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Aromatic ring dynamics, thermal activation and transient conformations of a 468 kDa enzyme by specific ^1H - ^{13}C labeling and fast-MAS NMR

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Abstract

Aromatic residues are located at structurally important sites of many proteins. Probing their interactions and dynamics can provide important functional insight but is challenging in large proteins. Here, we introduce approaches to characterize dynamics of phenylalanine residues using ^1H -detected fast magic-angle spinning (MAS) NMR combined with a tailored isotope-labeling scheme. Our approach yields isolated two-spin systems that are ideally suited for artefact-free dynamics measurements, and allows probing motions effectively without molecular-weight limitations. The application to the TET2 enzyme assembly of ~0.5 MDa size, the currently largest protein assigned by MAS NMR, provides insights into motions occurring on a wide range of time scales (ps-ms). We quantitatively probe ring flip motions, and show the temperature dependence by MAS NMR measurements down to 100 K. Interestingly, favorable line widths are observed

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down to 100 K, with potential implications for DNP NMR. Furthermore, we report the first ^{13}C $R_{1\rho}$ MAS NMR relaxation-dispersion measurements and detect structural excursions occurring on a microsecond time scale in the entry pore to the catalytic chamber and at a trimer interface that was proposed as exit pore. We show that the labeling scheme with deuteration at ca. 50 kHz MAS provides superior resolution compared to 100 kHz MAS experiments with protonated, uniformly ^{13}C -labeled samples.

Introduction

Many cellular processes such as protein synthesis, chaperoning or enzymatic reactions rely on protein complexes of hundreds of kilodalton in size. Understanding how these machineries function at the atomic scale requires the determination of high-resolution structures, generally by X-ray crystallography or cryo-electron microscopy, as well as the characterization of intramolecular motions. NMR spectroscopy plays a central role in deciphering dynamics at atomic resolution, and thus often providing the crucial link between structure and function.¹ While solution-state NMR has been very powerful for many such structure-dynamics-function studies, including the structural characterization of short-lived “excited states”,^{1,2} it has inherent physical limitations when dealing with large molecules in excess of ca. 50-70 kDa. Their slow overall tumbling in solution results in rapid signal decay, and the low sensitivity and resolution renders atom-specific observation difficult. The use of selective CH_3 -labeling,³⁻⁵ deuteration and methyl-TROSY NMR⁶ has lifted these protein-size limitations partly, allowing the study of dynamics and interactions of methyl-bearing residues even in proteins as large as 1 MDa.^{3,7,8} Moieties other than methyls have been thought to be generally undetectable in large proteins by solution-NMR, until recent breakthrough isotope labeling methods allowed observing any kinds of aromatic and aliphatic moieties for a 82 kDa protein malate synthase G (MSG) in solution.⁹

Aromatic residues have been the subject of much interest since the early days of protein NMR.¹⁰⁻¹² They are overrepresented at protein interfaces and play a prominent role in guiding enzyme mechanisms.¹³ Understanding their mobility can therefore provide key functional information. Characterizing the mobility of buried aromatics, and in particular ring-flip dynamics can reveal ‘breathing’ motions of proteins and thus point towards (local) unfolding events.¹⁴ Furthermore, aromatics are important structural probes, as their involvement in hydrophobic core regions implies that they have numerous short-distance contacts that are quite useful for determining protein structures. Therefore, the global fold of the 41-kDa maltodextrin-binding protein (MBP) determined by solution NMR, using the NOEs between the amide and methyl protons, together with the residual dipolar couplings for the polypeptide backbone and hydrogen-bond restraints, resulted in RMSDs larger than 3.8 Å for the side-chain heavy atoms of the N- and C-terminal domains.^{15,16} On the other hand, the corresponding side-chain RMSDs, obtained exclusively on the basis of the NOEs including aromatic and aliphatic protons other than the methyl moieties, were 2.3 Å for both domains.^{17,18} These considerations have been at the origin of long-standing interest in aromatics over several decades.^{11,19-23}

The usefulness of solution-NMR studies of aromatics is limited by several factors; first, for large proteins the spectra of aromatics are of low quality. The use of advanced labeling, such as deuteration and introduction of ^1H - ^{13}C labels with the SAIL scheme^{17,18,24} or ^{19}F labeling^{25,26} may extend the limits to several tens of kDa, but slow tumbling necessarily always leads to loss of spectral quality. Second, the overall molecular tumbling represents an inherent obstacle for detecting internal motion. Specifically, internal motions can only be detected when they are either faster than the overall-tumbling correlation time (i.e. ca. 5-30 ns) through spin-relaxation experiments, or slower than ca. 10 μs , where line-broadening effects can be quantified by relaxation dispersion experiments. Consequently, the time scale of ring flips is notoriously difficult to quantify by solution-state NMR studies.²⁰ An additional difficulty for dynamics measurements, and for the ability to even detect and resolve aromatic signals, is the presence of strong scalar couplings between adjacent carbons in uniformly ^{13}C -labeled samples.

Under magic-angle spinning (MAS) NMR conditions, where overall molecular tumbling is absent, atomic-resolution information can be obtained for all backbone and side chain sites, irrespective of the molecular weight, and without an inherent “blind spot” on the time scale of motions, thus resolving both limitations of solution-state NMR. The structure and dynamics of aromatics in peptides and small proteins have been in the focus of many MAS NMR studies over the last decades.^{21,27–32} The possibility to study samples at very low temperature in MAS NMR furthermore opens avenues for studying fundamental properties of proteins, such as temperature-induced activation of motions.^{33–35} An inherent challenge for solid-state NMR is related to the presence of dipolar couplings which – although they can be exploited to study dynamics and structure – lead to line broadening, in particular for nuclei with large gyromagnetic ratio (in particular ^1H). This problem can be alleviated by faster magic-angle spinning (the term fast evolved with time as the hardware evolved and currently lies around 100 kHz at most), specific isotope labeling, including deuteration, or combinations thereof, as shown herein.

