A Simple Approach to Membrane Protein Secondary Structure and Topology based on NMR Spectroscopy

Francesca M. Marassi
The Wistar Institute, Philadelphia, Pennsylvania 19104 USA

ABSTRACT This paper describes a simple, qualitative approach for the determination of membrane protein secondary structure and topology in lipid bilayer membranes. The approach is based on the observation of wheel-like resonance patterns observed in the NMR ^{1}H-$^{15}N/^{15}N$ polarization inversion with spin exchange at the magic angle (PISEMA) and $^{1}H/^{15}N$ heteronuclear correlation (HETCOR) spectra of membrane proteins in oriented lipid bilayers. These patterns, named Pisa wheels, have been previously shown to reflect helical wheel projections of residues that are characteristic of α-helices associated with membranes. This study extends the analysis of these patterns to β-strands associated with membranes and demonstrates that, as for the case of α-helices, Pisa wheels are extremely sensitive to the tilt, rotation, and twist of β-strands in the membrane. Therefore, the Pisa wheels provide a sensitive, visually accessible, qualitative index of membrane protein secondary structure and topology.

INTRODUCTION

The recent availability and rapidly increasing number of genomes from several organisms has spurred a great deal of effort in the area of structural genomics (www.ncbi.nlm.nih.gov:80/Entrez/Genome/main_genomes.html). The goal of structural genomics is to obtain structural data about all known proteins, based on the identification of protein families and folds, with the intent of gaining functional information (Sali, 1998). To this end, advances in NMR spectroscopy and x-ray crystallography have rendered high-throughput structure determination of many soluble proteins highly feasible. However, a recent statistical analysis of the complete or partial genomes from several organisms identifies as many as 30% of all open reading frames encoding membrane proteins (Wallin and von Heijne, 1998), molecules for which high-throughput structure determination is far from within reach. In light of these statistics, the continuous development of NMR spectroscopy and x-ray crystallography for membrane protein structure determination is of the utmost importance. Although the goal is to obtain atomic resolution structures for membrane proteins, approaches that also provide easily accessible, qualitative, structural information can be extremely useful for identifying common folds and in guiding complete structure determination. This paper presents a simple NMR spectroscopic approach for identifying the secondary structure and topology of membrane proteins in lipid bilayer membranes.

Overall, four main types of folds are observed for the membrane-associated domains of proteins: transmembrane α-helices, membrane interfacial α-helices, transmembrane β-barrels, and membrane interfacial β-sheets (White and Wimley, 1999). Regardless of fold, all membrane proteins adopt three-dimensional structures with a unique direction in space defined by the membrane normal. Because this directionality is an intrinsic component of membrane protein structure and function, it is highly desirable to carry out structure determination within the context of lipid bilayer membranes. NMR spectroscopy of uniaxially oriented lipid bilayer samples is ideally suited for this task (Marassi and Opella, 1998). Sample orientation preserves the directional quality of membrane protein structures and also provides the mechanism for resonance line narrowing. The majority of membrane proteins incorporated in lipid bilayers are immobile on time scales longer than milliseconds, so that the relevant dipolar coupling and chemical shift interactions, present in the polypeptide backbone sites, are not motionally averaged, and the angular information inherent in the anisotropic spin interactions is retained for structural analysis. In nearly all applications of NMR spectroscopy to oriented membrane protein samples, the spin interactions between directly bonded nuclei are utilized, and the bond length is assumed to be a fixed value, enabling angular constraints to be measured and interpreted directly. The measurement of multiple, orientation-dependent frequencies for nuclei at each residue, especially ^{15}N- and ^{13}C-labeled amide sites, enables complete protein structure determination (Ketchem et al., 1993; Opella et al., 1999). This requires the resolution and assignment of the resonances in multidimensional solid-state NMR spectra (Marassi et al., 1997, 1999a; Tan et al., 1999).

The polarization inversion with spin exchange at the magic angle (PISEMA) experiment gives high-resolution ^{1}H-^{15}N dipolar coupling/^{15}N chemical shift separated local field spectra of oriented membrane proteins where the individual resonances contain orientational constraints for structure determination (Wu et al., 1994; Marassi et al., 1997). Two recent studies showed that, on the path toward structure determination, the PISEMA spectra of membrane proteins in oriented lipid bilayers are sensitive qualitative

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Address reprint requests to Dr. Francesca M. Marassi, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. Tel.: 858-646-3100; Fax: 858-646-3199; E-mail: fmarassi@burnham.org.

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indices of α-helical structure and topology (Marassi and Opella, 2000; Wang et al., 2000). For helical proteins, these spectra exhibit characteristic wheel-like patterns of resonances, so-called Pisa wheels, that reflect helical wheel projections (Schiffer and Edmunson, 1967) of residues in both transmembrane and in-plane α-helices. When a Pisa wheel spectrum is observed, no assignments are needed to determine the tilt of a transmembrane helix, and a single resonance assignment is sufficient to determine the helix rotation in the membrane (Marassi et al., 1999b). This information is crucial for determining the supramolecular architectures of membrane proteins and their assemblies.

Pisa wheels can be observed in the PISEMA spectra of several oriented, ^{15}N-labeled, α-helical membrane proteins (Marassi et al., 1997, 1999b; Kovacs and Cross, 1997; Kim et al., 1998; Kovacs et al., 2000). The spectrum of the uniformly ^{15}N-labeled pore-lining M2 transmembrane α-helix of the acetylcholine receptor (AChR) is the most conspicuous example (Opella et al., 1999). The M2 α-helix is tilted by 12° in lipid bilayers and rotated about its long helix axis so that the hydrophilic residues face the N-terminal side of the membrane. This leads to the assembly of a symmetric, pentameric, funnel-like pore, with its wide opening at the N-terminal side of the membrane. All of these conclusions about the qualitative structure of the peptide in bilayers are immediately apparent from inspection of its assigned PISEMA spectrum before complete structure determination (Marassi and Opella, 2000).

