SOLID-STATE NUCLEAR MAGNETIC RESONANCE INVESTIGATION OF PROTEIN AND POLYPEPTIDE STRUCTURE

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KEY WORDS: orientational constraints, oriented samples, distance constraints, magic angle spinning, torsional constraints

ABSTRACT

Solid-state nuclear magnetic resonance (NMR) is rapidly emerging as a successful and important technique for protein and peptide structural elucidation from samples in anisotropic environments. Because of the diversity of nuclei and nuclear spin interactions that can be observed, and because of the broad range of sample conditions that can be studied by solid-state NMR, the potential for gaining structural constraints is great. Structural constraints in the form of orientational, distance, and torsional constraints can be obtained on proteins in crystalline, liquid-crystalline, or amorphous preparations. Great progress in the past few years has been made in developing techniques for obtaining these constraints, and now it has also been clearly demonstrated that these constraints can be assembled into uniquely defined three-dimensional structures at high resolution. Although much progress toward the development of solid-state NMR as a routine structural tool has been documented, the future is even brighter with the continued development of the experiments, of NMR hardware, and of the molecular biological methods for the preparation of labeled samples.
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PERSPECTIVES AND OVERVIEW

Fifteen years ago, a paper was published that demonstrated that high-resolution structural constraints could be obtained by solid-state nuclear magnetic resonance (NMR) of proteins in hydrated but anisotropic environments (15). This review documents the progress made since that early paper, with a focus on the very recent accomplishments in this field. The potential of this approach has been rapidly evolving through the development of new types of constraints, the collection of many constraints simultaneously, and the determination of the first three-dimensional structure for the Protein Data Bank (accession code #1mag).

Knowledge of high-resolution protein structure is severely biased toward water-soluble proteins. Membrane and structural proteins, as well as proteins that exist in very-high-molecular-weight complexes, are not well represented in the data banks. Structural proteins are often long filaments that aggregate, making crystallization nearly impossible for X-ray crystallography. Similarly, such characteristics make solution NMR methods nonviable. Although the structure of Bombyx mori silk fibroin was one of the first protein structures solved (72), the fiber diffraction characterization represented a low-resolution structure that has not been substantially improved until recently (18). Work with β-amyloid peptides that form complex aggregates represent another example where the standard protein structural methods are not successful because of an inability to crystallize and/or solubilize the protein in a structurally relevant environment. Despite some recent spectacular successes by diffraction (19, 85), membrane proteins pose similar problems for both solution NMR and X-ray crystallography. Membrane proteins represent as much as one third of the Mycoplasma genitalium proteome (25). With so many critically important proteins that are exceptionally difficult to crystallize or solubilize into isotropic solution, a new structural approach has been needed (12, 17, 83).
Solid-state NMR has the inherent ability to detect single atomic sites and, hence, the potential to yield high-resolution data. At high field, sensitivity is vastly improved, to the extent that reasonable amounts of isotopically labeled protein are required for obtaining high-quality spectra. The restriction for solution NMR, that samples must be isotropically tumbling, does not apply to solid-state NMR: for solid-state NMR, the more rigid the samples the better. Indeed, this reflects the distinction between solid-state NMR (observation of anisotropic systems) and solution NMR (observation of isotropic systems). Consequently, the "correlation time problem" so frequently discussed as a molecular weight limit for solution NMR does not apply to solid-state NMR (83). The molecular weight range that can be studied by NMR is nearly without bounds, hence studies of silk fibroin (18), of the 16-MDa filamentous virus fd (94), and of colicin E1 P190 in planar lipid bilayers (58).

There are many approaches for gaining structural insights into proteins from NMR. Long before the NOESY experiment was developed for solution NMR, structural information was being sought from spectra of protein solutions. Similarly, in solid-state NMR there are many approaches for gaining structural insight. Characterizations of pKₐs, hydrogen-deuterium exchange, and interpretation of isotropic chemical shifts are a few such approaches. This review, however, focuses on three types of structural constraints that have the greatest demonstrated capacity for playing a major role in the elucidation of complete three-dimensional protein structures: orientational, distance, and torsional constraints. As the interpretation of isotropic chemical shifts and the inherent chemical shift anisotropy becomes a more precise science, it is entirely possible that constraints from these observations will become much more important. Indeed, very significant progress in this arena has been made recently (40, 41, 93, 111). Hydrogen-deuterium exchange has also developed into an important tool, from the work of Harbison et al (39) to the recent folding studies of a membrane bound polypeptide (9).

However, currently the primary structural tools for solid-state NMR spectroscopists are the measurement of specific site orientations with respect to the laboratory frame from aligned samples and, using unoriented samples, the measurement of intramolecular distances and the measurement of specific torsion angles. Orientational constraints are observed in samples that have a unique orientation with respect to the magnetic field axis of the NMR spectrometer. The data from an orientation-dependent nuclear spin interaction constrains the orientation of a specific molecular site with respect to the axis of alignment. By obtaining numerous constraints, all with respect to the same axis, three-dimensional structures can be achieved. Distance constraints that define the relative separation of two atoms can be obtained through observation of
residual dipolar interactions. Both homonuclear and heteronuclear interactions can be observed with considerable precision. As is routine in solution NMR, three-dimensional structures can be solved by distance constraints. Torsional constraints are defined through the observation of the relative orientation of spin interaction tensors in adjacent sites within the protein. Such constraints lead directly to the definition of the structural torsion angles, the ultimate goal for defining three-dimensional structure.

The assembly of three-dimensional structures is similar to solution NMR where distances and torsional constraints are involved; however, orientational constraints are unique in this regard. These latter constraints are absolute and independent, unlike the torsional and distance constraints, which are relative and dependent constraints. The independent nature of the orientational constraints means that the errors associated with individual constraints do not sum when multiple constraints are used for defining protein structure. Moreover, because the constraints are very precise, the search of conformational space consistent with the constraints is challenging because of the high-penalty barriers between penalty function minima (57). Although each of these types of constraints has advantages, in combination there is great synergy. Orientational and torsional constraints can determine the relative orientation of molecular groups, and for orientational constraints this applies even to groups vastly separated in the protein, but neither constraint is appropriate for precisely defining the separation of these groups, a task directly solved by distance constraints. Conversely, even precise distance measurements may leave the relative orientation of groups poorly defined. To date, few examples exist in the literature where combinations of structural constraint types have been used in solid-state NMR (6, 9, 30). Until the spectroscopy for each of these constraint types becomes more routine, such papers will be rare. Moreover, the number of constraints typically developed by solid-state NMR for any one structure has been relatively small; however, the precision of the constraints is much higher than in solution state NMR. Consequently, even with relatively few constraints, high-resolution structure is achievable.