MAS NMR experiments that probe molecular dynamics are commonly based either on spin-relaxation measurements, or on probing the dynamically-averaged anisotropic interactions, most often the dipolar coupling strengths.³⁶ In fully labeled, protonated proteins, the experimental accuracy of both types of approaches is, however, compromised: (i) in relaxation measurements, the presence of multiple spin-spin couplings may lead to an additional apparent spin decay which is due to either proton-driven spin diffusion (for the case of longitudinal relaxation)^{37–40} or so-called dipolar dephasing^{41–43}, or scalar-coupling-induced signal modulations.^{44,45} (ii) Even though robust methods for measuring dipolar couplings in fully labeled samples are being actively developed,^{46–49} the measurement of dipolar coupling tensors with precision remains challenging in the presence of multiple spin-spin couplings and experimental imperfections.^{50,51} In addition to challenging the precision of dynamics measurements, uniform labeling also compromises spectral resolution, because of the large number of aromatic resonances and the presence of scalar couplings that are difficult to remove. Over the last years, several groups have shown the advantages of combining deuteration and sparse protonation which result to good approximation in two-spin systems, high MAS frequencies (40-100 kHz) and sensitive proton-detected MAS NMR experiments to gain insight into structure and dynamics of the protein backbone^{43,52–54} and

side-chain methyl sites.^{55,56} Aromatic labeling schemes, with or without deuteration, have been used for solution-NMR or MAS NMR at modest MAS frequencies.^{24,30,57–61}

Herein we demonstrate that specific aromatic labelling^{24,58} and deuteration with ¹H-detected NMR experiments at high MAS frequencies (>40 kHz) allows probing dynamics occurring over many orders of magnitude in time, as exemplified here with the 468 kDa large dodecameric TET2 aminopeptidase TET2 from *Pyrococcus horikoshii*.⁶² TET2 forms hollow tetrahedral-shaped particles with a large central cavity (Figure 1A),⁶³ in which peptides of up to ca. 40 amino acids in length⁶⁴ are degraded to amino acids. Access to this large catalytic chamber is enabled through four ~18 Å-large entry pores on the faces of the tetrahedral structure. How amino acids produced by the catalytic reaction are removed from the cavity is subject to debate.

We probe several aspects of phenylalanine dynamics in TET2 with site-specific labeling. (i) Using dipolar-coupling measurements at room temperature, we investigate the ring flip motions, as well as the motion of the Phe ring axis. (ii) Relaxation measurements provide the time scale of these dynamics. A heterogeneity of ring-flip rates is observed, with the fastest flips occurring in the nanosecond range. (iii) By performing 40 kHz MAS NMR experiments at temperatures from 100 K to room temperature, we investigate how the ring flips become activated, and we furthermore report high-quality spectra at low temperature, an interesting finding for dynamic-nuclear polarization (DNP) studies. (iv) We furthermore introduce a ¹³C R_{1ρ} relaxation-dispersion experiment and show its ability to probe microsecond (μs) mobility; we detect in particular residues at the entry pore and the trimerization interface, which undergo μs motions, and investigate their potential functional relevance by activity measurements with mutants. We furthermore show that the labeling scheme provides superior resolution compared to fully labeled (protonated) samples, even if highest-possible (100 kHz) MAS frequencies are used, and discuss the potential for obtaining structural restraints.

Methods

Sample preparation

For selective Phe labelling of TET2 in an otherwise deuterated environment, TET2 was produced by bacterial overexpression in *E. coli* BL21(DE3) in D₂O-based M9 minimal medium. At an OD₆₀₀ of ca. 0.6, one hour prior to induction of over-expression with 1 mM IPTG, specifically labelled L-phenylalanine (SAIL Technologies Inc.) with ¹H-¹³C spin pairs either at the *para*-CH or the two *ortho*-CH sites and otherwise fully ²H, ¹⁵N labeled, was added at a concentration of 20 mg/L. Prior to addition, the phenylalanine powder was dissolved in a small amount of water with potassium hydroxide. The single-point mutants for assignment were labeled by adding the *para*-CH labelled ketoacid precursor⁵⁸ instead of the amino acid (30 mg/L). The incorporation of these (cheaper) compounds is similar to the one of the amino acids. Note that a more conservative mutation (Tyr rather than Ala) may be used for mutagenesis-based Phe assignment; we find that Ala substitution does not lead to structural perturbations as controlled through 2D H-N spectra. Deuterated glucose (2 g/L, not ¹³C-labeled) and ¹⁵N-labelled ammonium chloride (1 g/L) were added as carbon and nitrogen sources. The protein purification was described previously⁶⁵. Briefly, it involved

heat shock at 85 °C and gel filtration. The purified protein was dissolved in buffer A (20 mM Tris, 50 mM NaCl, pH 7.5) and concentrated to 20 mg/mL using a centrifugal concentration device. To prepare a sample suitable for solid-state NMR, 50% (v/v) of 2-methyl-2,4-pentanediol (MPD) was added, resulting in a white precipitate (possibly nanocrystalline). The samples were filled into 1.3 mm Bruker rotors using an ultracentrifugation device in a SW-32 swinging-bucket rotor at 60000 g for 2 hours.

NMR spectroscopy

All NMR spectra were recorded on Bruker Avance-III spectrometers operating at 14.1 T (600 MHz ^1H Larmor frequency; 1.3 mm probe, 55 kHz MAS), with the exception of the low-temperature spectra, collected at 800 MHz ^1H Larmor frequency (40 kHz MAS), and the spectra shown in Figure 5, collected at 950 MHz (55 kHz in a probe for 1.3 mm rotors or 103 kHz in a probe for 0.7 mm rotors). Except for the low-temperature experiments, the sample temperature, regulated with a cooling gas flow, was estimated from the ^1H bulk water line position relative to the CH resonance of MPD at 4.1 ppm which is almost independent of temperature over the range considered here (see Figure S1).

The low-temperature experiments were performed on a widebore 800 MHz Bruker Avance-III spectrometer at CRMN Lyon. Sample cooling was achieved with pre-cooled bearing (2.8 bar), drive and variable-temperature (1200 L/h) gas flows. The reported temperatures are the gas temperatures; we estimated that the 40 kHz MAS rotation leads to an effective sample heating of ca. 10 K.

