.

We demonstrate how these techniques have provided a concise picture of the internal dynamics in a popular model system, the SH3 domain of α-spectrin. T_1-based methods have shown that large-amplitude bond orientation fluctuations in the picosecond range and slower 10 ns low-amplitude motions coexist in these structures. When we include $T_{1\rho}$ data, we observe that many residues undergo low amplitude motions slower than 100 ns. On the millisecond to second scale, mostly localized but potentially cooperative motions occur. Comparing different exchange experiments, we found that terminal NH_2 groups in side chains can even undergo a combination of ultraslow large-angle two-site jumps accompanied by small-angle fluctuations that occur 10 times more quickly.

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1. Introduction

Conformational transitions within a protein structure represent the basic processes mediating its function. Therefore, detailed knowledge of the molecular-level dynamics of proteins is required for an in-depth understanding of their biological activity. Proteins are not at all static structures: driven by fast thermal motion of their structural elements and within the hydration shell,[1] the cooperativity arising from their polymeric nature leads to dynamics covering a vast spectral range. Internal motions on the microsecond to second time scale are of particular interest, as this is the time scale of many biologically relevant events, such as catalysis, recognition, folding, and so forth. It is well-known that, in solution, the direct NMR assessment of such slow motions is challenged by the overall Brownian tumbling that averages anisotropic interactions, and enables observation of motions on the time scale slower than the Brownian tumbling only by means of isotropic chemical shift exchange-based methods. This restriction is lifted in the solid state, and consequently, developing and applying corresponding NMR approaches using the named interactions to address the whole frequency range of internal protein dynamics is at the focus of current intense activities.

To date, most studies of site-resolved internal dynamics in solid proteins have been limited to the picosecond to microsecond range,[2−5] relying mostly on ^{13}C or ^{15}N T_1 relaxation times. For the lower end of this window and its extension to still slower dynamics, there is still a considerable lack of experimental tools that can provide reliable quantitative information. In this Account, we review a number of methodological approaches and applications thereof, focusing on molecular motions on the microsecond to second time scales. We place a specific focus on the use of heteronuclear $^{13}C-^{1}H$ and in particular $^{15}N-^{1}H$ dipole−dipole couplings to detect the changes in the respective bond orientation. Their use is on the one hand convenient, because these interactions are readily measurable in samples with the common isotope labeling schemes. The respective tensor magnitude and orientation is very well-known, requiring no additional knowledge as is for instance the case for the chemical-shift anisotropy. By way of their magnitude in the 10−20 kHz range, the effects of XH dipole−dipole interactions on transverse and rotating-frame relaxation phenomena are on the other hand particularly sensitive to slow (more specifically: "intermediate") motions in the reciprocal time range, that is, about 1−100 μs. The qualitative presence of any appreciably faster motions is further identified by fast-limit motional averaging and thus reduced couplings, most often quantified by an order parameter $S < 1$. Slower motions are the domain of specific exchange experiments, which can of course also be conducted on the basis of isotropic chemical-shift changes, which is the other most useful NMR interaction. ^{2}H NMR based on the quadrupolar interaction allows for very detailed insights into localized dynamics,[6] but requires specific isotope labeling and is thus less attractive as a potential routine tool.

2. New and Not-so-New Solid-State NMR Tools to Study Slow Protein Dynamics

2.1. The Fast End of Slow: Spin−Lattice Relaxation in the Rotating Frame. The measurement of the relaxation time $T_{1\rho}$ (or its inverse, the corresponding rate $R_{1\rho}$) is a well-known routine tool for studying molecular motions. The frequency window of this method is determined by the nutation frequency of the rf spin-lock field which usually has a value of several tens of kilohertz. Since the amplitude of the spin-lock field can be varied within a relatively wide range, one can measure $T_{1\rho}$ dispersions, which significantly enhances the capability of this technique. Here, we shall discuss a few important challenges and limitations.

In practice, a quantitative interpretation of $T_{1\rho}$ is often challenged by the so-called spin−spin (or coherent) contribution to the relaxation rate arising from the additional relaxation pathway from the rotating frame Zeeman reservoir to the lattice through the proton dipolar reservoir.[7] It is further enhanced by (easily avoided) rotational resonance conditions. The proton-related coherent contribution strongly depends on the ratio between spin-lock and local dipolar field: the higher this ratio, the smaller this contribution. For rigid molecules, this contribution can be quite appreciable even for relatively strong spin-lock fields, and thus, one should always be aware of this effect and try to set the spin-lock field as high as possible. This leads to the self-evident limits of the application of strong and long rf pulses: they can be dangerous both for the hardware and for the sample. However, using the resonance offset, the effective amplitude of the spin-lock pulse can be increased without increasing the rf power.