This paper extends the application of the Pisa wheel index to include β-stranded structures in lipid bilayers. Furthermore, the occurrence of Pisa wheels is demonstrated not only in the PISEMA spectra of oriented membrane proteins, but also in their ^{1}H/^{15}N heteronuclear correlation (HETCOR) spectra (Ramamoorthy et al., 1995). Thus, the NMR spectra of membrane proteins in oriented lipid bilayers provide a powerful index, which can be used to qualitatively identify structure and topology on the path toward structure determination. This approach is analogous to the chemical shift indices used to identify secondary structure in solution NMR studies of globular proteins (Wishart et al., 1992; Kuszewski et al., 1995).

MATERIALS AND METHODS

The NMR resonance frequencies (^{15}N chemical shift, ^{1}H-^{15}N dipolar coupling, ^{1}H chemical shift, and ^{13}C chemical shift) of proteins immobilized in membranes, oriented with their normal parallel to the magnetic field, depend on the protein orientation within the membrane, the magnitudes and orientations of the principal elements of the amide ^{15}N, ^{1}H, and ^{13}C chemical shift tensors, and the NH bond length. The chemical shift tensors and bond length are characterized reasonably well, and so it is possible to calculate the NMR spectra for specific models of proteins in oriented samples.

NMR spectra were calculated on a Silicon Graphics O_2 computer (Mountain View, CA), using the FORTRAN program FINGERPRINT (Marassi et al., 1999b; Marassi and Opella, 2000). For the ideal α-helices and β-strands, the inputs to the program are the protein orientation, its backbone dihedral angles, ideal peptide geometry, and the appropriate NMR tensors. The protein orientation is prescribed by the polar angles α_i and β_i that define the orientation of the peptide plane for residue i, relative to the magnetic field direction and to the membrane (Tycko et al., 1986; Opella et al., 1987). Starting with the orientation (α_i, β_i), and the dihedral angles (ϕ_i, ψ_i) of residue i, the orientation of (α_{i+1}, β_{i+1}) of residue $i + 1$ is calculated. This process is repeated to obtain the orientations (α, β) of all other residues in the protein. The NMR frequencies of each residue are calculated from the corresponding values of (α, β). Identical backbone dihedral angles were used for all residues of the ideal 20-residue α-helix ($\psi = -65$, $\phi = -40$) and the two 13-residue β-strands ($\psi = -140$, $\phi = 135$ and $\psi = -135$, $\phi = 140$).

For any protein whose Protein Data Bank (PDB) coordinates incorporate membrane directionality, the inputs to the program are the PDB coordinates and the appropriate NMR tensors. The protein orientation is extracted from the PDB coordinates and written in terms of α and β of its residues. The NMR frequencies of each residue are calculated from the corresponding values of (α, β). The PISEMA spectrum of porin from *Rhodobacter capsulatus* was calculated from the coordinates of its x-ray structure (Weiss et al., 1991; PDB file 2POR).

The principal values and molecular orientation of the ^{15}N ($\sigma_{11} = 64$ ppm, $\sigma_{22} = 77$ ppm, $\sigma_{33} = 217$ ppm, and σ_{11}NH = 17°) and ^{1}H ($\sigma_{11} = 3$ ppm, $\sigma_{22} = 8$ ppm, $\sigma_{33} = 17$ ppm, and σ_{11}NH = 0°) chemical shift tensors and the NH bond distance (1.07 Å) were as previously determined (Wu et al., 1995). The ^{15}N chemical shifts of the Gly residues in the porin molecule were calculated using the tensor principal values and orientation ($\sigma_{11} = 41$ ppm, $\sigma_{22} = 64$ ppm, $\sigma_{33} = 210$ ppm, and σ_{11}NH = 20°) determined by Drobny and co-workers (Oas et al., 1987). The ^{15}N and ^{1}H chemical shifts were referenced to 0 ppm for liquid ammonia and tetramethylsilane, respectively. In all the calculated spectra the resonances are represented by circles whose radius is 5 ppm in the ^{15}N chemical shift dimension and 300 Hz in the ^{1}H-^{15}N dipolar coupling dimension. These values are representative of experimentally observable linewidths.

RESULTS AND DISCUSSION

NMR spectra of oriented α-helices and β-strands

The PISEMA spectra calculated for an α-helix and two β-strands of different twist, placed at several orientations relative to the magnetic field, are shown in Fig. 1. The spectra of α-helices and β-strands are characteristically different, and thus provide a basis for qualitative structural characterization before resonance assignment and complete structure determination. Because PISEMA spectra are symmetric, and do not distinguish between positive and negative dipolar coupling frequencies, the resonances for only one-half of the spectrum are shown. In both cases, when the helix and strand long axes are oriented parallel to the membrane normal (Fig. 1, *A*, *H*, and *O*), all of the amide sites have an identical orientation relative to the magnetic field, and therefore all of the resonances overlap, with unique ^{1}H-^{15}N dipolar coupling and ^{15}N chemical shift frequencies. Because the backbone amide NH bonds in an α-helix are nearly but not exactly parallel to the helix axis, the resonance frequencies approach, but do not reach, their maximum attainable values of 10 kHz. In contrast, the amide NH bonds in β-strands are nearly perpendicular to the strand axis; therefore, the resonance frequencies approach, but do not reach, their maximum attainable values of 5 kHz.