The spectrum on fully protonated TET2 was obtained at 950 MHz with a 0.7 mm HCN probe (Bruker Biospin). 103 kHz MAS was achieved with 2.66 bar bearing, 3.06 bar drive (ambient temperature) and the sample cooling gas (200 L/h) was at 273 K, leading to an effective sample temperature of ca. 35°C. All experiments employed in this study are based on direct ^1H detection with 5 kHz ^{13}C WALTZ decoupling during signal acquisition. All pulse sequences are displayed in Figure S2. All spectra were processed with nmrPipe⁶⁶ and visualized with CCPNMR⁶⁷. Quantitative peak integrals in series of 2D spectra (relaxation and REDOR experiments) were extracted with nmr-View (OneMoon Scientific). Integrals from 1D spectra were extracted with in-house written python programs, using the nmr-glue package⁶⁸ for handling of the processed NMR data. All analyses of relaxation data (mono-exponential fits) and REDOR fitting were performed with in-house written python scripts. The REDOR analysis was similar to previously employed strategies^{52,56}, based on GAMMA⁶⁹ simulations. All error estimates were obtained from standard Monte Carlo simulations. Briefly, 100-500 “noisy” data sets were generated in which random noise according to 3 times the spectral noise level was added to the back-predicted data points from exponential relaxation fits or from REDOR simulations. These data sets were subsequently fitted, and the reported error margins reflect the standard deviation over these data sets.

Molecular dynamics simulations

All simulations were performed using GROMACS⁷⁰ MD code using the Amber ff99SBws force field with balanced protein-water interactions⁷¹. Equations of motion were integrated

with a time step of 2 fs. All covalent bonds were constrained to their equilibrium values using the LINCS algorithm⁷². The electrostatic interactions were calculated by the Particle Mesh Ewald algorithm, and a cutoff of 1.0 nm was used both for Lennard–Jones interaction and for the real-space coulomb contribution. The starting structure was obtained integrating the high-resolution X-ray structure of TET2 (PDB code: 1y0r) with lower resolution cryo-EM data for modelling the flexible loop (120–132), as reported in the PDB entry 6F3K⁷³. The dodecamer was solvated with 77847 TIP4P/2005⁷⁴ water molecules and electro-neutralized with 262 Na⁺ and 178 Cl[−] ions in a rhombic dodecahedral box with periodic boundary conditions. After being solvated, the system was energy minimized and then equilibrated while restraining all the protein heavy atoms first in the NVT (T=300 K) ensemble for 1 ns and later in the NPT (P=1 bar, T=300 K) ensemble for 10 ns. Structural restraints on protein atoms were then released for the 1-μs production run in the NPT ensemble (P=1 bar, T=300 K). Constant temperature simulations were performed using by means of a stochastic thermostat⁷⁵ and Parrinello-Rahman scheme⁷⁶ was used for NPT runs. During all the simulations, the correct conformation of the catalytic site was preserved by artificially restraining the distances between the zinc ions and the coordinating oxygen/nitrogen atoms to their experimental values.

Results

High resolution MAS NMR of *ortho*- and *para*-CH Phe sites

TET2, formed by twelve copies of a 39 kDa subunit, is one of the largest systems studied quantitatively by NMR methodologies and to the best of our knowledge the largest system for which near-complete MAS NMR assignments have been achieved⁷³. The large number of aromatic sites represents a challenge for resolving individual sites. For high-resolution aromatic MAS NMR experiments, we labeled Phe residues specifically with an isolated ¹H-¹³C spin pair in an otherwise deuterated environment. The strong dilution of the ¹H-¹H dipolar-coupling network in such a sample as well as the removal of ¹³C-¹³C dipolar and scalar couplings leads to greatly enhanced sensitivity and resolution and facilitates artefact-free analyses of dynamics. Phe labeling at high levels is achieved by addition of suitably-labeled Phe, or its biosynthetic precursor, phenylpyruvate, to a ²H, ¹²C, ¹⁵N labeled expression medium^{24,58} (see Methods section for details). We explored two different labeling schemes, where ¹H-¹³C pairs are placed either at the C_ξ site (henceforth called *para*-CH), or the two C_δ sites (*ortho*-CH; see insert of Figure 1B). The two different moieties are sensitive to different types of Phe motion: the *para*-CH moiety is insensitive to ring rotations but senses motions of the Phe ring axis, arising as a consequence of χ_1 dihedral-angle fluctuations and backbone dynamics; the *ortho*-CH spin pairs are additionally re-oriented by ring flips (i.e., rotation around the χ_2 dihedral, see insert in Figure 1B).^{10–14}

The spectrum of *para*-CH shows ten well-resolved resonances, as expected for the ten Phe sites in TET2. The *ortho*-CH labeled sample only features four signals, while the remaining 6 sites are unobservable at temperatures between ca. 0 and 55°C, an observation that we ascribe to ring flips, as investigated further below.

The excellent resolution of these spectra opens the way to site-resolved studies of dynamics. Resonance assignment is necessary for site-specific studies, but far from trivial for a protein

of 39 kDa. We have recently achieved near-complete assignment of backbone $^1\text{H}^{\text{N}}$, ^{13}C , ^{15}N , as well as Ile- $\delta 1$, Leu- $\delta 1$, Val- $\gamma 1$ (ILV) methyl sites.^{73,77,78} However, we found that coherence transfer into the aromatic rings was inefficient, such that none of the Phe ^{13}C *ortho* or *para* carbons had been assigned. We employ here an assignment strategy that combines (i) experiments that probe the spatial proximity between the Phe hydrogens, ILV methyl groups and amide- ^1H spins, using the known structure⁶³ for assignment of cross-peaks and (ii) a mutagenesis-based assignment approach. For the former approach, we recorded a 3D ^1H - ^1H radiofrequency-driven recoupling⁷⁹ (RFDR) experiment with two simultaneously edited $^{13}\text{C}/^{15}\text{N}$ frequencies and ^1H detection, using a deuterated TET2 sample with ^1H spins at exchangeable sites (amides), $^{13}\text{CHD}_2$ -methyls on ILV residues and *para*-CH sites. The experiment simultaneously reveals distances between amide, methyl and aromatic sites; for the *ortho*-CH sample we collected 2D (^{13}C -filtered) and 3D (^{15}N -filtered) versions of the experiment. Examples of through-space contacts of the *para*-CH sites of F232 with amide sites in the vicinity are shown in Figure 1C, and displayed on the structure in Figure 1D. The full data set for *para*-CH is shown in Figure S3, and the corresponding data for the *ortho*-CH assignment are shown in Figure S4.