The alternative approach to suppress the undesired contribution is proton decoupling during the spin-lock pulse applied on X nuclei.[7] This enables decreasing the amplitude of the spin-lock pulse and thus studying slower motions. This type of experiment is a heteronuclear equivalent of the homonuclear relaxation in the doubly rotating frame.[8] However, a theoretical background of this experiment has not been worked out yet. The H−X dipolar relaxation mechanism vanishes to first order in this case, but still it is

effective in higher orders, and the quantitative description of this is still missing. Another option to suppress the spin−spin contribution to the relaxation rate is to perform the $T_{1\rho}$ experiments under very fast MAS. At spinning rates of 50−60 kHz and more, the interproton dipolar interaction is scaled down to a significant degree, and thus the couplings within the proton dipolar reservoir effectively diminish. The applicability of this approach has been recently demonstrated[9] and reviewed in this issue.[10]

The spin−spin contribution can also be suppressed by the replacement of protons by deuterons.[11] This also makes the proton dipolar reservoir and the dipole−dipole couplings within it negligible and enables analyses of $T_{1\rho}$ relaxation times of X nuclei at low spin-lock fields.[12] This claim may be challenged by the observed residual MAS rate dependence of $T_{1\rho}$ in such as samples,[9,10] yet is on the other hand convincingly validated by the fact that the proton line width is only 20−30 Hz at 10−20 kHz spinning in partially deuterated proteins,[11] while it is still around 150−200 Hz at 60 kHz MAS in a fully protonated protein.[13] Since proton line width is a direct measure of the $^1H-^1H$ coupling and hence the efficiency of the spin−spin contribution, its almost 1 order of magnitude lower value in deuterated proteins even at moderate MAS renders the contribution safely negligible. Any residual $R_{1\rho}$ dependence on the MAS rate ($\omega_R = 2\pi\nu_R$) can straightforwardly be explained by its additivity to the effective spin lock field ω_e in the spectral density functions, $J(\omega_e \pm \omega_R)$ and $J(\omega_e \pm 2\omega_R)$, which further depend on both amplitude and time scale of molecular motion. Also, rotary resonance effects may play a role.[10] Recently, the $R_{1\rho}$ equations for the CSA and heteronuclear dipolar relaxation mechanisms were rigorously derived,[14] taking into account (i) the resonance offset of the spin-lock pulse and (ii) spin-lock and MAS frequencies of the same order of magnitude. The latter is especially important for the experiments at high MAS rates. It is noted that coherent effects at the (relatively narrow) rotary resonance conditions[9] are not included in the treatment and should thus be avoided.[9,14]

2.2. Intermediate Dynamics: Motions on the Interaction Time Scale. Besides $T_{1\rho}$ experiments, the toolbox of solid-state NMR contains line shape- and transverse-evolution-based experiments, and, as highlighted here, separated-local-field (SLF) methods, in order to address intermediate motions. Lineshape experiments are among the earliest dynamic solid-state experiments performed in biological solids.[15] They rely on the partial averaging of the quadrupolar interactions or the CSA. The sensitive dynamic range is about 2 orders of magnitude in correlation time, centered around the inverse of the interaction constant. For a specifically labeled sample, even a single static NMR spectrum may already reflect the occurrence of a dynamic process and its characteristic time. However, the inherently low signal-to-noise ratio of such experiments, in combination with the requirement for site-selective isotope labels, renders line-shape experiments hardly suitable to obtain a comprehensive picture of the dynamics of a protein.