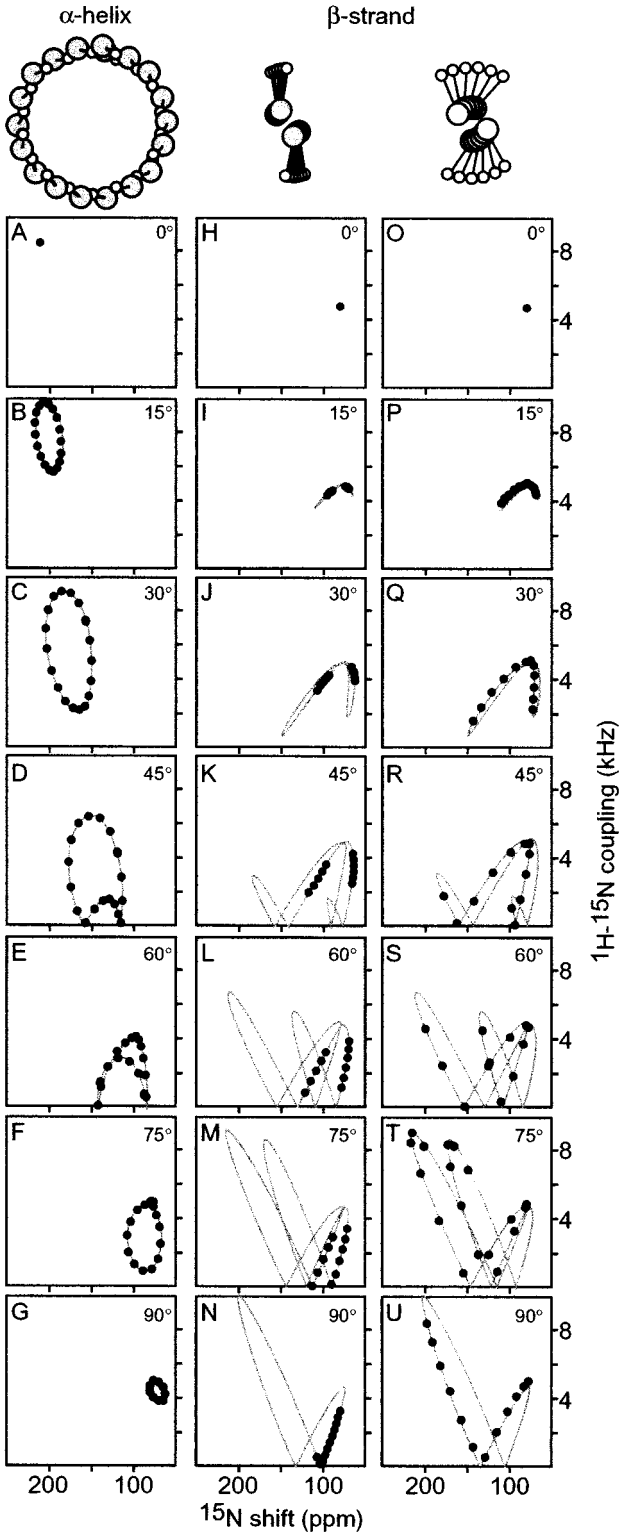

FIGURE 1 ^{1}H-^{15}N dipolar coupling/^{15}N chemical shift PISEMA spectra calculated for a 20-residue α-helix (*A–G*) and 13-residue β-strands (*H–U*) at different helix and strand tilts relative to the membrane normal. *A*, *H*, and *O*, 0°; *B*, *I*, and *P*, 15°; *C*, *J*, and *Q*, 30°; *D*, *K*, and *R*, 45°; *E*, *L*, and *S*, 60°; *F*, *M*, and *T*, 75°; *G*, *N*, and *U*, 90°. The α-helix had uniform dihedral angles of $\phi = -65$, $\psi = -40$. The β-strands had uniform dihedral angles of $\phi = -140$, $\psi = 135$ (*H–N*), or $\phi = -135$, $\psi = 140$ (*O–U*). The gray lines trace out the Pisa wheels characteristic of helices or strands associated with membranes. Views of the α-helix and β-strands, from their respective helix or strand axes, are shown at the top of the figure.

Tilting the structures away from the membrane normal breaks the symmetry and introduces variations in the orientations of the amide NH vectors relative to the magnetic field, with concomitant dispersion of both the ^{15}N chemical shift and ^{1}H-^{15}N dipolar coupling frequencies. Indeed, Fig. 1 indicates that the prospects for resolving the NMR spectra of tilted α-helices and β-strands are quite good. For all orientations different from 0°, the PISEMA spectra trace out striking resonance patterns, whose shapes and frequency breadths reflect both the secondary structure and its degree of tilt in the lipid bilayer membrane (Fig. 1, gray traces). These characteristic patterns result from the orientation of the amide NH bond vectors relative to the α-helix or β-strand axis and from the noncoincidence of the σ_{33} component of the ^{15}N chemical shift tensor and the NH bond vector (Harbison et al., 1984; Hartzell et al., 1987; Oas et al., 1987; Teng and Cross, 1989; Hu et al., 1993; Mai et al., 1993; Shoji et al., 1993; Wu et al., 1995).

For α-helices, the PISEMA spectra trace out wheel-like resonance patterns, named Pisa wheels, that mirror helical wheel projections (Marassi and Opella, 2000; Wang et al., 2000). In an α-helix with 3.6 residues per turn, adjacent amide residues are separated by 100° of the arc of the wheel (Schiffer and Edmunson, 1967), resulting in 18 different amide NH bond orientations that can be sampled for any given helix orientation and 18 corresponding different resonances around the Pisa wheel in the spectrum. Because transmembrane helices are typically ~20 amino acids long and are nearly all tilted with respect to the bilayer normal (Bowie, 1997), it is possible to resolve many resonances from residues in otherwise uniform helices and to observe characteristic Pisa wheel patterns in the PISEMA spectra of many different uniformly ^{15}N-labeled membrane peptides and proteins (Marassi et al., 1997, 1999b, 2000a; Kim et al., 1998; Opella et al., 1999; Kovacs et al., 2000; Wang et al., 2000).

In contrast, for β-strands, where the amide NH bonds are nearly perpendicular to the strand axis, the PISEMA spectra trace out twisted wheel-like resonance patterns, or twisted Pisa wheels. As for the case of transmembrane helices most of the strands in β-barrels are tilted, foretelling excellent prospects for resolving the solid-state NMR spectra of β-stranded structures in oriented lipid bilayers. The position of the resonances along the characteristic trace is important because it reflects both the polarity and the twist of the β-strand in the bilayer membrane. The effect of twist is readily assessed by comparing the spectra for a β-strand with modest twist ($\phi = -140$, $\psi = 135$; Fig. 1, *H–N*) versus those for a more twisted β-strand ($\phi = -135$, $\psi = 140$; Fig. 1, *O–U*). As illustrated in the cartoons at the top of Fig. 1,

increasing the degree of twist relieves the NH bond vectors in the β-strand of their highly overlapped orientations and results in PISEMA spectra with higher resolution, although the characteristic twisted Pisa wheel patterns remain virtually unchanged, at least for these two cases. For a β-strand with 1.99 residues per turn ($\phi = -140$, $\psi = 135$), adjacent amide residues are separated by $\sim$180° of the arc of the backbone wheel projection, resulting in 104 different amide NH bond orientations that can be sampled for any given strand orientation and 104 corresponding, different resonances in the characteristic PISEMA spectrum (Fig. 1, *I–N*). Similarly, for a β-strand with 1.92 residues per turn ($\phi = -135$, $\psi = 140$), adjacent amide residues are separated by 187° of the arc of the wheel projection of the backbone, resulting in 25 different amide NH bond orientations that can be sampled for any given strand orientation and 25 corresponding, different resonances in the characteristic PISEMA spectrum (Fig. 1, *P–U*). Because the strands in β-barrels are typically only $\sim$8–12 residues long, their resonances occupy only a portion of the spectrum (Fig. 1, *I–N* and *P–U*).