We complemented these experiments with a mutagenesis approach of *para*-CH samples. We obtained spectra of three TET2 mutant samples in which individual Phe residues were replaced by Ala, one at a time, and the disappearance of the peak corresponding to the mutated Phe allows straightforward assignment (Figure S3A). Using these approaches, we unambiguously assigned 8 out of 10 *para*-CH sites. The unambiguously assigned sites include the structurally and functionally most interesting sites located close to the substrate entry pore of TET2 (F84, F140) and trimerization interface (F224) at the apices of the tetrahedral assembly structure.

Phe rings in TET2 have a rigid ring axis

With these assignments at hand, we probed the dynamics of Phe side chains over a wide range of time scales, from picoseconds to milliseconds, using dipolar-coupling and nuclear spin relaxation measurements. The dipolar ^1H - ^{13}C couplings provide direct information about the amplitude of the motion of the CH bonds, averaged over time scales up to a few hundred μs .³⁶ Comparison of the measured dipolar-coupling tensor parameters compared to the tensor parameters in the absence of motion provides information about the amplitude and geometry of the motion. The amplitude is often expressed as order parameter S (ranging from 1 for rigid sites to 0 for fully flexible sites), as well as the geometry and asymmetry of the motion. The order parameter is obtained from comparing the tensor anisotropy δ_{meas}^D to the rigid-limit value anisotropy δ_{rigid} as $S = \delta_{\text{meas}}^D / \delta_{\text{rigid}}$. Motion without axial symmetry (i.e., lower than C3 symmetry) such as two-site ring flips, leads to an asymmetry of the resulting dipolar-coupling tensor,⁵⁶ and the detection of tensor asymmetry can thus be related to the geometry of the motion. We have recently shown that specific labeling, fast MAS (>50 kHz) and an optimized REDOR experiment allows measuring these tensor parameters at high precision and accuracy.⁵⁶

Figures 2A,B and S5 show REDOR-based^{52,80} dipolar-coupling measurements of the *para*-CH sites. We find high order parameters ($S > 0.9$) for all ten *para*-CH sites, indicating that the

ring axis of all phenylalanines in TET2 undergo only smallscale motions. The good fit of the simulated curves with a single fit parameter, S , and zero tensor asymmetry to the experimental data confirms that the motion can be described by a small-amplitude motion of the axis without significant population of alternative rotamer states around the $C\alpha$ - $C\beta$ axis. When describing the motion in the framework of the diffusion-in-a-cone model, the opening half-angle of the cone, θ , is 21° at most for the ten Phe sites ($S = 0.5 \cdot \cos \theta (1 + \cos \theta)$).^{81,82} This view of little motional freedom of the ring axes in TET2 is in good qualitative agreement with MD simulations described below.

Phenylalanine ring flips in TET2

The above-described *para*-CH REDOR data report only on ring-axis motion (i.e., motions around the side chain χ_1 dihedral angle and additional backbone motion), but not on ring flips around χ_2 . In contrast, the *ortho*-CH site are sensitive reporters of such motions. The *ortho*-CH Phe spectrum of TET2 (red in Figure 1B) shows only four cross-peaks, assigned to F26, F84, F176 and F232 (Figure S4). We performed 1H - ^{13}C dipolar coupling measurements to probe the motions of these observable *ortho*-CH sites. Rotations of the Phe ring by a half-turn reorient the two *ortho*-CH sites by 120° . The dynamically-averaged 1H - ^{13}C dipolar-coupling tensor resulting from fast ring flips has a reduced tensor anisotropy (order parameter $S=0.625$) and it is asymmetric⁵⁶ ($\eta=0.6$; see Figure S6 for illustration of the averaging). The experimental REDOR recoupling data, shown in Figure 2C, are in excellent agreement with the behavior expected for ring flips and small-amplitude local motion: first, the tensor anisotropy is considerably lower than the one of the *para*-CH sites, as evidenced immediately by the slower buildup of I/I_0 (compare the recoupling curves of Figures 2A and 2C). Second, the dipolar-coupling tensors are asymmetric: fits of REDOR curves that assume a symmetric dipolar-coupling tensor ($\eta=0$ and individually best-fit δ , grey curves in Figure 2C) lead to systematic deviations from the experimental data. Because the REDOR experiment has a built-in normalization taking into account the relaxation decay,⁸⁰ such deviations of the experimental data from the simulations can be ascribed to an asymmetric dipolar-coupling tensor due to the two-site jump.⁵⁶ We used two alternative descriptions of these REDOR data. In a first approach we fitted a general dipolar-coupling tensor with an anisotropy δ and asymmetry η (dashed red line in Figure 2C). The best-fit order parameters for the observed sites are ca. 0.5 (Figure 2D) and the asymmetry is $\eta=0.55$. Both values are slightly lower than those expected for jumps between the two ring orientations ($S=0.625$ and $\eta=0.6$), showing that additional motions are present on top of the ring flips, which reduce the dipolar-coupling asymmetry ($\eta<0.6$) and anisotropy ($S<0.62$).

