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Structure and dynamics of a membrane protein in micelles from three solution NMR experiments

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Abstract

Three solution NMR experiments on a uniformly ^{15}N labeled membrane protein in micelles provide sufficient information to describe the structure, topology, and dynamics of its helices, as well as additional information that characterizes the principal features of residues in terminal and inter-helical loop regions. The backbone amide resonances are assigned with an HMQC-NOESY experiment and the backbone dynamics are characterized by a ^{1}H-^{15}N heteronuclear NOE experiment, which clearly distinguishes between the structured helical residues and the more mobile residues in the terminal and interhelical loop regions of the protein. The structure and topology of the helices are described by Dipolar waves and PISA wheels derived from experimental measurements of residual dipolar couplings (RDCs) and residual chemical shift anisotropies (RCSAs). The results show that the membrane-bound form of Pf1 coat protein has a 20-residue trans-membrane hydrophobic helix with an orientation that differs by about 90° from that of an 8-residue amphipathic helix. This combination of three-experiments that yields Dipolar waves and PISA wheels has the potential to contribute to high-throughput structural characterizations of membrane proteins.

Introduction

The anisotropy of the dipole-dipole and chemical shift interactions map protein structures onto NMR spectra of aligned samples. As a result, it is possible to determine the structures of proteins in aligned samples by NMR spectroscopy (Opella et al., 1987; Bax et al., 2001; Prestegard et al., 2001). The intimate relationship between spectroscopic indices of regular secondary structures, such as PISA wheels (Marassi and Opella, 2000; Wang et al., 2000) and Dipolar waves (Mesleh et al., 2002), and sequential resonance assignments (Marassi and Opella, 2003) accelerates the structure determination process. Further, the incorporation of angular restraints from dipolar couplings measured on aligned samples enables the fold of the protein to be determined with a high degree of confidence based on limited information relating the orientations of separate structural elements such as helices.

It is possible to prepare both completely and weakly aligned samples for NMR studies of membrane proteins associated with lipids that assemble into micelles, bicelles, or bilayers. The high helix content of the principal class of membrane proteins is an advantage in the context of aligned samples. By contrast, this is typically a disadvantage in isotropic samples where the limited dispersion of chemical shifts is compounded by the relatively large number of similar hydrophobic side chains and lack of detectable 'long-range' NOEs. Completely aligned samples of membrane protein in lipid bilayers have enabled the structures of several membrane proteins to be determined by solid-state NMR spectroscopy (Ketchem et al., 1993; Opella et al., 1999; Wang et al., 2001), including with the recently implemented 'Shotgun' approach (Marassi and Opella, 2003) where the three-dimensional structure of a membrane protein was determined from the spectra of one uniformly

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and four selectively ^{15}N- labeled samples by utilizing the mapping of the structure onto the spectra for simultaneous resonance assignment and structure determination. In this article, we describe an adaptation of this approach for solution NMR studies of membrane proteins in weakly aligned micelle samples. As an example, we describe the backbone structure and dynamics of the membrane-bound form of Pf1 coat protein with solution NMR experiments on uniformly ^{15}N labeled samples in sodium dodecylsulfate (SDS) micelles. We have previously described some aspects of the structure and dynamics of this protein in dodecylphosphocholine (DPC) micelles and in phospholipid bilayers; in these environments, Pf1 coat protein shows characteristics of a typical membrane protein with a long hydrophobic transmembrane helix and a short amphipathic in-plane helix (Schiksnis et al., 1987; Shon et al., 1991). The dynamics of the protein have been described by molecular dynamics simulations and relaxation and line shape measurements (Tobias et al., 1995; Shon et al., 1991). Its overall features are similar to those of the better-studied fd coat protein (Almeida and Opella, 1997; Marassi and Opella, 2003), although there is no apparent sequence homology between them.

