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Structural studies of proteins by paramagnetic solid-state NMR spectroscopy

Christopher P. Jaroniec*

Department of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210, USA

Abstract

Paramagnetism-based nuclear pseudocontact shifts and spin relaxation enhancements contain a wealth of information in solid-state NMR spectra about electron-nucleus distances on the ~20 Å length scale, far beyond that normally probed through measurements of nuclear dipolar couplings. Such data are especially vital in the context of structural studies of proteins and other biological molecules that suffer from a sparse number of experimentally-accessible atomic distances constraining their three-dimensional fold or intermolecular interactions. This perspective provides a brief overview of the recent developments and applications of paramagnetic magic-angle spinning NMR to biological systems, with primary focus on the investigations of metalloproteins and natively diamagnetic proteins modified with covalent paramagnetic tags.

Keywords

Solid-state NMR; Magic-angle spinning; Paramagnetic relaxation enhancement; Pseudocontact shift; Protein structure

1. Introduction

Biological magic-angle spinning (MAS) solid-state NMR spectroscopy has matured tremendously throughout the last decade or so, yielding unprecedented structural, dynamic and mechanistic insights for a broad range of biomacromolecules and macromolecular assemblies as discussed in detail in comprehensive reviews [1–22]. While this rapid growth continues apace, for many biological systems the solid-state NMR studies aimed at elucidating the tertiary, quaternary or supramolecular structure are hampered by a limited number of distance restraints on the order of ~5–10 Å that can be derived from measurements of weak dipolar couplings among ¹H, ¹³C, ¹⁵N and other nuclei [23–31].

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*Tel.: +1 614 247 4284; Fax: +1 614 292 1685; jaroniec@chemistry.ohio-state.edu.

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While the magnitudes of nuclear gyromagnetic ratios place a fundamental limit (of approximately 10 Å) on the range of distances that can realistically be probed in practice using conventional dipolar coupling based solid-state NMR approaches, as well as on the quality and quantity of such long distance restraints due to low intensities of the associated resonances in multidimensional correlation spectra, both the length scale and number of accessible structural restraints may be considerably increased by taking advantage of the presence of native or nonnative paramagnetic centers in the biological system of interest. Such paramagnetic centers cause hyperfine interactions between the unpaired electron spins and surrounding nuclei, which far exceed in magnitude the internuclear dipolar couplings, and manifest themselves in NMR spectra as electron-nucleus distance dependent pseudocontact shifts (PCSs) [32] and/or paramagnetic relaxation enhancements (PREs) [33] of the nuclear spins that are detectable for distances on the order of ~10-20 Å and beyond [34]. Fig. 1 illustrates conceptually how site-resolved nuclear PRE and PCS phenomena can be measured in proteins using two-dimensional NMR.

In contrast to solution NMR, where pseudocontact shifts and paramagnetic relaxation enhancements have been utilized successfully for many years to study biomacromolecular structure and interactions [35–39], until quite recently, solid-state NMR studies of paramagnetic systems have focused nearly exclusively on small inorganic compounds [40–53] with only a few early investigations aimed at selectively isotope labeled peptides and proteins [54–63]. This trend began to shift dramatically around 2007, however, with the initial reports describing applications of MAS solid-state NMR to uniformly ^{13}C , ^{15}N -enriched paramagnetic proteins, including the resonance assignments of Cu(II)-Zn(II) superoxide dismutase (SOD) [64], measurement of ^{13}C PCSs in a domain of a matrix metalloproteinase-12 (MMP-12) loaded with Co(II) in place of the native Zn(II) [65], and observation of residue-specific transverse PREs in nitroxide spin-labeled analogs of the B1 immunoglobulin binding domain of protein G (GB1) [66]. Since that time, the unique, long-range nature of these paramagnetic phenomena has opened up multiple avenues for solid-state NMR protein structure determination, characterization of molecular interfaces and interactions, as well as fast acquisition of solid-state NMR data, and many of these new developments have been discussed in prior reviews [67–73]. This perspective focuses on the recent advances in application of paramagnetic MAS solid-state NMR to the structural studies of metalloproteins and natively diamagnetic proteins modified with paramagnetic tags.