A more economic alternative is to apply separated-local field (SLF) experiments which provide information on the anisotropic heteronuclear HX dipolar couplings, correlated with the isotropic chemical shift of the X nucleus which assures site resolution. Such experiments have found numerous applications, for example for the determination of torsion angles or the amplitude of fast-limit dynamics.[16] The actual experimental implementation varies in various details, but a common feature is the central role of the a homonuclear dipolar decoupling block, for example a variant of Lee−Goldburg irradiation (LG), or MREV-8, which suppresses homonuclear dipole−dipole interactions among the abundant proton spins and thus ensures the measurement of a localized interaction through a modulation of spectral intensity monitored in an indirect t_1 dimension. The main use of such experiments in biomolecular NMR up to now was to determine on an absolute scale the dynamic order parameter S which quantifies the amplitude of molecular reorientations[16] through a reduction of the static-limit dipolar coupling in case the dynamics is in the fast limit, that is, when the correlation time of motion is much shorter than the inverse of the strength of the NMR interaction, that is, $\tau_c \ll 100\ \mu s$.

Yet, SLF experiments have not yet found many applications in determining the actual time scale of the dynamics, which we consider an unrecognized potential. Recent work in our group has thus focused on developing strategies to extract motional correlation times. We have explored the use of the classic DIPSHIFT variant, which is based on a rotor-syncronized Hahn echo on the X channel and Lee−Goldburg irradiation for ^1H homonuclear decoupling in t_1, to quantify intermediate-range motions. We further stress the use of simple analytical formulas based upon the Anderson−Weiss approximation[17] to quantitatively analyze the intermediate-motional T_2-type signal decay and the shape changes of the modulation curves found under these conditions, enabling the determination of correlation times in the range of 1 ms to 100 ns. An alternative is provided by LG-CP, where we found that the most economic way to extract the dynamic information is to record Hartmann−Hahn matching curves as a function of the rf power on one of the two channels.[18] The latest development was concerned

with weak XH couplings, for which the original DIPSHIFT experiment can be adapted by implementing elements of REDOR-type recoupling, where the issue is the exact way in which information on the time scale of the dynamics is retained.[19] These developments have paved the way for the successful application of DIPSHIFT-type experiments in the field of biologic solids, and different studies along these lines are currently underway in our laboratory.

Methods utilizing other types of T_2 effects, which are generally based upon the interference of motion with transverse coherence evolution (dephasing and rephasing), now start appearing in the literature. As an example, the popular Carr–Purcell–Meiboom–Gill transverse relaxation rate (R_2) dispersion technique, providing information on isotropic chemical-shift exchange and being frequently applied in solution-state NMR studies of conformational dynamics,[20] can also be applied to solid proteins.[21] In the same reference, Schanda and co-workers have further demonstrated that the difference in R_2 of XH zero- and double-quantum coherences is in principle sensitive to intermediate-time scale changes of not only the isotropic chemical shift, but also of the CSA tensors of the two involved nuclei. Its application to a conformational exchange process in microcrystalline ubiquitin suggests that in this case, isotropic shift variations of the involved ^{15}N and ^{1}H nuclei dominate over reorientations of either of the two CSA tensors.[21]

2.3. Ultraslow Motions: Exchange NMR. In the exchange experiment, the precession frequencies of a nucleus are compared before and after the mixing time t_m; see Figure 1. If a magnetic interaction determining the precession frequency is modulated by molecular motion during the mixing time, then the precession phases are not equal anymore ($\phi_1 \neq \phi_2$), and the signal decreases with increasing t_m, permitting the determination of the exchange rate.

Apart from the *J*-coupling, all other relevant NMR interactions, such as dipolar couplings, the (isotropic and anisotropic) chemical shift can be modulated by molecular reorientations and thus can be used in this experiment. Different modifications of the exchange experiment select different useful interactions and suppress the remaining ones. Again, heteronucleus-based detection under high-resolution MAS conditions is imperative. As 2D exchange spectroscopy based upon, for example, the observation of exchange signals among the CSA sideband manifold is not economic, many different 1D versions became available, which commonly work with fixed evolution times for the phases ϕ_1 and ϕ_2, and create a stimulated-echo type of signal function. One specific variant, the so-called time-reversed

