Protein Structure Determination with Paramagnetic Solid-State NMR Spectroscopy

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CONSPECTUS

Many structures of the proteins and protein assemblies that play central roles in fundamental biological processes and disease pathogenesis are not readily accessible via the conventional techniques of single-crystal X-ray diffraction and solution-state nuclear magnetic resonance (NMR). On the other hand, many of these challenging biological systems are suitable targets for atomic-level structural and dynamic analysis by magic-angle spinning (MAS) solid-state NMR spectroscopy, a technique that has far less stringent limitations on the molecular size and crystalline state.

Over the past decade, major advances in instrumentation and methodology have prompted rapid growth in the field of biological solid-state NMR. However, despite this progress, one challenge for the elucidation of three-dimensional (3D) protein structures via conventional MAS NMR methods is the relative lack of long-distance data. Specifically, extracting unambiguous interatomic distance restraints larger than \( \sim 5 \text{ Å} \) from through-space magnetic dipole–dipole couplings among the protein \(^1\text{H}, ^{13}\text{C}, \text{and } ^{15}\text{N}\) nuclei has proven to be a considerable challenge for researchers. It is possible to circumvent this problem by extending the structural studies to include several analogs of the protein of interest, intentionally modified to contain covalently attached paramagnetic tags at selected sites. In these paramagnetic proteins, the hyperfine couplings between the nuclei and unpaired electrons can manifest themselves in NMR spectra in the form of relaxation enhancements of the nuclear spins that depend on the electron–nucleus distance. These effects can be significant for nuclei located up to \( \sim 20 \text{ Å} \) away from the paramagnetic center.

In this Account, we discuss MAS NMR structural studies of nitroxide and EDTA-Cu\(^{2+}\) labeled variants of a model 56 amino acid globular protein, B1 immunoglobulin-binding domain of protein G (GB1), in the microcrystalline solid phase. We used a set of six EDTA-Cu\(^{2+}\)-tagged GB1 mutants to rapidly determine the global protein fold in a de novo fashion. Remarkably, these studies required quantitative measurements of only approximately four or five backbone amide \(^{15}\text{N}\) longitudinal paramagnetic relaxation enhancements per residue, in the complete absence of the usual internuclear distance restraints. Importantly, this paramagnetic solid-state NMR methodology is general and can be directly applied to larger proteins and protein complexes for which a significant fraction of the signals can be assigned in standard 2D and 3D MAS NMR chemical shift correlation spectra.

Introduction

Many proteins implicated in fundamental biological processes or development of disease are an integral part of high molecular weight complexes that contain other proteins, nucleic acids, carbohydrates, or lipids. Notable examples of such macromolecular assemblies include membrane proteins, amyloids, microtubules, and chromatin. While biological systems of this type frequently defy analysis using the classical tools of structural biology (X-ray crystallography and solution-state nuclear magnetic resonance (NMR) spectroscopy), they can be probed with atomic-resolution detail by magic-angle spinning (MAS) solid-state NMR, which suffers from fewer...
limitations on molecular size and crystalline state. Historically, structural and dynamic studies of biological solids by solid-state NMR have employed tailored one- or two-dimensional (1D or 2D) radio-frequency (RF) pulse schemes in combination with samples containing isolated NMR-active nuclei, such as $^{13}$C, $^{15}$N, $^2$H, or $^{31}$P, located at a few specific sites. Over the past decade, however, thanks to major strides in high-field magnet (≥20 T) and probe technologies, multidimensional RF pulse sequences, and methods for preparation of homogeneous samples, MAS solid-state NMR has been utilized with great success to yield high-resolution structural models of peptides and proteins in microcrystalline, fibrous, or membrane-bound states, as well as insights into backbone and side-chain conformational dynamics. For a discussion of structural studies of proteins using complementary static solid-state NMR methods, the reader is referred to recent reviews.

