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Myristoylation as a general method for immobilization and alignment of soluble proteins for solid-state NMR structural studies

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Abstract

N-terminal myristoylation of the immunoglobulin-binding domain of protein G (GB1) from group G Streptococcus provides the means to bind the protein to aligned phospholipid bilayers for solid-state NMR structural studies. The myristoylated protein is immobilized by its interactions with bilayers, and the sample alignment enables orientationally dependent ^{15}N chemical shifts and ^{1}H-^{15}N-dipolar couplings to be measured. Spectra calculated for the average solution NMR structure of the protein at various orientations with respect to the magnetic field direction were compared to the experimental spectrum. The best fit identified the orientation of the myristoylated protein on the lipid bilayers, and demonstrated that the protein adopts a similar structure in both its myristoylated and non-myristoylated forms, and that the structure is not grossly distorted by its interaction with the phosholipid bilayer surface or by its location in the restricted aqueous space between bilayer leaflets. The protein is oriented such that its charged sides face the phosphatidylcholine headgroups of the lipids with the single amphiphilic helix running parallel to the bilayer surface.

Introduction

The NMR method of choice for determining the structure of a protein depends on its physical properties and the available methods of sample preparation. Soluble proteins that reorient rapidly in solution are generally good candidates for multidimensional solution NMR spectroscopy. In contrast, solid-state NMR of aligned samples has been most useful for determining the structures of membrane-associated proteins (Opella et al., 2001). The intrinsic physical limitations of the solution method are such that successful applications to slowly reorienting or immobile proteins or complexes are difficult and highly problematic for insoluble proteins. In these cases, solid-state NMR methodology may become of interest since it can be effectively applied to insoluble and immobile proteins. Solid-state NMR of aligned samples requires that the protein be immobilized and uniaxially oriented parallel to the direction of the applied magnetic field. For proteins with hydrophobic membrane-spanning helices, this is readily achieved by incorporating the protein in aligned lipid bilayers (Marassi et al., 1997). For soluble proteins, however, this strategy is not feasible and appears to be limited to using either crystals or frozen solutions for solid-state NMR studies.

In this article, we present a strategy that may be generally applicable for immobilizing and aligning soluble proteins, transforming them into suitable candidates for structure determination by solid-state NMR spectroscopy. The protein of interest is attached, via a short linker, to a myristoyl group which anchors it to a hydrated, planar, aligned lipid bilayer. The lipid bilayer alignment is achieved by the interaction of the hydrated phospholipid headgroups with the silica surface of glass slides. Provided that the linker is

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kept reasonably short, the motion of the protein relative to the membrane is highly restricted, and the protein is uniaxially oriented along with the lipids. Immobilization and orientation enable the measurement of orientation-dependent spin interactions that serve as restraints for protein three-dimensional structure determination (Opella et al., 1987).

Application of the general strategy is illustrated for the immunoglobulin-binding domain of protein G from group G Streptococcus (GB1). The high-resolution three-dimensional structure of this domain has been determined by solution NMR spectroscopy (Gronenborn et al., 1991). The structure is composed of a four-stranded sheet, covered on one face by an α-helix. The central strands, β1 and β4, of the two hairpins run parallel, with β2 and β3 connected by the single helix. The structure contains an extensive hydrogen-bonding network and a tightly packed hydrophobic core that contributes to the extreme thermal stability of this small domain.