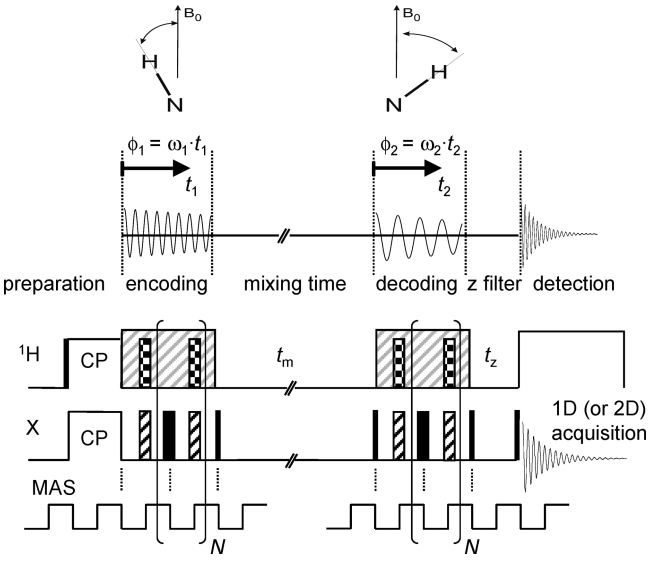

FIGURE 1. Principle of an exchange experiment (top) and schematic pulse sequence for different CODEX experiments (bottom), where the wider bars denote π pulses. The phase cycle of the other pulses and the signal detection can be adapted to either pick up the cosine or the sine component of the magnetization precessing under the interaction of choice in periods 1 and 2 which flank the mixing time. The resulting site-resolved intensity corresponds to a stimulated echo, where often the phase cycle combines both components: $\cos(\phi_1)\cos(\phi_2) + \sin(\phi_1)\sin(\phi_2) = \cos(\phi_1 - \phi_2)$. For the original CSA CODEX, the solid and hatched π pulses are used along with homonuclear decoupling on ^{1}H (gray hatch). For dipolar CODEX applied to partially deuterated proteins, the solid and checkered π pulses are used, and for nonrecoupled CODEX for isotropic shift exchange no π pulses but only dipolar decoupling are applied during t_1 and t_2.

ODESSA experiment, was the first to be applied to solid proteins.[22]

Soon after, the development of the now most popular centerband-only detection of exchange (CODEX) experiment by Schmidt-Rohr and colleagues[23] permitted the accurate determination of both exchange rate and amplitude of motion. This approach was quickly applied to biopolymeric systems.[24,25] In the original CODEX, the CSA interaction is reintroduced during the encoding periods by a train of 180° pulses applied every half of the MAS rotor period in analogy to the well-known REDOR pulse sequence; see Figure 1. Putting one of the 180° pulses to another rf channel, the CSA-based CODEX can be turned into its dipolar variant,[26,27] enabling a simpler interpretation of the results, which are now based upon the reorientation of a XH or XY dipolar tensor. In this case, the cosine and sine contributions to the stimulated echo (see caption of Figure 1) are associated with in-phase and antiphase magnetization terms, respectively. A last important variant of a MAS exchange experiment is of course based on isotropic shifts, detecting

simple chemical exchange, that is, the modulation of the isotropic chemical shifts by conformational transitions. In this case, simply no recoupling pulses are applied, but either one 180° pulse on the proton channel in the middle of the recoupling periods or conventional dipolar decoupling in order to remove undesirable *J*-coupling. The dipolar and CSA interactions are then averaged out by MAS. This version of the exchange experiment may be referred as "nonrecoupled CODEX", yet it is fully equivalent to the many well-known variants of stimulated echo experiments.

There are some important issues for the CODEX variants and related exchange experiments that deserve some attention. First, one must distinguish between the exchange process (i.e., molecular motion) and longitudinal relaxation that both simultaneously occur during the mixing time. A correction of the data for T_1 relaxation can be done either by swapping the *z* storage/filter periods in a pulse sequence of the type shown in Figure 1,[23,27] or via a separate measurement.[26] Second, the observed exchange process can be caused not only by molecular motion but also by spin diffusion. The latter can be reduced by suitable isotopic dilution, and/or can be identified and corrected for via its usually absent temperature dependence. Third, the exchange process can also be caused by the so-called RIDER (relaxation-induced dipolar exchange with recoupling) effect,[28] which is based on the loss of heteronuclear spin states (antiphase magnetization) during the mixing time due to the longitudinal relaxation of adjacent, not irradiated but dipolar-coupled spins (often quadrupolar nuclei such as ^{14}N). The recipe to suppress this important effect is either dipolar decoupling from these additional nuclei, or accumulation of only the cosine (in-phase) components of the magnetization.[26]