It is important to recognize that for characterizing protein conformation, the determination of the torsion angles within the protein is adequate for the structural solution. Even for high-resolution crystal structures the elucidation of bond angles and bond lengths is rarely obtained from the crystal data. Consequently, the structural problem from this solid-state NMR view does not involve the independent characterization of the relative position of all atoms. Rather, it is the characterization of torsion angles that connect such relatively rigid structural elements as an indole group, a methylene, or a peptide plane. Even in this latter example, the plane could be considered as two halves separated by the \( \omega \) torsion angle.
ORIENTATIONAL CONSTRAINTS

Orientational constraints are derived from observations of a wide range of anisotropic nuclear spin interactions, such as chemical shifts, homo- and heteronuclear dipolar interactions, and quadrupolar interactions. The quality of the constraints is dependent on many parameters: how well the orientation of the spin interaction tensors are known with respect to the molecular frame, how well the samples are aligned, how well the dynamics are characterized for the site, relaxation rates of the observed nuclei, and the specific orientation of the site. These parameters can be characterized independently, and many of them have only a modest impact on the accuracy of constraint interpretation. Consequently, the precision and accuracy of these constraints can be very high.

Some interaction tensor orientations, such as the dipolar interactions between covalently attached nuclei, are accurately known whereas a deuteron bound to a nitrogen atom or many chemical shift tensors are less well known. Amide $^{15}$N chemical shift tensors have been extensively studied and although the tensor element magnitudes vary by 10–20%, the tensor orientation with respect to the molecular frame is invariant except for glycine. The glycine tensor orientation differs by 4–6° compared with the other amino acids (70, 80). Tensor element magnitudes can be characterized through observation of the unoriented spectrum of a single-site–labeled peptide. It is important to realize that different crystal forms of the same peptide can yield substantially different tensor element magnitudes (44). Consequently, it is best to characterize tensors not from model compounds, but from the compound of interest in the environment of interest. Tensor orientations have been determined using single crystals of model compounds, as well as through a characterization of the relative orientation of a known tensor (e.g. dipolar interaction) to a less-well-defined tensor (e.g. chemical shift). This can also be done for peptides in the environment of interest (100). As more tensors are characterized and more orientational constraints interpreted, the variability in the tensors will be realized and the need for numerous tensor characterizations reduced.

Much effort has gone into the improvement of sample alignment. A dispersion of molecular orientations with respect to the magnetic field axis will broaden the observed resonances because the resonance frequencies reflect this orientation with respect to the axis. Consequently, a broad resonance will decrease the accuracy and precision with which the molecular orientation can be determined. However, these NMR studies have a tremendous advantage in that they are performed in a high magnetic field, where the anisotropy of the diamagnetic or paramagnetic (for some samples) susceptibility can aid sample alignment. This magnetic influence was responsible for the first protein orientational constraints observed through aligned microcrystals of myoglobin (82).
and liquid crystalline filamentous viral solutions (15). Even in bilayer preparations where mosaic spreads as small as ±0.3° have been observed, it is clear that shear alignment between glass plates is not solely responsible for the excellent alignment (10). Prosser et al (87) took advantage of paramagnetic susceptibility to reorient lipid disc-like aggregates known as bicelles (90) so that the vector, perpendicular to the bilayer surface, is parallel to the magnetic field axis. The diamagnetic susceptibility of the lipids alone is such that the normal aligns at 90° with respect to the field, but with the addition of paramagnetic ions, the weak diamagnetic term is dominated by the paramagnetic susceptibility. Such preparations are actively being pursued as orienting matrices for membrane proteins.

Characterization of molecular dynamics is one of the major strengths of solid-state NMR. When samples have significant dynamic flexibility, it is essential that the influence of structure on the observed spectra be isolated from the influence of dynamics. Wide-line spectroscopy of unoriented samples can lead to detailed characterization of the molecular motion, especially when combined with temperature dependence. Below 200°K, almost all motions around the torsion angles cease. Raising the temperature above 200°K permits the assessment of motional averaging of the anisotropic spin interactions by such motions. These characterizations differ from relaxation studies because all motions faster than the interaction magnitude (e.g. 10^4 Hz for some chemical shift interactions) influence the spectra independent of motional frequency (48), whereas the influence on relaxation properties by molecular motion has a severely non-linear frequency dependence. Consequently, it is possible to define the axis of molecular motion, and its amplitude with wide-line NMR and with relaxation rates define the frequency of the molecular motion without assuming a motional model (78).

If the resonances being observed as a structural constraint suffer from efficient T2 relaxation, the line will be substantially broadened, reducing the precision with which the orientation of the site can be assessed. Fortunately, there are numerous spin interactions to choose from and severe problems can be avoided. Finally, the orientation of the tensor with respect to B0 influences the accuracy with which the orientation of the site can be determined. For instance, the dipolar interactions have an orientational dependence such that the observed dipolar splitting, Δνobs, equals the interaction magnitude, ν∥, multiplied by the orientation dependence:

\[ Δν_{obs} = ν∥(3 \cos^2 θ - 1). \]

θ is the angle of the unique tensor axis with respect to B0. For an N-H bond, the unique axis is aligned parallel to the covalent bond. For this interaction, the difference in Δνobs for θ = 0° and 5° is 240 Hz, whereas the difference for
\[ \theta = 40^\circ \text{ and } 45^\circ \text{ is } 2790 \text{ Hz. Although the former difference is on the border of detectability, the angles determined in the vicinity of } 40^\circ \text{ have a much smaller error bar.} \]

