Probing Lipid–Protein Interactions by Solid-state NMR Spectroscopy of Fast Frozen Samples

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Solid-state NMR spectroscopy of fast frozen samples of ^{15}N indole labelled gramicidin A has revealed specific hydrogen bonding between the indole N–H and the solvating environment of the gramicidin channel state in the liquid-crystalline phase of hydrated lipid bilayers.

The role of tryptophan in stabilizing membrane protein conformation is thought to be very important. Recently, it has been argued that tryptophan will partition at the bilayer interface[1] and now tryptophan has been shown to be located near the bilayer surface of several transmembrane proteins.[2–5] Furthermore, it has been suggested in a computational study that the indole N–H of Trp_{15} in the membrane-spanning dimer of gramicidin A may hydrogen bond to the lipid, thereby forming a specific lipid–protein interaction.[6] Electrophysiological studies also suggest that these interactions may exist for the tryptophans of gramicidin.[7] Here we present spectroscopic evidence that the Trp_9 indole of gramicidin in the channel state, which is closer to the bilayer centre, may also be hydrogen bonded.

Chemical shift tensors reflect the electronic environment of the nuclear site and consequently, they have the potential to report on interactions, such as hydrogen bonding and ring stacking, as well as structural constraints such as torsion angles.[8–10] Making these interpretations has been hampered by the lack of computational techniques, high quality experimental data and well-defined structural characterizations. However, recently there has been progress in the calculation of isotropic ^1H chemical shifts for the purposes of structural characterization.[11,12] Furthermore, correlations between the N···O hydrogen bond length and chemical shift have been established.[9] As this length shortens in a series of oligopeptides the ^{15}N isotropic frequency shifts substantially downfield. Here we have obtained accurate values for the chemical shift tensor elements of the indole ^{15}N site in several different environments. The qualitative result is that these tensor elements differ substantially between these environments and that the shifts in frequency suggest hydrogen bonding for this site when it is in a lipid bilayer.

Shown in Fig. 1 are the chemical shift powder patterns for the $^{15}N_{\varepsilon 1}$ labelled amino acid, tryptophan and $^{15}N_{\varepsilon 1}$-Trp_9 labelled gramicidin A. The tensor elements, which have been determined from spectral simulations, are very similar for these two indole sites (Table 1). Once gramicidin is mixed with lipid and bilayers formed by the addition of water, the indole site becomes motionally averaged, owing to global rotational motion of the channel about the bilayer normal, as shown in Fig. 1(*e*). Consequently, the values of the tensor elements are not directly comparable with the dry powder samples. However, the isotropic average of the tensor elements in these

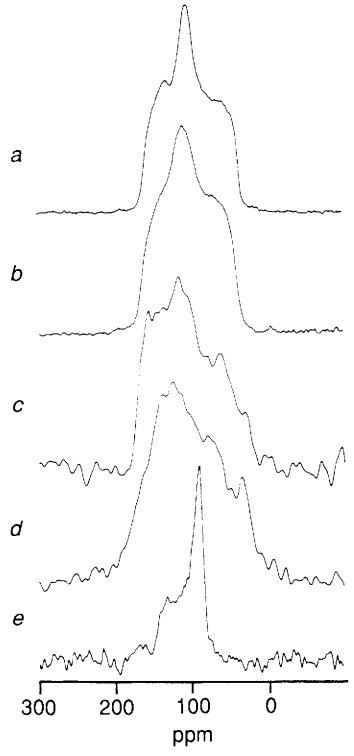

Fig. 1 ^{15}N NMR powder pattern spectra of $^{15}N_{\varepsilon 1}$ labelled tryptophan. All spectra were acquired at room temp. [except (*c*) and (*d*) which were recorded at 150 K] with a resonant frequency of 40.62 MHz. Cross polarization from abundant ^{1}H was used with a Hahn echo to minimize probe ringing, using: 1 ms mixing time, 6.5 μs 90° pulse width, 48 μs echo delay and a 7 s recycle delay. (*a*) Powder pattern of a dry sample of the amino acid; 6144 acquisitions. (*b*) Powder pattern of Trp_9 labelled gramicidin A precipitated from an aqueous solution and dried at room temp.; 11568 acquisitions. (*c*) Sample in (*e*) that has been fast frozen in small aliquots by plunging into liquid propane prior to further cooling in liquid nitrogen. An accurate determination of the σ_{11} tensor element is somewhat compromised by natural abundant intensity from the lipid choline headgroup; 6544 acquisitions. (*d*) Sample in (*e*) that has been frozen slowly in bulk by blowing cold nitrogen gas over the sample in the NMR probe; 3520 acquisitions. (*e*) Powder pattern of a fully hydrated lipid bilayer sample obtained at 308 K above the gel to liquid-crystalline phase transition temperature; 896 acquisitions.

two preparations can be compared (Table 1) and they are substantially different. To change the isotropic averages, the static tensor elements must also change significantly.