The structure and dynamics of the membrane-bound form of Pf1 coat protein in SDS micelles are described with the results of three solution NMR experiments, two of which, the HMQC-NOESY (Shon and Opella, 1989) and heteronuclear ^{1}H-^{15}N NOE (Bogusky et al., 1987) experiments, have been applied previously to Pf1 coat protein in DPC micelles. However, the chemical shift differences between spectra obtained in DPC micelles and SDS micelles are sufficient to require the reassignment of the resonances. The sequential assignment of all backbone amide resonances was accomplished with a single two-dimensional HMQC-NOESY experiment on a uniformly ^{15}N labeled isotropic sample. The heteronuclear ^{1}H-^{15}N NOE experiment is particularly sensitive to the internal backbone dynamics of membrane proteins, because of their distinctive pattern of structured helical segments interspersed with mobile connecting loop and terminal segments. The results of these two experiments are complemented by measurements of residual dipolar couplings (RDCs) from an IPAP-HSQC experiment (Ottiger et al., 1998) and residual chemical shift anisotropies (RCSAs), which are obtained from the differences in the frequencies of the nitrogen resonances in isotropic and weakly aligned polyacrylamide gel samples. The RDCs and RCSAs enable PISA wheels and Dipolar waves to be used in the analysis of solution NMR studies of membrane proteins in micelles. Previously, they have been used in solid-state NMR studies of membrane protein in bilayers (Marassi and Opella, 2000; Wang et al., 2000; Opella et al., 2002). An essential step is the preparation of weakly aligned micelle samples, and this can be done in several ways, including through the addition of lanthanide ions (Ma and Opella, 2000; Veglia and Opella, 2000) or the incorporation of the protein-containing micelles into strained polyacrylamide gels (Tycko et al., 2000; Sass et al., 2000; Chou et al., 2001).

Experimental methods

Sample preparation

Uniformly ^{15}N-labelled Pf1 coat protein was prepared as described previously (Schiksnis et al., 1987). The weakly aligned sample was obtained by soaking a solution of 1 mM Pf1 coat protein in 500 mM SDS and 40 mM NaCl into a 7% acrylamide gel made from a stock solution containing 30% w/v acrylamide and 0.8% w/v N, N′-methylenebisacrylamide. The polyacrylamide gel was cast in a 3.5 mm-inner diameter glass tube, washed for about two hours, cut to a length of 25 mm, and dried at 35 °C overnight. The protein solution was then soaked into the dry gel overnight with the length of the gel restricted to 18 mm. Isotropic samples were prepared in the same way except for being cast in a 4.2 mm tube and having no restriction on the length. The pH of the samples was adjusted to 4.0, and all experiments were performed at 313K.

NMR spectroscopy

The NMR experiments were performed on Bruker Avance 750 and DRX 600 spectrometers. The two-dimensional HMQC-NOESY spectrum was obtained on an isotropic sample using a 250 ms mixing time. The steady-state heteronuclear ^{1}H-^{15}N NOE measurements were also made on this sample using the experiment described by Farrow et al. (1994) with and without ^{1}H irradiation time of 2.2 s. IPAP-HSQC spectra (Ottiger et al., 1998) obtained with suppression of the NH_2 signals (Ishii et al., 2001) on isotropic and weakly aligned samples of protein-containing SDS micelles in a polyacrlamide gel were used to measure the ^{1}H-^{15}N residual dipolar couplings. For the sample in the polyacrylamide gel, it was necessary to use a