**Specific Site Isotopic Labeling**

The advent of peptide synthesis has had a profound influence on the development of orientational constraints, because in this way the above parameters can be extensively characterized (13). Utilizing this technology and the more than 120 orientational constraints observed from $^{15}$N and $^{13}$C chemical shifts, from $^{15}$N-$^1$H, $^{15}$N-$^2$H, and $^{15}$N-$^{13}$C dipolar interactions, and from $^2$H quadrupolar interactions, it has been possible to solve the structure of the polypeptide gramicidin A (gA) (55, 57). This peptide has an alternating sequence of 15 amino acid residues with D and L stereochemistry. The structure is a $\beta$-strand with all side chains on one side, forcing the strand into a helix. A monovalent cation-selective channel is formed by a dimer of this structure across lipid bilayers. Many laboratories have contributed to the characterization of the tensors, development of the experimental constraints, and characterization of the dynamics (e.g. 48, 59, 86, 97). The interpretation of the orientational constraints is complicated because of the $\cos^2 \theta$ dependence leading to multiple orientational solutions for each observable. Consequently, despite the precision, even accuracy of the constraints, there are ambiguities that must be resolved. Multiple constraints often eliminate some of these ambiguities, as does the restricted covalent geometry of the structural elements. With 120 constraints for 37 structural elements in this 15-amino acid polypeptide, it has been possible to uniquely define the orientation of each structural element with respect to $B_o$ and the channel axis of this peptide. The assembly of these orientations into a structure shows both the weaknesses and the strengths of this solid-state NMR approach. Gramicidin has 6.5 residues per turn, and if the errors of each peptide plane orientation accumulated in assembling the structure, there would be considerable ambiguity as to the hydrogen bond pattern, e.g. between residue 1 and 6, 7, or 8. Instead, the hydrogen bond pairs are all uniquely defined within 1 Å root mean square deviation (rmsd) of ideal hydrogen bond geometry, illustrating the advantage of using absolute structural constraints. However, in assembling the individual side chains, some van der Waals overlap is generated between side chains, similar to the 1-Å error in hydrogen bond distances described above. Consequently, the initial structure is not perfect. A refinement against all the experimental constraints, hydrogen bond distances, and CHARMM (4a) energy results in a unique structure, with each torsion angle defined to within a few degrees and virtually all the constraint ambiguities solved. An example of the high-precision orientational constraints is shown in Figure 1 for a 5-fluoro-$d_4$-Trp$_{13}$-labeled gA preparation in dimyristoylphosphatidylcholine bilayers. The
fluorinated indole rings are being studied to further characterize the influence of the indole dipoles on the potential energy barrier for cation transport at the center of the bilayer (5, 47). The refined structure of the gramicidin channel is also shown in Figure 1.

Single-site labels have also been used to study the 26–amino acid peptide melittin (96) and the 25–amino acid transmembrane peptide from M2 protein (60). Melittin is a bee venom toxin that forms a voltage-dependent anion-selective channel in lipid bilayers. The peptide is known to be predominantly \(\alpha\)-helical. The 10 backbone carbonyl \(^{13}\text{C}\) labels incorporated individually into melittin were used to show that the peptide adopts a helical conformation and a transbilayer orientation in membranes. Although crystal studies have suggested that the peptide has two helical segments with an angle of 120° between them.
there is little evidence of a different orientation for the two helical segments from the orientational constraints. Therefore, the solid-state NMR data are more consistent with the solution NMR results in micelles, where the angle is closer to $160^\circ$. M2 protein from influenza A virus is a proton channel in the viral capsid that plays a key role in infection. Although the protein has only 97 amino acid residues, the channel structure is formed by a tetramer of the protein. The nine $^{15}$N chemical shifts observed for the single M2 transmembrane peptide (M2-TMP), which is known to be $\alpha$-helical, define the helical orientation with respect to the bilayer normal as $33^\circ$, assuming an ideal helix. Moreover, it is shown that the known tetramer is symmetric or pseudosymmetric, because multiple resonances for individual labels are not observed. If it is also assumed that the hydrophilic residues are on the interior of the four-helix bundle, then the bundle must be left-handed.

In a related approach, Ulrich et al have specific-site–labeled retinal bound to bacteriorhodopsin (107) to determine details of the bound ligand conformation. It was further shown that a change in retinal orientation occurs between the initial and M states of the complex (106). Similarly, 11-\textit{cis} retinal bound to rhodopsin has been characterized (34). These studies illustrate the ability of solid-state NMR to characterize the bound conformation of ligands to membrane receptors.

**Amino Acid Specific Labeling**

Many of the initial protein structural studies by solid-state NMR were conducted by uniformly labeling an amino acid type through bacterial cultures. Many constraints obtained for the 50-amino acid major coat protein of the filamentous virus, fd, were shown to be consistent with the $\alpha$-helical “shingling” of the virion (16, 94).

Recent studies by Demura et al (18) on *Bombyx mori* silk fibroin have led to a significant refinement of the original fiber diffraction of the protein (72). This protein is dominated by the repeating sequence of Gly-Ala-Gly-Ala-Gly-Ser. Through labeling with $^{15}$N and $^{13}$C, $^{15}$N-$^1$H and $^{15}$N-$^{13}$C dipolar interactions, as well as chemical shifts, have been observed from oriented samples. Furthermore, because the silk fibroin does not undergo axial reorientation, the orientation dependence of the sample can be recorded for the asymmetric chemical shift tensors, providing a unique solution for the orientation of these tensors with respect to the fiber axis (Figure 2). With these constraints, specific bond orientations were determined for the Ala and Gly residues. By combining these solid-state NMR results with the most reliable constraints from X-ray fiber diffraction, the orientation of the major axis of the unit cell relative to the fiber axis, the backbone torsion angles were precisely determined. As structural biology focuses on challenging molecular structures, this illustrates an important
paradigm: The ability to use multiple types of constraints from diverse techniques will be important for solving structures of the highest complexity and at the highest possible resolution.

Simmons et al (95) have labeled spider dragline silk with d$_3$ Ala. Excellent orientation was achieved and the results were interpreted in terms of highly oriented and weakly oriented domains, consistent with two crystalline domains embedded in a glycine-rich amorphous domain.

**Uniform Labeling**

To make this structural approach practical for membrane proteins, it is necessary to obtain more than a few constraints at a time, hence uniform $^{15}$N and $^{13}$C labeling. With this comes the challenge of resolving the numerous resonances and assigning them. The development of the PISEMA (polarization inversion with spin exchange at the magic angle) experiment greatly improves the resolution of static solid-state chemical shift–dipolar correlation spectra (110). Marassi et al recorded PISEMA spectra of uniformly $^{15}$N-labeled fd coat protein (50 amino acids) (71) and of the colicin E1 P190 protein (190 amino acid residues) (58). A majority of the resonances are resolved in these spectra (the colicin E1 P190 spectrum is shown in Figure 3), but an additional spectral dimension has also been demonstrated, thereby gaining even more resolution (88). This use of the $^1$H chemical shift dimension has been illustrated using the fd coat protein (83). These $^{15}$N spectra even without assignments provide useful information for membrane-bound proteins. Resonances with near maximal N-H dipolar coupling and maximal chemical shift suggest an $\alpha$-helix aligned near parallel to the bilayer normal. Resonances with half-maximal N-H dipolar coupling and minimal chemical shifts suggest $\alpha$-helices parallel to the bilayer surface. Clearly, the assignment of these resonances is an essential next step. Homonuclear spin diffusion is a likely choice that has been demonstrated on model peptides (14, 89).

