Structure and dynamics from solid state NMR spectroscopy

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NMR methods designed to observe biological samples in anisotropic environments (eg. membrane-bound proteins or structural proteins) have now been available for two decades. These 'solid state' NMR methods used to be called 'wide-line' NMR spectroscopy because typical observations were of very broad resonances. Today, however, there are approaches for achieving high resolution structures of high molecular weight biological complexes using solid state NMR.

As there is no need to crystallize samples or to have isotropic solutions, there is no intrinsic molecular weight limit for this structural approach. This advantage can be combined with the determination of dynamics at high resolution (one of the traditional strengths of biological solid state NMR). Recently there have been significant advances in our ability to characterize specific-site dynamics, and this is leading to important insights into protein function.

In NMR spectroscopy of molecules in solution, molecular motions provide the line narrowing mechanism. To obtain high resolution solid state NMR spectra, it is necessary either to spin the sample mechanically, or to orient it. High-speed sample rotation is used in the rotational resonance [1] and rotational echo double-resonance [2] methods for achieving distance constraints. In another approach, uniformly aligned samples have been used to achieve orientational constraints [3]. These constraints are possible because the NMR observables, such as chemical shift, dipolar and quadrupolar interactions, are orientation-dependent with respect to the static magnetic field of the NMR spectrometer. Furthermore, because these interactions have a definable orientation with respect to the molecular frame (i.e. the molecular bonds) it is possible to constrain the orientation of these bonds with respect to the magnetic field and the alignment axis, which is a unique molecular axis.

From orientational constraints to structure

Over the past few decades considerable effort has been expended in trying to align samples for a wide range of spectroscopies. For the gramicidin channel the orientational dispersion has been measured to be less than $\pm 0.3°$ in the best samples [4]. While such optimal alignment can be put to good use, it is not essential for this solid state NMR method. Dispersions of the order of 10° for preparations of bacteriorhodopsin [5], viral coat protein [6] and silk fibroin [7] have not prevented the determination of unique structural information from these systems.

Biosynthetic and chemical methods for isotopic labelling at single or selective sites have been used to assign NMR observables to a specific molecular site. The advances in solid-phase peptide synthesis have made it a very important tool for studies of polypeptides and protein fragments. Not only is there efficient (and economical) use of labels, but high incorporation of the label can be achieved for efficient double labelling (for example, the incorporation of a $^{13}C_1$ labelled amino acid and an adjacent ^{15}N-labelled amino acid).

A plethora of NMR observables is possible. All atomic sites can be labelled in proteins with NMR-active nuclei and, therefore, not only the individual observation of chemical shifts and quadrupolar interactions, but a wide range of dipolar interactions are potentially available for observation. Some of these interactions are much more easily observed and interpreted than others. The structure of the polypeptide backbone of the gramicidin channel has been determined using three interactions for each peptide linkage: the ^{15}N chemical shift (Fig. 1a), the ^{15}N–^{1}H and ^{15}N–^{13}C dipolar interactions [4]. The conformation of the indole rings has been determined with a combination of ^{15}N chemical shift, ^{15}N–^{1}H dipolar and ^{2}H quadrupolar interactions [8], while the other aliphatic side chains have been solved with constraints derived solely from ^{2}H NMR (K-C Lee, S Huo and TA Cross, unpublished data). Each interaction constrains the structure of the molecule with respect to the orientational axis (large arrow) as shown in Fig. 2, where each experimental constraint is illustrated by a small arrow indicating the Z component of the NMR observable.

An initial structure for the gramicidin channel was assembled from these constraints by assuming that each peptide linkage is planar. Determining the backbone structure thereby reduces to a challenge of solving for 28 dihedral angles. From the 44 orientational constraints in the backbone it was possible to solve for these torsion angles. Interestingly, two possible solutions were achieved each with the same folding motif (i.e. the same hydrogen bonds, residues per turn and helix sense). The difference is a subtle one, in that they have opposite patterns for the 'in' (toward the channel axis) and 'out' (toward the lipid environment) alternating orientation of the carbonyl bonds in the peptide planes. The exact orientation of these carbonyl groups is of great interest since these oxygens provide much of the cation solvation environment when cations are in the channel. An initial structure has also been solved for each of the side-chains using a total of 68 additional

constraints ([8]; and K-C Lee, S Huo and TA Cross, unpublished data).

The refinement of this structure has begun using three separate approaches: a simulated annealing protocol to use the constraints optimally; modification of the interaction magnitudes based on detailed dynamics described below; and improved interaction characterizations using fast-frozen sample preparations. The initial computational efforts have achieved a set of conformations with only a $\pm 3°$ root mean square deviation range in the backbone torsion angles including the ω torsion angle which was allowed to deviate from 180° in the computational refinement [4].

