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Solid State Nuclear Magnetic Resonance Spectroscopic Approaches to the Study of the Molecular Organization of Biological Membranes

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Abstract. This paper gives an historical overview of the development of nuclear magnetic (NMR) resonance spectroscopic methods to the investigation of the static and dynamic structures of lipids and proteins in biological membranes, with special emphasis on recently developed solid state NMR methods applicable to these systems. Topics covered include NMR studies of phospholipids, sterols, peptides and proteins in model lipid bilayer membranes, as well as biological membranes themselves.

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is one of the more versatile methods currently available for the study of molecular structure and dynamics. Apart from the well known contributions of NMR to the study of the structures of small molecules in solution, which have had such a profound impact on all branches of chemistry, one of the more impressive recent achievements has been the elucidation of the complete three-dimensional structures of macromolecules in solution through the use of multidimensional NMR methods [1,2]. This approach, applicable to molecules having molecular weights of up to 25-30 kD [1], complements classical x-ray crystallographic methods since many macromolecules do not readily crystallize under normal laboratory conditions, although of course in several specific instances spectacular structural information on isolated membrane proteins has been achieved when suitable crystals or crystalline arrays have been obtained [3-7]. Biological membranes, however, pose a special challenge since neither of these powerful methods have been routinely useful in their study. Most membrane components (including both lipids and proteins) do not form highly crystalline arrays under physiological conditions, prohibiting x-ray studies, and the size of these native macromolecular aggregates are too large to use conventional solution high resolution multidimensional NMR methods. In response to these problems, new solid state NMR methods are currently being developed, which can provide unique information on molecular structure and dynamics in non-crystalline solids with atomic resolution, and no theoretical limit imposed by relaxation on the size of the molecules involved. In this Chapter we review the theoretical bases of some of these solid state NMR methods, together with recent applications to the study of the molecular organization of lipids and proteins in model and biological membranes.

2. General considerations

The strong intra- and intermolecular interactions present in rigid solids, as well as in more mobile polymeric materials and in liquid crystals (among which we include the smectic lipid mesophases which form the basic matrix of biological membranes), together

with the large molecular weights of many of the molecules involved, combine to produce generally very slow and anisotropic motions in membranes. These preclude the use of conventional high resolution solution NMR methods, which require fast, isotropic reorientation at the molecular level in order to produce complete averaging of most magnetic interactions. In contrast, solid state NMR spectra express fully or in part the tensor properties of many interactions of magnetic nuclei (dipolar, quadrupolar, chemical shift anisotropy), where the values of the NMR observables strongly depend on the orientation of the relevant interaction with respect to the applied magnetic field. Although these quantities in principle contain valuable information on the molecular structure of the material involved, their combined expression most often leads to strong overlapping of spectral features, producing uninterpretable spectra, compounded by a severe loss of signal to noise ratio, due to the dispersion of spectral frequencies over a wide range (tens to hundreds of kilohertz). Modern solid state NMR methods combine the separation and/or removal of most of these interactions to achieve high resolution spectra, using either mechanical, magnetic or spin-excitation procedures. In some cases, the interaction of interest may then be selectively reintroduced, in order to obtain detailed atomic level structural information.

Such methods can also provide valuable information on the dynamical properties of individual molecules and aggregates, since they can sample an impressive (10^{12}) range of frequencies, ranging from very fast (intramolecular) motions, in the range of 10^9 to 10^{12} Hz (which can be analyzed via spin-lattice relaxation methods), to motions near the resonance frequencies (ω_0) of the nuclei involved (10^7-10^9 Hz with currently attainable magnetic fields), to intermediate motions ($\sqrt{M_2} \ll \tau_c^{-1} \ll \omega_0$, where $\sqrt{M_2}$ is the square root of the second moment, the "NMR time scale", on the order of $\sim 10^5$ Hz for most magnetically active nuclei) which can be studied by lineshape analysis, to slow ($\sqrt{M_2} \approx \tau_c^{-1}$) or ultra slow ($\sqrt{M_2} \gg \tau_c^{-1}$) motions ($10^3$ to 10^0 Hz), which can be detected via transverse relaxation or 1- and 2-D chemical exchange methods. So, comprehensive information on molecular structure and dynamics can be simultaneously achieved, a feat that no other methodology can currently match. In the following sections we will briefly outline the theoretical bases of several of these approaches, and give recent applications in the field of membrane biophysics.