2.4. Tackling Side Effects Related to ^{1}H−^{1}H Dipole−Dipole Couplings. One of the substantial difficulties in the NMR-characterization of the protein dynamics is proton driven spin diffusion (PDSD) between ^{13}C/^{15}N nuclei. While the effect is helpful for structural studies, it is a severe obstacle in dynamic NMR since (i) PDSD averages the relaxation rates of various nuclei in a protein if the PDSD rate is faster or comparable to the relaxation rates, thus deteriorating the selectivity of the dynamic information; and (ii) in the exchange NMR experiments, PDSD overlaps molecular dynamic processes, if PDSD is faster or comparable to molecular motions, thus making determination of the motional parameters uncertain.[22] Only the T_2-type experiments (e.g., signal loss in DIPSHIFT experiments) are unaffected by PDSD. There are two options to suppress PDSD in proteins: very fast MAS, which makes the diffusion slower;[29,30] and/or preparation of a proton diluted (deuterated) sample.[11,31] Note that proton decoupling is not useful for this purpose because (i) proton decoupling in some cases may even accelerate PDSD[32] and (ii) typical relaxation delays and mixing times are of the order of seconds and for practical reasons applying high power decoupling for such time periods is not feasible.

3. Case Studies of the SH3 Domain

The first (and until 2010, the only) protein sample prepared according to the proton-depletion protocol was the α spectrin SH3 domain, which is also relatively easy to prepare in the form of microcrystals. It is thus among the proteins most studied by solid-state NMR, and has served as a benchmark sample for the majority of methodological developments. In the following chapter, sample applications of some of the NMR techniques presented above will be discussed and compared with results from more established techniques revealing fast (ns−ps) motions.

3.1. The Big Picture: Combination of Methods Probing Faster Motions. The list of the site-specific ^{15}N NMR experiments on studying dynamics in SH3 includes (i) T_1 measurements at different resonance frequencies,[5] (ii) ^{15}N−^{1}H dipolar couplings[33] and CSA cross correlated relaxation rate $\eta^{DD/CSA}$ measurements,[34] (iii) dipolar CODEX experiments,[26] and (iv) $T_{1\rho}$ measurements at different spin-lock fields.[12] The combined analysis of dipolar couplings, $\eta^{DD/CSA}$, and T_1's[35] as well as the comparison of the solid- and liquid-state ^{15}N−^{1}H order parameters[36] have shown that relatively large-amplitude motions in the protein occur mostly on the picosecond time scale. At the same time, practically all residues undergo slower low amplitude motions with a correlation times ranging from few ns to ∼100 ns. Approximately 5−6 residues reveal mobility in the time range of few hundred nanoseconds.

The simultaneous fitting of R_1- and R_2-type (transverse evolution-based) data, $\eta^{DD/CSA}$ and order parameters, the latter just characterizing amplitudes of fast motions, is poorly sensitive to the motions on the microsecond time scale. The R_1 and R_2 only depend on the values of the spectral density function at the resonance frequency (several tens/hundreds MHz) and zero, respectively, and it must be noted that a proper analysis of R_2 data requires dedicated intermediate-motional theories that are not yet well developed. The gap can, however, be narrowed substantially by $R_{1\rho}$ data. The SH3 domain by now is the only protein for which all sets of data were measured. In our group we conducted a simultaneous analysis of ^{15}N R_1, $R_{1\rho}$ relaxation rates measured at