For helices with tilts greater than 40° and β-strands with tilts greater than 30°, some amide NH bonds adopt orientations relative to the magnetic field equal to the magic angle (54.7°) and have corresponding resonances with ^{1}H-^{15}N dipolar coupling frequencies of 0 kHz (Fig. 1, *D*, *E*, *K–N*, and *R–U*). Amide NH bonds that are oriented with angles greater than 54.7° relative to the magnetic field have corresponding resonances with ^{1}H-^{15}N dipolar coupling frequencies whose sign is inverted. These can be observed in the spectra of β-strands (Fig. 1, *K–N* and *R–U*) and of helices (Fig. *D–G*) tilted by more than 45°, and result in all or a portion of the PISEMA spectrum being apparently reflected through the 0-kHz axis. Helices oriented parallel to the membrane surface have their amide NH bonds and σ_{33} components of the ^{15}N chemical shift tensor nearly orthogonal to the magnetic field direction and give highly overlapped PISEMA spectra in the region around 5 kHz and 75 ppm (Fig. 1 *G*). This case is distinct from that of β-strands parallel to the membrane surface where the NH bonds and ^{15}N shift σ_{33} components have orientations to the magnetic field that range from orthogonal to parallel. As a result, the spectra display much greater resonance frequency dispersion (Fig. 1, *N* and *U*).

Because α-helices and β-strands in oriented bilayers give rise to distinct PISEMA spectra, the latter provide excellent qualitative indicators of protein secondary structure and topology. Even without resonance assignments, the spectra provide an index of secondary structure and a direct measure of its tilt relative to the bilayer normal. Variations in local twist along a β-strand are expected to influence the spectral resolution, but not the shape of the characteristic Pisa wheel. Variations in the backbone dihedral angles distort the helix or strand axis linearity and lead to spectra that deviate from the ideal patterns described above. Likewise, variations in the amide NH bond length and ^{15}N chemical shift tensor anisotropy and orientation all lead to deviations from the ideal spectra. Large variations in the NH bond length of $\pm$0.02 Å lead to modest excursions of 0.6 kHz in the ^{1}H-^{15}N dipolar coupling frequency. The amide ^{15}N chemical shift tensors of several helical model peptides have been measured (Harbison et al., 1984; Hartzell et al., 1987; Oas et al., 1987; Teng and Cross, 1989; Hu et al., 1993; Mai et al., 1993; Shoji et al., 1993; Wu et al., 1995). Although the magnitudes of the principal elements can vary by as much as 20 ppm from site to site in a polypeptide (Mai et al., 1993), no significant variation has been observed for the tensor orientation in the peptide molecular frame. Typically, $\sigma_{11} = 64$ ppm, $\sigma_{22} = 77$ ppm, $\sigma_{33} = 217$ ppm, and σ_{33}, in the peptide plane, makes an angle of 17° with the NH bond (Wu et al., 1995). The fact that Pisa wheels are observed experimentally for many α-helical membrane proteins in lipid bilayers demonstrates that residue-dependent tensor variations are not sufficiently large to overshadow the prominent structure-dependent features of the PISEMA spectra. Although the case of β-strands remains to be examined experimentally, the observations that the isotropic ^{15}N chemical shift is only moderately sensitive to backbone conformation (Wishart et al., 1991) and that the ^{15}N chemical shift anisotropy is only weakly correlated to backbone conformation (Fushman et al., 1998) suggest that Pisa wheels are also likely to be experimentally observed for oriented β-strands.

The ^{1}H/^{15}N heteronuclear correlation (HETCOR) NMR spectra calculated for α-helical and β-stranded proteins in oriented lipid bilayers are shown in Fig. 2. As for the case of PISEMA, clear structure-dependent Pisa wheel patterns are observed in these spectra, suggesting that they might also serve as useful indices of secondary structure and topology. The spectra also reflect α-helix or β-strand tilt and rotation as well as the degree of strand twist. The chemical shift tensor of the amide ^{1}H in α-helical peptides is reasonably well characterized, and the ^{1}H chemical shift frequency is extremely valuable for resolving among resonances in uniformly ^{15}N-labeled proteins, providing an additional orientational constraint for structure determination (Gerald et al., 1993; Wu et al., 1995; Ramamoorthy et al., 1995; Marassi et al., 1997, 2000a). The observation of Pisa wheels in the HETCOR spectra of oriented membrane proteins would go a long way toward aiding the resonance resolution and assignment as well as the structure determination processes. Pisa wheels have been observed experimentally in the ^{1}H/^{15}N HETCOR spectra of the coat protein from fd bacteriophage and the surface-active peptide magainin-2 in lipid bilayers (Marassi and Opella, unpublished results). In addition, Pisa wheels can be predicted, but have not yet been observed, in the ^{1}H-^{13}C/^{13}C PISEMA (Gu and Opella, 1999a) and ^{13}C/^{15}N HETCOR (Gu and Opella, 1999b) spectra of oriented proteins. It is likely that these spectra may also serve as useful structural indices.