The remarkable recent progress notwithstanding, the elucidation of three-dimensional protein structures by conventional MAS solid-state NMR techniques continues to be a formidable undertaking. More specifically, this methodology generally relies on the availability of numerous site-resolved structural restraints in the form of distance-dependent magnetic dipole–dipole couplings among the $^{13}$C, $^{15}$N, and $^1$H nuclei. Given the magnitudes of the relevant nuclear gyromagnetic ratios and the inverse third power dependence of the dipolar coupling constants on internuclear separation, the couplings of interest become exceedingly small (typically on the order of few to tens of hertz) for interatomic distances in the range of ~5–10 Å and above as shown in Figure 1. Consequently, the detection and quantification by the established dipolar recoupling techniques of multiple $^{13}$C–$^{15}$N, $^{13}$C–$^{13}$C, and $^1$H–$^1$H couplings corresponding to interatomic distances longer than ~5 Å in highly $^{13}$C,$^{15}$N-enriched proteins constitutes a major challenge due to the presence of many stronger couplings associated with shorter, structurally less-interesting contacts as well as nuclear spin relaxation phenomena. While this limit can be pushed to ~10 Å by detecting $^1$H–$^1$H contacts in proteins prepared with sparse methyl and amide proton labeling or couplings between $^{19}$F and $^{13}$C or $^{15}$N nuclei in proteins containing a $^{19}$F-labeled amino acid at a specific position, the majority of MAS NMR structural studies that employ the usual dipolar-coupling-based methods nevertheless suffer from a relative shortage of critical unambiguous long-range distances that constrain the three-dimensional protein fold.

**FIGURE 1.** Dependence of $^1$H–$^1$H, $^{13}$C–$^{13}$C, and $^{13}$C–$^{15}$N dipolar coupling constant magnitudes on the internuclear distance. The variable shading of the plot area serves as a rough representation of the relative difficulty with which a particular distance can be accessed by using conventional MAS NMR techniques; the lighter the shading, the more difficult the dipolar couplings corresponding to that distance are to detect and quantify.

**Fundamentals of Paramagnetic Solid-State NMR Spectroscopy**

The quantity of long-range distance restraints can be dramatically boosted by including in the NMR study one or more isostructural paramagnetic variants of the protein of interest. Indeed, paramagnetic NMR spectroscopy has been broadly utilized over the past several decades to investigate the structures of soluble proteins and, much more recently, analogous MAS based approaches have also been demonstrated for protein molecules in the solid phase. In metalloproteins, which possess an intrinsic high-affinity metal binding site, a suitable paramagnetic center may already be present in the wild-type form of the protein or otherwise may be introduced through metal exchange. On the other hand for natively diamagnetic proteins, which are the central focus of this Account, a variety of covalent chemical tags containing stable radicals or capable of binding paramagnetic transition metal or lanthanide ions may be incorporated at specific locations, most commonly via cysteine site-directed mutagenesis followed by thiol-disulfide chemistry. Figure 2 shows two representative tags of this type, which are also particularly relevant to the studies discussed here.

Unpaired electrons, which possess magnetic moments that exceed nuclear magnetic moments by several orders of magnitude, couple strongly to the surrounding nuclei through the hyperfine interaction. These couplings can appear in NMR spectra in the form of paramagnetic shifts or relaxation enhancements of the nuclear resonances, both of which have well-defined dependencies on the distance...
between the nucleus and the paramagnetic center.21–25 Most importantly, these effects can be considerable for electron–nucleus separations of up to ∼20 Å or beyond, which exceeds by at least 3–4-fold the length scale of structural restraints accessible via traditional NMR methods. In this Account, we will concentrate mainly on the use of nuclear paramagnetic relaxation enhancements (PREs), which are the most significant paramagnetic effect for nitroxide radicals, Cu2+ ions, and other paramagnetic centers characterized by isotropic or weakly anisotropic magnetic susceptibility tensors, in the structural studies of proteins by MAS solid-state NMR, but note that paramagnetic pseudo-contact shifts are also a valuable source of long-range restraints on biomolecular structure.26,30 In the context of such studies, Solomon dipolar relaxation,35 which arises from the fluctuations of local magnetic fields generated by the paramagnetic center and experienced by the nuclear spins, is the primary mechanism responsible for causing longitudinal (Γ1) and transverse (Γ2 or Γ1F) nuclear PREs. Assuming that in the solid phase the electron–nucleus couplings are modulated largely by rapid electron spin relaxation, with negligible contributions from overall molecular tumbling and slow chemical exchange type motions, the longitudinal and transverse nuclear PREs can be approximated as follows:21,24,25

\[
\Gamma_1 \approx \frac{2C}{r^6} \left( \frac{3T_{1e}}{1 + \omega_1^2 T_{1e}^2} + \frac{7T_{1e}}{1 + \omega_e^2 T_{1e}^2} \right)
\]