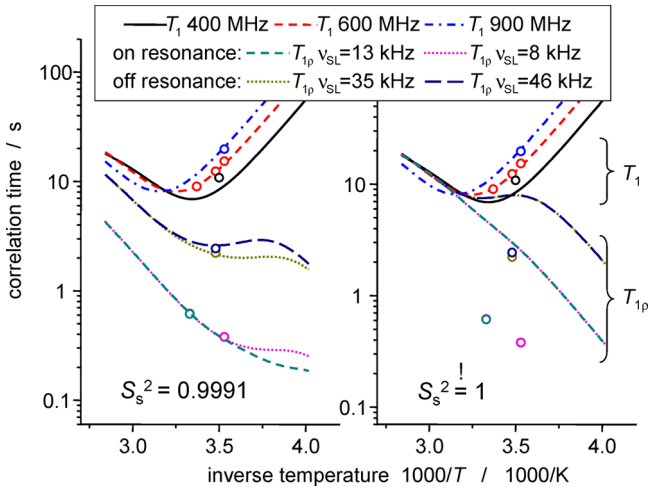

FIGURE 2. Experimental (circles) and simulated (curves) T_1 and $T_{1\rho}$ relaxation times for the residue Q16 of SH3, with the latter taken at different spin lock frequencies ν_{SL} and spectral offsets. The fits assume a three-component correlation function with $S_f^2 = 0.78$, $\tau_f < 10^{-11}$ s, $S_i^2 = 0.956$, $\tau_i = 5.1 \times 10^{-9}$ s, $\tau_s = 2.9 \times 10^{-6}$ s, and slow-motion order parameters as indicated. The surprising sensitivity to such small apparent amplitudes simply arises from the fact that in the fast-motion branch of $T_{1\rho}$ ($\nu_{SL}\tau \ll 1$), a Lorentzian spectral density $J(2\pi\nu_{SL}) \approx (1 - S^2)\tau_s$, that is, the low amplitude is offset by a relatively large correlation time. Data reproduced from ref 37.

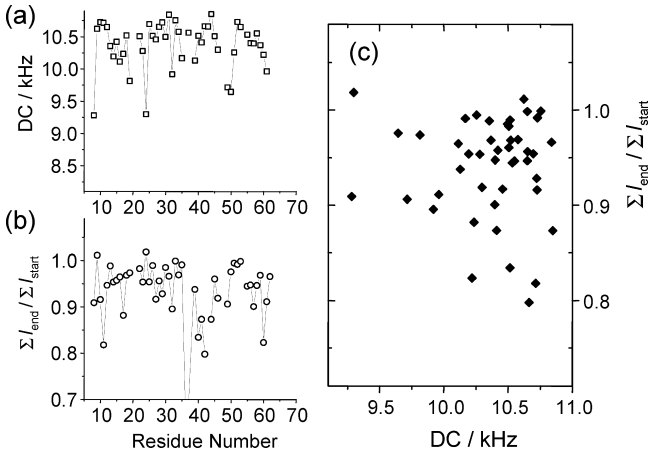

FIGURE 3. $^1H-^{15}N$ dipolar couplings reflecting the NH order parameters (a) and ratios of the peak intensities taken at the end vs the beginning ($t_m = 0$) of the ^{15}N dipolar CODEX mixing time dependencies (b) as a function of a residue number in SH3, as well as a correlation plot of both quantities (c). The data are taken from refs 26 and 33, respectively. The physical meaning of the CODEX intensity ratio is similar to a dipolar order parameter, yet it is not fully equivalent since the former depends not only on the motional parameters but also on the encoding interval, while the latter reflects the cumulative amplitude of all motions with the correlation time around few microseconds and faster. The error margins of both correlated quantities are around 5−10%.

different resonance frequencies and spin-lock fields, and order parameters from $^{15}N-^1H$ dipolar couplings.[37] The results suggest that the inclusion of $R_{1\rho}$ data in the fitting makes the dynamic picture appreciably more certain and exact, as it allows gauging the number of motional modes (components of the correlation function) or the presence of broad correlation time distributions. It turns out that there is more intermediate dynamics in the protein than it follows from the analysis without $R_{1\rho}$ data: not just 5−6, but about half of all residues in the SH3 domain undergo motions with a correlation time longer than 100 ns. The amplitude of these slow motions is very small in some cases, the order parameter S_s being larger than 0.99, which is most likely the consequence of a lowly populated excited state and large-angle motion rather than of very small actual amplitude. Nevertheless, using the $R_{1\rho}$ data these motions can be reliably identified, as demonstrated in Figure 2. It can be also demonstrated that for some residues the assumption of two discrete motional modes does not work very well and thus, a more complex form of the correlation function is needed for an adequate description of the data.

3.2. Motions on the Microsecond to Second Time Scale and Correlation of Different Methods. Recently we reported on the observation of residue-specific slow motions in the SH3 domain using the dipolar CODEX experiment.[26] It has been demonstrated that most of the residues in this protein are rigid on the millisecond to second time scale, however, some of them reveal appreciable millisecond dynamics. Particularly high-amplitude millisecond motions were mainly observed for the terminal NH_2 groups of two Gln residues. The comparison of the dipolar CODEX "amplitudes" and dipolar order parameter data for the same protein presented in Figure 3 shows that the mobilities on the sub-microsecond (fast-limit averaging) and millisecond to second time scales are essentially different phenomena: there is no correlation between the amplitude of such fast motions and the presence of slower motions. At the same time, CODEX, fast-limit dipolar order parameters and also $R_{1\rho}$ data complement each other, providing a clearer picture of the protein dynamics.[37]