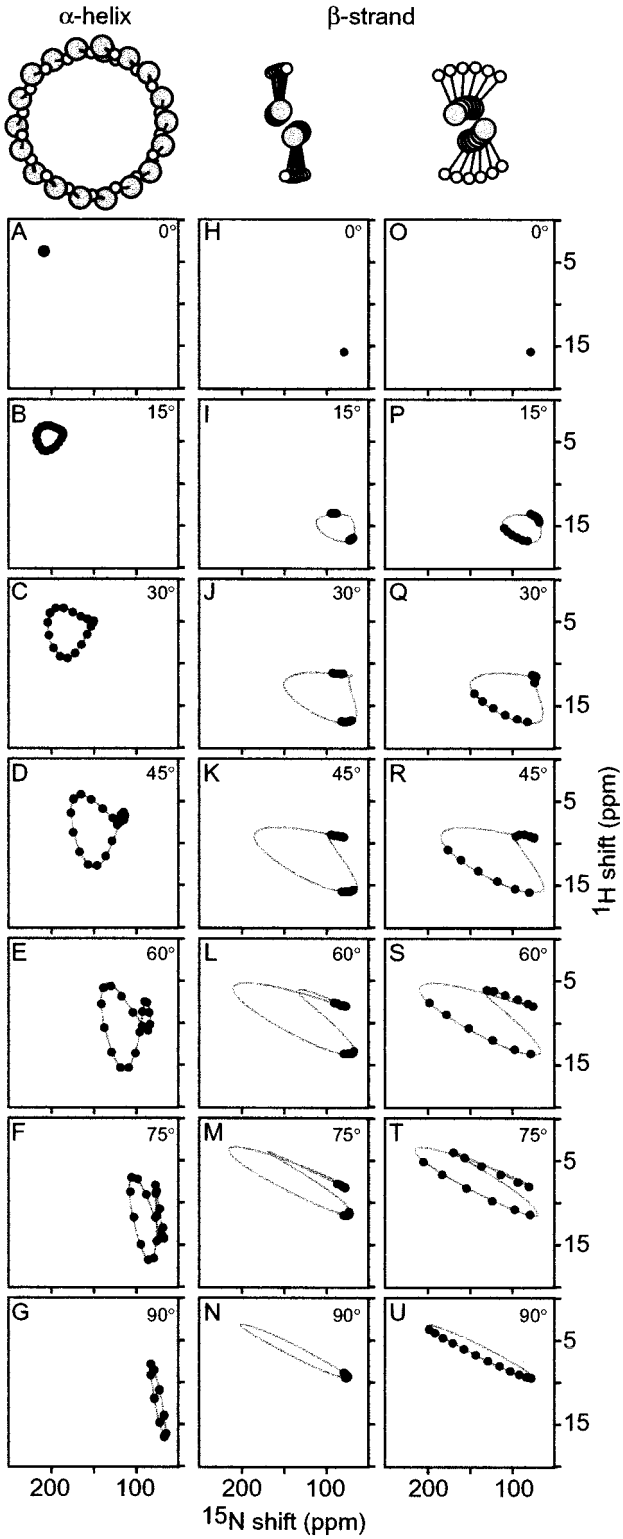

FIGURE 2 $^{1}H/^{15}N$ chemical shift HETCOR spectra calculated for a 20-residue α-helix (*A–G*) and 13-residue β-strands (*H–U*) at different helix and strand tilts relative to the membrane normal. *A*, *H*, and *O*, 0°; *B*, *I*, and *P*, 15°; *C*, *J*, and *Q*, 30°; *D*, *K*, and *R*, 45°; *E*, *L*, and *S*, 60°; *F*, *M*, and *T*, 75°; *G*, *N*, and *U*, 90°. The α-helix had uniform dihedral angles of $\phi = -65$, $\psi = -40$. The β-strands had uniform dihedral angles of $\phi = -140$, $\psi = 135$ (*N–H*), or $\phi = -135$, $\psi = 140$ (*O–U*). The gray lines trace out the Pisa wheels characteristic of helices or strands associated with membranes. Views of the α-helix and β-strands, from their respective helix or strand axes, are shown at the top of the figure.

Transmembrane β-strands

The characteristic Pisa wheel patterns of resonances observed in the PISEMA spectra of α-helices have been described (Wang et al., 2000; Marassi and Opella, 2000). They reflect helical wheel projections of the backbone residues and provide a direct measure of both the helix tilt and the angle of rotation about the helix axis in the membrane. Similarly, the PISEMA spectra of β-strands in oriented lipid bilayers reflect the strand tilt, twist, and angle of rotation about the strand long axis within the membrane. As for α-helices, the resonances in the spectra of β-strands mirror wheel projections of the residues in the strand backbone. Fig. 3 demonstrates that the rotation and tilt of a transmembrane β-strand can be obtained directly from its PISEMA spectrum.

In a mutually orthogonal laboratory frame of reference (*x*, *y*, *z*), with the *z* axis parallel to the lipid bilayer normal (n) and to the direction of the applied magnetic field (B_o), the angle $\rho = 0°$ for a strand axis (SA) aligned parallel to *z*, and with the amide nitrogen of residue 2 aligned on *x*. The strand polarity is set by a right-handed rotation of the wheel projection about the *z* axis, through the angle ρ (Fig. 3, *A* and *B*). The β-strand tilt from the bilayer normal is prescribed by a right-handed rotation of the SA about *y*, through an angle τ (Fig. 3, *E* and *F*). Therefore, τ is the angle between SA and the direction of B_o, and ρ is the polar angle between the projection of B_o on the wheel and a vector drawn between residue 2 and the center of the wheel. These angles (ρ, τ) are sufficient to define the orientation of a β-strand in the lipid bilayer membrane.

In Fig. 3, *I* and *J*, the resonances in the PISEMA spectra for two different β-strand orientations, with equal values of $\tau = 30°$, but different values of ρ, fall on the same curve (gray trace), prescribed by τ. However, the spectra differ in the positions of their resonances on this trace, which mirror exactly the polarity ρ of their corresponding wheel projections. In Fig. 3 *A*, rotation through ρ orients the amide NH bonds of residues 5 and 6 nearly parallel to the laboratory *y* axis. The strand is then tilted through $\tau = 30°$ around *y* so that these same NH bonds align nearly orthogonal to the magnetic field. The corresponding resonances have ^{1}H-^{15}N dipolar coupling frequencies around 5 kHz. In Fig. 3 *B*, on the other hand, the amide NH bonds of residues 5 and 6 are nearly orthogonal to the laboratory *y* axis after rotation through ρ and make angles smaller than 90° with the magnetic field direction after a tilt through $\tau = 30°$. Their corresponding resonances have ^{1}H-^{15}N dipolar coupling frequencies that are less than 5 kHz.