2. Paramagnetic effects in solid-state NMR spectra

For paramagnetic metal ions with anisotropic magnetic susceptibility tensors, such as Co^{2+} and most lanthanides other than Gd^{3+} , the PCSs of NMR signals for a polycrystalline solid sample undergoing MAS have the same form as in solution [34,68–70]:

$$\delta^{\text{PCS}} = \frac{1}{12\pi r^3} \left[\Delta\chi_{ax}(3\cos^2\theta - 1) + \frac{3}{2}\Delta\chi_{rh}\sin^2\theta\cos 2\varphi \right] \quad (1)$$

where r is the electron-nucleus distance, χ_{ax} and χ_{rh} are the axial and rhombic components of the magnetic susceptibility tensor of the metal, and angles θ and φ describe

the orientation of the electron-nucleus vector in the frame of the magnetic susceptibility tensor. The PCS magnitude is independent of nucleus type, with typical values ranging from a few tens of a ppm to several ppm—furthermore, due to its orientational dependence the PCS can be positive or negative. It is also worth noting that for nuclei located in immediate proximity to the paramagnetic center, a non-zero electron spin density at the nucleus can lead to additional Fermi contact shifts of the NMR signals [32,34]. While these contact shifts can far exceed the PCS contributions for the affected nuclei, they are generally negligible beyond the coordination sphere of the metal ion and hence of limited utility in the context of long distance measurements in biomolecules.

The presence of a paramagnetic center in the molecule of interest also invariably leads to enhanced relaxation rates of the nuclear spins due to the modulation of electron-nucleus dipolar couplings [34]. In the solid phase this modulation is largely associated with electron spin relaxation (described to a reasonable approximation by the longitudinal relaxation time constant T_{1e}), and the longitudinal (Γ_1) and transverse (Γ_2 or $\Gamma_{1\rho}$) nuclear PREs can be expressed as follows [68–70]:

$$\Gamma_1 \approx \frac{2C}{r^6} \left(\frac{3T_{1e}}{1+\omega_n^2 T_{1e}^2} + \frac{7T_{1e}}{1+\omega_e^2 T_{1e}^2} \right) \quad \Gamma_2 \approx \Gamma_{1\rho} \approx \frac{C}{r^6} \left(4T_{1e} + \frac{3T_{1e}}{1+\omega_n^2 T_{1e}^2} + \frac{13T_{1e}}{1+\omega_e^2 T_{1e}^2} \right) \quad (2)$$

where r is the electron-nucleus distance, ω_n and ω_e are the nuclear and electron Larmor frequencies, and C a pre-factor that depends on fundamental constants, the nuclear gyromagnetic ratio and the spin quantum number for the paramagnetic center [68–70]. For centers with isotropic or nearly isotropic magnetic susceptibility, including nitroxides and Mn^{2+} , Cu^{2+} and Gd^{3+} ions, longitudinal and/or transverse PREs constitute the primary paramagnetic effects while the PCS contributions are negligible. Importantly, notably absent in the solid state [74] is the Curie relaxation mechanism [75,76], which stems from the interaction between the nuclei and the average magnetic moment of the paramagnetic center and which can dominate transverse nuclear spin relaxation in soluble biomacromolecules containing rapidly relaxing metal ions [34].

3. Applications to structural studies of metalloproteins

The first MAS solid-state NMR measurements of ^{13}C PCSs in the 159 amino acid MMP-12 by Bertini, Luchinat and co-workers [65] are shown in Fig. 2a. Nearly 250 experimental shifts, in the range of -3 to 3 ppm, could be detected for nuclei located up to ~ 20 Å from the paramagnetic Co^{2+} center. These PCSs were found to be in good agreement with the corresponding shifts calculated using the known three-dimensional structure and magnetic susceptibility tensor parameters for Co(II) -MMP-12. Notably, a small number of the experimental PCSs displayed substantial deviations from the calculated values, which could be quantitatively accounted for by considering the contributions to the PCS from several Co^{2+} ions in neighboring protein molecules within the crystal lattice [65]. Subsequent studies demonstrated that the intra- and intermolecular PCSs could be determined independently using MMP-12 microcrystals consisting of physical mixtures of Co(II) and Zn(II) proteins to yield valuable structural restraints [77], and that rapid (~ 60 kHz) MAS permits paramagnetic shifts to be quantified for nuclei as close as ~ 6 Å to the Co^{2+} center