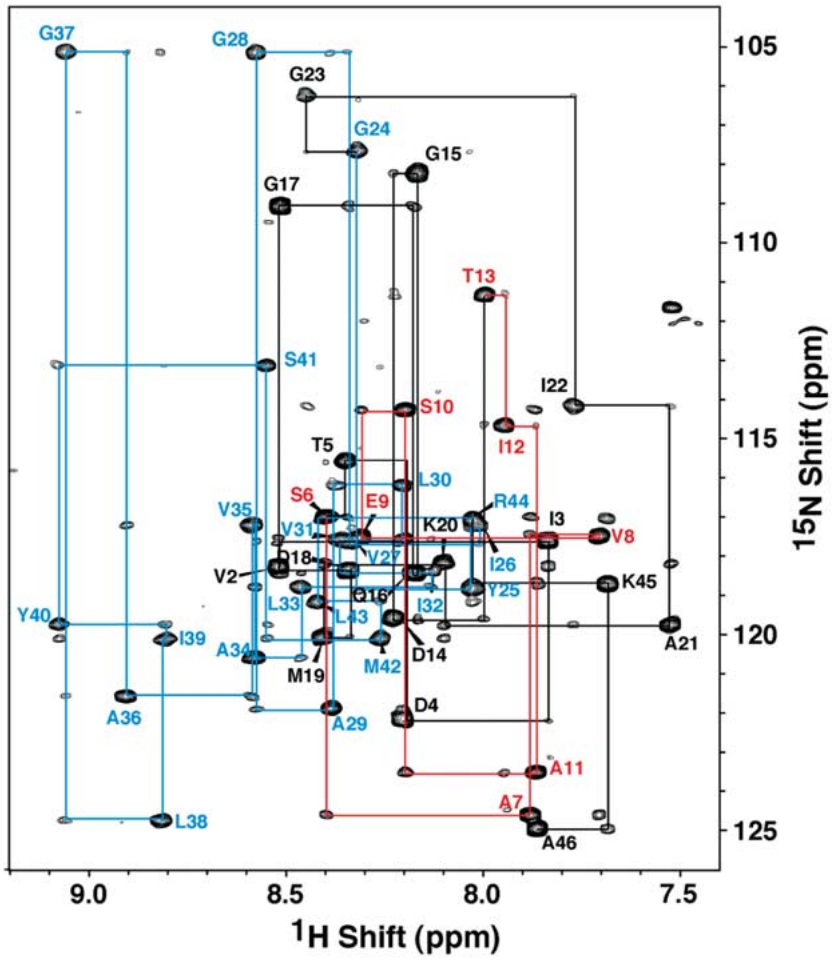

Figure 1. HMQC-NOESY spectrum of Pf1 coat protein in SDS micelles obtained with a mixing time of 250 ms. The lines show the sequential connectivities between amide protons. The spectrum was obtained using a recycle delay of 1.5 s and 512 t_1 points. The spectrum was processed using the program NMRPipe (Delaglio et al., 1995) and a sine-bell function was applied in both dimensions.

version of the IPAP-HSQC experiment modified for suppression of the NH_2 resonances from acrylamide (Ishii et al., 2001). While the positions of the peaks shift slightly in the uncompressed gel samples of the protein relative to the isotropic samples, this does not affect the measurement of the ^1H-^{15}N RDCs. The minor isotropic chemical shift differences in the gel environment did not interfere with the measurement of residual dipolar couplings, however, it was essential to have a control experiment under the same gel sample conditions for the accurate measurement of alignment-induced chemical shifts.

Results

First experiment

The amide region of the two-dimensional ^1H-^{15}N HMQC-NOESY spectrum of uniformly ^{15}N labeled Pf1 coat protein in SDS micelles is shown in Figure 1. The ^1H-^1H homonuclear mixing interval of 250 msec enabled the observation of cross-peaks from all of the backbone H_N-H_N NOEs between adjacent residues in the protein. The sequential NOE connectivities used to assign the resonances are represented with lines between cross-peaks. We previously applied this experiment to Pf1 coat protein in DPC micelles (Shon et al., 1991) and observed NOEs only between residues in the helical segments. The results obtained in SDS micelles differ in that it is possible to observe