In these spectra, the resonances from either side of the β-strand occupy separate wings of the twisted Pisa wheel, as

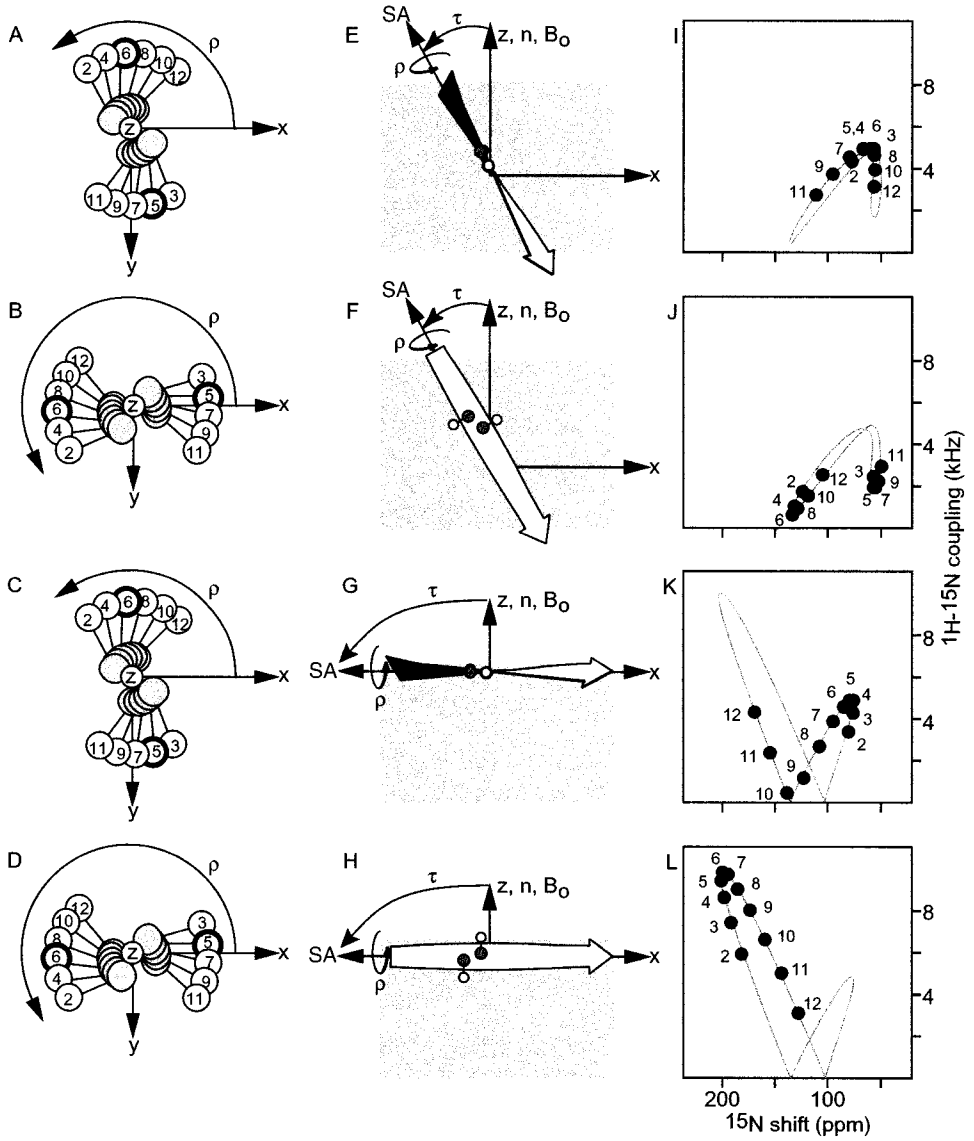

FIGURE 3 Correspondence between β-strand tilt and polarity, and the resulting PISEMA spectra, for a uniformly ^{15}N-labeled, β-stranded protein in oriented lipid bilayers. The wheel projections of backbone amide NH bonds were rotated by different values of the polar angle ρ (*A–D*). The β-strands were rotated through different values of ρ about their strand long axes (SA) and then tilted away from the membrane normal (n) by an angle $\tau = 30°$ (*E* and *F*) or $\tau = 90°$ (*G* and *H*). The amide NH bonds of residues 5 and 6 are shown: N atoms (gray balls) and H atoms (white balls). The PISEMA spectra were calculated from the described helix rotations and tilts (*I–L*). The gray lines trace out the Pisa wheel patterns characteristic of β-strands tilted by $\tau = 30°$ (*I* and *J*) or $\tau = 90°$ (*K* and *L*).

dictated by the rotation angle ρ. Thus, the peptide planes from residues 2, 4, 6, 8, 10, and 12, on one face of the β-strand, have a similar orientation with respect to the magnetic field direction and have corresponding resonances that occupy one separate wing of the spectrum. Residues 3, 5, 7, 9, and 11, on the opposite face of the β-strand, have peptide planes with a similar orientation relative to the magnetic field direction and corresponding resonances that occupy the other wing of the spectrum (Fig. 3, *I–J*). As illustrated in Fig. 1, the degree of strand twist also affects the position of resonances on the twisted Pisa wheel.

β-Strands parallel to the membrane surface

In a second mode of membrane association, β-strands align with their axes parallel to the bilayer surface. For example, the formation of extended β-sheets on the membrane surface has been implicated in the aggregation of β-amyloid fibrils (Benzinger et al., 2000; Lansbury et al., 1995; Yanagisawa et al., 1995; Fletcher and Keire, 1997; Terzi et al., 1997). As shown in Fig. 3, *K* and *L*, the PISEMA spectra of in-plane β-strands also exhibit characteristic resonance patterns that reflect the strand tilt and rotation described in terms of the angles ρ and τ. These spectra were calculated for two strands parallel to the membrane surface ($\tau = 90°$) but with different rotations of ρ about their respective SAs. Both spectra trace out the circular pattern predicted for a strand tilt of 90° (gray traces); however, the positions of the resonances are markedly different and mirror both the strand rotation on the membrane surface (Fig. 3, *G* and *H*) and their corresponding wheel projections (Fig. 3, *C* and *D*).