\[
\Gamma_2 \approx \Gamma_1 \rho \approx \frac{C}{r^6} \left( 4T_{1e} + \frac{3T_{1e}}{1 + \omega_1^2 T_{1e}^2} + \frac{13T_{1e}}{1 + \omega_e^2 T_{1e}^2} \right)
\]  (1)

where \(r\) is the electron–nucleus distance, \(T_{1e}\) is the relaxation time constant for the paramagnetic center, \(\omega_1\) and \(\omega_e\) are the nuclear and electron Larmor frequencies, respectively, and \(C\) is a prefactor that depends on fundamental constants, the nuclear gyromagnetic ratio, and the spin quantum number (\(S\)) for the paramagnetic center. The variation of longitudinal and transverse PREs with the electron–nucleus distance and electron relaxation time is illustrated in Figure 3 for the case of a 15N nucleus and a \(S = 1/2\) paramagnetic center (e.g., nitroxide radical or Cu2+) at 11.7 T. Notably, these plots show that the magnitudes of longitudinal and transverse nuclear PREs strongly depend on the identities of both the nuclear spin and the paramagnetic center, as well as the external magnetic field, and that under typical experimental conditions one or both of these PRE effects can be sizable for nuclei located ∼10–20 Å or farther away from the paramagnetic center. Moreover, given that reasonable relaxation time estimates are available in the literature for various paramagnetic species21 (if necessary these values can be further refined during the course of the protein structure calculation), the experimentally determined PRE values can in principle be directly translated into electron–nucleus distances in a quantitative manner.
In Figure 4, we illustrate the basic concept for how these nuclear PRE phenomena can be harnessed to yield a large number of site-resolved distance restraints for a natively diamagnetic protein. Namely, this is attainable by recording a set of conventional 2D NMR chemical shift correlation spectra (e.g., \( ^{15}\text{N} - ^{13}\text{C} \) or \( ^{13}\text{C} - ^{13}\text{C} \), or analogous 3D data sets) for a reference diamagnetic protein and several of its variants containing covalently linked paramagnetic tags at different positions. The PREs are then extracted by monitoring individual cross-peak amplitudes in spectra that contain correlations for directly bonded backbone or side-chain nuclei and are similar to those normally used for resonance assignment purposes. In the schematic 2D NMR spectra in Figure 4, we consider signals for two nuclei of a particular type (e.g., \( ^{15}\text{N} \)), located in different regions of the protein and colored accordingly in green and blue. While for the diamagnetic protein sample both cross-peaks have similar amplitudes and linewidths, their relative intensities differ substantially in the spectra for the two paramagnetic analogs based on the proximity of each nucleus to the unpaired electron spin.

**Qualitative PRE Measurements in Spin-Labeled Proteins**

Nitroxide radicals, which in the context of NMR experiments on hydrated proteins at room temperature are characterized by relatively long electron spin relaxation times (on the order of \( \sim 100 \text{ ns} \)), are expected to generate significant transverse PREs for the neighboring nuclear spins. Indeed, these PRE effects were successfully observed in a recent MAS NMR study of uniformly \( ^{13}\text{C}^{15}\text{N} \)-enriched analogs of the model 56-residue B1 immunoglobulin binding domain of protein G (GB1) in the microcrystalline solid phase, containing nitroxide spin labels at two solvent-exposed surface residues. The key results of that study are summarized in Figure 5, which shows a comparison of 2D \( ^{15}\text{N} - ^{13}\text{C} \) chemical shift correlation spectra for the spin-labeled T53C mutant of GB1 and a reference diamagnetic protein tagged at the same residue with an analog of the nitroxide radical containing an acetyl group in place of the paramagnetic oxyl moiety. The foremost feature of these spectra is that a number of signals present for the control sample are severely attenuated or altogether missing in the spectrum of the spin-labeled protein. The most strongly affected resonances are invariably associated with residues found in strands \( \beta_1 - \beta_4 \) and the intervening loops, predicted to be in the spatial proximity (within \( \sim 10 - 12 \text{ Å} \)) of the radical, based on the structural model of GB1. In contrast, the least affected residues are located in the \( \alpha \)-helix and removed from the spin label by \( \sim 15 - 20 \text{ Å} \) or more. These variations in signal intensities are generally consistent with the PRE values calculated for \( ^1\text{H}, ^{13}\text{C}, \text{and} ^{15}\text{N} \) nuclei. Specifically, these calculations predict that cross-peaks for amino acids located within \( \sim 10 \text{ Å} \) of the nitroxide radical should be effectively fully suppressed, owing mainly to the large transverse PRE for the amide proton that leads to rapid coherence decay during the initial \( ^1\text{H} - ^{15}\text{N} \) cross-polarization period of the 2D experiment (e.g., the transverse magnetization for a \( ^1\text{H} \) nucleus located 5 Å away from the radical is expected to decay completely within a period of only \( \sim 20 \mu\text{s} \), while for a 10 Å electron-\( ^1\text{H} \) distance the magnetization decays to 10% of its initial value in \( \sim 500 \mu\text{s} \)). It is important to note here that for the studies described in this section...
Protein Structure Determination
Sengupta et al.

