

## Solid State NMR Strategy for Characterizing Native Membrane Protein Structures

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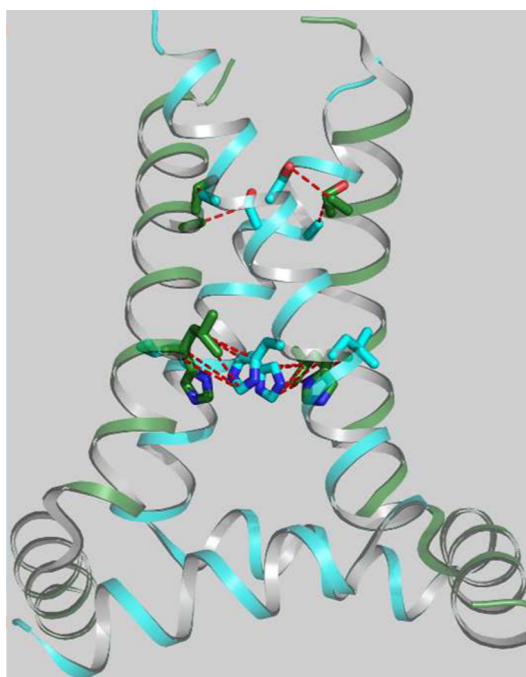
### CONSPECTUS

Unlike water soluble proteins, the structures of helical transmembrane proteins depend on a very complex environment. These proteins sit in the midst of dramatic electrical and chemical gradients and are often subject to variations in the lateral pressure profile, order parameters, dielectric constant, and other properties. Solid state NMR is a collection of tools that can characterize high resolution membrane protein structure in this environment. Indeed, prior work has shown that this complex environment significantly influences transmembrane protein structure. Therefore, it is important to characterize such structures under conditions that closely resemble its native environment.

Researchers have used two approaches to gain protein structural restraints via solid state NMR spectroscopy. The more traditional approach uses magic angle sample spinning to generate isotropic chemical shifts, much like solution NMR. As with solution NMR, researchers can analyze the backbone chemical shifts to obtain torsional restraints. They can also examine nuclear spin interactions between nearby atoms to obtain distances between atomic sites. Unfortunately, for membrane proteins in lipid preparations, the spectral resolution is not adequate to obtain complete resonance assignments.

Researchers have developed another approach for gaining structural restraints from membrane proteins: the use of uniformly oriented lipid bilayers, which provides a method for obtaining high resolution orientational restraints. When the bilayers are aligned with respect to the magnetic field of the NMR spectrometer, researchers can obtain orientational restraints in which atomic sites in the protein are restrained relative to the alignment axis. However, this approach does not allow researchers to determine the relative packing between helices.

By combining the two approaches, we can take advantage of the information acquired from each technique to minimize the challenges and maximize the quality of the structural results. By combining the distance, torsional, and orientational restraints, we can characterize high resolution membrane protein structure in native-like lipid bilayer environments.



### Introduction

The characterization of native-like structures of small helical transmembrane (TM) proteins is particularly challenging. The high fraction of hydrophobic amino acid residues in the TM domain leads to weak interactions between helices and

an increased significance for the interactions of the protein with its lipid environment.<sup>1</sup> Consequently, the characterization of these proteins in membrane mimetic environments that do not provide interactions with the protein similar to those of the native environment can lead to non-native-like

structures.<sup>2</sup> The detergent based environments used in X-ray crystallography have resulted in very few crystal structures of proteins with less than four TM helices due, in part, to the lack of a stabilizing membrane mimetic environment. While there are more solution NMR structures of such proteins, the validity of detergent micelles as an adequate environment for stabilizing native-like structures has been questioned.<sup>2,3</sup> Solid-state NMR (ssNMR) spectroscopy has a unique capability to characterize these structures in a native-like lipid environment, even a liquid crystalline lipid bilayer environment. While such promise has been extant for more than a decade,<sup>4,5</sup> there have been significant challenges to overcome before this potential could be routinely achieved. Today, these challenges have been addressed and ssNMR's potential for achieving native-like structures validated.

In the past year, it has become clearer what properties of the native membrane protein environment need to be adequately modeled in the membrane mimetic environment for structural characterization.<sup>2</sup> While the hydrophobic thickness of the membrane mimetic can be modulated by the protein, it is also clear that the membrane can influence the tilt of the TM helices.<sup>6</sup> Dual hydrophilic surfaces constraining the TM helices to span a bilayer environment can be important in contrast to the single surface of a detergent micelle that permits hydrophilic side chains from the center of a TM helix to interact with the polar surface without disrupting the interaction of the terminal regions with the aqueous interface. Similarly, it is necessary for the membrane mimetic to have a dramatic dielectric gradient and water concentration gradient, such that a span of at least 20 Å is very hydrophobic and so the interfacial region is well-defined.<sup>2</sup> It may also be important for the membrane mimetic to accurately model the lateral pressure profile of the native membrane.