3. The lipid matrix

Lipids are by far the most studied component of biological membranes, both in situ, and in pure artificial membranes. Early work, pioneered by Chapman and coworkers (reviewed in ref [8]) used classical wide line NMR methods combined with calorimetric techniques to detect thermotropic transitions in lipid mesophases. High resolution spectra could only be obtained by reducing particle size by sonication, and although interesting structural information was derived from these studies, the potential effects of sonication on structure and function in these systems precluded its further use. However, detailed information on lipid order profiles and dynamics was then derived from ^{2}H NMR studies [9-12] and the use of double resonance (cross-polarization, CP) techniques [13-15]. When combined with "magic-angle" spinning (MAS, [16]) and high power proton decoupling, it is now possible to obtain high resolution ^{13}C and ^{1}H NMR spectra of both non-sonicated natural and artificial membranes, from which dynamical information with atomic resolution has been derived [17-19]. Finally, ^{31}P NMR has proven particularly useful in the study of the orientational and dynamic properties of lipid headgroups [20]and lipid polymorphism [21,22].

Despite these advances, we are still far from understanding the physical and biological (passive permeability, facilitated and active transport, enzyme activities, signal transduction) properties of biological membranes based on the properties of their individual molecular components and their interactions. Current solid state NMR methods are, nonetheless, beginning to provide previously unattainable static and dynamic structural information which can help solve these long standing problems in molecular biophysics, and several of these developments are detailed below.

3.1 Lipid dynamics and the physical properties of membranes

A detailed model of the molecular dynamics of lipid membranes, obtained from longitudinal and transverse ^{2}H NMR spin relaxation measurements [23,24], concluded that three main types of motion can be distinguished in these systems: internal, overall and collective lipid motions. The collective motional modes are ultra-slow motions which are responsible for the transverse relaxation behavior seen under conditions generated by a version of the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence appropriate to ^{2}H NMR [25]. Although a previous study suggested that diffusion through the curved membrane surfaces was the most plausible mechanism for this relaxation process [25], the authors of the latter study found that the same type of relaxation process could also be observed in oriented planar bilayers, and therefore concluded that collective order director fluctuations were the dominant relaxation process [23,24]. From the experimental relaxation rates, the average elastic constant for phosphatidylcholine (PC)-cholesterol membranes was estimated to be 10^{-11} N; this value increased almost four-fold in the presence of (40 mol%) cholesterol. An independent study which investigated the angular dependence of deuterium spin-lattice relaxation rates of oriented PC bilayers obtained results consistent with the three motional classes described above [26]. A multinuclear NMR study then used ^{2}H NMR of specifically labeled phospholipid molecules, combined with relaxation-edited ^{13}C CP/MAS [27] and ^{31}P NMR to compare the effects of cholesterol, ergosterol and lanosterol on the order and dynamics of PC lipid bilayers [28]. It was concluded that the effects seen depended on both the sterol structure and the type of fatty acids esterified to the phospholipid. For the saturated dimyristoyl-PC (DMPC), the overall ordering effect was ergosterol>cholesterol>>lanosterol, while for the unsaturated 1-palmitoyl-2-oleoyl-PC (POPC), the ordering effect was cholesterol>ergosterol≈lanosterol. In the saturated system, ergosterol and cholesterol (at 30 mol %) increased the local order of the phospholipid molecules without affecting the collective motions (order director fluctuations), while lanosterol disrupted lipid packing in the bilayer. For the unsaturated system, it was found that both ergosterol and lanosterol (at 30 mol %) decreased the elastic constant and/or the coherence length (thickness) of the bilayers, and increased the frequency of the ultraslow collective motional modes, suggesting altered lipid packing within a bilayer configuration (confirmed by ^{31}P NMR).

Slow motions in lipid bilayers have also been studied by two dimensional exchange ^{2}H spectroscopy [29-31] and ^{31}P NMR [32,33]. Lipid lateral diffusion was found to be the most likely explanation for the exchange effects observed in the 2D-^{2}H NMR spectra [29-31], and excellent agreement was obtained between the experiments, computer simulations and numerical calculations in the case of phospholipid bilayers on a spherical solid support [31]. ^{2}H NMR has the disadvantage, however, of very short T_1's (on the order of milliseconds) for deuterium nuclei in fluid lipid bilayers, which limits the applicability of this methodology, based on longitudinal magnetization exchange. On the other hand, ^{31}P NMR, which has relatively long longitudinal relaxation times (on the order of seconds for phospholipids), combined with large chemical shift anisotropies and inherently high sensitivity (a 100% abundant magnetically active isotope) offers a particularly advantageous approach to study slow lipid motions in membranes. It has been used to detect and measure lipid lateral diffusion in curved lipid bilayers (vesicles), while 2D spectra of oriented planar membranes show no exchange effects [32]. Both the ^{2}H and ^{31}P NMR studies provide estimates of lipid lateral diffusion constants in good agreement with those obtained by fluorescence recovery after photobleaching, without the uncertainties derived from the introduction of large fluorescent reporter groups, or the requirement of optically transparent samples. On the other hand, 2D-^{31}P NMR spectroscopy was unable to detect lipid exchange between coexisting bilayer (L_α), hexagonal (H_{II}) or cubic (I_2) phases, placing constraints on the stability or kinetic behavior of proposed transition intermediates [33].