Materials and methods

Biosynthetic myristoylation

Myristoylation of GB1 was achieved by adding a five amino acid signal sequence at the N-terminus of the protein by oligonucleotide directed mutagenesis of the gene. Expression of a polypeptide with the GNAAS linker preceding the original N-terminal methionine residue was confirmed by mass spectrometry (data not shown). Co-expression of this construct with N-myristoyl CoA transferase (Duronio et al., 1990) in *E. coli* yielded varying amounts of myristoylated and non-myristoylated protein, depending on growth conditions. Uniformly ^{15}N-labeled myristoylated GNAAS-GB1 was prepared by growing *E.coli* carrying the GB1 expression plasmid and pBB131 (kindly provided by J.I. Gordon) using $^{15}NH_4Cl$ as the sole nitrogen source, and adding myristic acid (15 mg l^{-1}) prior to induction. Cells (2 g) were suspended in 5 volumes (10 ml) of PBS, heated at 80 °C for 5 min, and then chilled in ice for 10 min. The lysed cell suspension was centrifuged at 16 000 rpm (SS-34 rotor, Sorvall, Newtown, CT) for 30 min at 4 °C. The supernatant was dialyzed against excess water at 4 °C and lyophilized. The protein was dissolved in 2 ml of 7.5 M guanidine-HCl, 50 mM Tris-HCl at pH 8 and 5 mM EDTA, centrifuged at 40 000 rpm (Type 75 TI, Beckman Coulter, Fullerton, CA) for 45 min and applied at ambient temperature and a flow-rate of 3 ml min^{-1} to a Superdex-75 column (HiLoad, 2.6 × 60-cm; Amersham Biosciences, Buckinghamshire, UK) equilibrated in 50 mM Tris-HCl, pH 8, 4 M guanidine-HCl, 5 mM EDTA. The myristoylated GB1 was separated from the non-myristoylated GB1 by reverse-phase HPLC on PorosII resin (Perceptive Biosystems, Clearwater, MN) using a linear gradient of 0 to 60% acetonitrile/0.05% trifluoroacetic acid. Pure protein (>95% as assessed by mass-spectrometry) was folded by dialysis into the appropriate buffer and concentrated.

Solid-state NMR sample preparation

Aligned bilayer samples were prepared by co-dissolving 2.5 mg of the purified ^{15}N-labeled protein with 50 mg of 1,2-Dimyristoyl-*sn*-Glycero-Phosphatidylcholine (DMPC) (Avanti Biochemicals, Alabaster, AL), in 2 ml of chloroform, 0.5 ml of trifluoroethanol and 5 drops of water. This yields a lipid:protein mole ratio of 200:1. The solution was bath-sonicated until clear and spread on the surface of 25 11 × 11 mm glass slides (Paul Marienfeld, Lauda-Königshofen, Germany) that had been etched in hydrofluoric acid to reduce their nominal 0.06 mm thickness (Prosser et al., 1995). After removing the solvents under vacuum, the slides were stacked and placed in a sealed chamber maintained at 94% relative humidity with a saturated ammonium phosphate solution. Oriented bilayers formed after overnight equilibration of the sample. The sample was sealed in a thin plastic film before insertion into the coil of the NMR probe.

Solid-state NMR experiments

One-dimensional ^{15}N and ^{31}P solid-state NMR spectra were obtained on samples of myristoyated-GB1 in aligned lipid bilayers. The experiments were performed on a home-built NMR spectrometer with Magnex 700/62 magnet (Magnex Scientific, Oxfordshire, UK). The flat-coil, double resonance ^{1}H/^{15}N and ^{1}H/^{31}P NMR probes were home-built, with coil dimensions of 11 × 11 × 3 mm. One-dimensional ^{31}P NMR spectra of the oriented lipid bilayers on glass plates were recorded using a single-pulse experiment with ^{1}H decoupling during acquisition. One dimensional ^{15}N NMR spectra of the same protein sample in lipid bilayer preparations were recorded using single-contact CPMOIST (*c*ross-*p*olarization with *m*ismatch-*o*ptimized *IS t*ransfer) (Levitt et al., 1986; Pines et al.,

1973), with a cross-polarization radio frequency (rf) field strength of 52 kHz, and a ^{1}H decoupling rf field strength 63 kHz during the 5 ms acquisition time. The recycle delay was 7 s to prevent radio frequency heating of the hydrated samples. The temperature was controlled at 30 °C.