Figure 4 shows $^{15}$N homonuclear spin diffusion between selected sites in gramicidin oriented in lamellar-phase lipid bilayers (64). Although experimental development is still required, both spectral resolution and homonuclear spin diffusion appropriate for resonance assignments have been demonstrated.
Figure 3  A two-dimensional PISEMA spectrum of uniformly $^{15}$N-labeled colicin E1 P190 in oriented and fully hydrated lipid bilayers of dimyristoylphosphatidylcholine and dimyr- 

histoylphosphatidylglycerol. The sample contained just 6 mg of protein and was observed at 28°C with a $^{15}$N frequency of 70.97 MHz. A dipolar slice at 242 ppm is shown (left), and the spectral region for alpha helices aligned parallel and perpendicular to the bilayer surface are indicated (top). [Reprinted with permission from Kim et al (58).]

DISTANCE CONSTRAINTS

The magnitude of nuclear dipole-dipole (DD) interactions between two spins is proportional to their gyromagnetic ratios and the orientation of their internuclear vector with respect to the applied magnetic field and is inversely proportional to the cube of their through-space distance. Direct measurements of the dipolar couplings thus lead to distance constraints between nuclear spins, providing one of the most universal approaches to accurately determining molecular
structures. In contrast to solution state NMR, where the DD interactions are usually averaged by fast molecular motions so that they can only be detected through cross relaxation or nuclear Overhauser effects (75), solid-state NMR experiments enable the direct measurement of dipolar couplings and, thus, of distance constraints between spin pairs. In unoriented powder samples, dipolar interactions lead to typical powder patterns, which allow determination of internuclear distances in a straightforward manner. Unfortunately, the existence of other interactions, such as the chemical shift anisotropy (CSA) and DD interactions with abundant nuclei such as $^1$H and $^{14}$N, tends to obscure the more informative homo- and heteronuclear dipolar interactions between dilute spins.

A powerful technique to yield high-resolution chemical shift isotropic NMR spectra from unoriented samples is magic angle spinning (MAS), in which the sample is spun around the axis tilted at $\theta_m = \cos^{-1}(1/\sqrt{3})$ from the applied external magnetic field. MAS averages the broadening from both the CSA as well as weak dipolar couplings between dilute spins while strong DD interactions with abundant nuclei are removed by irradiation at the resonance frequency of the abundant nuclei, providing solution-like high-resolution solid-state NMR spectra of dilute spins. High-resolution solid-state MAS NMR spectroscopy has become an important tool for structural studies of a variety of biopolymers, including polypeptides and proteins.

A significant feature of solid-state MAS NMR is that although the anisotropic interactions containing rich structural information (for example, CSA represents the distribution of electrons around nuclei, and dipolar interactions are related to internuclear distances) are suppressed by the mechanical spinning, they can be selectively recovered by spectroscopic manipulations. Extensive efforts have been focused on how to interfere with the effect of sample spinning on these interactions while utilizing the advantage of high-resolution spectra, thereby allowing for the precise and accurate measurement of individual interactions of interest. The weak DD couplings between dilute spins are of particular interest, because the dilute spins such as carbon, nitrogen, and oxygen are essential elements for constructing the backbone of polypeptides and proteins, fundamental to structural determination of these biopolymers. Many experimental techniques have been developed to prevent these weak dipolar couplings from being averaged out by MAS, so that precise measurements of long-range internuclear distances can be obtained, providing a way to determine the secondary structure of polypeptides and proteins.

Generally, with caution taken, the precision and accuracy of distance constraints can be very high. Measurement of distance constraints by solid-state NMR spectroscopy usually requires labeling with rare spin isotopes such as $^{15}$N and $^{13}$C at specific sites of interest in order to arrange for DD interactions to be present and observable, thus providing the desired distance constraints.
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![Spectroscopic Graph](image)

- Gly2 (114 ppm)
- Val8 (145 ppm)
- Trp9 (198 ppm)
between these specific sites. However, the isotopic labels also increase the probability of intermolecular dipolar couplings with neighboring molecules (74), particularly when long-range distances are expected or peptides aggregate (6, 62, 76, 98). Dilution of the isotope labels is thus required in order to attenuate the intermolecular effect so as to obtain accurate distances (6). Significantly, the dilution effect allows for a calibration of these intermolecular influences, thereby providing a way to access the packing arrangement. Molecular dynamics is another factor that could seriously deteriorate the accuracy of distance constraints when such motions partially average the dipolar coupling. For instance, the measured distance between $^{13}$C and $^{15}$N in alanine by NMR is longer than that obtained by X-ray, owing to the fast motion in the amino group (37). Molecular vibrations also affect the accuracy of distance measurements (50). Lowering sample temperature is an effective way to minimize the effect of molecular motion, although it may induce conformational rearrangements, as in the case of enkephalins (54). On the other hand, the motionally reduced dipolar coupling may also be used (a) to characterize the orientation of the internuclear vector with respect to the motional axis if the magnitude of the dipolar coupling can be determined independently (42), or (b) to determine the dipolar coupling if the orientation of the internuclear vector with respect to the motional axis can be obtained in separate experiments (9).

Distance measurements by solid-state NMR have been increasingly used to characterize three-dimensional structures. Depending on the source of distance information, experimental techniques for distance measurements can be divided into two general categories: homonuclear and heteronuclear dipolar recouplings.

**Homonuclear Distance Measurements**

Rotational resonance ($R^2$) spectroscopy is an important method for measuring homonuclear distances between two isolated spins, such as $^{13}$C, that have different isotropic chemical shifts. $R^2$ is a resonance phenomenon achieved when the time dependence induced by sample spinning interacts with internal interactions such as chemical shifts and DD interactions. When the chemical shift difference between two spins matches a multiple of the sample spinning speed—i.e. $\Delta \omega_{iso} = n \omega$, where $\Delta \omega_{iso}$ is the chemical shift difference, $\omega$ is the sample spinning speed, and $n$ is an integer—then a resonance occurs. This phenomenon is observed as a peak in the $R^2$ spectrum at the spinning speed $\omega$.