3.2 Lipid order profiles in membranes

The "order profile" of the methylene segments and other regions of phospholipids in membranes has been investigated in detail using ^{2}H NMR of selectively labeled molecules [10-12]. The segmental order parameters, S_{cd}, can be directly obtained from the

frequency separation ($\Delta\nu_q$) of the singularities of the experimental spectra of unoriented samples by using the relationship:

$$\Delta\nu_Q = 3/4(e^2qQ/h).S_{cd}$$

where $\Delta\nu_q$ is the observed quadrupole splitting (the separation between the major singularities in the ^2H NMR powder pattern), and e^2qQ/h is the nuclear quadrupole coupling constant for ^2H, typically about 168 kHz for C-D bonds.

Despite the simplicity of this approach, the difficulty and cost of selective deuteration of large molecules has limited its application. To circumvent this problem, other approaches have been sought in order to obtain order profiles of lipid molecules in a variety of systems. One of these approaches proposes a procedure (termed "smoothing") to extract order profiles from ^2H NMR spectra of perdeuterated molecules in both bilayer [34,35] and hexagonal phases [36], assuming a monotonic decrease of S_{cd} as a function of segment position. However, it has recently been shown [28] that smoothed order profiles may differ significantly from actual profiles obtained with specifically ^2H labeled lipids, indicating that structural conclusions drawn from smoothed profiles need to be made rather carefully.

As an alternative to ^2H labeling schemes, several methods have been proposed to extract order profiles from natural abundance, high resolution ^{13}C spectra, using two-dimensional variations of the CP/MAS experiment. Most of these methods were inspired by the early work of Waugh and co-workers [37] in which dipole-dipole spectra of spins with different chemical shifts were separated in a 2D experiment: the so-called separated local field experiment. The original procedure was restricted to single crystals and uniaxially oriented fibers, in which the limited number of molecular orientations allowed some resolution of the resonances in the chemical shift dimension, despite the presence of large chemical shift anisotropies (CSA). For polycrystalline or amorphous materials, MAS is required to average out the CSA's, but the generally weak heteronuclear dipolar information is then lost, since it is also scales by a factor of $P_2 = 1/2(3\cos^2\theta - 1)$, where θ is the angle between the spinner axis and the external magnetic field. Several procedures have been proposed in recent years to recover the information on dipole-dipole interactions from high resolution CP/MAS spectra of dilute spins (^{13}C, ^{15}N, ^{31}P) in solids [38]. For example, Spiess and coworkers proposed a 2D-wideline separation (WISE) NMR experiment in which proton (abundant I spin) magnetization is allowed to decay in the x-y plane under all dipolar interactions during the evolution period (t_1), and is then used to cross-polarize (CP) the directly attached ^{13}C nuclei (rare S spins), which are then detected with high resolution (proton decoupling) during t_2 [39]. The resulting 2D-spectrum provides the total ^1H dipolar spectra for protons directly attached to each carbon site, resolved in the chemical shift dimension. However, the total dipolar spectrum includes both ^1H-^1H and ^1H-^{13}C interactions, and although valuable qualitative information has been obtained in a variety of polymer [39] and lipid membrane [40] systems, is not possible to obtain a quantitative estimate of the value of S_{ch}. A second approach has been recently proposed [28] in which protons are allowed to evolve under the influence of the pulse sequence $(\pi/2)_0$-τ-$(\pi/2)_{90}$, which leads to the appearance of a "solid echo" at a time $t=2\tau$ after the first echo [41]. At the echo maximum, CP contact is established to transfer magnetization to directly coupled ^{13}C nuclei, which are then detected with high resolution, under MAS and with proton decoupling. By systematically varying the inter-pulse interval, τ, a 2D NMR spectrum is obtained which correlates the chemical shift of the different ^{13}C sites with the inter-proton pair dipolar spectrum of the directly attached protons [42-45], from which the corresponding inter-pair order parameters $S_{ch(inter)}$ can be calculated by the relationship [44,45]:

$$M_{2(inter,av)} = S^2_{ch(inter)}.M_{2(inter)}$$

$M_{2(inter,av)}$ is the motionally averaged interpair second moment, obtained experimentally, and $M_{2(inter)}$ is the interpair second moment for an isolated trans-palmitoyl chain, 5.5×10^9 s^{-2} [46]. The inter-proton pair order parameters are to be distinguished from their intra-pair analogs (which provide information equivalent to that obtained from quadrupolar splittings in ^2H NMR), because the inter-pair values are influenced both by

whole chain flexing and twisting motions, as well as by local motions of the methylene segment during the NMR time scale [45]. They can, however, provide detailed information on the type and degree of motion of the different parts of the phospholipid molecules, and are useful where no specifically ^2H-labeled molecules are available.

Several other methods have also been developed recently to selectively recover heteronuclear dipolar interactions (from which the corresponding order parameters can be directly calculated) from natural abundance high resolution ^{13}C NMR CP/MAS spectra of lipids. In one of the most successful approaches (labeled proton-detected local field spectroscopy , PDLF [47]), protons are allowed to evolve only under the effect of (scaled) ^1H-S spin (^{13}C, ^{31}P) interactions, because the concurrent application of an MREV-8 [48] multiple pulse sequence removes the proton homonuclear dipolar interaction, and two simultaneous π pulses on the ^1H and S spins in the middle of the t_1 evolution period refocus the chemical shifts without affecting the ^1H-S interactions. The ^1H magnetization, modulated by the ^1H-S dipolar interaction, is then transferred through CP to the S spins, which are detected under conditions of high power proton decoupling. The experiment has been performed in static samples consisting of small molecules magnetically oriented in nematic liquid crystals and in polycrystalline samples with frequency selection [49] or by means of switched-angle sample spinning (SAS), which involves rotation off the magic-angle during the evolution period (t_1), in order to retain the ^1H-S interaction, followed by MAS during detection to produce high resolution [47,50]. Another useful approach has been to use off magic-angle spinning (OMAS) during the whole experiment, which retains the dipolar interaction but removes most of the relatively small CSA of the methylene carbons [40]. Figure 1 shows the PDLF 2D-NMR spectrum of egg-PC obtained using the SAS procedure [50]:

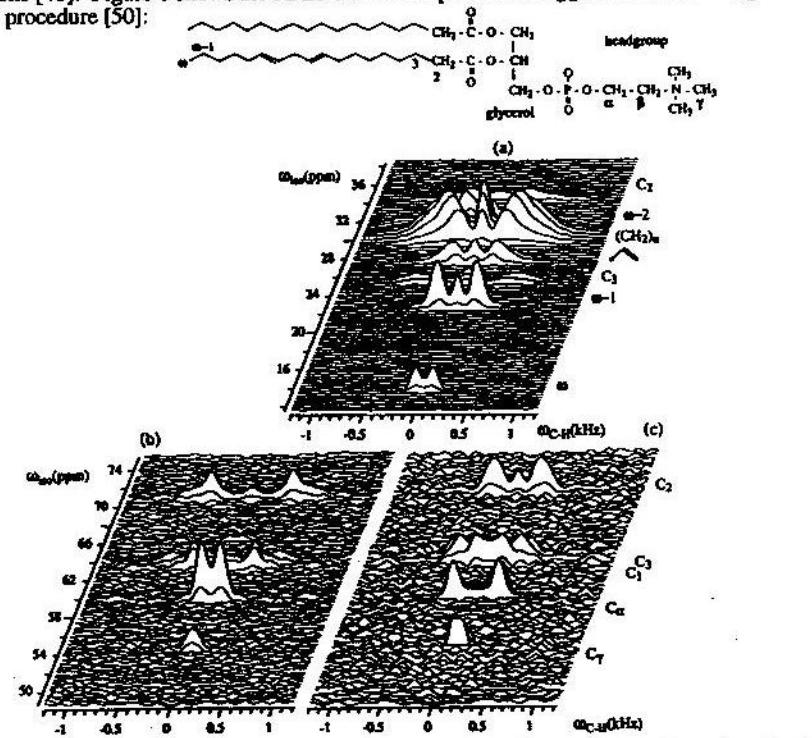

Figure 1. PDLF 2D spectra of egg phosphatidyl-choline liquid crystalline membranes with a schematic of the lipid molecule. The spectra were taken with the pulse sequence of Nakai and Terao [47] with SAS. (a) and (b) were taken with P2=-0.2, (c) P2=0.2. The Pake doublet-like dipolar spectra at each ^{13}C frequency allows the determination of the ^{13}C -1H dipolar coupling from which the sign and value of S_{ch} can be calculated. Taken from Hong et al. [50], with permission.

while Figure 2 compares the values of the segmental order parameters (S_{ch}) derived from these types of experiment [40,50] with those obtained by ^{2}H NMR of specifically deuterated molecules.