Two ssNMR approaches have been used for achieving atomic resolution structural restraints of membrane proteins. Proteoliposome preparations for magic angle spinning (MAS) spectroscopy have been used for torsional and distance restraints, resulting in numerous studies of membrane proteins.<sup>7–12</sup> Recently, MAS spectroscopy has been used to obtain orientational restraints.<sup>13</sup> Indeed, the structure of the G-protein coupled receptor, CXCR1, has recently been characterized using orientational restraints from MAS spectroscopy of a proteoliposome preparation.<sup>14</sup> Uniformly oriented samples through magnetically aligned bicelles or mechanically oriented bilayers on glass surfaces have more frequently been used to obtain orientational restraints from oriented sample (OS) NMR.<sup>15–19</sup> Each of these approaches

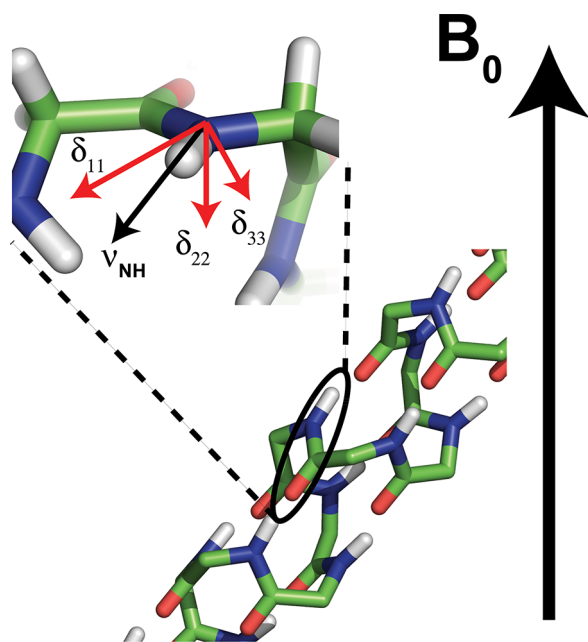
for structural characterization has advantages and significant challenges, but by combining the two approaches we can take advantage of both techniques to minimize the challenges and maximize the quality of the structural results.<sup>20,21</sup>

Numerous publications on the expression, isotopic labeling, purification, and reconstitution of membrane proteins have been published in recent years, demonstrating that the production of enough protein for solid state NMR spectroscopy is routinely possible.<sup>22</sup> Detailed protocols for the preparation of high *q* (ratio of lipid to detergent) bicelle samples necessary for achieving uniform orientation have been published.<sup>23</sup> Uniform orientation of bilayers on glass slides had been more of an art than a science, but recently with a greater understanding of how to minimize detergents from the purification and reconstitution steps for the final samples this art form has been transformed into a science (Murray et al., unpublished). Currently, we are working on six full length membrane proteins that have been uniformly oriented, one in bicelles and five using glass slides. As a result, the preparation of such oriented samples does not appear to be a significant limitation for the structural characterization of small helical membrane proteins. Furthermore, in the preparation of the mechanically oriented samples, proteoliposomes are prepared that can be used directly as a MAS sample. As a result, it is not necessary to develop two different sample preparation protocols in order to take advantage of the structural restraints obtained from both techniques.

The proteins used as examples here include the M2 protein from Influenza A. This is a proven drug target that has multiple functions including a proton channel formed by a tetramer of the single TM helix of this protein.<sup>24–26</sup> The conductance domain (residues 22–62) has the same proton conductance properties as the full length protein.<sup>27</sup> In addition, we discuss two membrane proteins from *Mycobacterium tuberculosis*, CrgA, that has two TM helices and is involved in cell division<sup>28</sup> and Rv1861 that has three TM helices and binds nucleotide triphosphates.<sup>29</sup>

## Structural Restraints

Orientalional restraints are typically obtained from separated local field (SLF) spectroscopy when the anisotropic chemical shift and dipolar interactions are correlated. More specifically, the PISEMA and SAMPI4 experiments<sup>30,31</sup> (and recent enhancements on these experiments<sup>32,33</sup>) are routinely used to obtain <sup>15</sup>N–<sup>1</sup>H dipolar interactions and the anisotropic <sup>15</sup>N chemical shifts. These experiments have focused on the <sup>15</sup>N spins in proteins because the homonuclear <sup>15</sup>N–<sup>15</sup>N spin interactions are small, whereas <sup>13</sup>C–<sup>13</sup>C homonuclear



**FIGURE 1.** From an  $^{15}\text{N}$  labeled site, such as in the inset image of a peptide plane from a TM helix, the component of the chemical shift tensor ( $\delta_{ii}$ ) and dipolar interaction ( $\nu_{\text{NH}}$ ) parallel to the magnetic field ( $B_0$ ) can be assessed as structural restraints.

interactions in uniformly labeled protein are substantial, thereby further complicating the spectra. Considerable amino acid specific labeling is often required for resolving resonances from multiple helices and for achieving the residue specific resonance assignments. While this approach has been criticized for being laborious, the need for native-like structures warrants such an effort. Furthermore, the expression and sample preparation for this spectroscopy is no longer such a time-consuming process, and therefore, the preparation of multiple samples is not a major impediment for structural characterization. The orientational restraints result from observations of the anisotropic spin interaction component parallel to the magnetic field axis (Figure 1). The orientational dependence of the spin interaction ( $(3\cos^2\theta - 1)/2$ , where  $\theta$  is the angle of the spin interaction tensor element with respect to the magnetic field) leads to a ready interpretation of the data as a structural restraint for the atomic site. The analysis of multiple sites leads to accurate characterizations of individual TM helices. Typically, the helix tilt and rotation angles are determined first with the data also supplying precise backbone torsion angles for atomic resolution structure determination. However, these restraints do not provide tertiary structural restraints between helices.