**Figure 4** Two-dimensional $^{15}$N-$^{15}$N spin diffusion experiment for three $^{15}$N-labeled sites in the gramicidin A channel structure uniformly aligned with respect to the magnetic field axis: Gly$_2$, Val$_8$, and Trp$_9$ (64). Although Gly$_2$ is removed significantly in terms of the primary sequence, this helical structure has 6.5 residues per turn, and hence Gly$_2$ is close to both Val$_8$ and Trp$_9$. The cross-polarization pulse sequence involves mixing of the $^1$H-coupled $^{15}$N magnetization on the $Z$ axis for 5 s, approximately equal to the $^{15}$N $T_1$ relaxation time.
the spinning speed, and \( n \) is an integer—this rotational resonance significantly enhances the dipolar exchange rate between the two spins, which is attenuated by MAS. The enhanced dipolar exchange rate leads to spectral line broadening and splitting in one-dimensional spectra (79) if the homonuclear coupling is relatively strong, such as that between two directly bonded nuclei, containing structural information about the internuclear distance (66, 108). When the homonuclear dipolar coupling is weak, it is necessary to generate a longitudinal polarization difference between the two spins by selectively inverting one of the two homonuclear resonance lines in order to monitor their subsequent longitudinal magnetization exchange between the two spins as a function of time. The trajectory of the population difference may then be simulated to yield an accurate internuclear distance up to 6 Å for \(^{13}\text{C}-^{13}\text{C}\) pairs. Depending on the integer number \( n \), the trajectory is also related to other parameters, such as the chemical shift tensors of the two spins, relative orientations of chemical shift and dipolar tensors, and the zero-quantum relaxation time (\( T_{2ZQ} \)), which can be independently measured or estimated. \( R^2 \) experiments have been widely used to measure the internuclear distances in protein structures.

Griffin and coworkers (6, 62, 98) have put great effort into characterizing the complete structure of the \( \beta \)-amyloid peptide fibril (\( \beta32-42 \)) by applying the \( R^2 \) technique to \(^{13}\text{C}-^{13}\text{C}\) pairs systematically labeled in the peptide chain. This peptide is derived from the \( \beta \)-amyloid protein that is the primary constituent of the amyloid plaques, associated with Alzheimer’s disease (AD). Based on the distance constraints from intramolecular \(^{13}\text{C}-^{13}\text{C}\) pairs in combination, with knowledge of the \(^{13}\text{C}\) chemical shift frequencies and individual absorption frequencies (amide I absorption band of 1660–1650 cm\(^{-1}\)) by isotope-edited infrared spectroscopy, an initial structural analysis for the \( \beta34-42 \) amyloid was obtained. It was also shown that the intermolecular couplings severely affected the observed \( R^2 \) exchange curves, as demonstrated in Figure 5a. Three typical selectively labeled \(^{13}\text{C}\) pairs in the peptide chain are indicated by I, II, and III for intramolecular distances. Their \( R^2 \) exchange curves with 100% labeled peptide and 1:5 isotope diluted peptide are also shown to illustrate the intermolecular effects. No effect of isotope dilution is observed for the \( R^2 \) exchange curve (Figure 5aI). On the other hand, the \( R^2 \) exchange curve (Figure 5aIII) results solely from the intermolecular distance, because the \( R^2 \) exchange for the 1:5 isotope dilution is entirely abolished. Both intra- and intermolecular distances are responsible for the \( R^2 \) exchange curve (Figure 5aII) in the 100% labeled sample. The results are consistent with an antiparallel \( \beta \)-sheet structure, as shown (Figure 5b). A set of constraints from the distance measurements eliminates many of the structures from a library generated by molecular dynamics simulated annealing calculations with energy minimization. The center region of the peptide is still less clear. The earlier distance measurements between the
Figure 5  (a) Three typical magnetization exchange results obtained from $R^2$ experiments for \(\beta\)-amyloid peptide fibril ($\beta32-42$) showing the effect of isotope dilution. (I, II, III) Correspond to labels on right. (Solid circles) The results for the 100% doubly labeled samples; (open circles) results for the 1:5 diluted samples. (b) The isotope-labeled pairs indicated I, II, and III for the intramolecular distances and $I'$ (not shown), $II'$, and $III'$ for intermolecular distances. [Reprinted with permission from Lansbury et al (62).]

\(\alpha\)-carbon of Gly$_{37}$ and carbonyl carbon of Gly$_{38}$, both of which are located in the center region, were not able to provide a clear distinction between the cis or trans configuration for the Gly$_{37}$-Gly$_{38}$ amide bond (62, 98). It is believed that the labeled sample was not dilute enough to attenuate the intermolecular effect. Recent spin-echo experiments and $n = 2$ $R^2$ measurements clearly suggest that the Gly$_{37}$-Gly$_{38}$ peptide bond is in the trans configuration, rather than the previously reported cis bond (6).

Although $R^2$ experiments have been extremely successful, the $R^2$ condition is narrow and tends to be impractical to fulfill in some cases, particularly where the chemical shift difference is small, requiring slow spinning speeds and yielding low-sensitivity spectra with many spinning sidebands. Many improved techniques have been developed. Terao and coworkers (99, 101) proposed an $R2TR$ (rotational resonance in a tilted rotating frame) technique that extends the $R^2$ from the rotating frame to the double tilted rotating frame by off-resonance irradiation. The rotational resonance condition in the tilted rotating frame becomes the difference, or the sum of amplitudes of two effective fields, rather than the chemical shift difference, as in conventional $R^2$, is equal to $n\omega_r$. The former can be used for scaling down the large chemical shift difference and the latter for enlarging the small chemical shift difference. It is successfully demonstrated...
that the resonance condition can be switched with complete control by the pulse sequence, allowing different spectroscopic manipulations within the sequence (52). Rotational resonance tickling (R2T) (7) by ramping the radio frequency (RF) field through the R2 condition greatly reduces the dependence of spin dynamics on zero-quantum parameters. Moreover, broadband homonuclear dipolar recoupling techniques are designed for recoupling dipolar couplings of spins across a broad range of chemical shifts and have been accomplished by several groups. An attractive advantage of these broadband recoupling techniques is to allow correlation spectra in solid-state systems with uniformly labeled isotopic spins (e.g. 13C), akin to NOESY and TOCSY spectra in solution NMR, to be obtained, providing connectivity of the spin networks (1, 2, 20, 81). However, few structures of uniformly labeled samples have been determined by these broadband recoupling techniques, because the distances cannot be accurately determined, owing to the contribution of dipolar couplings from neighboring spins.