For illustration purposes we also used an alternative description of the problem at hand. We simulated REDOR traces for a spin system undergoing explicit jumps between the two equally populated states, as well as an adjustable order parameter which reflects additional motion such as low-amplitude librations within each of the two ring orientations and motion of the backbone and the $C\alpha$ - $C\beta$ axis. This two-site jump simulation is also in good agreement with the experimental data when assuming an order parameter of ca. $S=0.8$ (Figure 2E). This value is slightly lower than the ones detected for the ring axis (i.e., the *para*-CH order parameters, Figure 2B) and also lower than the typically observed range for $C\alpha$ bond motion.⁸⁴ These observations suggest that the *ortho*-CH sites sense (i) ring flips,

(ii) motions of the ring axis, just as the *para*-CH site does, and (iii) reorientational motion of the ring within each of the two rotamer states. Taken together, the dipolar-coupling data unambiguously establish that the four visible sites undergo ring flips on time scales shorter than ca. 100 μ s, the time scale over which dipolar-coupling averaging is effective.³⁶

We used ^{13}C $R_{1\rho}$ relaxation measurements at different temperatures (25, 48°C) and spin-lock radio-frequency (RF) field strengths to estimate the time scales of Phe motion more precisely (Figure 2F,G,H). The $R_{1\rho}$ rate constant depends on the spectral density function describing the underlying motion, in particular the spectral density at frequencies corresponding to sums and differences of MAS frequency and spin-lock frequency (all in the tens of kHz range),³⁶ and it is therefore most sensitive to motions in the range from several nanoseconds to milliseconds (i.e. the inverse of these frequencies). The sloped solid lines in Figure 2H show calculated $R_{1\rho}$ rate constants for the simple 2-site ring-flip motion (obtained in this case by fitting numerical spin simulations of the fate of ^{13}C coherence under a spin-lock RF field and explicit jumps). These curves show that $R_{1\rho}$ is low when the motion is too fast (faster than ns) or too slow (slower than ms), and that the highest $R_{1\rho}$ arises when the motion is ca. 1 μ s. As $R_{1\rho}$ depends on spectral density functions at sums and differences of the spectral density function, the $R_{1\rho}$ rate constant is different when a different spin-lock RF field strength is used experimentally, which is shown by orange and green curves in Figure 2H. This difference is only visible when the motion occurs in the μ s-ms range, where the rate is close to this frequency difference ($\nu_{\text{MAS}} - \nu_{\text{RF}}$), i.e. only in the “right branch” of the curve. In addition, when increasing the temperature, the ring flips are expected to be accelerated, which means that $R_{1\rho}$ is either increased (when the motion occurred on the nanosecond time scale) or decreased (if it is in the μ s-ms range). Of note, these experiments exploit exclusively relaxation due to the dipolar coupling and ^{13}C CSA; fluctuations of the isotropic chemical shift are disregarded, because the strong spin-lock RF fields (15 kHz at least) suppress any effects that isotropic chemical-shift fluctuations may have. (Isotropic chemical-shift fluctuations are explored in Bloch-McConnell-type relaxation-dispersion experiments considered in the last part of this manuscript.)

Exploiting the dependency of the observed $R_{1\rho}$ rate constants on RF field and temperature (Figure 2F) allows us to unambiguously establish that the motion of the four *ortho*-CH sites occurs on the nanosecond time scale, and not on the μ s-ms time scales (Figure 2H). We used the ring flip rate constants from the $R_{1\rho}$ measurements to provide a rough estimate of the activation energies of ring flips, which is of the order of ca. 25-70 $\text{kJ}\cdot\text{mol}^{-1}$ (Figure 2I). We note that this estimation is based only on two temperatures, and it is, thus, to be considered as a rough approximation, but it is noteworthy that the values are in good agreement with previously reported values from solution-state NMR^{12,20,85,86} and MAS NMR^{28,87,88} of peptides and small proteins, which are in the range 37 to 90 $\text{kJ}\cdot\text{mol}^{-1}$. As expected from a decreased $R_{1\rho}$ rate constants at increasing temperature (see Figure 2F), the peaks of the four observable Phe sites sharpen up at higher temperature (Figure S8).

The *para*-CH sites, which are insensitive to ring flip motions and have restricted motion (Figure 2B), have $R_{1\rho}$ rate constants about an order of magnitude lower ($\sim 10 \text{ s}^{-1}$) than those of the *ortho*-CH sites (100-250 s^{-1}), as well as overall significantly lower R_1 rate constants (Figure 2F,G).

MD simulations provide a rationale for observed ring flips

As shown from the experimental data reported above, the four visible *ortho*-CH sites in TET2 are flipping on a nanosecond time scale; however the other six sites are unobservable around room temperature (from ca. 0 to 55°C). The signals of these sites maybe broadened by static disorder or dynamics. Given that all *para*-CH sites are observed, static disorder of the *ortho*-CH sites appears somewhat less likely. We speculated that they may be undergoing slower ring flips than the observable phenylalanines: large-amplitude motion on the μ s time scale would lead to strong *dipolar relaxation*; additionally, an *isotropic chemical-shift* difference between the two rotamer states would induce further line broadening.³⁶ Fast (sub-microsecond) ring flips, in contrast, should lead to more favorable line widths, rendering the peaks observable.

We sought to understand what discriminates the four observable sites from the unobservable ones and turned to all-atom molecular dynamics (MD) simulations of the entire dodecameric 468 kDa particle over a simulation time scale of 1 μ s. The simulation of a ~half-megadalton-large particle for time scales extending to microseconds is computationally costly; the explicit simulation of the twelve symmetric subunits improves the sampling statistics considerably, such that we effectively have a longer observation window. Figures 2J and S9 show the time traces and rotamer populations of the χ^2 angles of the Phe residues (ring flips), and Figure S10 displays the equivalent data for χ^1 dynamics (ring axis dynamics). Along the simulation, three of the four observable Phe rings (F26, F84, F232) undergo rapid ring flips on the tens-of-nanosecond time scale, and the fourth observable residue, F176, undergoes several jumps in our simulation window. The fact that the latter residue undergoes less frequent jumps coincides well with the observation that it has the lowest peak intensity. (High-ns to low- μ s motion induces the fastest transverse relaxation; cf. Figure 1H.) Four of the non-observable Phe sites (F103, F112, F140, F224) do not undergo any ring flips along the simulation and another two (F176, F206) undergo only one or two ring flips over the simulation time scale. Taken together, our experimental observations allow quantifying ring flip motions, and the MD simulations provide a rationale for all of the four observed and six unobserved signals. The MD simulation also shows that eight Phe rings in TET2 populate a single rotamer state, and two of them show rare excursions to an alternate conformation, a finding that is in good qualitative agreement with the high order parameters observed for the *para*-CH sites (Figure 2B).