Torsional restraints are often obtained from extensively  $^{13}\text{C}$  and  $^{15}\text{N}$  labeled samples with MAS spectroscopy.<sup>34</sup> The isotropic chemical shifts of the carbonyl,  $\text{C}\alpha$ , and  $\text{C}\beta$  carbons

provide significant  $\phi, \psi$  torsion angle restraints for the polypeptide backbone. However, MAS resonance assignments are particularly challenging in the TM helices of membrane proteins because of broad line shapes and the uniformity of both the helical structures and their environment, all of which result in very little frequency dispersion for each amino acid type. The dominance of nonpolar amino acid residues contributes to the uniformity of the resonance frequencies. Typically the best linewidths for membrane proteins have been achieved from crystallized samples in detergent environments as opposed to proteoliposome preparations. However, Ladizhansky has demonstrated excellent linewidths from proteoliposome preparations of sensory rhodopsin following extensive lipid screening efforts.<sup>35</sup> Whether this spectral resolution is unique to the rhodopsin family of membrane proteins has yet to be determined.

Distance restraints have also been obtained from extensively  $^{13}\text{C}$  and  $^{15}\text{N}$  labeled samples by MAS spectroscopy, and like the torsional restraints they are dependent on resonance assignments. Here, the assignments are even more challenging as the interhelical distances are typically obtained between side chain resonances. The dispersion in the TM helices for the side chains is even less than in the polypeptide backbone, confounding the challenges for spectral interpretation. Once again, this appears to be the result of the relatively uniform apolar environment. As a result, it is difficult to collect the large number of distance restraints necessary for characterizing a tertiary structure of a helical membrane protein by MAS ssNMR alone.

The precision and accuracy of the orientational restraints are dictated by knowledge of the various spin interaction tensor element magnitudes and orientations relative to the molecular frame, the local and global dynamics present for a given set of sample conditions, and the quality or mosaic spread of the sample orientation. While the  $^{15}\text{N}$ - $^1\text{H}$  dipolar interaction magnitude and orientation is highly uniform from site to site, the magnitude of the  $^{15}\text{N}$  chemical shift tensor elements varies significantly, especially that for glycine compared to the other amino acids.<sup>36</sup> The dynamics of the helices in TM proteins includes the global rotational dynamics about the bilayer normal, which has no impact on the orientational restraints from mechanically oriented samples and is a requirement for bicelle oriented samples. Local motions in the helical backbone are minimal as reflected in an order parameter of  $>0.95$ . The quality of the sample alignment can influence the spectral linewidths; however, typical linewidths in both the dipolar and chemical shift

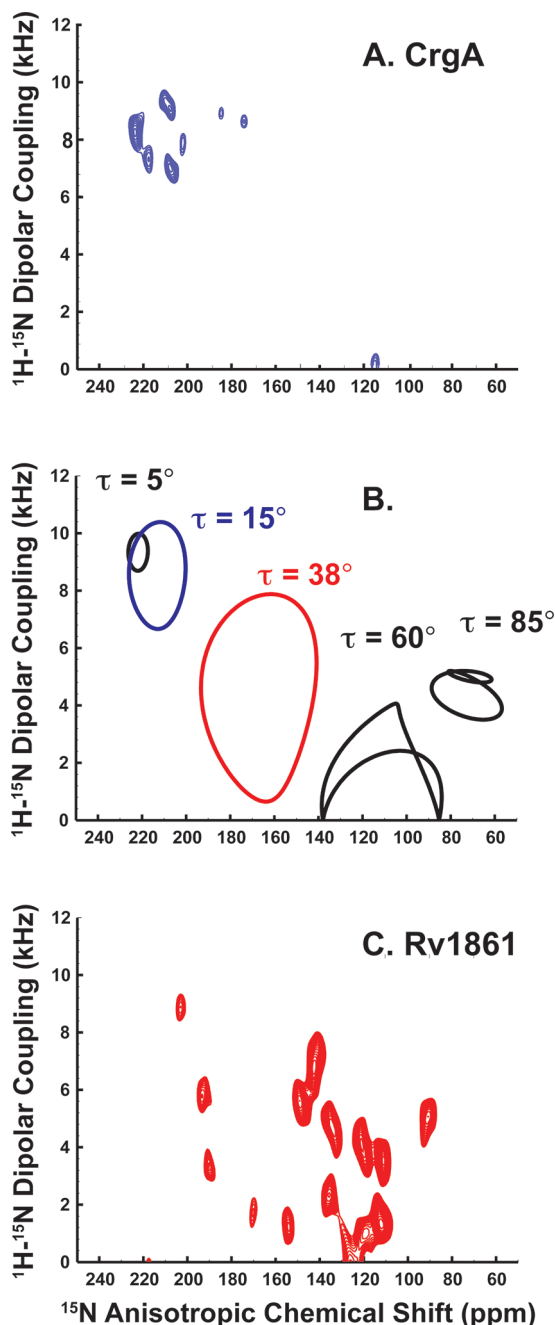
dimensions suggest a mosaic spread of bilayer normal orientations of less than  $1^\circ$  both for mechanically aligned samples and for magnetic alignment of bicelles.<sup>37</sup>

As a result of the uniform alignment of the samples and the high sensitivity of the observed resonances to the orientation of the tensors with respect to the magnetic field, the orientational restraints have high structural precision. Even with the 10 ppm variation in the chemical shift tensor element magnitudes, typical  $^{15}\text{N}$  chemical shift orientational restraints have an error bar of less than  $3^\circ$ .<sup>38</sup> In comparison, the torsional restraints from the isotropic chemical shifts have a larger error bar and the distance restraints obtained from proton driven spin diffusion experiments, while very important, are qualitative.