**Heteronuclear Distance Measurements**

Rotational-echo double resonance (REDOR) NMR spectroscopy (37) is a powerful method for measuring weak heteronuclear dipolar couplings between unlike spins (labeled I and S) such as 13C, 15N, and 31P. REDOR utilizes RF pulses at the resonance frequency for the I or S spins to interfere with the effect of MAS on the dipolar interactions. REDOR uses rotational echoes generated by sample spinning. A series of π pulses are applied to the I or S spins to prevent the dipolar IS coupling from refocusing at the time of the rotational echoes. Usually two π pulses are required in each rotational period of the magic angle spinning to ensure that the sign of the heteronuclear dipolar coupling is the same at the beginning of each rotational period, so that the resulting phase angle can be accumulated from one sample revolution to the next. The resonance intensities of the I spin are then monitored as a function of the rotor cycles with and without irradiation at the resonance of the S spin. The difference between two intensities depends exclusively on the heteronuclear dipolar coupling, yielding the internuclear distance between the two spins.

REDOR has several attractive features. Different from homonuclear distance measurements, REDOR relies only on the dipolar coupling between two spins and requires no knowledge of the chemical shift tensors of the spins and their relative orientation, and therefore the data analysis for extracting dipolar distances from REDOR experiments becomes simple. REDOR is also capable of measuring relatively long distances, up to 10 Å, depending on the labeled spin pairs being studied, e.g. 13C-15N, 15N-31P, or 13C-31P. REDOR spectroscopy is versatile and has been modified extensively for various systems. For example, transferred-echo double-resonance (TEDOR) NMR spectroscopy (43) utilizes coherence transfer between two coupled spins to eliminate unwanted
background signals from uncoupled spins. Rotational-echo, adiabatic-passage, double resonance (REAPDOR) (35), derived from the combination of the principles for REDOR with that for transfer-of-populations-double-resonance (TRAPDOR) (33), is capable of measuring the distance between spin-1/2 and quadrupolar nuclei such as $^{13}\text{C}-^{14}\text{N}$, $^{13}\text{C}-^{2}\text{H}$, and $^{13}\text{C}-^{23}\text{Na}$ pairs. These techniques have great potential for studies in biological systems, including polypeptides and membrane and structural proteins. For instance, nitrogen occurs in every backbone structural element of proteins and 99.63% of naturally abundant nitrogen is $^{14}\text{N}$; thus it would be convenient to utilize this spin in distance measurements, thereby avoiding isotopic labeling with two different nuclei. Many proteins bind cations, and REAPDOR provides an important technique that could be used for measuring distances between the cation and various sites within the proteins.

REDOR and its related methods find their great utility in characterizing diverse systems. Nishimura et al (76) determined the complete three-dimensional structure of the pentapeptide Leu-enkephalin dihydrate for the first time, on the basis of six accurately measured interatomic $^{13}\text{C}^{15}\text{N}$ distances from REDOR NMR spectroscopy, combined with some additional constraints from $^{13}\text{C}$ chemical shifts (54). Enkephalin is an endogenous morphine-like peptide, composed of two pentapeptides, Tyr-Gly-Gly-Phe-Met-OH (Met-enkephalin) and Tyr-Gly-Gly-Phe-Leu-OH (Leu-enkephalin). The sequence of the first four amino acids is commonly found in the N-terminal region of endorphins and is responsible for their biological activity through binding to δ-, µ-, or κ-receptors. For the Leu-enkephalin study, six samples were systematically synthesized with different pairs of $^{13}\text{C}^{15}\text{N}$ labels in the pentapeptide backbone (Figure 6), and then distance constraints were measured between the $^{13}\text{C}^{15}\text{N}$ pairs. By using standard bond lengths and angles, these constraints were then converted topologically into local torsion angles of constituent amino acid residues. This metric method (11) was described by Brenneman & Cross (4) for structural determination of polypeptides and was also used by others (27, 62, 30). It is important to note that in order to derive accurate distance constraints, as discussed with homonuclear constraints, when measuring long-range distances, dipolar contributions from labeled nuclei of neighboring molecules must be considered. Nishimura et al (76) found that the dilution effect on II (see Figure 6) is smaller than on I and III and, thus, concluded that the peptides possibly form an antiparallel β-sheet structure such that one peptide interacts with two other peptides.

The REDOR experiments are usually implemented at low spinning speeds and over many rotor cycles so as to increase the size of the difference signal, especially when the distance between two spins of interest is long or molecular motions are present, resulting in weak dipolar couplings. Like many multiple-pulse sequences, phase cycling (36, 67, 69) is employed in the REDOR
Figure 6: Six pairs of $^{13}$C/$^{15}$N labels for the determination of the three-dimensional structure of Leu-enkephalin. [Reprinted with permission from Nishimura et al. (76).]
experiments to partially compensate for errors due to $\pi$ pulse imperfections such as inaccuracy of the flip angle and the resonance offset effect, but not for the finite duration of the pulses. The REDOR experiments require analysis of the complete signal intensity to yield accurate distance information. The whole signal intensity includes the centerband as well as all sidebands if the spinning speed is not much larger than the size of the CSA for the spin under study. It becomes difficult to monitor the sideband intensities when the spinning is low and the CSA is large, especially when sensitivity becomes a factor. The reason for this is that the dephasing of spinning sidebands strongly depends on the relative orientation of the chemical shift tensor and the internuclear vector (29). The dephasing behavior of individual sidebands may be different, so that any individual sideband dephasing does not represent the accurate distance. It is thus preferable to implement the REDOR experiments at high spinning speed in order to concentrate the signal intensity into the centerband. High-field NMR is attractive because of greatly enhanced sensitivity as well as gains in resolution, but the CSA also increases, thereby increasing the demands on spinning speed. However, at very high spinning speed, REDOR tends to become inefficient due to the fact that the finite $\pi$-pulse lengths correspond to a significant fraction of the rotational period (73, 103).

Recently, simultaneous frequency and amplitude modulation (SFAM) NMR spectroscopy (26) was proposed as an alternative to measuring internuclear distances. For this experiment, the carrier frequency of one of the spins (e.g. $^{15}$N) is modulated cosinusoidally while its RF amplitude is modulated as a sine wave. Different from REDOR, SFAM introduces a time dependence via modulations to interfere with sample spinning and thus prevents the heteronuclear dipolar couplings from being averaged out by sample spinning. Unlike the REDOR experiments, SFAM has no limitation on the strength of irradiation and thus can be used at high field where high spinning speed is desirable. SFAM has been proven to be very efficient over a broad range of spinning speeds and to be insensitive to the RF inhomogeneity and resonance offset due to the fact that the carrier frequency and the RF amplitude are modulated simultaneously (26, 51).