Dynamics

Large amplitude dynamics can be readily defined by the averaging of powder pattern spectra (which are spectra of unoriented samples, not necessarily dry samples). Relatively small amplitude librational motions (limited amplitude motions about the torsional axes) can also be characterized in detail from these spectra. In Fig. 1b it is shown that the averaging of the powder pattern spectra for gramicidin between 200 K and 283 K (just below the lipid phase transition temperature) is anisotropic [10]. In fact, significant librational motion about χ_1 can be ruled out while motion about χ_2 is entirely consistent with the data. Furthermore, from the spectral simulations, the amplitude for the librational motions of this site is shown to be $\pm 25°$. With this detailed dynamic model in place, it is possible to measure the ^{15}N T_1 relaxation rates for specific sites [9] and interpret these rates in light of this model rather than having to assume a model for the local motions, as is done in most studies. This approach reduces the number of variables to be solved with relaxation data, and therefore unique frequency solutions can be achieved.

Advantages, disadvantages and prospects for the future

The use of solid state NMR spectroscopy for characterizing high resolution structure and dynamics has been recently reviewed [11]. The combination of high resolution structure and dynamics has the potential to lead to important correlations with functional properties of proteins in general. For gramicidin, it has been shown that the librational rate for the peptide linkages [9] in the backbone is occurring on the same timescale as the calculated translational rate for the cations between carbonyl solvation clusters. The librational amplitudes in the indole side chains cause dramatic fluctuations in the radial component of the dipole moment which is considered to be important for stabilizing cations in the channel. Therefore, the combination of high resolution dynamics and structure brings new insights to the functional understanding of this relatively simple molecule, gramicidin A, and demonstrates the potential for achieving similar insights for proteins in general.

There are hurdles that need to be overcome before this approach can be used to solve macromolecular structures on a routine basis, however. Improvements in the procedures for alignment will come from the application of very high magnetic fields [12]. Although the first 750 MHz NMR spectrometers have just recently been put in place, the next generation of magnets are already under development with the location of a 850 MHz (10^{-6} to 10^{-7} homogeneity) magnet in the National High Magnetic Field Laboratory in 1994. The ^{15}N spectral resolution in the aligned samples of gramicidin (3 ppm linewidth out of an anisotropy of 150