Oriental restraints are absolute restraints,<sup>38</sup> meaning that the error from one site does not add to the error of other sites along the helix. For orientational restraints, the structure at each site is restrained independently to the laboratory frame of reference defined by the axis of the NMR magnetic field, which is also the protein alignment axis. However, for distance restraints, the error accumulates across the molecular structure, in other words the error between points A and B, and between B and C are added when discussing the error between points A and C, and so these restraints are known as relative restraints. Similarly, torsional restraints based on the isotropic chemical shifts from MAS spectroscopy are relative restraints. The number of restraints needed to adequately define a structure is significantly greater when using relative restraints alone. By using absolute restraints, the total number of restraints required for a well-defined structure can be substantially reduced.

### Obtaining Orientational Restraints

The interpretation of orientational restraints is a two stage process. The spectra of TM helices provide images of helical wheels in the SLF spectra with 3.6 resonances per turn around a wheel-like pattern of resonances known as a PISA (polar index slant angle) wheel (Figure 2).<sup>39,40</sup> The spectral dispersion is dependent on the helical tilt angle with respect to the bilayer normal:  $0\text{--}10^\circ$  provide very little dispersion, while  $10\text{--}20^\circ$  provide more, and  $30\text{--}60^\circ$  provide maximal dispersion. Amphipathic helices on the bilayer surface are further complicated, because the plane orthogonal to the magnetic field is also a symmetry plane for the resonance frequencies. Therefore, a helix at exactly  $90^\circ$  to the bilayer normal displays a pattern of resonances in which a full circuit of the PISA wheel is achieved in a  $180^\circ$  arc of the helical wheel. Wheels associated with helical tilts either less than or more than  $90^\circ$  morph this

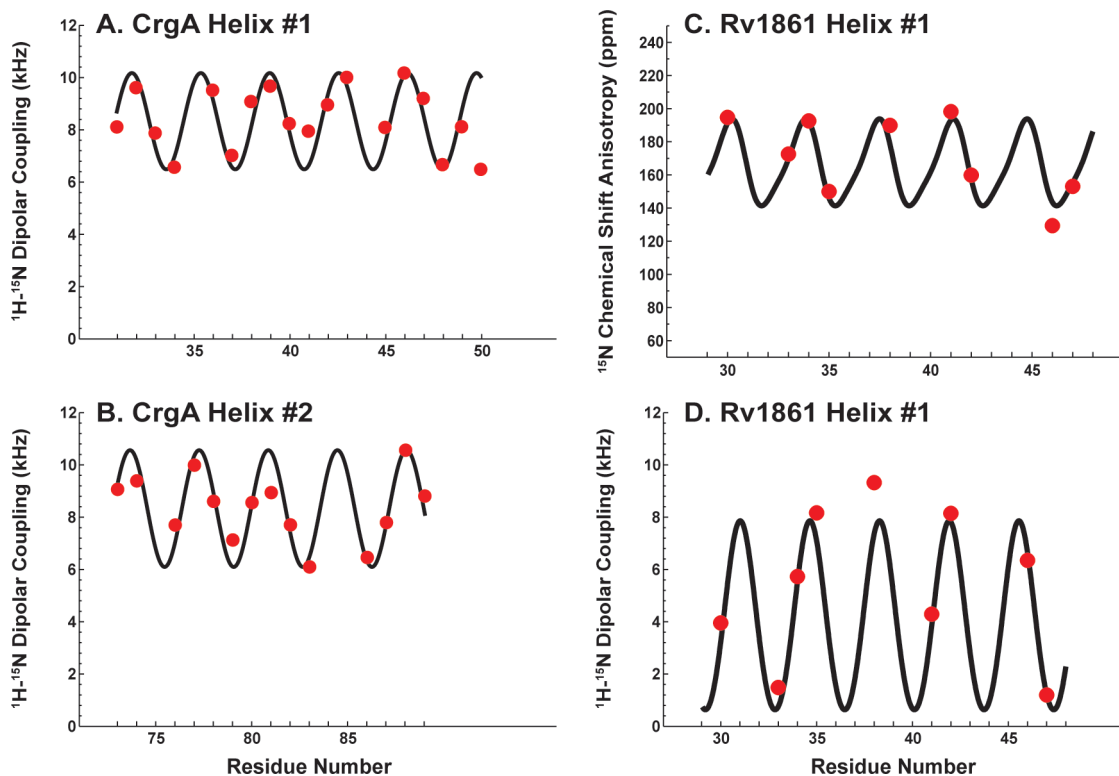


**FIGURE 2.** Initial analysis of SLF spectra. (A) PISEMA spectrum of CrgA  $^{15}\text{N}$  Phe labeled sample (residues: 33, 37, 51, 79, and 81). (B) Calculated PISA wheels for different helical tilt angles.  $\tau = 15^\circ$  is consistent with the CrgA data in (A) and  $38^\circ$  is consistent with the data in (C). (C) SAMPI4 spectrum of Rv1861  $^{15}\text{N}$  Val labeled protein.