(and other analogous experiments aimed at the detection of intramolecular electron–nucleus contacts discussed later in this Account), the $^{13}$C, $^{15}$N-enriched paramagnetic proteins are routinely diluted within the microcrystalline lattice to a mole fraction of $\sim$25% or less by coprecipitation with a diamagnetic, natural abundance protein in order to minimize the effects of intermolecular PREs. Furthermore, comparisons of chemical shifts for the backbone $^{15}$N and $^{13}$C atoms are used to confirm that the different mutants exhibit the same overall fold as the wild-type protein.

While somewhat qualitative in nature, MAS solid-state NMR experiments of proteins modified with spin label tags are capable of yielding vital information about intramolecular protein architecture or intermolecular protein–protein interactions on length scales that elude conventional techniques. Indeed, this spin-labeling methodology has very recently been employed by Ladizhansky and co-workers to probe long-range intermolecular contacts and determine the oligomerization interface for a membrane protein in lipid bilayers.\(^3\)

Quantitative PRE Measurements in EDTA–Cu$^{2+}$-Tagged Proteins

Although clearly beneficial for certain types of applications, the use of nitroxides as relaxation agents in the context of high-resolution structural analysis of uniformly $^{13}$C, $^{15}$N-labeled proteins by solid-state NMR suffers from a major drawback. Namely, the presence of large transverse PREs, which lead to severely attenuated signal intensities for numerous residues, precludes quantitative PRE and electron–nucleus distance measurements for those residues. As suggested by the results of PRE calculations shown in Figure 3, this problem may be overcome by using tags containing more rapidly relaxing paramagnetic centers, and Cu$^{2+}$ ions appear to be especially promising in this regard. Specifically, in analogy to spin labels, Cu$^{2+}$ centers have $S = 1/2$ and do not elicit significant paramagnetic shifts (due to a near isotropic magnetic susceptibility tensor).\(^2\) However, with typical $T_1$ of $\sim 1$–5 ns, in contrast to nitroxides, Cu$^{2+}$ ions are expected to cause transverse PREs that are roughly 1–2 orders of magnitude lower, while concurrently generating substantial longitudinal PREs for the common biological low-$\gamma$ nuclei such as $^{15}$N and $^{13}$C at moderate to high ($\sim 10$–20 T) external magnetic fields. The latter is related to the fact that the inverse of the electron relaxation time is on the order of the relevant nuclear Larmor frequencies in angular units.

The viability of this approach was demonstrated for several GB1 cysteine mutants, each modified with an EDTA-based Cu$^{2+}$ binding tag shown in Figure 2. The initial study by Nadaud et al.\(^4\) showed that the combination of relatively small transverse PREs and sizable longitudinal PREs associated with the Cu$^{2+}$ center enables quantitative measurements of the longitudinal PRE values for backbone $^{15}$N nuclei at moderate ($\sim 10$ kHz) MAS frequencies. This was achieved by using standard 2D $^{15}$N–$^{13}$C chemical shift correlation based methods to determine, in a residue-specific manner, the $^{15}$N longitudinal relaxation rate constants ($R_1$) for proteins containing an EDTA–Cu$^{2+}$ side chain, as well their diamagnetic EDTA–Zn$^{2+}$ counterparts; for each $^{15}$N site, the longitudinal PRE is obtained by taking the difference between the $R_1$ values obtained for the Cu$^{2+}$ and Zn$^{2+}$ proteins. Most importantly, measurable longitudinal PRE effects could be detected for $^{15}$N nuclei located up to $\sim 20$ Å from the Cu$^{2+}$ center, and the experimental PRE
magnitudes and $^{15}$N--$^{15}$Cu$^{2+}$ distances were found to be in good agreement with those predicted based on the known protein structure. In subsequent studies, taking advantage of the intrinsic relaxation properties of proteins containing covalent paramagnetic tags, it was shown that paramagnetic relaxation-assisted condensed data collection type approaches can be employed at high ($\sim 40$ kHz and greater) MAS rates to rapidly determine residue-specific longitudinal $^{15}$N PREs for samples containing as little as $\sim 100$ nmol of $^{13}$C,$^{15}$N-labeled protein. Note that the use of fast MAS comes with an additional benefit of allowing longitudinal $^{13}$C PREs to be accessed in quantitative fashion by analogous methods, which is not possible at lower MAS frequencies due to interference from proton-driven $^{13}$C spin diffusion phenomena.