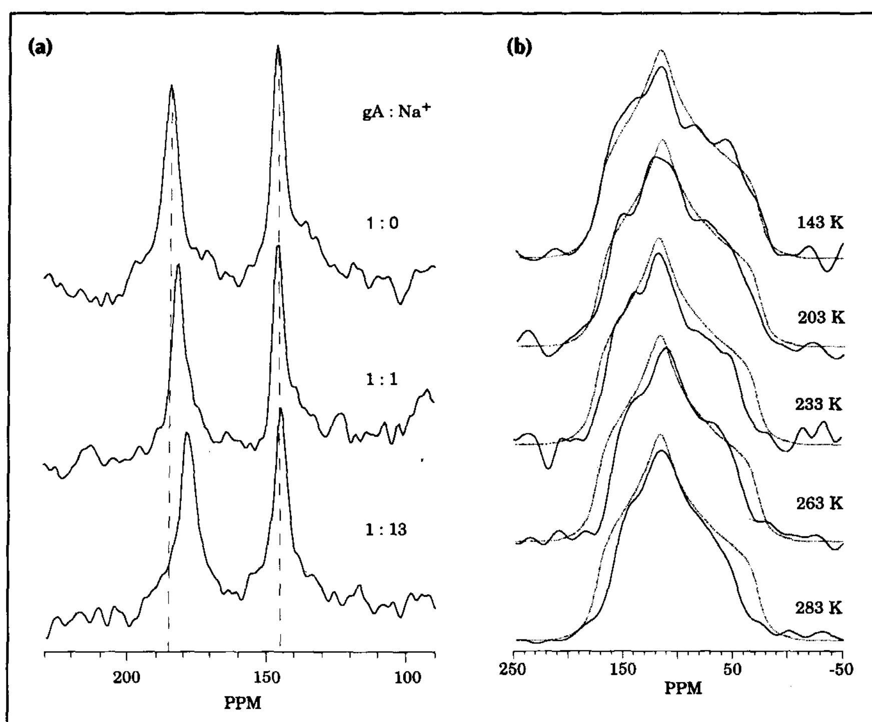

Fig. 1. ^{15}N chemical shift NMR spectra of Trp13 labelled gramicidin A in hydrated lipid bilayers. **(a)** Spectra of uniformly aligned $^{15}N_{\alpha,\epsilon1}$ Trp13 labelled gramicidin in the presence and absence of cations. Small changes in the chemical shifts occur at high enough molar ratios to have 80 % occupancy of one (1:1) and two (1:13) binding sites in the channel. These small differences in chemical shift suggest a change of a few degrees in the orientation of the peptide plane. **(b)** Spectra of fast-frozen bilayer preparations (unoriented) of $^{15}N_{\epsilon1}$Trp13 labelled gramicidin as a function of temperature between 143 K and 283 K. The dotted line is the spectral simulation of the static spectrum at 143 K. Above 200 K, librational motions average the chemical shift interaction in a specific way that can yield both the amplitude of the motion and the orientation of the motional axis to the molecular frame.

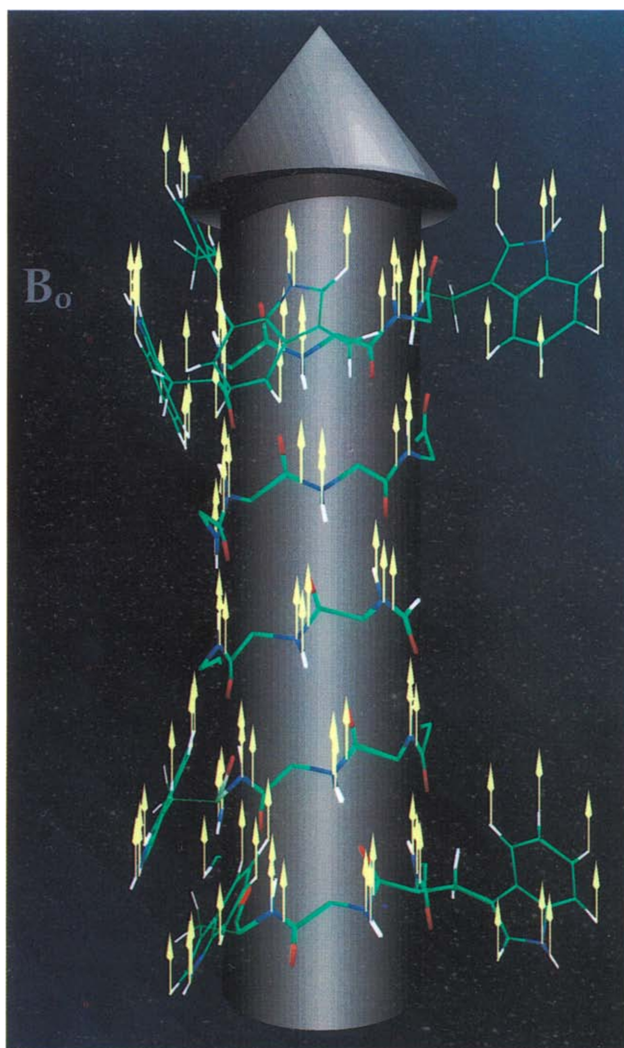

Fig. 2. A portion of the gramicidin backbone structure and indole side chains shown in a side view. This structure was determined from solid state NMR-derived orientational constraints. Specific site NMR observables were recorded in uniformly aligned preparations of the channel in hydrated lipid bilayers. The Z component of each of these observables (small arrows) can be used to align the specific nuclear site with respect to the magnetic field of the NMR spectrometer (large arrow). The channel axis and bilayer normal have been aligned in these samples to be parallel with respect to the magnetic field.

ppm) is comparable to the ^{15}N spectral resolution in solution NMR (0.3 ppm out of an isotropic dispersion range of 15 ppm), therefore sufficient spectral resolution exists for the development of multi-dimensional solid state NMR methods [13], that offer an opportunity to avoid specific site labelling. These new methods will permit the study of proteins from cloned genes and allow the collection of numerous constraints at one time. This structural approach would also be enhanced by the addition of distance constraints [14]. The orientational constraints have been shown to be very good local constraints, and even the errors summed over seven residues for a turn of the gramicidin helix did not interfere with the unambiguous determination of the inter-turn hydrogen bonding pattern. While distance and orientational constraints have yet to be combined, there is no intrinsic barrier to accomplishing this task. A final concern involves the need for a detailed characterization of the interaction coordinate systems in the molecular frame. By judicious choice of the NMR observables to be interpreted, this problem can be minimized.

Solid state NMR can not only describe the complete structure of macromolecules, but it also has the capability to determine the structure of bound ligands [5]. The potential also exists to investigate a region of a macromolecule, such as the active site, without the need for complete structural elucidation. Solid state NMR now provides a proven tool for structural biologists to characterize the detailed conformation and dynamics of membrane-bound and structural proteins.

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