The two-dimensional ^{1}H-^{15}N PISEMA (*p*olarization *i*nversion with *s*pin *e*xchange at the *m*agic *a*ngle) spectrum (Wu et al., 1994) was obtained using similar conditions, rf field strengths, and signal averaging as in the one-dimensional experiments. The frequency-switched Lee–Goldburg condition for spin-lock of the ^{1}H magnetization at the magic angle (Lee and Goldburg, 1965; Bielecki et al., 1990) was obtained with a 34.5 kHz frequency jump. A total of 1024 transients were acquired for each of 64 t_1 values for a total evolution of 2.5 ms in t_1. The data were zero filled twice, in both the t_2 and t_1 dimensions, yielding a 512 × 512 real matrix. A 400 Hz exponential multiplication was applied in t_2, while a 90° phase shifted sine bell multiplication was applied in t_1. The data were processed using Felix97 (Accelrys, San Diego, CA). The ^{15}N and ^{31}P chemical shifts were referenced to 0 ppm for external liquid samples of ammonia and phosphoric acid, respectively.

Calculation of PISEMA spectra

The ^{15}N chemical shift and ^{1}H-^{15}N dipolar coupling frequencies form the basis for protein structure determination in uniaxially oriented samples, since they depend on the orientation of the molecular site with respect to the direction of the applied magnetic field, and on the magnitudes and orientations of the principal elements of the spin-interaction tensors as shown in Figure 1. The ^{15}N chemical shift frequency is given by:

$$\nu_{\text{N shift}} = \sigma_{11}\sin^2(\alpha-\delta)\sin^2\beta + \sigma_{22}\cos^2\beta + \sigma_{33}cos^2(\alpha-\delta)\sin^2\beta, \quad (1)$$

where σ_{11}, σ_{22}, and σ_{33} are the principal elements of the ^{15}N chemical shift tensor, and δ is the angle between the σ_{33} axis and the NH bond. The ^{1}H-^{15}N dipolar coupling frequency is given by:

$$\nu_{\text{NH cplg}} = \mu_0\gamma_H\gamma_N h/8\pi^3 r^3 (3\sin^2\beta\cos^2\alpha - 1), \quad (2)$$

where γ_H and γ_N are the gyromagnetic ratios of the two spins, μ_0 is the permittivity of free space, h is Planck's constant, and r is the NH bond length. The dipolar interaction results in a doublet, with maximal splitting when the bond vector is aligned parallel to the magnetic field direction, and half maximal when the bond vector is aligned perpendicular to the field. In both equations [1] and [2] α and β are the polar angles that describe the peptide plane orientation in the magnetic field: α is the angle between the NH bond and the projection of the magnetic field direction on the peptide plane, and β is the angle between the normal to the peptide plane and the direction of the magnetic field (Opella et al., 1987).