$180^\circ$  wheel into a  $360^\circ$  wheel associated with helical tilt angles of less than  $70^\circ$  or more than  $110^\circ$  (e.g., Figure 2B,  $85^\circ$  helical tilt). Also note that spectra are symmetric about 0 kHz and that the PISA wheels retain their asymmetry when they span this symmetry axis for the data.

The uniformity of the helical structure is seen more clearly in the dipolar and chemical shift waves in Figure 3. The





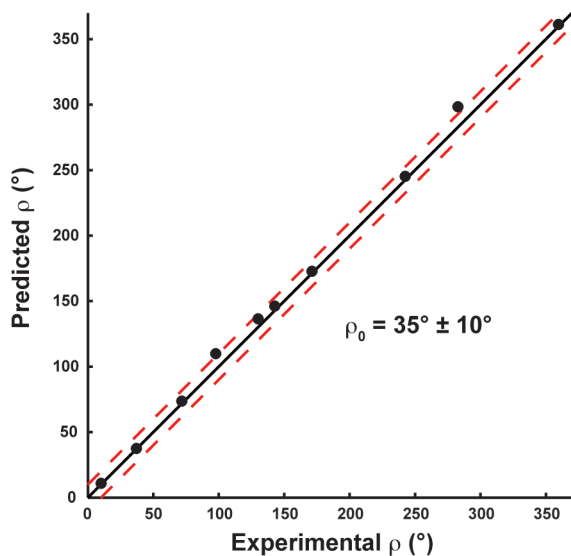
**FIGURE 3.** The uniform oscillation of the anisotropic chemical shift (C) and dipolar interactions (A, B, and D) is displayed more clearly with these wave patterns with exactly 3.6 residues per cycle. CrgA has two helices (A and B). Rv1861 has three helices, but only the data from helix #1 is displayed here (C and D).

influence of varying tensor element magnitudes, tensor orientations, and torsion angles have all been studied.<sup>41</sup> Indeed, the helical torsion angles in a lipid environment ( $\phi$ ,  $\psi = -60$ ,  $-45^\circ$ ) are significantly different from helices in an aqueous environment ( $\phi$ ,  $\psi = -65$ ,  $-40^\circ$ ), where there is competition for the amide hydrogen bonding by water that is absent in the lipid environment. The uniformity of the structure in helical segments has been confirmed by high resolution crystal structures, as has the shift in helical torsion angles.<sup>41,42</sup>

The dependence on helical tilt for the pattern of resonances causes not only a change in the dispersion (the size of the wheel), but also in the center of mass of the resonances (Figure 2B). A kink in the helix would typically result in a change in the helical tilt and a change in the center of mass for the helical segment with a different tilt angle. As a result, such deformations are readily identified in the spectra.<sup>43</sup> While it is not necessary to have the resonance assignments to determine the helical tilt, it is necessary to have minimal assignments for characterizing the rotational orientation of the helix.<sup>39</sup> Because of the resonance pattern, it is only necessary to assign a single sequence specific resonance to determine the rotational orientation of a uniform helical segment. Typically, a single amino acid specific labeled

sample is adequate to accomplish this goal. Likewise, a change in rotational orientation induced by a kink or bend within a helix can typically be assessed with a second amino acid specifically labeled protein.

The complete spectral assignments can be achieved through multiple amino acid specific labels and through some reverse labeling (growing bacteria on uniform  $^{15}\text{N}$  labeling media with unlabeled amino acids added to avoid labeling these residues).<sup>44,45</sup> Often resonances in the first turn or the last turn of a TM helix display less helical uniformity, potentially the result of a single hydrogen bond from within the helix per peptide plane and a more polar environment resulting in secondary hydrogen bonds to the amide backbone sites. The result is to induce more scatter in the resonance frequencies about the helical wheel. Consequently, the resonance assignments are best defined from the core of the helical segment toward the ends of the helical segments. Assignments are facilitated by plotting theoretical  $\rho$  values ( $100^\circ$  per residue) versus the experimentally characterized values from the PISA wheel analysis (Figure 4). Occasionally, two resonances that are very close to each other (such as two Ala residues in  $i$  and  $i + 7$  positions in the sequence) may be difficult to assign with this approach.



**FIGURE 4.** Experimental  $\rho$  values from PISA wheel analysis are plotted against predicted values ( $100^\circ/\text{residue}$ ) for the same helix #1 residues of Rv1861 shown in Figure 3C and D.

Interestingly, a mistaken assignment for two such resonances in a PISA wheel has very little impact on the structure, because resonances that are so close to each other give rise to nearly identical orientational restraints and hence only a marginal difference (less than  $1-2^\circ$ ) in the orientations for these sites.