(Fig. 2b and 2c) [78]. Interestingly, the latter study showed that for ^{13}C nuclei located 6.2 Å or more from the Co^{2+} site the calculated PCS values were a close match to the experimental shifts (Fig. 2b). On the other hand, considerable discrepancies were found between the experimental and calculated shifts for the detectable nuclei belonging to histidine residues coordinating the metal (Fig. 2c), and attributed to a combination of the Fermi contact contribution stemming from partial delocalization of the Co^{2+} unpaired electrons onto the coordinating ligands and a possible breakdown of the pointdipole approximation implicit to PCS calculations using Eq. (1) [78].

The use of PCS restraints in protein structure determination was initially demonstrated by Bertini and co-workers for MMP-12 [79], where in addition to yielding long distance information these data were also found to be useful for resolving ambiguities in resonance assignments and conventional nuclear distances. The data set used to derive the protein structural ensemble (Fig. 2d) consisted of over 300 ^{13}C PCSs for the Co(II) protein and ~800 unambiguous ^{13}C - ^{13}C and ^1H - ^1H distance restraints extracted with the help of PCS data, supplemented by chemical-shift based backbone dihedral restraints. Remarkably, Luchinat et al. [80] recently showed that by supplementing the internuclear distance and total (i.e., combined intra- and intermolecular) PCS restraints measured for an undiluted paramagnetic Co(II) -MMP-12 sample with unit cell parameters available from powder X-ray diffraction it is possible to determine both the protein structure and its packing within the crystal lattice (Fig. 2e).

The MAS solid-state NMR studies of another microcrystalline paramagnetic metalloprotein, human Cu(II) - Zn(II) SOD, initiated by Pintacuda, Emsley, Bertini and coworkers [64] demonstrated that nearly complete ^{13}C and ^{15}N resonance assignments for this 153-residue protein could be established and revealed that NMR signals from nuclei located as close as ~5 Å to the Cu^{2+} center could be detected. The use of protein perdeuteration followed by complete reprotonation of the exchangeable amide protons combined with 60 kHz MAS [81–84] enabled very high resolution and sensitivity ^{15}N - ^1H spectra to be recorded for Cu - Zn SOD in both the Cu^+ (diamagnetic) and Cu^{2+} (paramagnetic) oxidation states (Fig. 3a), which in turn facilitated the determination of residue-specific longitudinal relaxation rates for backbone ^{15}N and ^{13}CO nuclei (Fig. 3b and 3c) [85]. Altogether, these experiments furnished a set of ~200 PRE restraints suitable for use in protein structure refinement, consisting primarily of quantitative longitudinal ^{15}N and ^{13}CO PREs corresponding to $^{15}\text{N}/^{13}\text{CO}$ - Cu distances of ~10–20 Å (obtained as differences between the relaxation rate constants measured for Cu(II) - Zn and Cu(I) - Zn SOD) supplemented by 25 upper distance restraints for residues with resonances observable in spectra of Cu(I) - Zn SOD but not the Cu(II) protein [85].

A complementary study [86] demonstrated that it is possible for SOD to exchange the metal ions, resulting in a protein having one metal binding site occupied by Co(II) with the second site being empty, and to use this Co(II) -SOD sample to determine site-specific PCS restraints. With the aid of perdeuteration, rapid MAS and ^1H -detected triple-resonance spectra, a set of ~450 PCSs could then be determined including, for the first time, amide ^1H paramagnetic shifts [86]. With large sets of conventional ^1H - ^1H distances (~300 total derived from 3D HNN spectra) [84], and paramagnetic PRE and PCS restraints available for

SOD it became possible to quantitatively assess the effect of different types of restraints on the quality of the resulting NMR structural ensembles as illustrated in Fig. 4. Specifically, it was found that when used independently the PRE (Fig. 4b) or PCS (Fig. 4c) restraints are able improve the precision of the protein structure by roughly a factor of two relative to that derived using ^1H - ^1H distances alone (Fig. 4a), with the backbone atom RMSD being reduced from 3.1 Å without any paramagnetic restraints to 1.6 Å and 1.7 Å with PREs or PCSs, respectively. The simultaneous use of both PRE and PCS data results in further improvement in the structure quality (backbone RMSD of 1.4 Å), with the protein backbone conformation in the vicinity of the metal binding sites being particularly well-defined (Fig. 4d).