Ring flips as those observed here and the associated line broadening, may be more generally an explanation for the fact that aromatic ring spin systems are often difficult to detect and assign by MAS NMR.⁸⁹

High-resolution low-temperature NMR and thermal activation of ring flips

The fact that the *ortho*-CH sites of six Phe residues are unobservable suggests motions on the μ s time scale. We reasoned that higher temperature would accelerate the ring flips such that the associated transverse relaxation rate constant would decrease. However, even at 55°C sample temperature we were unable to detect additional cross-peaks (Figure S7), suggesting that in the accessible temperature range the motion cannot be accelerated sufficiently. We, thus, used a low-temperature MAS NMR setup to cool the sample to 100 K,

at a MAS frequency of 40 kHz in a 1.3 mm rotor. Figure 3A shows ^1H - ^{13}C correlation spectra at six temperatures over this range. While at 280 K only four *ortho*-CH cross-peaks are seen, the complexity of the spectrum is greatly increased at temperatures of 240 K and below, with at least 10 distinguishable peaks at 100 K. Although we do not have assignments of these peaks, the observation of a large number of peaks strongly suggests that the aromatic ring flips are slowed down sufficiently to shift them out of the μs time window where the motion induces fast transverse relaxation. Remarkably, the line widths remain similar to those at room temperature, all the way down to 100 K. This is in stark contrast to the spectra of amides, which are severely broadened at low temperature, hampering site-specific observation (Figure S11).

We used temperature-dependent *ortho*-CH ^{13}C spin relaxation measurements to study the thermal activation of the Phe ring flips (Figure 3B,C). Systematic analyses of NMR relaxation rate constants have been used previously to obtain activation energies of protein motions from one-dimensional spectra without site-specific resolution.^{34,35} While in principle the quantitative analysis requires that individual sites are measured at variable temperatures, such an analysis is complicated by the fact that the *ortho*-CH spectra become complex and overlapped at low temperature, and have not been assigned here. In 1D spectra the relative contributions of different phenylalanine sites to the NMR intensity varies greatly with temperature, as illustrated by the fact that only 4 out of 10 Phe rings contribute at all to the signal at ambient temperature. Interpreting 1D integrals over the entire temperature range, thus, necessarily induces some bias, hampering quantitative analysis. We therefore analyze the data only qualitatively. Both ^{13}C R1 and $R_{1\rho}$ show a biphasic temperature dependence, with very low relaxation rates at low temperatures and a strong increase at temperatures at ca. 220-240 K ($R_{1\rho}$) or 240-280 K (R1). Qualitatively, this behavior is expected for a model where ring flips and small-amplitude motions – librations within each of the rotamer states – coexist. At low temperatures the ring flips are expected to be very rare and they do not contribute to spin relaxation, such that both R1 and $R_{1\rho}$ are low. The onset of spin flips is observed by increased relaxation; hereby, the raise of $R_{1\rho}$ is observed at lower temperatures than the one of R1. This behavior is expected, because $R_{1\rho}$ senses slower motions (tens-of-ns to ms, see Figure 2H), while sizeable R1 relaxation is induced only when the motions are in the ns range. Our data indicate, qualitatively, that ring flips enter the ms- μs regime at temperatures of ca. 220-240 K, and are accelerated to the ns regime above 240 K. The temperature of this apparent onset of aromatic ring flips is in good agreement with a dynamical transition observed by Mössbauer spectroscopy,⁹⁰ neutron scattering,⁹¹ MD simulations,⁹² x-ray crystallography⁹³ and NMR.^{34,94}

The remarkably favorable line widths of aromatic sites found at low temperature point out a strong potential of this labeling scheme for dynamic nuclear polarization experiments, where line widths of backbone sites may otherwise become broad (cf. Figure S11).

Relaxation-dispersion experiments reveal short-lived (μs) states at the entry pore and trimer interface

Having studied in detail ring flips and ring-axis motions, occurring primarily on sub-microsecond time scales, we explored the potential of the labeling approach to unravel short-

lived alternate conformations by relaxation-dispersion NMR. Many functional processes in proteins, such as enzymatic catalysis^{95,96} or allosteric signal transduction^{97,98} are linked to the presence of transient “active” functional states in dynamic equilibrium with the “ground” state. In solution-state NMR, a well-established arsenal of methods for detecting such motions has been developed, in particular relaxation-dispersion (RD) experiments.^{2,99,100} In MAS NMR, RD methodologies have been introduced only recently.^{43,101–104} The primary experimental challenge for measuring RD MAS NMR data is the presence of coherent decay mechanisms (dipolar dephasing) which contribute to the observed spin relaxation, thereby complicating the extraction of parameters reflecting dynamics. Deuteration and high MAS frequencies were proposed as solutions for ¹⁵N Carr-Purcell-Meiboom-Gill (CPMG) RD¹⁰¹ and ¹⁵N R_{1ρ} RD experiments,^{43,102,103} but to our knowledge these approaches have not been extended to ¹³C RD experiments.