In Fig. 3 *C*, rotation through ρ orients the amide NH bonds of residues 5 and 6 nearly parallel to the laboratory *y* axis. A subsequent tilt of the strand, through $\tau = 90°$ around

y, aligns these NH bonds nearly orthogonal to the magnetic field, so that their resonances have ^{1}H-^{15}N dipolar coupling frequencies around 5 kHz. In Fig. 3 *D*, on the other hand, the NH bonds of residues 5 and 6 align nearly orthogonal to the *y* axis after rotation through ρ and nearly parallel to the magnetic field direction after a tilt through $\tau = 90°$. Their corresponding resonances have ^{1}H-^{15}N dipolar coupling frequencies around 10 kHz. As for the previous case of transmembrane strands, the positions of resonances on the twisted Pisa wheel reflect the degree of β-strand twist angle.

Outer membrane porin from *R. capsulatus*

The β-barrels, typified by the bacterial outer-membrane porins, are water-filled pores made up of 14–18 transmembrane β-strands, with tilts of 30°–60°. Each strand contains 7–12 amino acids with alternating hydrophobic and polar residues in the membrane-buried region. Hydrophobic residues face the membrane interior and polar residues line the pore (Seshadri et al., 1998). The sensitivity of Pisa wheels to the tilt and polarity of β-strands could be particularly useful for determining the mode of membrane insertion of these molecules. The PISEMA spectrum calculated for porin from *R. capsulatus* (Weiss et al., 1991; PDB file 2POR) is shown in Fig. 4. The spectrum from uniformly ^{15}N-labeled porin displays only modest resolution among its 269 resonances (301 residues excluding the N terminus and the 38 prolines) and demonstrates the need for higher spectral dimensionality (Fig. 4 *B*). However, the majority of resonances do fall on a common twisted Pisa wheel, characteristic of β-strands tilted by 30°–60°, whose trace is clearly visible even in the absence of substantial resolution. Thus, it is possible that even a crowded spectrum such as this one may offer some qualitative information about struc-

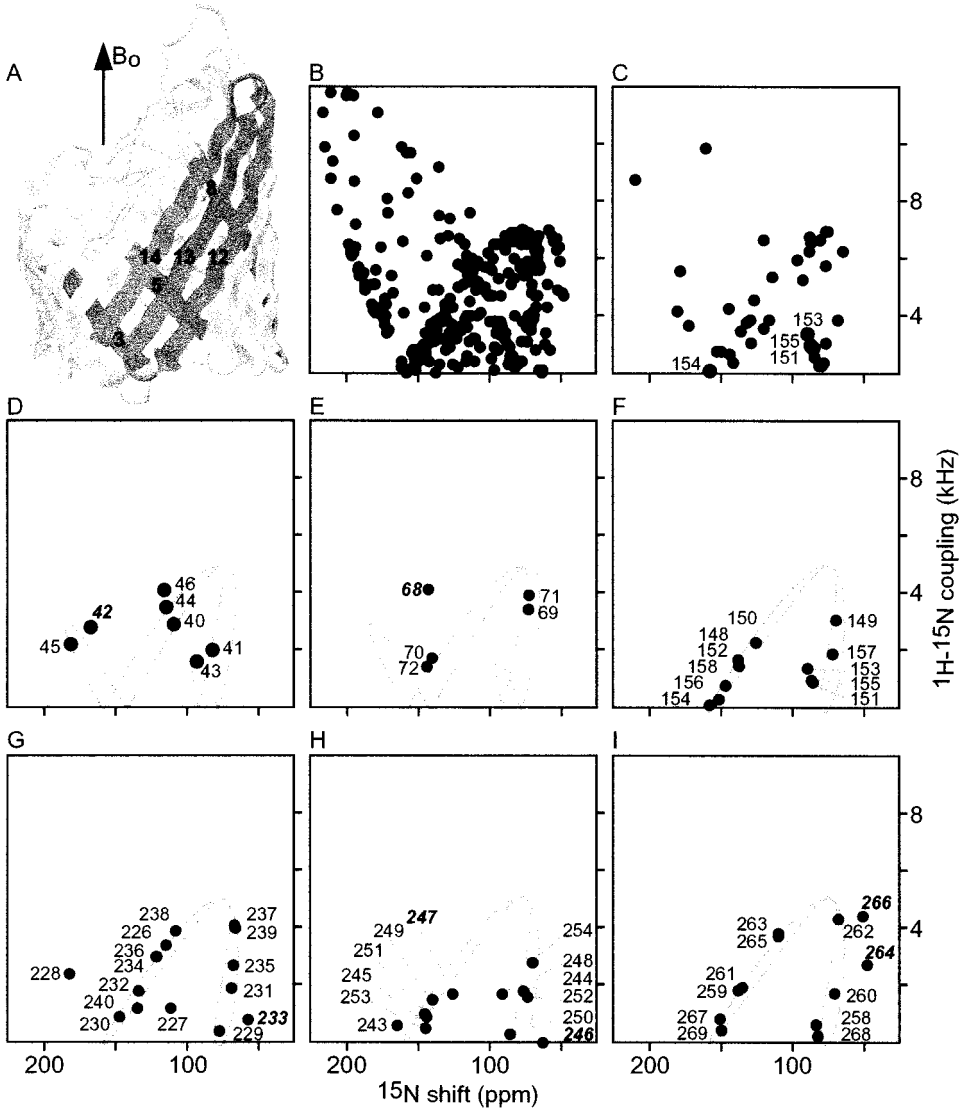

FIGURE 4 ^{1}H-^{15}N dipolar coupling/^{15}N chemical shift PISEMA correlation spectrum calculated for outer membrane porin from *R. capsulatus* (Weiss et al., 1992; PDB file 2POR). The side view of the porin structure has the PDB *z*-coordinate axis parallel to the membrane normal and to the direction of the magnetic field (B_o) (*A*). The spectra for uniformly ^{15}N-labeled porin (*B*) and [^{15}N]Ala-labeled porin (*C*) both display the twisted Pisa wheel pattern predicted for β-strands. Resonances from Ala151, 153, 154 and 155, in β-strand 8, are labeled. The spectra for individual β-strands (*D–I*) were extracted from the uniformly ^{15}N-labeled spectrum in *B*. The spectra for β-strands 3 (*D*) and 5 (*E*) overlay on a twisted Pisa wheel calculated for a tilt of 60° (*gray line*). The spectra for β-strands 8 (*F*), 12 (*G*), 13 (*H*), and 14 (*I*) overlay on a twisted Pisa wheel calculated for a tilt of 35° (*gray line*). The glycine resonances are labeled in bold italic.

ture. The spectrum was calculated assuming that the porin molecule is completely immobilized by its membrane environment, and although residues in the loop and terminal regions of the molecule could display varying degrees of mobility, this assumption is likely true for the membrane-buried β-strands, suggesting that the prominent twisted Pisa wheel feature of the spectrum would likely prevail.