4. Applications to structural studies of natively diamagnetic proteins

The successful measurements of site-specific solid-state NMR PREs and PCSs in uniformly ^{13}C , ^{15}N -labeled paramagnetic metalloproteins and their use in the context of protein structure refinement naturally raise the possibility of extending these investigations to natively diamagnetic proteins, where the paramagnetic centers are introduced in the form of covalent tags (Fig. 5). Indeed, such studies were commenced by our group [66] concurrently with the metalloprotein work described above, and subsequently pursued by us [73,87–91] and others [92,93]. The fundamental approach was initially demonstrated for a model 56-residue globular protein GB1, where solvent-accessible residues were mutated to cysteines and tagged with a nitroxide spin label (Fig. 5a) [66]. Additionally, given the small size and dense crystal packing of GB1, the ^{13}C , ^{15}N -labeled paramagnetic proteins in these studies were diluted to a mole fraction of ~25% by co-precipitation with natural abundance diamagnetic protein in order to attenuate the intermolecular electron-nucleus couplings.

For nitroxide radicals present within hydrated proteins at ambient temperature, the relatively long electron spin relaxation time ($T_{1e} \approx 100$ ns) [34,36] generates considerable transverse nuclear PREs in the solid phase and these effects could be detected on a residue-specific basis by monitoring the NMR signal intensities in 2D ^{15}N - ^{13}C chemical shift correlation spectra. These experiments revealed that for amino acids located within ~10–12 Å of the radical the cross-peak intensities were significantly reduced or suppressed altogether, largely due to rapid amide proton relaxation during the initial ^1H - ^{15}N cross-polarization period. For more distant nuclei the electron-nucleus distances could be estimated by monitoring the relative signal intensities, with significant effects observed for distances up to ~20 Å (Fig. 6a and 6b). Although rather qualitative in nature these transverse PRE based restraints are capable of yielding valuable information about the protein fold and intermolecular interactions on a length scale that is not accessible by conventional means. An excellent recent demonstration of the utility of this spin-labeling based solid-state NMR approach for probing intermolecular contacts in large proteins has been provided by Ladizhansky and co-workers [92], who were able to use it to determine the oligomeric assembly of a seven-helix sensory rhodopsin in a membrane environment (Fig. 6c).

The direct correlation between the electron spin relaxation (T_{1e}) and the transverse PRE magnitude in the solid state (c.f., Eq. (2)) indicates that this phenomenon may be quenched by employing more rapidly relaxing paramagnetic species. The latter can be highly

The ability to record long distance PRE data for multiple paramagnetic mutants of the target protein opens up the possibility of determining its three-dimensional structure in a *de novo* manner in the absence of conventional internuclear distance restraints. This was pursued in a recent study by our group [90] that employed six mutants of GB1 containing Cys-EDTA-Cu²⁺ side-chains distributed throughout the protein at surface residues 8, 19, 28, 42, 46 and 53. Collectively, a set of 231 longitudinal ¹⁵N PREs could be determined (Fig. 7c), corresponding to ~4-5 restraints per amino acid residue on average—it is noteworthy that, due to their long range nature, even a fairly limited set of these PRE restraints provides extensive coverage of the protein structure. About half of the measured PREs exceeded 0.1 s⁻¹ and were used directly in the course of the structure calculations, while the remaining PREs were converted to lower-limit distance restraints in order to improve the convergence of the calculation procedure. In Fig. 8 we demonstrate the utility of these ¹⁵N PRE data for elucidating the three-dimensional fold of GB1. Most remarkably, these results show that by using a largely unrestricted calculation protocol that includes the PREs and chemical-shift based backbone torsion angles as the only experimental restraints it is possible to determine a protein fold that is in good agreement with the high-resolution crystal structure (RMSD of 1.8 Å for the backbone atom coordinates) (Fig.8b and 8c).