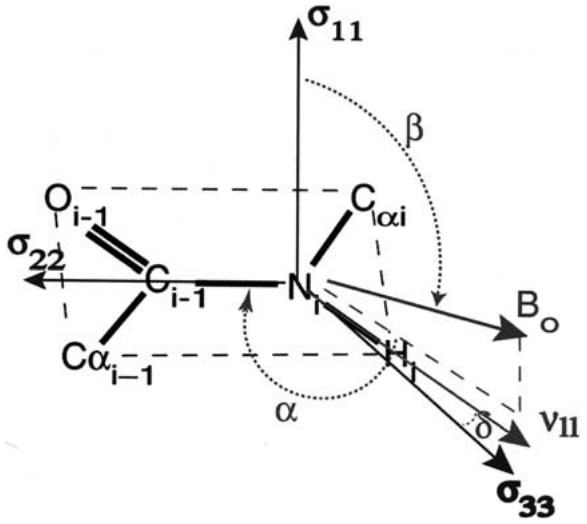

Figure 1. Orientations of the ^{15}N chemical shift (red arrows) and ^{1}H-^{15}N dipolar coupling (NH bond) spin interaction tensors in the peptide plane molecular frame of reference. The σ_{33} and σ_{11} principal elements of the ^{15}N chemical shift tensor are in the peptide plane, σ_{22} is orthogonal to the peptide plane, and the angle δ between σ_{33} and the NH bond is 17° (Wu et al., 1995). The polar angles α and β describe the peptide plane orientation in the magnetic field: α is the angle between the NH bond and the projection of the magnetic field direction (Bo) on the peptide plane, and β is the angle between the normal to the peptide plane and the direction of the magnetic field (Opella et al., 1987).

The amide ^{15}N chemical shift tensor and the NH bond length are characterized reasonably well (Wu et al., 1995), so that it is possible to calculate the NMR spectra for specific models of proteins at specific orientations in oriented samples, using Equations 1 and 2 (Marassi et al., 2000; Marassi, 2001). The principal values of the ^{15}N chemical shift tensor (σ_{11} = 64 ppm; σ_{22} = 77 ppm; σ_{33} = 225 ppm) were measured from the powder pattern of uniformly ^{15}N-labeled myristoylated-GB1 in unoriented lipid bilayers (Figure 2B). The tensor orientation (δ = 17°) and the NH bond distance (1.07Å) were as previously determined (Wu et al., 1995). The ^{15}N chemical shifts of the Gly residues, were calculated using the tensor principal

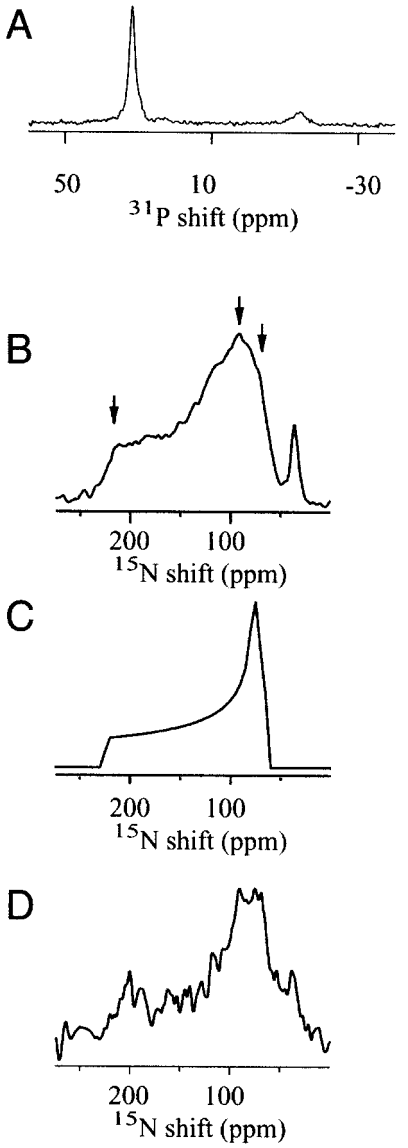

Figure 2. (A) One-dimensional ^{31}P NMR spectrum of oriented DMPC bilayers containing Myr-GB1. (B–D) One-dimensional ^{15}N NMR spectra of uniformly ^{15}N-labeled Myr-GB1 in DMPC lipid bilayers. (B) Experimental spectrum of Myr-GB1 in unoriented bilayers. The arrows indicate the three principal elements of the ^{15}N chemical shift tensor (σ_{11} = 64 ppm; σ_{22} = 77 ppm; σ_{33} = 225 ppm). (C) Calculated powder pattern of immobilized Myr-GB1 in DMPC bilayers. (D) Experimental spectrum of Myr-GB1 in oriented bilayers.

values and orientation ($\sigma_{11} = 41$ ppm; $\sigma_{22} = 64$ ppm; $\sigma_{33} = 210$ ppm; $\delta = 18°$) determined by Oas et al. (1987). The ^{15}N chemical shifts were referenced to 0 ppm for liquid ammonia.