Figure 6 shows representative 2D $^{15}$N--$^{13}$C correlation spectra recorded with short and long longitudinal $^{15}$N relaxation delays (labeled as $t_{\text{relax}}$ in the pulse scheme shown in the figure) for the K28C--EDTA--$^{15}$Cu$^{2+}$ and --$^{15}$Zn$^{2+}$ mutants of GB1. These data clearly illustrate that for most $^{15}$N nuclei the intrinsic relaxation rates are relatively low for the diamagnetic protein. On the other hand, in the presence of Cu$^{2+}$, the individual amide $^{15}$N relaxation rates vary widely as a function of the distance from the paramagnetic center as seen qualitatively in the spectra and quantitatively in the complete relaxation trajectories for selected residues. The summary of these measurements (Figure 7) shows that the magnitudes of the experimental longitudinal $^{15}$N PREs are indeed strongly correlated with predictions based on the fold of GB1, with the largest relaxation effects observed for the $\alpha$-helix residues found in closest proximity to the EDTA--Cu$^{2+}$ tag at position 28. Additionally, these quantitative data also enable the direct visualization of finer protein structural features, such as the individual turns of the helix (as seen from the modulation of the PRE magnitudes for residues $\sim 24$--$33$ with a period of $\sim 3$--$4$ amino acids).

It is noteworthy that several residues in K28C--EDTA--$^{15}$Cu$^{2+}$ GB1 that are not proximal to the Cu$^{2+}$ center, particularly...
Protein Structure Determination

Sengupta et al.

amino acids D40–W43 in loop 3 and strand β3, appear to show somewhat elevated 15N PREs. A study by Nadaud et al.43 demonstrated that these relatively minor effects can be attributed mostly to the presence of native aspartate and glutamate residues in GB1, the side-chain carboxylate groups of which act as intrinsic low-affinity metal binding sites capable of interacting with any slight excess of Cu2+ ions used for the sample preparation and not chelated by the EDTA tag, with a smaller additional contribution from residual intermolecular 15N–Cu2+ couplings due to insufficient dilution of the paramagnetic protein in the diamagnetic matrix. The influence of these effects on quantitative intramolecular PRE measurements can be minimized by loading the high-affinity metal binding tag with stoichiometric or slightly substoichiometric amounts of the metal and diluting the paramagnetic protein to a mole fraction of ~15–20%, with the latter providing a reasonable compromise between the spectral sensitivity and the suppression of intermolecular PREs.

Protein Fold Determination Using PRE Restraints

In this section, we illustrate how 15N longitudinal PREs can be used as structural restraints to elucidate the global three-dimensional fold of GB1, without having to rely on the availability of internuclear distances. In a recent study,28 Sengupta et al. determined a collection of 231 15N PREs, as described above, for six GB1 cysteine point mutants containing EDTA–Cu2+ tags at solvent-exposed residues distributed throughout the protein at positions 8 (β1 strand), 19 (β2 strand), 28 (α-helix), 42 (β3 strand), 46 (β3 strand), and 53 (β4 strand). This corresponds to approximately 38 out of 55 possible backbone 15N PREs per EDTA–Cu2+ variant or about 4–5 restraints per residue on average. Moreover, we note that roughly half of the measured PREs were smaller than 0.1 s⁻¹. To improve the convergence of the protein structure calculations, these PREs were converted to repulsive “NOE-type” distance restraints that prevented the 15N and Cu2+ atoms in question from approaching each other closer than 15.1 Å. The larger PRE values were not translated to 15N–Cu2+ distances, but rather used directly in the calculations. Figure 8 shows the summary of all 15N longitudinal PRE restraints determined for six GB1 variants containing cysteine EDTA–Cu2+ tags at positions 8, 19, 28, 42, 46, and 53 superimposed onto the GB1 X-ray structure. The Cu2+ centers (blue spheres) are shown at their approximate positions, and for clarity, the EDTA–side chains have been omitted.
Protein Structure Determination