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shown in Fig. 5c [91]), which offer significant advantages over the longer and more flexible EDTA-based tags including the generation of more pronounced nuclear PRE effects and reduction of the uncertainty in the position of the metal ion with respect to the protein. Recently, the measurements of solid-state NMR PCS restraints for several GB1 mutants modified with the 4MMDPA tag [94] (Fig. 5d) loaded with Co^{2+} and several lanthanides, as well as their use in three-dimensional protein fold determination using a Rosetta based approach [95] have also been demonstrated by Yang and co-workers [93]. The latter study found that for each GB1 analogue investigated the use of ~70–100 PCS restraints per mutant resulted in the lowest energy PCS-Rosetta protein models exhibiting backbone RMSDs on the order of ~1 Å relative to the high-resolution X-ray structure.

5. Concluding remarks

In this perspective we have outlined the recent progress in protein structural analysis by paramagnetic solid-state NMR. The ability to detect and quantify a multitude of site-specific nuclear PCSs and PREs in multidimensional MAS NMR spectra coupled with the long-range nature of these phenomena has already enabled, in a relatively short period of time, an array of applications ranging from structure refinement of metalloproteins to *de novo* structure determination of natively diamagnetic proteins tagged with paramagnetic side-chains and characterization of intermolecular contacts in a large membrane protein complex. Furthermore, although not elaborated upon here in detail, the solid-state NMR studies of biological samples containing extrinsic or covalently-bound paramagnetic centers are inherently compatible with condensed data acquisition schemes that yield spectra with enhanced sensitivity [88,96–103], and have also been utilized in a host of other applications including the characterization of Cu^{2+} ion binding to amyloid peptide assemblies [104,105], solvent-accessibility of peptide and protein surfaces [59,97,106,107], and architecture of membrane-bound small molecule aggregates [108]. Given the rapid pace of advances in paramagnetic solid-state NMR, numerous new applications of this methodology are likely to emerge in the coming years.

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Highlights

- Protein magic-angle spinning solid-state NMR
- Measurements of paramagnetic nuclear pseudocontact shifts and relaxation enhancements
- Structure determination of paramagnetic metalloproteins and diamagnetic proteins modified with covalent paramagnetic tags

Fig. 1. Schematic two-dimensional NMR chemical shift correlation spectra for a diamagnetic protein (a) and two of its structural analogs, each containing at a specific location a paramagnetic center with either an isotropic (b) or an anisotropic (c) magnetic susceptibility tensor. For the isotropic or nearly isotropic paramagnetic species (e.g., nitroxide spin label, Mn^{2+} or Cu^{2+}) in panel (b), the intensities of NMR signals (green and blue contours) are modulated by longitudinal and/or transverse PREs according to the proximity of the corresponding nuclear spins (green and blue spheres, denoted n) to the paramagnetic center

(red sphere, denoted e^-). The PRE magnitude, represented by the large sphere around the paramagnetic center, decreases from red to blue. For the anisotropic paramagnetic species (e.g., Co^{2+}) in panel (c), the frequencies of NMR signals are modulated by PCSs that depend on both the proximity of the nuclei to the paramagnetic center as well as their location in the frame of the magnetic susceptibility tensor. The susceptibility tensor is illustrated using isosurfaces identifying the locations of positive (blue) and negative (red) PCS, with successively more transparent surfaces corresponding to decreasing PCS magnitude. The transparent green and blue contours in the spectrum in panel (c) indicate the positions of the corresponding NMR signals for the diamagnetic protein in panel (a).

Fig. 2.

(a) Two-dimensional ${}^{13}\text{C}$ - ${}^{13}\text{C}$ proton-driven spin diffusion spectra of Co(II)-MMP-12 (red contours) and Zn(II)-MMP-12 (blue contours) recorded at 700 MHz ${}^1\text{H}$ frequency and 11.5 kHz MAS rate. The paramagnetic pseudocontact shifts are indicated by green arrows. Figure reproduced with permission from Ref. [65]. Copyright (2007) American Chemical Society.