Experimentally, the observation of PISEMA spectra from membrane proteins in oriented lipid bilayers requires the production of milligram quantities of isotopically labeled protein, followed by protein reconstitution in oriented lipid bilayers. Substantial progress has been made in both the areas of recombinant protein expression and oriented lipid sample preparation, and the spectra of a number of membrane proteins have now been recorded (Marassi et al., 2000b). The ability to express proteins in bacteria, or other organisms, allows a variety of isotope labeling schemes to be used for NMR spectroscopy. For example, porin can be expressed in bacteria, grown on media supplemented with all the individual amino acids, of which only one type is labeled. Such a selectively labeled protein sample can be extremely useful in providing resolution and, in some cases, some resonance assignments. The advantages of selective labeling are illustrated with the PISEMA spectrum calculated for [^{15}N]Ala-labeled porin (Fig. 4 *C*). There are 39 alanines in the porin molecule, and the spectrum displays excellent resolution. Because 24 of the alanines are in β-strands, the characteristic twisted Pisa wheel pattern is very well defined in this spectrum. For example, Ala151, 153, and 155, located on one face of β-strand 8, occupy one wing of the twisted Pisa wheel, whereas Ala154, on the opposite face of the β-strand, occupies the other wing. Experimentally, these resonances from neighboring residues can be assigned by means of ^{15}N spin exchange spectroscopy on a [^{15}N]Ala-labeled sample. This process applied to the case of β-strands would be aided by the large chemical shift difference between resonances from nearest neighbors (i.e., 154 and 155). However, even without resonance assignment, it is possible to identify a β-stranded structure from the predominant twisted Pisa wheel pattern.

The PISEMA spectra of individual β-strands in the structure of porin are shown in Fig. 4, *D–I*. Strands 8, 12, 13, and 14 are tilted by ~35°, and this tilt angle is immediately apparent from the spectra, which overlay remarkably well on the spectrum calculated for an ideal β-strand with a 35° tilt (Fig. 4, *F–I*, *gray trace*). Similarly, the spectra from strands 3 and 5 have a tilt of ~60° and overlay rather well on the spectrum calculated for an ideal β-strand with a 60° tilt (Fig. 4, *D* and *E*, *gray trace*). Once again, residues from opposite sides of each strand occupy different wings of the spectrum. For example, in strand 13, residues 244, 246, 248, 250, 252, and 254 face the polar pore interior and occupy the upfield wing of the twisted Pisa wheel, whereas residues 243, 245, 247, 249, 251, and 253 face the hydrophobic lipid core and occupy the downfield wing. This segregation of resonances from pore-facing or lipid-facing residues is apparent in the spectra from all strands.

The ^{15}N chemical shift tensor for glycine is significantly different from those of other residues and has been well characterized (Munowitz et al., 1982; Harbison et al., 1984; Oas et al., 1987; Mai et al., 1993; Shoji et al., 1993; Lee et al., 1998). There are 38 glycines in the porin molecule, and because the Gly resonance frequencies were calculated using Gly-specific values of the ^{15}N amide chemical shift tensor (Oas et al., 1987) they display the most significant deviations from the predicted Pisa wheel trace (10–20 ppm upfield; Fig. 4, *B* and *D–I*, bold italic). It is encouraging to see that the general features of the characteristic twisted Pisa wheel pattern are preserved, even in the crowded spectrum from uniformly ^{15}N-labeled porin, despite this residue-dependent tensor variation.

CONCLUSION

The definitive answer about the topology of a membrane protein can only come from its three-dimensional structure, obtained from the resolution and sequential assignment of resonances from all residues. However, the data presented in this study demonstrate that the NMR ^{1}H-^{15}N/^{15}N PISEMA and ^{1}H/^{15}N HETCOR spectra of uniformly ^{15}N-labeled proteins, associated with oriented lipid bilayers, yield characteristic patterns of resonances, so-called Pisa wheels, that provide a powerful, qualitative and visually accessible measure of membrane protein secondary structure and topology. Although Pisa wheels are observed for many α-helical membrane proteins in lipid bilayers, the case of β-strands remains to be examined experimentally. The present analysis predicts that Pisa wheels are also likely to be observed for oriented β-strands.

The experimental observation of Pisa wheels in the PISEMA spectra of several ^{15}N-labeled, α-helical membrane proteins in oriented lipid bilayers leads to an important conclusion for structural biological applications of solid-state NMR spectroscopy, i.e., that residue-dependent variations of the amide ^{15}N chemical shift tensor and residue-localized dynamics of the protein backbone are not sufficiently large to overshadow the prominent structure-dependent features in the NMR spectra of oriented membrane proteins.

In solution NMR protein structure determination, the sensitivity of the isotropic ^{13}C chemical shift to local molecular geometry provides a method for determining secondary structural elements based on simple inspection of C_α and C_β resonance assignments (Wishart et al., 1992; Kuszewski et al., 1995). This chemical shift index is so effective that it is routinely used to identify macromolecular structural features and to guide NMR structure refinement. In analogy to the solution NMR chemical shift index, the ^{1}H-^{15}N/^{15}N PISEMA, and possibly ^{1}H/^{15}N HETCOR and ^{1}H-^{13}C/^{13}C PISEMA, spectra of uniformly ^{15}N-labeled pro-

teins in oriented lipid bilayers could be used as indices of membrane protein structure and topology. These would enable valuable structural information to emerge from the spectroscopic studies before complete three-dimensional structure determination. The latter is a longer task that requires the resolution and assignment of all resonances.

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