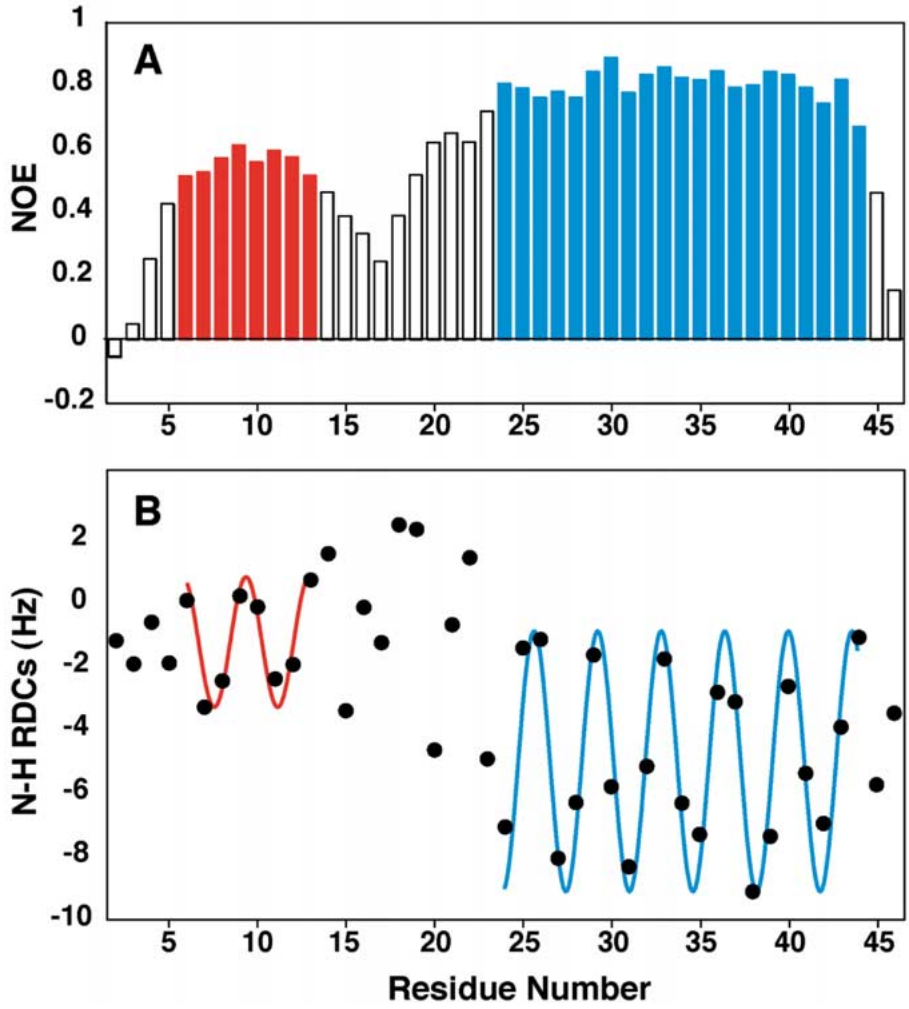

Figure 2. A. Steady-state heteronuclear ^{1}H-^{15}N NOE data for Pf1 as a function of residue number along the sequence. B. A plot of RDCs versus residue numbers for Pf1 in SDS micelle. Solid line represents the fitting of RDCs using sine-wave function (red; amphipathic helix, blue; trans-membrane helix).

the sequential NOE connectivities between residues in the loop connecting the hydrophobic and amphipathic helices in addition to those in the helices. As the structures of an increasing number of membrane proteins are being determined in various micelle, bicelle, and bilayer preparations, differences are being found in the structure and dynamics of residues that reside near the lipid head groups. In the longer term, this may become a compelling argument to perform all structure determinations of membrane proteins in lipid bilayers, which are the closest mimic to naturally occurring membranes. However, both micelles and bicelles provide model membrane environments compatible with native, functional membrane proteins and are useful for some NMR approaches to structure determination. Therefore, at present, there is considerable interest in determining the structures of membrane proteins in micelles by solution NMR spectroscopy, and comparisons between structures determined in various lipid environments will also be of interest until the effects of lipids and their assemblies on protein structure are better characterized. This is an area where there are likely to be differences between the results of x-ray crystallography and NMR spectroscopy, and the flexibility in choice of sample conditions is a valuable feature of NMR approaches to structure determination. One explanation for the increased number of backbone NOEs in Figure 1 compared to our earlier results (Shon et al., 1991) would be that the inter-helical loop residues of Pf1 coat protein