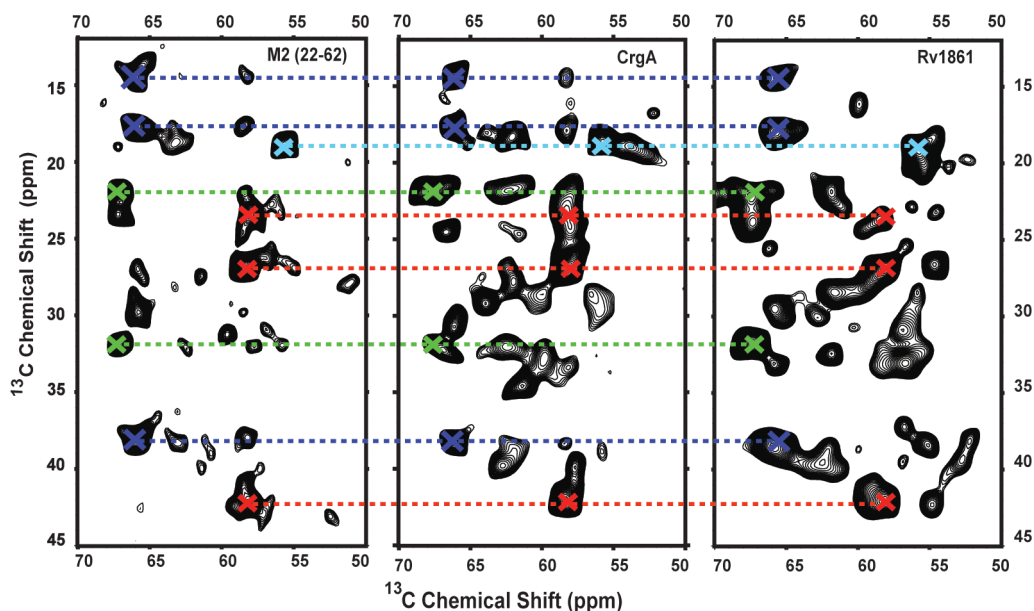
Of course, the resonance pattern(s) become more complex when there is more than one helix often causing the resonance patterns to overlap. Judicious choice for amino acid labels can lead to identification of the PISA wheels even when they are severely overlapped. Taking advantage of  $i$  to  $i + 4$  or  $i + 7$  patterns within a specific amino acid label or focusing on the distribution of the resonances at the core of the helix can be useful aids in identifying a PISA wheel within a TM helical sequence. We have recently assigned the resonances for Rv1861 having three TM helices, as well as the resonances from the two helices of CrgA (Murray et al., unpublished and Das et al., unpublished). These both represented challenging problems; the two helices of CrgA have small helical tilts ( $15^\circ$  and  $16^\circ$ ; Figures 2A and 3A and B). The fact that the tilt angles are small leads to only a modest dispersion in the resonances and both PISA wheels are severely overlapped. For CrgA, the presence of unique amino acids in both TM helices (valine in TM1 and alanine in TM2) was helpful for determining the tilt and rotation angles for both helices. For Rv1861, the helical tilt angles are much greater, but once again the PISA wheels are overlapped due to the similarity of the tilt angles,  $38^\circ$ ,  $40^\circ$ , and  $46^\circ$ . Virtually all hydrophobic amino acid labels were

needed to clinch the resonance assignments. There are two resonance correlation techniques that have recently been demonstrated,<sup>46,47</sup> suggesting that more sophisticated and less labor intensive approaches for resonance assignments or their validation may become routine with further enhancements in sensitivity.

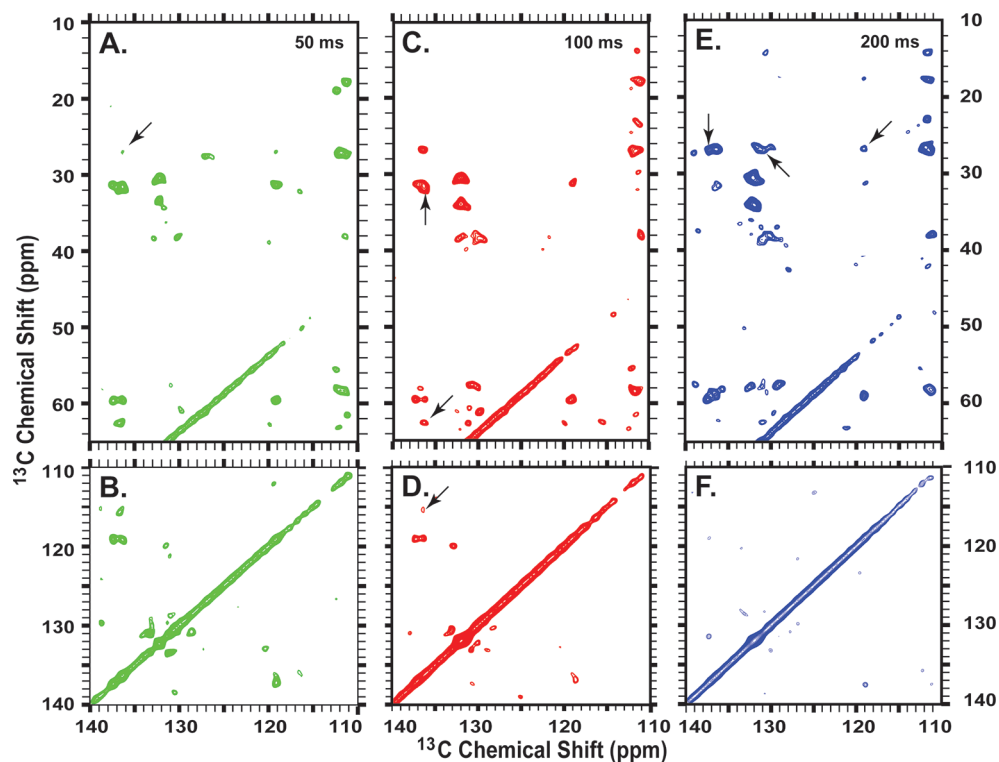
It has also been possible to characterize a helix that includes a bend or kink. The M2TM domain has a bent helix when the anti-flu drug, amantadine, is bound. Kinks may be required for the protein function, but exposure of polar atoms in the low dielectric environment will always be minimized for the sake of tertiary structural stability that is often marginal. As a result, the torsion angle space available for the bend or kink is limited. Mathematically this has been approached for the situation in which there is only two nonhelical torsion angles to show that unique solutions can be obtained if the tilt and rotational angles are known for the helical segments on either side of a kink.<sup>48</sup>