Figure 4 displays ¹³C R_{1ρ} RD profiles for *para*-CH sites in TET2. The majority of these sites (7 out of 10) display flat RD profiles over the RF field range (2 to 15 kHz; see Figures 4C and S12). Because μs motion would lead to non-flat RD profiles, if the motion modulates the isotropic chemical shift of the involved sites, these flat curves suggest that these sites do not undergo sizeable motion on the μs time scale. The observation of flat RD profiles for the majority of sites furthermore indicates that dipolar-dephasing contributions to these curves are efficiently suppressed with our approach, even at low RF field strengths. The finding that these artefactual contributions to RD profiles can be suppressed efficiently using deuteration and fast MAS mirrors findings in ¹⁵N RD experiments in deuterated proteins.¹⁰² Flat RD profiles without motion are required for quantitatively detecting μs motions. Three *para*-CH sites – F224, F84 and, to lower extent, F140, in immediate vicinity to F84 – show pronounced non-flat RD profiles, revealing μs-ms dynamics. These phenylalanines are located in two structurally and possibly functionally important parts of TET2: F224 is located at the trimer interface and occludes the pore between the neighboring subunits, which has been proposed as a possible product exit from the catalytic chamber.⁶⁴ F84 is located halfway between the substrate entry gate and the active site (Figure 4D,E). We have fitted a two-site exchange model to the RD data of F84, and of F224 and find an exchange rate constant of ca. $9800 \pm 3800 \text{ s}^{-1}$ and $11500 \pm 5400 \text{ s}^{-1}$ for these sites, respectively.

The above dipolar-coupling measurements (Figure 2B) have shown that these three Phe sites have a rigid axis over time scales up to a few hundred μs, i.e., including the time scale for which these relaxation-dispersion measurements detect motion. However, dipolar-coupling measurements report on the dipolar-coupling tensor averaged over all orientations, such that conformations populated to only a few percent would hardly be detectable in dipolar-coupling measurements. The combination of both views, i.e., the observation of high dipolar-coupling order parameters and pronounced relaxation-dispersion effects suggests that the observed RD profiles are due to exchange with a low-populated state, which might also involve alternate conformations of the backbone and other surrounding side chains. While the functional implications of these states are not within the scope of this study, they may be important for entry of substrates to the catalytic chamber (F84, F140), or product exit via the proposed pore that is occluded in the crystal structure by F224. We performed enzymatic activity experiments to assay the importance of F224 for product release from the cavity; the rationale is that if there is a transiently formed pore at the trimer interface (see

Figure 4D), opened by movement of F224, and if this pore enables release of products from the catalytic chamber, as proposed,⁶⁴ then the replacement of F224 by a very small (Ala) or large (Trp) residue would change the rate of product release. However, we find that the identity of this side chain (Phe, Ala or Trp) has negligible effects on the overall enzymatic activity (Figure S13), indicating either that the product release is not rate-limiting or that the possibly forming transient pore is not involved in release. Current work is directed towards deciphering the functional importance of the conformational-exchange process probed by the phenylalanines.

Discussion

We have demonstrated here the use of a specific Phe labelling scheme for MAS NMR, and shown its power to provide detailed insight into multiple types and time scales of Phe dynamics. Outstanding resolution, with ^1H (^{13}C) line widths of the order of ca. 35-50 Hz (^1H) and 30-45 Hz (^{13}C) has enabled site-specific assignment of Phe residues in TET2, one of the largest proteins studied to date by MAS NMR, and to our knowledge the largest protein, in terms of subunit size, for which near-complete assignments have been reported. Our study demonstrates that the presented approach can provide quantitative in-depth views of dynamics even in large and complex systems. We find that this specific labelling results in much superior spectral quality compared to fully protonated samples: we collected a ^1H - ^{13}C correlation spectrum of uniformly ^{13}C , ^{15}N -labeled TET2 at a MAS frequency of 100 kHz, which shows significantly larger linewidths than the specifically labeled sample, more cross-peaks and accordingly stronger signal overlap (Figure 5). The deuteration labeling scheme employed here will additionally benefit from higher MAS frequencies, as suggested by recent reports on deuterated, methyl-labeled proteins.¹⁰⁶ Additional biosynthetic precursors for labeling other aromatic sites, such as Tyr, His or Trp, are available,^{24,59,107} and may provide important insight also into more hydrophilic environments, such as membrane pores.

This study focused on the use of the specific Phe labels for the study of dynamics. The well-isolated ^1H - ^{13}C spin systems enable several experiments in a quantitative manner, which would be challenging in fully labeled systems because of the abundant dipole-dipole interactions:

- (i) relaxation-dispersion MAS NMR experiments, shown here for the first time with ^{13}C spins, probe exchange involving minor conformations exchanging on the μs -ms time scale. The usefulness of the RD technique to characterize in detail states with populations of a few percent has been shown previously for ^{15}N -based experiments in solids,^{103,104,109,110} and has an impressive track record in solution-state NMR.^{100,111} Like in solution-NMR, RD experiments would also enable the study of ring flips occurring on this time scale, as long as the two exchanging states have significantly different chemical shifts. In addition to RD experiments that exploit the fluctuation of isotropic chemical shifts, near-rotary resonance RD experiments (NERRD) can detect μs motion even in the absence of chemical-shift exchange.^{102,103,112}

- (ii) Dipolar-coupling tensors can be probed with high precision and accuracy using REDOR experiments, including the asymmetry of the dipolar tensor, which provides direct insight into the rotamer jumps, as exemplified here for ring flips. Although none of the ring axes in TET2 undergoes rotamer transitions (around χ^1), asymmetric dipolar-coupling tensors would also reveal such jumps, akin to the case of methyl side chains we reported earlier.⁵⁶
- (iii) Finally, the suppression of coherent effects (dipolar dephasing, spin diffusion, J-coupling evolution) allows accurate measurements of nuclear spin relaxation rate constants, additionally shedding light on the time scales of motion. Using such experiments at low temperature, we were able to reveal the onset of Phe ring flips, which enter the μ s-ms range at around 200 K.