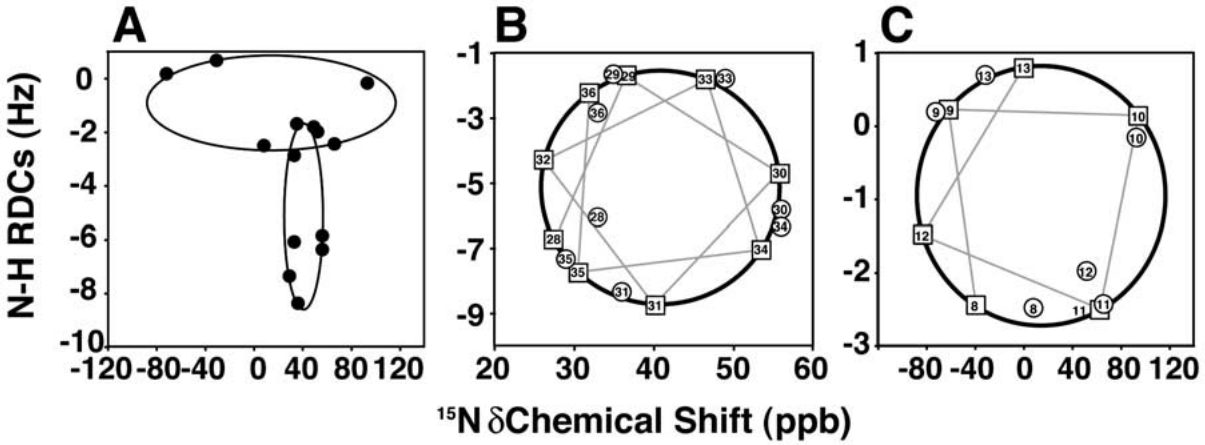

Figure 3. PISA wheel patterns for residues 8–13 and 28–36 of Pf1 coat protein in SDS micelles. ^{15}N chemical shift differences are calculated by subtracting the chemical shifts for isotropic sample from the chemical shifts for weakly aligned sample. The squares on the large circle represent the ideal PISA wheel pattern and the lines show the connectivity between the ideal PISA wheel pattern for consecutive residues.

are more 'structured' (i.e., they have smaller amplitude or lower frequency motions) in SDS micelles than in DPC micelles, although caution is needed in taking the timescales for both overall and local motions into account. Regardless of the explanation for the additional NOEs observed among backbone amide sites in SDS micelles, they make it possible to 'walk' through the entire protein sequence and assign all of the backbone amide resonances with one experiment on a uniformly ^{15}N labeled sample. Similar results were obtained from a ^{1}H-^{15}N HSQC-NOESY experiment modified from two-dimensional FHSQC (Mori et al., 1995) with the expected improvement in the resolution in the indirect dimension.

Second experiment

Backbone structure and dynamics are more obviously coupled in membrane proteins than they are in some other classes of proteins, and the heteronuclear ^{1}H-^{15}N NOE provides a remarkably direct and sensitive indicator of the local dynamics of membrane proteins in micelles (Bogusky et al., 1987). The experimental ^{1}H-^{15}N heteronuclear NOEs for all backbone amide sites of the membrane-bound form of Pf1 coat protein in SDS micelles are shown in Figure 2A. The values are taken from steady-state heteronuclear ^{1}H-^{15}N NOE spectra acquired using the method of Farrow et al. (1994) with and without 2.2 s of irradiation to saturate the ^{1}H magnetization. The regions with the slowest correlation times, as reflected in the positive ^{1}H-^{15}N heteronuclear NOE values, are highly correlated with the locations of the helices. The data in Figure 2A show that residues in the N- and C- terminal regions as well as in the loop connecting the helices are more mobile than residues in the helices. These data also indicate that the amphipathic helix on the surface of the micelles has some motions that are not present in the trans-membrane hydrophobic helix. The motions of the amphipathic helix have been described in some detail for the membrane-bound form of fd coat protein (Almeida and Opella, 1997).