The resonance frequencies for amide  $^{15}\text{N}$  sites of a TM helix can be used as high resolution orientational restraints, but there are multiple degeneracies both in the restraints defining the orientation of a peptide plane and in the *de novo* determination of torsion angles.<sup>37</sup> However, with knowledge that the data is from an  $\alpha$ -helix having torsion angles with a variation of  $\pm 5^\circ$  eliminates all of the degenerate solutions except for the trivial case in which the peptide plane is nearly parallel to  $B_0$  (within  $\pm 4^\circ$ ). Consequently, the interpretation of orientational restraints within a helix has a unique solution.

The result is a set of TM helices with accurately defined short-range structure, that is, torsion angles and precise orientational order with respect to the membrane environment. Recall that these restraints are absolute restraints and consequently the solution for a set of torsion angles results in a helix whose tilt and rotational angles cannot be changed during refinement. The remaining flexibility in packing a set of helices is limited to rotations about the bilayer normal and translations that are limited to the  $X,Y$  axes with a potential of a few angstroms of flexibility in the  $Z$  axis. Because the side chain conformations have not been determined, there is some additional complexity in packing the helices. However, because of the scarcity of interhelical hydrogen bonds, TM helices are typically packed so as to maximize the van der Waals interactions and interhelical backbone-backbone electrostatic interactions. In other words, the packing takes advantage of the small residues such as glycine, alanine, and serine to pack the backbones of adjacent helices closely together.<sup>49</sup> In fact, conserved glycines appear to be rarely, if ever, exposed to



**FIGURE 5.** DARR (dipolar assisted rotational resonance) MAS ssNMR spectra (243 K, 50 ms mixing, and 10 kHz spinning) from three proteins and their mixing times: (A) M2 protein, (B) CrgA, and (C) Rv1861. The aliphatic resonance envelopes for Leu (red), Val (green), Ile (blue), and Ala (cyan) are highlighted with dotted lines showing nearly identical positions for their resonance envelopes in the three spectra.



**FIGURE 6.** DARR MAS ssNMR spectra from M2 protein (residues 22–62) showing a number of crosspeaks correlated with interhelical distance restraints (arrows). (A and B) 50 ms mixing time; (C and D) 100 ms mixing time; (E and F) 200 ms mixing time.

the fatty acyl environment of the lipid bilayer, presumably because glycine residues would expose the polar atoms of the backbone to the apolar environment of the membrane.<sup>2</sup>

Consequently, it may be possible at this stage to generate models for helix packing based on these restraints. Such models can be used to predict distances (i.e., cross peaks in

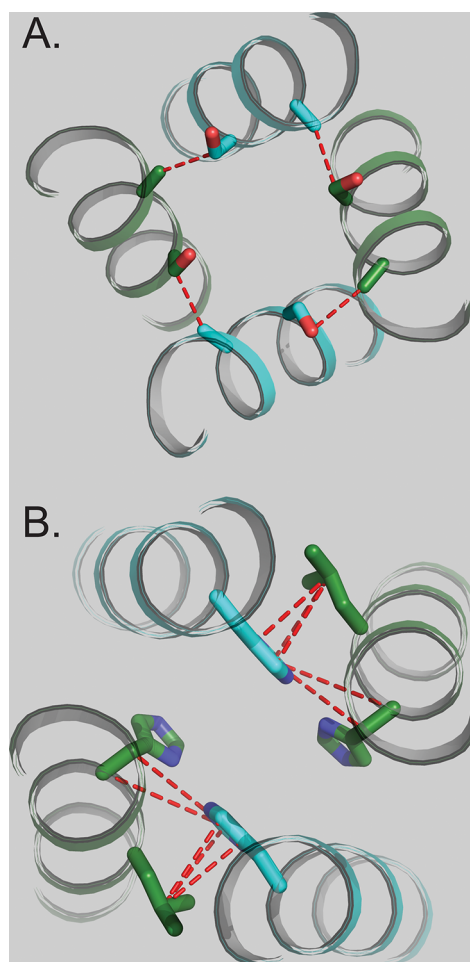
MAS spectra), thereby solving the docking problem with sparse distance restraints between the helices.

Oriental restraints can also be obtained for  $^{15}\text{N}$  histidine and tryptophan side chains.<sup>50</sup> Because both  $\chi_1$  and  $\chi_2$  are variables, it is rare that unique solutions can be obtained with just the anisotropic  $^{15}\text{N}$ – $^1\text{H}$  dipolar and  $^{15}\text{N}$  anisotropic chemical shift restraints. However, these side chains are substantially restrained by such data.