SFAM has great potential for measuring extremely weak residual dipolar couplings. An exclusive example of combining distance constraints with orientational constraints was demonstrated for characterization of gramicidin (9). The structure of gA in membranes has been derived using orientation-dependent solid-state NMR studies (55, 56), featuring an amino terminal to amino terminal hydrogen bonded single-stranded dimer with each monomer folding into a right-handed $\beta$-helix of 6.5 residues per turn. However, it is also well accepted that the left-handed antiparallel double-stranded structure adopted by gA in benzene/ethanol, used to cosolubilize peptide and lipid prior to bilayer formation, converts readily to the channel state when inserted into the bilayer. The four
tryptophan side chains may play an important role in the conversion process (M Cotten & TA Cross, unpublished results). Gramicidin M (gM), in which all Trp is replaced by Phe, is being used as a model to gain insight into the structural conversion for which it is speculated that gA is inserted as a double-stranded dimer and then unsgwells to form a single-stranded channel rather than inserted as monomers that dock to form the channel. Previous studies (8) indicated that gM features a left-handed double-stranded dimer structure in lipid bilayers. Distance measurements permit determination of whether the dimer is a parallel or antiparallel structure. As illustrated in Figure 7a, the amide nitrogen of Phe_{11} and the carbonyl carbon of Ala_{3} in the same monomer, specifically labeled, are 10 Å apart for both models, giving too small a $^{13}C/^{15}N$ dipolar coupling to be detected. The intermonomer distances between the labeled sites are 4 and 9 Å for antiparallel and parallel models, respectively. For the former model, the dimer structure results in a reasonable $^{13}C/^{15}N$ dipolar coupling, but not for the latter one, even though the dimer undergoes a fast global rotation around its helical axis, partially averaging the $^{13}C/^{15}N$ dipolar coupling. The dephasing of the $^{13}C$ signal at 173 ppm indicates a residual dipolar coupling of 19 ± 3 Hz. Taking into account the orientation of the internuclear $^{13}C/^{15}N$ vector with respect to the global motion axis (84), the distance between the $^{13}C/^{15}N$ pair is 4.5 ± 0.3 Å, consistent only with the antiparallel dimer structure.

TORSIONAL CONSTRAINTS

Although the measurement of relative tensor orientations was pointed out in 1980 (68), until recently torsional constraints from solid-state NMR characterizing protein structures had received only limited attention. Torsional or dihedral angles define the relative orientations of rigid structure elements such as an indole group, a methylene, or a peptide plane. Tensors are usually well defined or can be independently characterized with respect to their molecular frame. For example, the CSA of the backbone carbonyl carbon is reasonably well characterized, and the dipolar tensors are believed to be axially symmetric with respect to internuclear vectors. Thus, a correlation between two tensors on adjacent structural elements leads to torsional constraints between these adjacent elements. As discussed above, torsional information is indirectly available from $R^2$ (66) and REDOR experiments (29). However, the data analysis for extracting the torsional constraints is not straightforward; it depends on parameters such as $T_2^{0Q}$ in $R^2$ experiments. Two-dimensional (2D) exchange NMR spectroscopy (53) that correlates two NMR frequencies governed by selected interactions of interest in different time domains provides a direct approach for obtaining torsional constraints. However, the earlier 2D static exchange NMR spectroscopy finds limited applications because of low sensitivity and poor
Figure 7  (a) Two dimer structures of gramicidin M in hydrated dimyristoylphosphatidylcholine (DMPC) bilayers. (Solid circles) The labeled sites of $^{13}$C and $^{15}$N. (b) A set of $^{13}$C CPMAS spectra at different dephasing times with (left) and without (right) the simultaneous frequency and amplitude modulation (SFAM) irradiation on the $^{15}$N channel. The spectra were recorded at 315°K (above the phase transition temperature of DMPC). The peak at 174 ppm is not affected by dephasing and is therefore assigned to the carbonyl group of the lipids. The peptide resonance at 173 ppm is attenuated by the SFAM irradiation.
resolution associated with broad powder pattern line shapes. This is particularly a problem for complex systems such as peptides. MAS enhances sensitivity and improves resolution, but it also suppresses informative anisotropic interactions, including orientations with respect to the molecular frame.

Recent developments for measuring torsional constraints have focused mainly on how to avoid having informative anisotropies averaged by MAS. Significant progress has been made toward developing a powerful and attractive way to directly characterize local conformations of polypeptides and proteins. As demonstrated by Costa et al (6), direct measurement of torsion angles clarified that the Gly37-Gly38 amide bond in the β-amyloid fibril was in the trans configuration, which was ambiguous with just the distance constraints. Rotor-synchronized exchange spectroscopy (3) originally developed for studies of slow molecular motion and chemical exchange has been utilized in structural studies (105, 109). Low spinning speeds are required to generate spinning sidebands that contain chemical shift anisotropic information. With careful timing of pulses in synchrony with sample spinning, the chemical shift tensors of two spins cross-talk during the mixing time, developed by proton-driven 13C-13C spin diffusion, resulting in intersite cross peaks that contain orientational information between two chemical shift tensors. The torsional constraints can thus be extracted with knowledge of the orientations of the two tensors in their respective local molecular frame. The measurement on the 13C doubly labeled Ala1 and Gly2 of a model tripeptide, AGG, led to backbone torsion angles of φ,ψ = −78°, 168°, in excellent agreement with φ,ψ = −83°, 170° determined by neutron diffraction (105, 109).

Ishii and coworkers (49, 52) incorporated recoupling techniques under MAS with traditional static 2D exchange spectroscopy. The powder patterns for interactions of interest are restored in different time domains connected by a mixing time, during which the exchange mechanism is chosen. For instance, in an O-C1-Cα-H system, the CSA of C1 is restored by the 6π pulse sequence (104) in the t1 domain, and the magnetization exchange between C1 and Cα is driven by the R2TR (52). Finally, the Cα signal is selected and evolved under Cα-H dipolar coupling in the t2 domain, leading to a CSA-DD 2D correlation spectrum. This leads to characterization of the ψ torsion angle. For an H-15N-13C-H system, powder patterns of 15N-H and 13C-H dipolar couplings are restored in the t1 and t2 domains that are connected by cross polarization between 13C and 15N, resulting in a DD(NH)-DD(CH) correlation (49) and leading to characterization of the φ torsion angle. Hong et al (45, 46) proposed a 2D dipolar-chemical shift (DIPSHIFT) experiment in which dipolar sidebands composed by 13C-H and 15N-H dipolar couplings are present in the t1 domain, whereas the high-resolution 13C or 15N chemical shift evolves in the t2 domain. Because the 13C-H and 15N-H dipolar tensors are known to be axially symmetric with respect to
their internuclear vector, torsional constraints can be accurately extracted from 2D DD-DD or DIPSHIFT correlation spectra without major assumptions.