Third experiment

Dipolar waves are a direct consequence of the fact that the periodicity of α-helices maps to the measurement of anisotropic parameters in NMR spectroscopy (Mesleh et al., 2002). Figure 2B plots the experimental ^{1}H-^{15}N RDCs of the backbone amide sites of Pf1 coat protein as a function of residue number. The fits of the RDCs to sinusoids with a periodicity of 3.6 residues characteristic of a α-helix are also shown. The quantitative fit of two sine waves demonstrates that Pf1 coat protein has two α-helical segments, between residues 6 and 13 and between residues 24 and 44. The average values and the magnitudes of the dipolar couplings of the waves fit to the amphipathic helix region and the trans-membrane helix region are distinctly different, and can be used to determine the relative orientations of the two helices in the same molecular frame of reference (Mesleh et al., 2002).

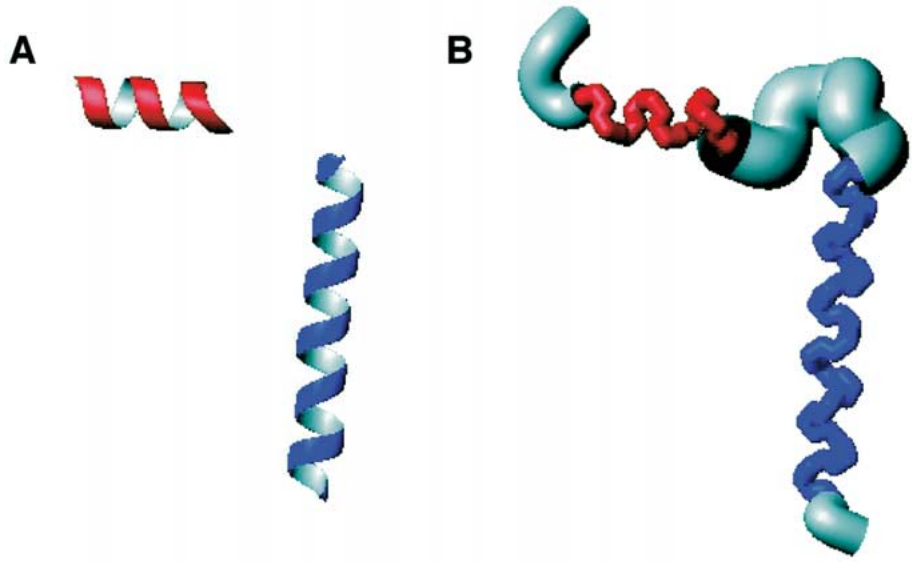

Figure 4. Backbone representation of the fold of the membrane-bound form of Pf1 coat protein in SDS micelles determined by the experimental data in Figures 2 and 3. (A) Ribbons showing the length and relative orientations of the helices. (B) Backbone bonds showing the three-dimensional structure of the protein. The thickness of the bonds is proportional to the positional variability of the backbone sites consistent with the orientational restraints. RMSDs for the residues in helices are about 0.5 Å while those for some residues in the interhelical loop region are an order of magnitude larger, as visualized using the program MOLMOL (Koradi et al., 1996).

PISA wheels and Dipolar waves from a weakly aligned sample

Figure 3A shows the PISA wheel patterns for the resonances from residues in the two helical segments of Pf1 coat protein in SDS micelles. In this plot each resonance is characterized by two frequencies, the ^{15}N RCSA and ^{1}H-^{15}N RDC. ^{15}N RCSAs are the difference between the chemical shifts observed in an isotropic sample and a weakly aligned sample. Since the ^{15}N RCSAs are very small, the isotropic values were measured for a protein-containing micelle solution in the same polyacrylamide gel that was cast in a NMR sample tube. The PISA wheel patterns in Figure 3A are analogous to those observed in solid-state NMR spectra of completely aligned proteins (Marrassi and Opella, 2000; Wang et al., 2000), and provide further evidence that there are two helical segments in the protein. The differences in position, size, and ellipticity are indicative of the presence of two differently oriented helices.