Conclusion

We have shown here that selective introduction of ^1H - ^{13}C spin pairs, in the present case into phenylalanine rings, combined with deuteration and ^1H -detected MAS NMR provides key insights into various motional processes. Selective protonation of other residue types in otherwise protonated proteins will allow extending this approach in a straightforward manner to, e.g. tyrosine, tryptophane or histidine, opening avenues to study their motions over many time scales at a similar level of detail as presented here. Although not the focus of the present study, the labelling scheme allows the detection of distance restraints to aromatic residues, as highlighted here with 13 distance restraints obtained to amides and methyl groups in TET2. Such restraints in the hydrophobic core of the protein often prove as highly valuable for structure determination.^{24,113} We are confident that the proposed labeling scheme in combination with high MAS frequencies (currently up to 111 kHz, and to increase within the next years) will prove very useful to study protein structure, interactions and dynamics essentially without limitations on molecular weight, possibly also coupled to DNP enhancement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proton-detected correlation spectra of the 468 kDa aminopeptidase TET2. (A) Cartoon representation of TET2; different subunits (39 kDa each) are shown in different colors. (B) ^1H - ^{13}C dipolar-coupling based 2D correlation spectra of para- ^1H - ^{13}C (blue) and ortho- ^1H - ^{13}C (red) Phe-labeled TET2 samples, obtained at 55 kHz MAS and a static magnetic field of 14.1 T. The assignment was obtained from mutagenesis and RFDR experiments. Typical line widths are of the order of ~ 35 Hz in ^1H and ~ 30 Hz in ^{13}C . (C) Example strips from through-space ^1H - ^1H RFDR TET2 with residue-specific assignments, obtained from a three-dimensional H-(C/N)-(C/N) experiment with simultaneous $^{13}\text{C}/^{15}\text{N}$ frequency editing in indirect dimensions, applied to a sample labeled on para-CH sites and Ile, Val, Leu $^{13}\text{CHD}_2$ methyl groups. All pulse sequences used in this study are shown in Figure S2. All detected distance restraints involving *para*-CH sites and *ortho*-CH sites are shown in Figures S2 and S3, respectively.

Figure 2.

Sub-microsecond dynamics of Phe residues in TET2 probed at *para*-CH (blue) and *ortho*-CH (red) sites using ^1H - ^{13}C dipolar couplings measured with time-shifted REDOR^{52,80} (A-E), and ^{13}C relaxation measurements (F-I). (A,C), representative REDOR dephasing curves, and best-fit simulations assuming either an axially symmetric tensor with a single fitted parameter, δ (grey), or a two-parameter fit of a tensor with anisotropy δ and asymmetry η (dashed red in C), or a two-site jump model by 120° (cf. panel I), and an additional fitted order parameter (solid red in C). (B,D,E) Fitted order parameters S ; for these calculations we assumed a rigid-limit C-H distance of $1.09 \text{ \AA}$.⁵⁷ All REDOR curves are shown in Figure S5. (F,G) Experimental ^{13}C $R_{1\rho}$ and R_1 rate constants. (H) The use of ^{13}C $R_{1\rho}$ rate constants to determine time scales of two-site jump motions, exemplified for *ortho*-CH site F232. The horizontal lines show the three experimentally measured rate constants, highlighted in bold face in panel (F). Sloped lines represent the theoretical $R_{1\rho}$ rate constants, calculated for the two-site jump model occurring at different exchange rate constants. These theoretical curves have been obtained from numerical spin simulations of the jumps in the GAMMA⁶⁹ package, as used and described earlier⁸³. Each experimentally determined $R_{1\rho}$ (horizontal lines) intersects the theoretical curve at two different rate constants, either on nanosecond (“left branch”) or micro-millisecond (“right branch”) time scales. Assuming that flips of a particular Phe can be described by one flip rate constant at a given temperature, then only a rate constant on the “fast” branch (ns), can explain all experimental data. This rate constant is shown for two temperatures for the case of F232 in panel (H). (I) Motional model, and estimated activation energy of two-site jump, using the rationale outlined in panel (H) and the two temperatures. Note that the activation energies are rough estimates, based on only two temperatures. (J) MD simulations of ring flips of three representative Phe in TET2.

Shown are the time traces of the twelve subunits in the dodecameric assembly. Two colors have been used to differentiate data pertaining to different subunits. The MD data for all Phe sites are shown in Figure S9 (χ^2 angle) and Figure S10 (χ^1 angle).

Figure 3.

Low-temperature MAS NMR measurements of *ortho*-CH sites in u - $[\text{}^2\text{H}, \text{}^{15}\text{N}]$, Phe-*ortho*- ^{13}C ^1H -labeled TET2. (A) CP-based ^1H - ^{13}C correlation spectra at temperatures down to 100 K (as indicated) and a MAS frequency of 40 kHz. (B,C) Temperature-dependent ^{13}C $R_{1\rho}$ and R_1 relaxation rate constants from one-di-mensional ^1H -detected measurements, using the pulse sequence shown in Figure S2D,E.

Figure 4.

Microsecond dynamics in TET2 probed by ^{13}C Bloch-McConnell $R_{1\rho}$ relaxation dispersion MAS NMR data of para-CH sites (A-C). Sizeable (non-flat) RD profiles for residues F224 (A) and F84 (B) point to microsecond dynamics in the trimerization interface (D) and entry pore (E), respectively. Pictorial representations of the dodecamer in the respective orientations are shown in (D) and (E). The solid lines show numerical fits to a two-site exchange model using the program *relax*.¹⁰⁵ The fitted exchange rate constants k_{ex} for F84 and F224 are $9800 \pm 3800 \text{ s}^{-1}$ and $11500 \pm 5400 \text{ s}^{-1}$ and the exchange parameters $\phi_{\text{ex}} =$

$p_{AB}(2\pi\delta)^2$ are below 1 ppm² in both cases. All *para*-CH ¹³C R_{1ρ} RD profiles are shown in Figure S12.

Figure 5.

Comparison of ^1H - ^{13}C correlation spectra obtained from deuterated, specifically Phe-labelled TET2 at 55 kHz MAS and of protonated, ^{13}C , ^{15}N -labelled TET2 at 103 kHz MAS frequency. The simplification of the spectra of the specifically labelled samples are due to narrower line widths, resulting from the removal of ^{13}C - ^{13}C scalar couplings and the better suppression of dipolar line broadening due to surrounding proton spins, and the absence of correlations other than those of *ortho*-CH or *para*-CH sites. Note that the spectrum at 103

kHz MAS was at an effective sample temperature of ca. 35°C, i.e., ca. 7°C higher than the ones collected at 55 kHz, which likely explains differences in peak positions.