(b,c) Experimental ${}^{13}\text{C}$ paramagnetic shifts for Co(II)-MMP-12 determined at ${}^1\text{H}$ frequencies of 850 and 900 MHz and MAS rates up to 60 kHz, plotted against calculated PCS values for (b) nuclei in the metal binding loop located as close as 6.2 Å to the Co^{2+} center and (c) nuclei from two histidine residues coordinating the metal. The MMP-12 ribbon diagram in (b) shows the “blind spheres” centered on the Co^{2+} in moderate (22 kHz; larger sphere) and fast (60 kHz; smaller sphere) MAS spectra within which NMR signals could not be detected, and the filled circles in panels (b) and (c) indicate paramagnetic shifts that could be obtained only at 60 kHz MAS. Figure reproduced with permission from Ref. [78]. Copyright (2010) American Chemical Society. (d) Ensemble of 20 low energy solid-state NMR structures of MMP-12 (PDB entry 2KRJ) [79] determined using a total of ~800 ${}^{13}\text{C}$ - ${}^{13}\text{C}$ and ${}^1\text{H}$ - ${}^1\text{H}$ distances and ~300 ${}^{13}\text{C}$ PCS restraints, complemented by the usual chemical shift-based dihedral angle restraints. The backbone atom RMSD between the mean

solid-state NMR structure and the high-resolution Xray structure is 1.3 Å. (e) Structure and crystal packing of Co(II)-MMP-12 determined using ~700 internuclear distances and ~470 total (i.e., intra- and intermolecular) ^{13}C PCSs measured in an undiluted paramagnetic sample combined with powder X-ray diffraction data [80]. The X-ray and NMR derived crystal lattices are shown in orange and blue, respectively. Figure reproduced with permission from Ref. [70]. Copyright (2013) The Royal Society of Chemistry.

Fig. 3. (a) Small regions from two-dimensional ¹⁵N-¹H spectra of diamagnetic Cu(I)-Zn(II) SOD (red contours) and paramagnetic Cu(II)-Zn(II) SOD (blue contours) recorded at 850 MHz ¹H frequency and 60 kHz MAS rate [85]. (b) Representative ¹⁵N and ¹³CO longitudinal relaxation trajectories and (c) ¹⁵N and ¹³CO longitudinal relaxation rates for Cu(I)-Zn(II) SOD (red) and Cu(II)-Zn(II) SOD (blue) [85]. The protein secondary structure is shown above the plots with locations of the histidine residues coordinating the copper ion indicated by asterisks. The gray rectangles indicate residues within 12 Å of the Cu²⁺ center according to the high-resolution crystal structure. Figure adapted with permission from Ref. [71]. Copyright (2013) American Chemical Society.

Fig. 4.

Ensembles of low energy solid-state NMR structures of SOD determined using ~300 ^1H - ^1H distances, chemical shift-based dihedral angle restraints, ambiguous hydrogen-bond restraints, and different types of paramagnetic restraints [85,86] as follows: (a) no paramagnetic restraints, (b) ~200 ^{15}N and ^{13}C PRE restraints recorded for the Cu(II)-Zn(II) protein [85], (c) ~450 ^1H , ^{13}C and ^{15}N PCS restraints recorded for the Co(II) protein [86], and (d) both PRE and PCS restraints. In each case the ribbon represents the mean structure and the Cu(II) and Co(II) ions are shown as violet and pink spheres, respectively. Figure reproduced with permission from Ref. [71]. Copyright (2013) American Chemical Society.

Fig. 5.

Paramagnetic tags utilized in the structural studies of natively diamagnetic proteins by solid-state NMR. The tags are covalently linked to a cysteine residue by reacting the protein with (a) (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)methanethiosulfonate [109], (b) N-[S-(2-pyridylthio)cysteamine]EDTA [110,111], (c) 1-(2-(pyridin-2-yl)disulfanyl)ethyl-1,4,7,10-tetraazacyclododecane [91], or (d) 4-mercaptomethyl-dipicolinic acid [94]. For brevity the resulting side-chains are referred to as R1, EDTA, TETAC, and 4MMDPA, respectively, where the latter three tags can be loaded with paramagnetic transition metal ions (M) such as Cu²⁺, Mn²⁺ or Co²⁺. Suitable diamagnetic reference samples required for quantitative measurements of PCS/PRE effects are generated by loading the metal binding tags with Zn²⁺ or tagging the protein with an analog of R1 containing a 1-acetyl group in place of 1-oxyl [112].

Fig. 6.