In order to evaluate the orientation of the myristoylated -GB1 from the experimental PISEMA spectrum, ^{1}H-^{15}N dipolar/^{15}N chemical shift spectra were calculated using the average solution NMR structure of protein G, (PDB file 2GB1; Gronenborn et al., 1991) at various orientations relative to the magnetic field direction, set to be coincident with the PDB coordinate z axis. The spectra were calculated and evaluated for best fit to the experimental data on a Silicon Graphics O_2 computer (Mountain View, CA), using the FORTRAN program SIMSPEC developed in our laboratory. For fitting spectra, the input to the program consists of the protein PDB coordinates, the ^{15}N chemical shift and ^{1}H-^{15}N dipolar coupling spin interaction tensors described above, and the ^{15}N chemical shift and ^{1}H-^{15}N dipolar coupling resonance frequencies measured in the experimental spectrum. SIMSPEC performs step-wise rotations of the PDB coordinates around the coordinate x- and y-axes, and calculates the ^{1}H-^{15}N PISEMA spectrum at each step. Since the lipid bilayer has macroscopic, uniaxial orientation around the direction of the magnetic field, which is set to be coincident with the z-axis, the results are invariant for rotations about the z-axis. At each step, the calculated spectrum is evaluated for best fit to the experimental spectrum by calculating a score (R) as the sum of the least squared distances between each experimental frequency (σ_{obs} and D^{NH}_{obs}) and the closest calculated resonance frequency (σ_{calc} and D^{NH}_{calc}) for all N resonances. Chemical shifts are compared by subtracting the isotropic values (105 ppm for Gly residues and 122 ppm for all others) from the calculated and experimental values. The two frequency dimensions are normalized using a generalization of the cross-validated scoring method so that the chemical shift and dipolar coupling contributions are weighted equally (Clore and Garrett, 1999). The score is given by

$$R^2 = \frac{1}{N}\sum_{i=1\ldots N}\left[\frac{(\sigma_{obs}-\sigma_{calc})^2}{\frac{2}{5}\sigma_{ax}^2(4+3\eta_R^2)} + \frac{(D^{NH}_{obs}-D^{NH}_{calc})^2}{\frac{8}{5}\nu_{II}^2}\right] \quad (3)$$

with N the number of experimental data measurements, σ_{ax} is the normalized magnitude of the full static chemical shift tensor (52.5 ppm for Gly and 51.5 ppm for other residues), η_R is the asymmetry

parameter (0.146 for Gly and 0.084 for other residues) of the chemical shift tensors given above, and ν_{II} is the maximum value of the dipolar coupling. Plots of the R score as a function of orientation indicate that the calculations converge to a unique minimum and its 180° rotation, so that it is possible to determine a unique protein orientation relative to the magnetic field direction. Rotations were performed in 1° steps from 0–180° around the x- and y- coordinate axes. Calculations for over 32 000 possible orientations were done in less than 10 min. The structure and orientation of myristoylated- GB1 were rendered using InsightII (Accelrys, San Diego, CA).