A comparative analysis of different experiments conducted on the same sample in general can be a very effective tool for revealing details of protein dynamics. Here we stress the use of the simultaneous analysis of different variants of the CODEX experiment on the example of the side chain NH_2 group of Gln 50 in SH3. Figure 4 presents the mixing time dependencies of intensity of the peak in 2D $^{15}N-^1H$ correlation spectrum assigned to this group for the dipolar and the nonrecoupled CODEX experiments, the latter being a simple stimulated echo based upon isotropic shift exchange. It is seen that both experiments reveal motions of this group in

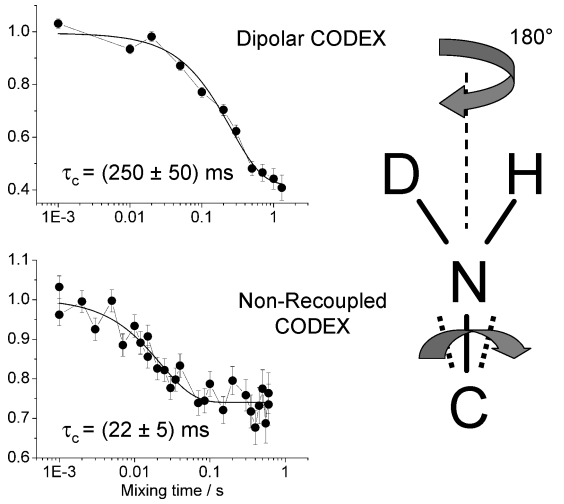

FIGURE 4. Dipolar and nonrecoupled CODEX (see Figure 1) mixing time dependencies of the side-chain ^{15}N of Gln 50 in SH3, conducted at 24 °C with encoding periods of 0.8 and 8 ms, respectively. The exponential fits (lines) and a schematic representation of the two types of motion of the actual NHD group are also shown.

the millisecond time scale, but the correlation times determined from the exchange decays differ by 1 order of magnitude.

This surprising result can be reasonably explained by considering that the two experiments are sensitive to different types of reorientations and that the NH_2 group simply takes part in two independent motions: first, a jumplike 180° turn around the C−N bond, and second a faster small-amplitude reorientation of this bond. The dipolar CODEX detects the ^{15}N−^{1}H bond reorientation, and thus, the faster small-amplitude motion cannot reliably be detected before the background of the 180° jumps because of an insufficient S/N ratio. On the other hand, isotropic shift exchange is not sensitive to the 180° jumps since it does not change the molecular conformation and the isotropic chemical shift of ^{15}N remains constant. We stress that without the comparative analysis of two types of exchange experiments the conclusion on two independent millisecond time scale motions would be impossible. Note that H/D exchange with the D_2O reservoir cannot explain the observation, because it would render the corresponding signal undetectable (the proton has to be in place before and after t_m). Thus, potential H/D exchange would only decrease the apparent T_1 relaxation time, which is accounted for in the analyses.

Finally, we address the (potential) differences of internal protein dynamics in the solid and solution states. While the solution state might provide the most "realistic" surrounding of a given functional protein, we stress that not only dedicated solid-state NMR data but also B-factors from X-ray crystallography have frequently been interpreted as carrying meaningful dynamic information. Thus, we comment on a recent critical assessment[38] which revealed that there is essentially no general correlation between B-factors and true dynamic information from solid-state NMR. The reason is that B-factors report on variations of atomic coordinates from one unit cell to the next without time scale information: static disorder and very fast conformational fluctuations contribute equally to large B-factors. Another point is that NMR is mainly sensitive to rotations, which may only involve significant displacements of weakly scattering hydrogen atoms. B-factors are thus to be interpreted with great care.

3.3. Solid- versus Solution-State Dynamics. Turning to more meaningful comparisons of dynamic information from solution- and solid-state NMR, relaxation experiments have demonstrated that the parameters of the fast (ps−ns time scale) internal dynamics of SH3 in the solid and liquid states are essentially the same.[39,40] As for the longer time scale, such a comparison is impossible because of the isotropic Brownian tumbling: the motions slower than the tumbling are not observable using the experiments employing anisotropic interactions. The only option for a direct comparison between the two is using isotropic chemical shift as a probe for conformational dynamics, as it is accessible in both the solid and liquid states. Using the mentioned nonrecoupled CODEX version, we have conducted such a study for SH3 in both states (to be published elsewhere), indicating that the millisecond motions are not identical in the two states. In solution, we observe no millisecond motions; however, they can be easily identified for some residues in the solid state.