### Obtaining Distance and Additional Torsional Restraints

The same liposome preparation used for preparing the oriented samples on glass slides can be used for MAS spectroscopy samples, although it is often possible to increase the protein/lipid ratio and hence the sensitivity of these samples. For sparse distance restraints we focus on the unique or rare amino acid residues in the TM helices for both assignments and restraints. As mentioned previously, the limited chemical shift dispersion within a residue type, especially for hydrophobic amino acid residues, coupled with many residues of the same type and linewidths that are relatively broad generates ambiguities for resonance assignments. Figure 5 shows multiple resonance envelopes for Leu, Val, Ile, and Ala cross-peaks that occur at essentially the same frequencies in the spectra of all three proteins. For these three proteins, the LVIA residues account for 50–70% of all the residues in the TM helices. In Rv1861, the three TM helices also include 16 glycine residues. However, with only sparse distance restraints needed to complete the structure, assignments can be achieved for the rare residues. Obviously, for those amino acids that are unique in the TM domain, a unique assignment can be achieved even if there are multiple residues of this type in the water-soluble domain. Chemical shifts, reflecting an  $\alpha$ -helix coupled to neighboring amino acids, can confirm this assignment. In addition, observation of residue pairs through NCOX or through CAN(CO)CX experiments with sparse labeling can lead to additional sequence specific assignments. Such labeling can be achieved with  $2$ - $^{13}\text{C}$  or  $1,3$ - $^{13}\text{C}$  glycerol or by reverse labeling as described above. From these assigned resonances, it is possible to search for a few specific interhelical distance restraints.

In studies of the M2 conductance domain that includes the single TM helix and the C-terminal amphipathic helix, we have been able to sequence specifically assign all of the TM helix backbone except for the numerous leucine residues. These assignments were achieved using uniform  $^{13}\text{C}$  and  $^{15}\text{N}$  labeled samples. However, even with these assignments, the side chain resonance overlap is so severe



**FIGURE 7.** Images of the M2 (22–62) protein structure showing the sparse interhelical distance restraints that uniquely constrain the quaternary structure.

between the side chain resonances of the hydrophobic residues that cross peaks cannot be unambiguously assigned and hence they lead to degenerate structural restraints. Unique residues in the TM helix, Ser31, Gly34, and His37, were then used to search for interhelical distances to restrain the tetrameric structure. None of these residues are exposed to the lipid environment, and therefore, they have the potential to generate interhelical restraints. In particular, the His37 side chain forms a dimer of dimers structure through imidazole-imidazolium hydrogen bonds that were originally characterized by  $\text{N}\delta_1$  and  $\text{N}\epsilon_2$  side chain labeling<sup>51</sup> and more recently through sparse labeling.<sup>52</sup> The unique chemical shifts of His37 displayed numerous DARR cross peaks with residues in the spatial vicinity<sup>20</sup> (Figure 6). While some of these distances were ambiguous in that the cross peaks could be assigned to multiple side chains, multiple uniquely assigned restraints were obtained to adequately restrain the tetrameric structure (Figure 7).



## Structural Refinement

Importantly, it is necessary to refine the structure in the same environment in which the structural restraints were obtained. The environment provides many of the interactions that stabilize the tertiary structure, and therefore, this should become a standard protocol for membrane protein structural refinement. All of the experimental restraints are used in a restrained molecular dynamics protocol. While only some of the side chains are experimentally restrained, the molecular dynamics force field can be anticipated to achieve a very realistic model of the remaining side chain orientations. Of course many of these side chain conformations are of little consequence since approximately half of them face the lipid environment and can be anticipated to have significant dynamics.

## Outlook

Especially for small helical membrane proteins the structural characterization in a native-like environment can be very important. Interhelical hydrogen bonds or Coulombic interactions between charged side chains are rare in the TM domain of these proteins. van der Waals interactions are nonspecific and consequently lead to relatively little stability for a given tertiary or quaternary structure. The result is that the interaction with the protein environment can often significantly influence the structure. OS and MAS ssNMR methods have been rapidly developing as a methodology for characterizing membrane protein structure in a lipid bilayer environment. By combining restraints from these two approaches, the limitations of each can be overcome to achieve a robust technique for the characterization of important membrane protein structures in environments that accurately reproduce the biophysical properties of the membrane environment and thereby lead to a native-like membrane protein structure.

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**Dylan T. Murray** received a B.S. in Physics from The State University of New York at Plattsburgh in 2004. From 2004 to 2007, he worked at the Center for X-ray Crystallography at the University of Vermont. Since 2007, he has been in the Molecular Biophysics Ph.D. program at Florida State University working in the laboratory of Prof. Cross. His research focuses on the development

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**Nabanita Das** received her B.Sc. and M.Sc. Biotechnology degrees from Bangalore University, India (2001–2006). Afterward, she worked as Scientist in Jubilant Biosys and BioBase International, Bangalore, India (2006–2008). She entered the Ph.D. Molecular Biophysics program at Florida State University in Fall 2008. Since 2009, she is pursuing her thesis research under the supervision of Prof. Cross on the *M. tuberculosis* membrane protein CrgA and CwsA drug target structure and functional studies by solid state NMR spectroscopy.

**Timothy A. Cross** received his Ph.D. from the University of Pennsylvania in 1981 studying membrane protein structure by solution NMR spectroscopy. Since 1985, he has been on the faculty of Florida State University in the Department of Chemistry and Biochemistry where his laboratory has been developing strategies for the characterization of membrane protein structure and dynamics in native-like environments using solid state NMR spectroscopy. He also heads up the NMR and MRI User Program at the National High Magnetic Field Laboratory in Tallahassee.

## FOOTNOTES

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The authors declare no competing financial interest.  
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