Results and discussion

One-dimensional solid-state NMR spectra of uniformly ^{15}N labeled myristoylated-GB1 in DMPC lipid bilayers are shown in Figure 2. The ^{31}P NMR spectrum of the lipids in an aligned sample (Figure 2A) displays a single peak at 32 ppm, as expected for highly aligned phospholipid bilayers (Herzfeld et al., 1978). The one-dimensional ^{15}N NMR spectrum of an unaligned (powder) sample in Figure 2B spans the full frequency range of the ^{15}N amide chemical shift anisotropy, as illustrated with the simulated ^{15}N amide chemical shift anisotropy powder pattern in Figure 2C, confirming that the protein is fully immobilized by its myristoyl anchor in the phospholipid bilayer. The narrow resonance intensity at 35 ppm arises from the amino nitrogens of the five Lys sidechains in the protein and is not of concern in this study. The experimental spectrum of uniformly ^{15}N-labeled myristoylated-GB1 in aligned DMPC bilayers (Figure 2D) also spans the full frequency range of the ^{15}N chemical shift anisotropy, but is substantially different in appearance from the powder patterns in Figures 2B and 2C. Although there is little resolution among the 61 backbone resonances, the distribution of intensities of the single-line resonances reflects the unique orientation of the protein relative to the lipid bilayer and magnetic field direction. The experimental two-dimensional ^{1}H/^{15}N PISEMA spectrum of the same sample shown in Figure 3A has many resolved resonances. The absence of intensity at the isotropic frequencies for the ^{15}N chemical shift ($\sim$120 ppm) and ^{1}H-^{15}N dipolar coupling (0 kHz) is highly significant, and serves to verify that the protein is immobile on the timescales (10^{-4} s) of both these anisotropic spin interactions. As a result, the orientational information of interest is fully manifested in the spectrum for structural analysis. The presence of individually resolved resonances, by itself, demonstrates that the protein is uniquely oriented by virtue of the interactions of the myristoyl group with the lipid bilayer. Thus, the spectrum in Figure 3A confirms the original hypothesis that myristoylation anchors the protein to aligned DMPC lipid bilayers in a way that immobilizes and orients the peptide bonds.

The ability to calculate solid-state NMR spectra for specific models of proteins in oriented samples (Marassi and Opella, 2001; Marassi, 2001) is fundamental for determining the orientation of myristoylated-GB1 relative to that of the aligned lipid bilayers. Both of the PISEMA resonance frequencies associated with each resonance depend on the protein structure and orientation, as well as the values and orientations of the principal elements of the relevant spin interaction tensors. The orientation of myristoylated-GB1 anchored to aligned DMPC bilayers was determined by calculating ^{1}H-^{15}N PISEMA spectra from the PDB coordinates of the average solution NMR structure (Gronenborn et al., 1991). Spectra were calculated for many different rotations of the protein xyz coordinates around the x- and y-axes relative to the coordinate z-axis. Representative spectra are shown in Figures 3B–E. These spectra reflect different orientations of myristoylated-GB1 relative to the magnetic field direction and the lipid bilayer. For each rotation around x and y, the calculated spectrum was compared with the experimental spectrum, and rejected or retained on this basis.

The two-dimensional PISEMA spectra (Figures 3B–E) calculated for four different orientations of GB1 are distinctly different from each other. The three protein orientations displayed in Figures 3G–I with their associated calculated spectra in Figures 3C–E reveal poor correspondence with the experimental spectrum (the R scores are 0.016, 0.022, and 0.023, respectively). The GB1 orientation (Figure 3F) that yields a calculated spectrum most similar to the experimental one (Figure 3A) corresponds to an orientation of GB1 with $x = 102°$ and $y = 142°$ and an R score of 0.0063. In this orientation the myristoylated-GB1 occupies the minimum distance between individual lipid bilayer leaflets and is well accommodated by the 28 Å interstitial aqueous space determined by X-ray diffraction for liquid-crystalline DMPC lipid bilayers at a similar temperature of 37° (Petrache et al., 1998). As shown, the single helix of GB1 lies on the surface of the lipid bilayer, and is oriented such that the