Particularly promising for determination of torsion angles in complex systems such as peptides is 2D double-quantum (DQ) NMR spectroscopy (91). DQ is typically used as a spectral filter to eliminate the unwanted signals from uncoupled spins, leaving only coupled spin pairs of interest, thus simplifying the observed spectra. The ability to efficiently generate DQ coherence under MAS (32, 65) has led to diverse applications of 2D DQ NMR spectroscopy in peptides and proteins, with the advantages of high sensitivity and high resolution. Figure 8 demonstrates the advantages of DQ filtration. Schmidt-Rohr (92) demonstrated torsion angle determination on static samples. In the experiment, DQ coherence was generated and evolved in the t1 domain under 13C-H dipolar couplings. This dipolar modulated DQ coherence was converted to single-quantum coherence for detection under 13C chemical shift and 13C-13C dipolar coupling. Similarly, Gregory et al (30) generated DQ coherence under MAS conditions by homonuclear dipolar recoupling using a windowless multi-pulse irradiation technique (DRAWS) (31) and leading to determinations of the mutual orientation of two or more CSA tensors with high accuracy, assuming that these tensors are well characterized with respect to the molecular frame. Levitt and coworkers used the C7 sequence (65) to efficiently generate and reconvert DQ coherence (22), independent of chemical shift tensors. The DQ coherence is evolved under 13C-H dipolar couplings in a H-13C-13C-H system in the t1 domain, and strongly depends on the orientation of the two 13C-H dipolar couplings, leading to extraction of torsional constraints without knowledge of chemical shift tensors (21, 23, 24).

FUTURE DIRECTIONS

The potential for solid-state NMR to become a major structural technique is beginning to be realized. However, the most critical hurdle in NMR remains sensitivity. Although sensitivity has improved by nearly 1000-fold over the past three decades, another order of magnitude would tremendously facilitate these experiments. Signal averaging time would be greatly reduced, sample size could be further reduced, and signal-to-noise ratios could be improved. NMR technology is on the brink of great advances: The first commercial 900-MHz instruments will be delivered in 1999, the first prototype high-temperature superconducting probes have been delivered, and cryocooled probes are on the horizon. Because signal-averaging time decreases in proportion to B0^3, a given signal-to-noise ratio achieved in 2 days at 600 MHz could be achieved in 14 h on a 900-MHz spectrometer. Even higher fields are possible (10, 77). Resistance magnets at the National High Magnetic Field Laboratory are being used
for NMR spectroscopy (Figure 9). At this time, resistive magnets operate at fields up to 33 T (1.4 GHz for $^1$H) and a 25-T, 52-mm bore magnet has been shown to be temporally stable to 1 ppm, and 1-ppm homogeneity over a spherical volume with a 1-cm diameter is anticipated by the end of 1998. High-temperature superconducting and cryocooled probes generate substantial sensitivity improvement for solution NMR (factors of 4 or 5), but the technology has yet to be developed for solid-state NMR. Polarization transfer methods such as dynamic nuclear polarization have demonstrated factors of 200 improvement in sensitivity on model systems (38). These advances in technology will greatly benefit structure determination by solid-state NMR.

Until recently, few membrane proteins had been cloned, overexpressed, and purified in sufficient quantity (5–10 mg) for NMR spectroscopy. However, this is rapidly changing, and new approaches for splicing protein domains (112) suggest that uniform labeling of domains within proteins rather than uniform labeling of the entire protein will soon be possible. This will improve spectral resolution and spectral interpretation.

The PISEMA experiments (110) have greatly improved the resolution of static solid-state spin interaction correlation spectra and have been successfully used to obtain orientational constraints from uniformly labeled proteins. In contrast, limited success has been achieved by the use of distance constraints and torsional constraints from unoriented uniformly labeled samples. A spin pair of interest needs to be isolated from the spin network so that the effect of neighboring spins in the network can be eliminated, providing accurate distance constraints and torsional constraints. A new methodology like QUIET NOESY (113) in solution NMR has yet to be developed for effectively quenching undesirable contributions and thus allowing for accurate measurements of distance constraints and torsional constraints in uniformly labeled samples.

The technology for structural characterization is also rapidly advancing. Many research groups have started to work in this area, developing new methods from magic angle spinning of oriented samples (28), to spin diffusion as a measure of depth in lipid bilayers (61), to a one-dimensional heteronuclear experiment (63). The utilization of more than one type of constraint is just beginning to happen (6, 9, 18, 30), and an efficient algorithm for solving structures has only just begun to be developed (4, 57). Yet a high-resolution structure of a peptide in a lamellar lipid phase has been solved where crystallographic
Figure 9. $^2$H nuclear magnetic resonance spectra of d$_4$-Ala$_5$-labeled gramicidin A in oriented lipid bilayers aligned with the bilayer normal parallel to the magnetic field axis. The high-field spectrum was obtained in a resistive magnet operating at 13 MW and temporally stabilized to 1 ppm. The field inhomogeneity was significantly worse than in the 14.1-T superconducting magnet contributing to the line width of these resonances. Despite this, excellent orientational constraints are obtained on a much smaller sample than was used for the 14.1-T spectrum. [Reprinted with permission from Cotten et al (10).]
efforts have been unsuccessful; high-resolution spectra of a 200-amino acid, uniformly $^{15}$N-labeled protein in a lipid bilayer has been observed; the structure of silk fibroin has been refined and the structure of a $\beta$-amyloid peptide characterized. These document that the potential for solid-state NMR is becoming a reality, and further advances in the technology will only enhance the general applicability of solid-state NMR (a) to a broad range of proteins that have not been readily approachable by other structural methods and (b) to enhance the efficiency with which structures can be determined.

ACKNOWLEDGMENTS

TAC gratefully acknowledges support from the National Institutes of Health (R01 AI-23007) and the National Science Foundation (MCB-9603935). A portion of the work reported in this review was performed at the National High Magnetic Field Laboratory, supported by NSF Cooperative Agreement DMR-9527035 and the State of Florida.

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