Sengupta et al.

of restraints, their long-range nature provides a wealth of information about the protein tertiary structure.

In order to assess the utility of solid-state NMR PRE restraints for protein structure determination, we first performed a set of idealized calculations for which the conformations of the backbone atoms in regular secondary structure elements were frozen as rigid bodies according to the GB1 crystal structure, and the backbone conformations of the remaining residues were randomized. The reference GB1 X-ray structure is shown in red. (top) Similar to top left, but showing 20 lowest energy structures calculated in a de novo manner using a largely unrestricted protocol with experimental restraints consisting of 15N PREs and backbone torsion angles based on NMR chemical shifts. (bottom) Comparison of the GB1 X-ray structure (red) with the regularized mean de novo solid-state NMR structure (blue) obtained from the 20 lowest energy structures shown in the top right panel. The gray cloud represents the conformational space occupied by backbone atoms in the 20 lowest energy NMR structures. Figure adapted from ref 28.

Concluding Remarks

The determination by MAS solid-state NMR techniques of numerous dipolar coupling based distance restraints significantly in excess of ∼5 Å is complicated for large biomolecules due to the inherent limitations of this methodology. However, this lack of long-range structural data, which acts as a bottleneck for protein structure determination, can be circumvented by using paramagnetic solid-state NMR methods that yield structural restraints in the ∼20 Å regime based upon dipolar interactions between the nuclear spins and a paramagnetic center embedded in the protein.

In this Account, we have outlined an approach to protein structure determination in the solid state, rooted in the measurements of site-specific nuclear paramagnetic relaxation.
enhancements in variants of the protein of interest modified to contain covalent paramagnetic tags at specific locations. This approach is general and expected to become widely applicable to protein molecules that are compatible with the introduction of non-native side-chain tags or other linkers containing paramagnetic moieties. A key advantageous feature of this methodology is that the extraction of site-resolved PRE data is relatively straightforward, consisting of monitoring of signal intensities in standard 2D or 3D NMR chemical shift correlation spectra displaying a minimal number of strong cross-peaks. These paramagnetic solid-state NMR methods are therefore relevant for larger protein systems, for which partial or complete resonance assignments can be established. Indeed, such applications are already beginning to emerge, as highlighted by a study of a seven-helix transmembrane sensory rhodopsin. In a recent series of studies, we have applied paramagnetic solid-state NMR to the structural analysis of microcrystalline model protein GB1. Most remarkably, we have been able to show that even as few as four to five longitudinal $^{15}$N PRE restraints per residue, recorded for several EDTA–Cu$^{2+}$ GB1 mutants and supplemented only by chemical shift based torsional restraints, are adequate for deriving, in a de novo fashion, a protein backbone fold that agrees closely with the X-ray structure. Although the resolution of the resulting structure does not correspond to the highest achievable one, such structures are likely to be of sufficient quality for many applications and, importantly, can be obtained rapidly using rudimentary data analysis procedures and samples containing limited amounts of isotopically labeled paramagnetic protein. Moreover, if necessary, these moderate resolution structures can be further refined by incorporating additional paramagnetic or conventional distance or dihedral angle restraints or by combining the existing PRE data with more sophisticated fragment based chemical shift–molecular mechanics structure calculation protocols.

Finally, we note that while the main focus of this Account was on the use of nuclear PREs as the sole long-range restraints in the structure determination of paramagnetically tagged proteins, the incorporation of paramagnetic restraints in the form of both nuclear PREs and dipolar shifts has also been recently demonstrated to significantly improve the quality of solid-state NMR structures for native metalloproteins derived by conventional internuclear distance based methods.

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**FOOTNOTES**

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The authors declare no competing financial interest.

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Protein Structure Determination  Sengupta et al.