(a) Two-dimensional ^{15}N - ^{13}C spectra of the T53C mutant of GB1 tagged with a nitroxide spin-label (red) and its diamagnetic analog (blue) recorded at 500 MHz ^1H frequency and 11.1 kHz MAS rate. Assignments for well-resolved NMR signals for representative residues proximal (I6, W43, T49, E56) and distal (V21, T25, V29) to the tag are indicated. (b) The crystal structure of GB1 showing the tag location (sphere) and the relative intensity of cross-peaks in the spectra of spin-labeled and reference diamagnetic proteins color coded as follows: strongly attenuated (red, relative intensity 0-0.33), moderately attenuated (green,

relative intensity 0.33-0.66), and least attenuated (blue, relative intensity 0.66-1). Figure adapted with permission from Ref. [66]. Copyright (2007) American Chemical Society. (c) Structural model of *Anabaena* Sensory Rhodopsin (ASR) trimer in a lipid environment. The individual ASR monomers are shown in different colors, with the nitroxide spin label tags introduced at the S26C sites shown in orange. The side-chains of residues spatially close to the spin labels and experiencing the largest intermolecular transverse PREs are shown in green. Figure reproduced with permission from Ref. [92]. Copyright (2012) American Chemical Society.

Fig. 7.

(a) Representative regions from two-dimensional ^{15}N - ^{13}C spectra and longitudinal ^{15}N relaxation trajectories for the K28C-EDTA- Zn^{2+} (blue contours and symbols) and K28C-EDTA- Cu^{2+} (red contours and symbols) GB1 mutants. The NMR data were recorded at 500 MHz ^1H frequency and 40 kHz MAS rate. Figure adapted with permission from Ref. [90]. Copyright (2012) Nature Publishing Group. (b) Longitudinal ^{15}N PREs for the K28C-EDTA- Cu^{2+} GB1 mutant plotted as a function of the residue number and mapped onto the crystal structure. Residues with ^{15}N PRE values $> 0.1 \text{ s}^{-1}$ (^{15}N - Cu^{2+} distances $< 15 \text{ \AA}$) are

colored in red, while those with ^{15}N PRE values $< 0.1 \text{ s}^{-1}$ (^{15}N - Cu^{2+} distances $> 15 \text{ \AA}$) are colored in blue. Figure adapted with permission from Ref. [88]. Copyright (2010) American Chemical Society. (c) Summary of longitudinal ^{15}N PRE restraints determined for a set of six GB1 mutants containing Cys-EDTA Cu^{2+} tags at positions 8, 19, 28, 42, 46, and 53. For clarity the EDTA side-chains have been omitted and the Cu^{2+} ions are shown as blue spheres at their approximate positions. Figure reproduced with permission from Ref. [73]. Copyright (2013) American Chemical Society.

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Fig. 8. Structure determination of GB1 using solid-state NMR PREs. (a) Ensemble of ten lowest energy structures (blue) generated using a protocol where residues in regular secondary structure elements were held fixed according to the crystal structure and the backbone conformations of the remaining residues were randomized. The reference X-ray structure is shown in red. (b) Similar to panel (a) but showing the ensemble of 20 lowest energy structures calculated in a *de novo* fashion using the experimental PRE data and chemical-shift based backbone dihedral angle restraints. (c) Comparison of the crystal structure (red)

with the mean solid-state NMR structure (blue) obtained from the ensemble of 20 lowest energy structures in panel (b). The gray cloud represents the conformational space occupied by the backbone atoms in the ensemble. Figure adapted with permission from Ref. [90]. Copyright (2012) Nature Publishing Group.

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Fig. 9.

Structure calculation of GB1 using solid-state NMR PCSs using PCS-Rosetta. (a) Combined score of PCS energy from three tags and Rosetta energy versus the C α RMSD to the high-resolution X-ray structure of GB1. Sampling from the mutants K28C-4MMDPA, D40C-4MMDPA and E42C-4MMDPA is represented in red, green and blue, respectively. The models with the lowest combined scores have C α RMSDs to the X-ray structure in the range 0.7-1.1 Å. (b) 3D representation of structural models obtained using PCS-Rosetta. The X-ray structure of GB1 is shown in gray, and K28C-4MMDPA, D40C-4MMDPA and E42C-4MMDPA mutants in red, green and blue, respectively. Figure reproduced with permission from Ref. [93]. Copyright (2013) American Chemical Society.