These findings are in an agreement with the recent results of Schanda and co-workers.[21] Using the technique mentioned in section 2.2, they studied conformational exchange in ubiquitin in the microcrystalline state and in solution, and revealed that slow internal motions are much slower in the former. This means that intermolecular contacts in microcrystalline proteins enhance energy barriers between conformational substates, which affect the rates of slow internal mobility. The experimental evidence of this phenomenon is still sparse and its detailed description is not yet available. Still, it is clear that one should be cautious in generalizing solid-state NMR results for an interpretation of protein behavior in solution.

4. Conclusions

We have reviewed a number of established as well as novel solid-state NMR techniques suited to study internal protein dynamics on the microsecond to second range. Rotating-frame $T_{1\rho}$ experiments cover the range below microsecond to milliseconds, and benefit from off-resonance spin lock irradiation

to increase the effective field. Line shape or T_2-type experiments address a complementary time range. We advocate the use of DIPSHIFT-type experiments for this purpose, combining site-resolution with the well-known orientation of X−H dipolar tensors. Ultraslow motions are the domain of exchange experiments, where, apart from traditional isotropic shift exchange, recoupling-based techniques such as variants of the CODEX experiment appear most promising. All techniques benefit hugely from the use of either proton-depleted samples or very high MAS rates in excess of 50 kHz, which interrupt the strong dipolar proton network and render dynamic information more localized.

Thanks to the absence of overall tumbling, the application of solid-state NMR methods thus offers great potential for generating new insights into biological function on the molecular level. The discussed approaches are naturally useful for proteins that are not soluble or very large, or are dissolved in very viscous solvents.[41] Interesting options are studies at variable degrees of hydration or studies of interactions with solid matrices such as certain polysaccharides[42] that render proteins robust against freezing or drying. It should, however, be kept in mind that solid-state NMR is often more laborious and time-consuming than liquid-state NMR, mainly due to the compromise in resolution, requiring longer experimental times, and/or elaborate sample preparation procedures to obtain at least microcrystalline samples. A new and promising route is study of sedimented samples in fast-spinning MAS rotors.[43] Also, it is an open question worthy of detailed investigation if and if yes under which conditions the internal dynamics of solid protein samples are comparable with the native, that is, dissolved or membrane-bound state.

In perspective, we expect that many of the methods discussed herein will be applied in combined fashion, making use of the specificity of different NMR interactions and methods to address certain ranges of correlation times and motional amplitude. This will enable the development of specific motional models and thus will help to understand proteins as molecular machines, and ultimately engineer these and tune their function and activity.

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Alexey Krushelnitsky graduated from the Physics Department of Kazan State University and worked in the group of Prof. V. D. Fedotov at the Russian Academy of Sciences since 1986. He has obtained his Ph.D. and Habilitation degrees in Kazan in 1991 and 2006, respectively. For many years, he has been commuting between Kazan and Halle, collaborating with the NMR group at the Institute of Physics of the Martin-Luther-University Halle-Wittenberg. In 2011, he switched completely to Halle and now holds a postdoctoral position there.

Detlef Reichert has studied physics at and obtained the doctoral degree in 1990 from the Technical University of Merseburg, East Germany. After a postdoctoral period in the lab of Zeev Luz at the Weizmann-Institute of Science, Israel, he joined the Institute of Physics of the Martin-Luther-University Halle-Wittenberg, Germany, from where he received his Habilitation in 2000. Since then, he has been a senior scientist in the Biophysics group.

Kay Saalwächter studied chemistry at the universities of Mainz and Freiburg, Germany, and the University of Massachusetts at Amherst, and obtained his doctoral degree for work on solid-state NMR methods with H. W. Spiess at the MPI for Polymer Research, Mainz. He then switched back to Freiburg university, receiving his habilitation on polymer and liquid-crystal applications of NMR in the lab of H. Finkelmann in 2004. Since 2005, he is Professor of Experimental Physics, heading the NMR group at the Institute of Physics of the Martin-Luther-University Halle-Wittenberg in Halle (Saale), Germany.

FOOTNOTES

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