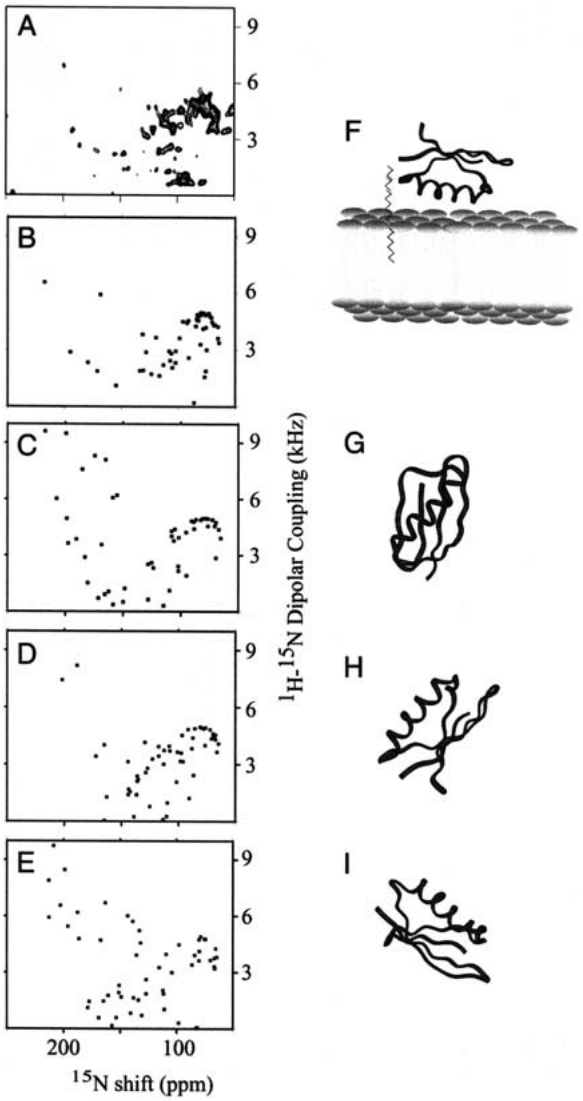

Figure 3. (A–E) Two-dimensional ^{1}H-^{15}N PISEMA spectra of uniformly ^{15}N-labeled Myr-GB1 in oriented DMPC bilayers. (A) Experimental spectrum. (B–E) Spectra calculated from the rotated PDB coordinates of the average solution NMR structure (PDB file 2GB1; Gronenborn et al., 1991). (F–I) Average solution NMR structure of Myr-GB1 at four different orientations relative to the magnetic field direction, B_0. (F) The structure of Myr-GB1 placed in its proper orientation relative to the lipid bilayer and B_0, as determined using the program SIMSPEC by least-squares fitting of the calculated spectrum to the experimental spectrum in (B). The PDB coordinate rotations were (B, F) x = 102°, y = 142°; (C, G) x = 20°, y = 120°; (D, H) x = 140°, y = 100°; (E, I) x = 50°, y = 50°. The laboratory z-axis is parallel to the magnetic field direction, B_0.

side chains of polar residues (Lys28, Glu32, Asn 35 and Asp 36) are able to interact with the zwitterionic phosphatidylcholine headgroup of DMPC. However, because the NMR spectra are invariant to inversion, it is also possible that the external side of the sheet interacts with the bilayer surface. This surface contains an abundance of polar residues (Gln2, Lys4, Asn8, Thr51, Thr53 and Thr55) equally well suited for such an interaction. Indeed, since the widths of GB1 is ∼25 Å in this dimension, both sides of the protein may interact with opposing bilayer surfaces and these interactions could contribute to stabilizing the overall orientation of the protein with respect to the membrane.

Conclusions

The ^{15}N chemical shift and ^{1}H-^{15}N dipolar coupling frequencies measured from a two-dimensional PISEMA spectrum of uniformly ^{15}N labeled myristoylated GB1 provide sufficient information to determine the structure of the protein. In the present application without resonance assignments it was possible to demonstrate that the protein is oriented such that it rests on the bilayer surface with its charged sides facing the phosphatidylcholine headgroups of the lipids. The similarity between the experimental ^{1}H-^{15}N PISEMA spectrum of myristoylated-GB1 with a spectrum calculated based on the solution NMR structure of GB1 confirms that the overall structure is not affected by its interaction with the phospholipid bilayer surface or by its occupation of the restricted aqueous space between bilayer leaflets.

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