By lowering the temperature to less than 150 K the global and local torsional motions for the indole site will cease, resulting in the potential for obtaining values for the static tensor elements. However, there are two signficiant consequences. For those ^{15}N sites involved in donating a hydrogen bond the isotropic chemical shift is temperature sensitive and consequently the tensor elements will also be temperature sensitive.[13,14] Secondly, in lowering the temperature relatively slowly (*e.g.* with cold gas) the environment for the nuclear site becomes heterogeneous resulting in severely broadened discontinuities of the powder pattern spectrum as shown in Fig. 1(*d*). Even for water soluble proteins slow freezing techniques result in heterogeneous broadening of the magic angle spinning spectra.[15] For membrane proteins the problem is considerably worse. When the temperature is lowered through the gel to liquid-crystalline phase transition the hydrophobic dimension of the bilayer changes considerably and with it the lipid–protein interactions. Samples that were frozen slowly showed so much heterogeneous broadening of the powder pattern as seen in Fig. 1(*d*) that it was not possible

Table 1 Chemical shift tensor elements for $^{15}N_{\varepsilon 1}$ tryptophan[a]

Sample condition	σ_{11}	σ_{22}	σ_{33}	σ_{iso}
Amino acid	35 ± 1	104 ± 1	158 ± 1	99 ± 1
Trp_9 Gramicidin A dry powder	36 ± 2	106 ± 2	161 ± 2	101 ± 2
Hydrated bilayer				
308 K		85 ± 2[b]	144 ± 2[b]	105 ± 2
150 K	36 ± 3	116 ± 2	171 ± 2	108 ± 2

[a] All chemical shifts given in ppm relative to $^{15}NH_4NO_3$. [b] Axially symmetric powder pattern with $\sigma_{\perp}$ at 85 ppm and $\sigma_{\parallel}$ at 144 ppm.

to determine accurately the tensor element magnitudes. Even if they could be determined it is not clear that it would reflect the environment of the indole above the lipid phase transition temperature.

To avoid these distortions, standard fast freezing techniques were adapted from electron microscopy,[16] *i.e.* freezing small quantities at a time of the hydrated lipid preparation in liquid propane before lowering the temperature further in liquid nitrogen. In so doing, it has been possible to trap the liquid-crystalline state of the bilayer system and a single conformational species of gramicidin resulting in the observation of well-defined discontinuities [Fig. 1(*c*)]. Both σ_{22} and σ_{33} differ by 10 ppm or more from these elements for the amino acid or the dry powder of Trp_9 gramicidin A. Furthermore, the isotropic average has shifted upon lowering the temperature, *i.e.* the isotropic average for the fast frozen powder pattern and that for the axially symmetric powder pattern differ by 3 ppm.

In both the non-crystalline powder of the amino acid and the polypeptide it is unlikely that the indole N–H is hydrogen bonded. However, the vey substantial changes in the tensor element magnitudes, the isotropic frequency, and the observation of a significant temperature dependence for the isotropic frequency strongly suggests that this indole N–H is hydrogen bonded when in the lipid bilayer environment. Since this tryptophan is well buried within the bilayer it may be that indole is donating a hydrogen bond to a trapped solvent molecule. This would be similar to the trapped ethanol that is hydrogen bonded to the indole N–H groups in crystals of gramicidin obtained from a benzene–ethanol cosolvent system.[17] Furthermore, it is possible in the bilayer that a water molecule forms a hydrogen bond bridge between the indole and the lipid ester. It is not known, at this time, what the exact location of this indole is within the bilayer.

The search for specific lipid–protein interactions has spanned several decades.[18] Through the use of fast-freeze techniques evidence for these interactions or those of peptide with solvent have been obtained. These techniques should have considerable utility in a variety of solid-state NMR applications. Recently, others have noticed that magic angle spinning linewidths can be improved by freezing fine droplets of a sample preparation in liquid nitrogen prior to lyophilization[19] and time resolved rapid reactions are being studied by fast freezing NMR techniques.[20] This fast freezing technology has the potential to improve many current experiments as well as the potential to solve one of the most fundamental problems facing biological solid-state NMR spectroscopists—the ability to resolve structural from dynamic effects on powder pattern spectra by the rapid quenching of molecular motions without causing conformational rearrangements.

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