Figure 3A shows the plot of RDCs versus ^{15}N RCSAs for residues in both the amphipathic helix and the transmembrane hydrophobic helix, in which the thin ovals superimposed on the data represent ideal PISA wheel patterns. Figure 3B and C separate the data from the two helices on scales that show circles that illustrate the correspondence between helical wheels and PISA wheels with the 100° angle between positions of adjacent residues in the helix. The squares are the points for an ideal PISA wheel pattern. The differences in positions of squares and circles in Figures 3B and C show that there are only minor deviations due to variations among the ^{15}N chemical shift tensors. The resonance from residue 32 is overlapped with another in the spectra, and is not included in the Figure. The data for the amphipathic helix and the transmembrane helix can be readily distinguish in the plots in Figure 3, and this feature may be useful in studies of larger, more complex membrane proteins in identifying helices. The PISA wheels can be used to assist in the assignment process, as demonstrated with analogous solid-state NMR data (Marassi and Opella, 2002).

Discussion

Comparisons between measurements on isotropic and weakly aligned samples enable a remarkable amount of information to be obtained from just three experiments on a membrane protein in micelles. The sequential resonance assignments are obtained from the homonuclear ^{1}H-^{1}H NOEs observed in a single two-dimensional HMQC-NOESY spectrum, since

the amide hydrogens in the polypeptide backbone are proximate in helices and in the turn between helices. The ^{1}H-^{15}N heteronuclear NOEs differentiate between residues in structured helices and more mobile loop and terminal segments. Complementary information about structure (time average positions) and dynamics (time dependent positions) emerges from the first two experiments, and set the stage for the orientational parameters from the third experiment. The experiments directly show the positions of the helices in the sequence and their relative orientations with intervening and terminal residues that display greater mobility indicative of internal dynamics that are completely consistent with the previous molecular dynamics calculations (Tobias et al., 1995).

The PISA wheels and Dipolar waves derived from the measurements of RCSAs and RDCs obtained by comparison of isotropic and weakly aligned samples measured with an IPAP experiment provide structural information in the form of the relative orientations of the helices in the molecular frame. If the amplitude and average value of the sinusoid fitted to the experimental RDCs are allowed to vary, it is possible to include additional residues at the N-terminal (19–23) or the C-terminal (45–46) in the hydrophobic helix from Figure 2B based solely on the score that reflects periodicity. However, the inclusion of these additional residues in the helices as well as the appearance of curvature of the N-terminal helix that would result from this altered fitting are unlikely, because the experimental ^{1}H-^{15}N NOEs in Figure 2B indicate that the local dynamics are increased for these same residues.

A structural model of the protein was built using strong dihedral restraints to define the location of the helices and the residual dipolar couplings to optimize their relative orientations. Using XPLOR-NIH (Schwieters et al., 2003), a simple one step protocol was implemented to generate an ensemble of 40 structures starting from an extended template using dihedral restraints ($\Phi = -65^{\circ}$ and $\Psi = -40^{\circ}$) and SANI restraints with force constants scaled gradually from 0 to 30 while the system is cooled from 3000 K to 25 K. Due to angular degeneracies inherent in the use of RDCs, there are four families of structures defined by the four symmetry-related arrangements of the helices consistent with the experimetntal data. However, the correct arrangement of helices is determined by reference to the solid-state NMR data on a completely aligned sample in lipid bilayers (Shon et al., 1991), since this provides absolute orientational information. The 14 structures in this family were then used to generate an average structure in which the relative orientations of the two helices are meaningful. The conformations of the loop and termini are under-restrained, therefore their conformation is less meaningful. Figure 4 shows the structure of Pf1 as determined with the information derived from these three experiments.

The determination of the structures of membrane proteins is an important aspect of contemporary structural biology. These proteins require the presence of lipids to maintain their structural and functional integrity, however the lipids complicate the preparation of samples for experimental measurements. As a result, it is of interest to make comparisons among various types of lipid assemblies, including micelles, bicelles, and bilayers. Structure determination of completely aligned membrane proteins in lipid bilayers has been demonstrated by solid-state NMR spectroscopy. Because there are several ways to prepare weakly aligned samples of membrane proteins in micelles, it is possible to make measurements and to analyze the residual chemical shift anisotropies and residual dipolar couplings in the backbone sites in terms of PISA wheels, Dipolar waves, and other indices of protein structure. This lends itself to rapid and accurate structure determination, setting the stage for high throughput analysis of the structures of membrane proteins by NMR spectroscopy.

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