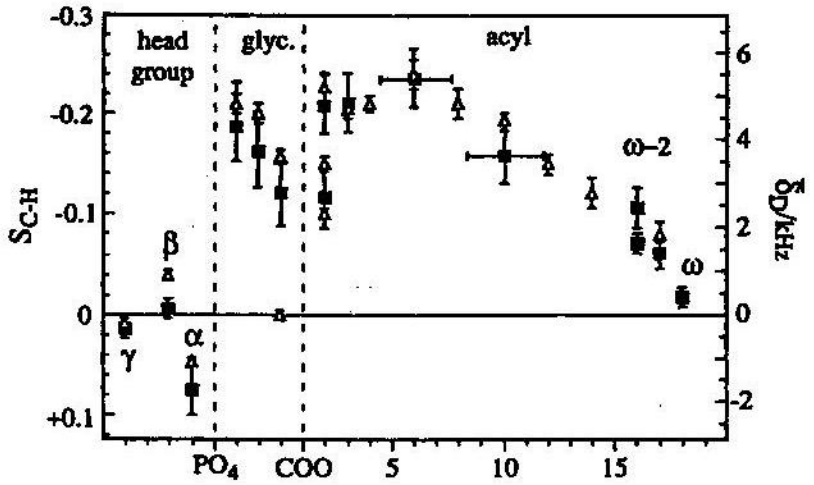

Figure 2. Motionally averaged C-H order parameters obtained from dipolar couplings through PDLF spectra (■) compared with those obtained from ^{2}H quadrupolar couplings (△). Taken from Hong et al. [50], with permission.

^{13}C-^{31}P dipolar couplings have also been measured by OMAS experiments [40] and from them conformational constraints have been obtained for the headgroup conformation. This approach is much simpler than that of previous studies which involved detergent titration of magnetically oriented bilayers [51]. Heteronuclear dipolar coupling/chemical shift 2D spectra have also been obtained by suppressing the I-I interaction during the evolution period, by spin locking the I spins at the magic angle and using flip-flop Lee-Goldburg phase and frequency shifted irradiation [52,53] followed by high resolution detection under MAS and proton decoupling (PISEMAMAS, [54]), as will be discussed in more details below. Recent work by Griffin and coworkers [55] uses MAS to eliminate I-I interactions (this is restricted to materials such as membranes and liquid crystals with fast molecular motions [19], together with rotor-synchronized π pulses to reintroduce I-S interactions (see section 4.2.1) during the evolution period, followed by high resolution detection under MAS and I-S decoupling. This method (labeled DROSS for Dipolar Recoupling On-axis with Sign and Shape preservation) also enables a determination of both the sign and magnitude of the ^{1}H-^{13}C dipolar interaction in liquid crystalline lipid membranes, but without the technical difficulties involved in the SAS and OMAS PDLF experiments. Typical results for DMPC are shown in Figure 3:

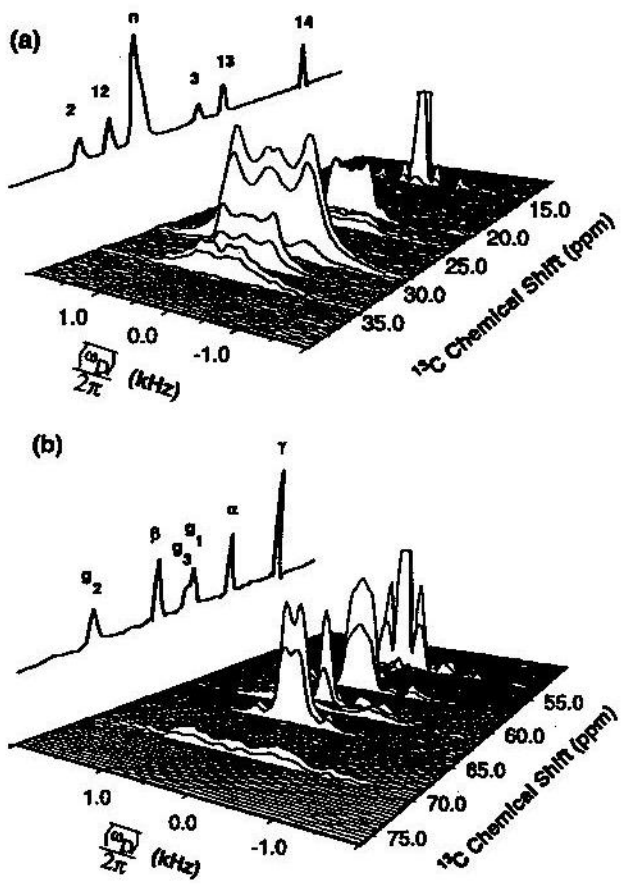

Figure 3. DROSS spectra of dimyristoylphosphatidyl-choline liquid crystalline membranes. a) Acyl chain region, b) headgroup and glycerol region. Taken from Gross et al. [55], with permission.

Two-dimensional solid state heteronuclear correlation spectroscopy [2,56,57] has also been used to obtain NMR constraints on the conformation of the phospholipid headgroups in liquid crystalline bilayers [58]. This 2D-experiment involves the x-y evolution of protons during t_1 under MAS (which, as mentioned above, removes I-I interactions in lipids), then the frequency labeled magnetization is transferred by CP to the directly attached ^{13}C nuclei, which are then detected with high resolution during t_2. Figure 4 shows a resulting 2D spectrum which illustrates the close proximity between the sn-2 carbonyl ^{13}C nucleus and the headgroup Hα protons, suggesting a bent-back, compact conformation for the phospholipid molecules, even in the liquid crystalline phase [58]:

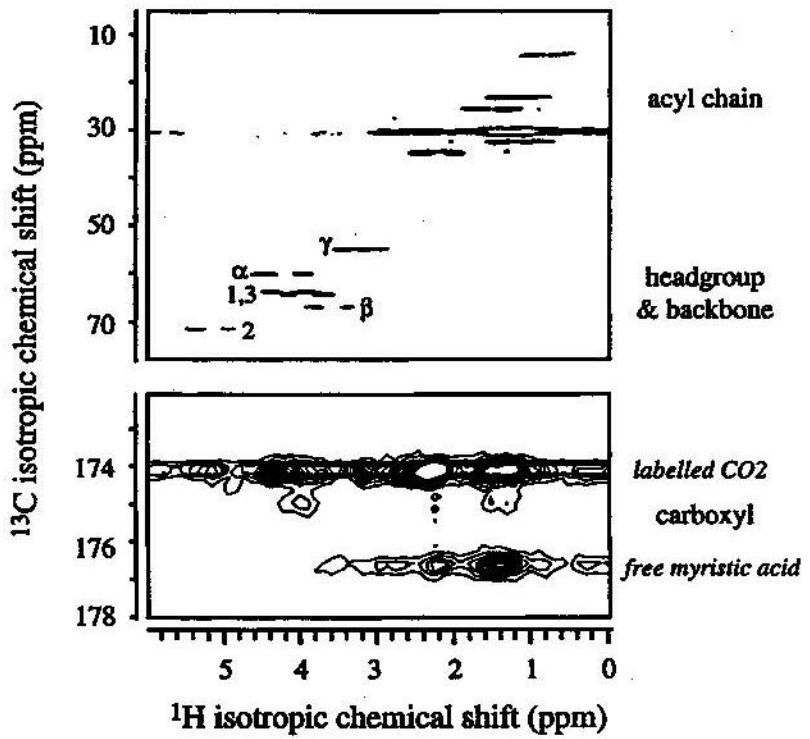

Figure 4. 2D ^{13}C-^{1}H chemical shift correlation spectrum of [^{13}C]-CO2-labeled dimyristoylphosphatidylcholine (DMPC) taken with the HETCOR pulse sequence. Note the strong correlation peaks of ^{13}C-CO2 with H2 and Hα, indicating a "bent-back" conformation of the headgroup. Taken from Hong et al. [58], with permission.

Motionally averaged CSAs can also provide valuable information on the type and extent of motions present in phospholipid bilayers [15,59]. They can be recovered from high resolution CP/MAS spectra through experiments in which the ^{13}C magnetization, obtained by CP from protons, is allowed to evolve under MAS and the effect of rotor-synchronized π pulses during t_1 and is detected with high resolution in t_2 [60]. With judicious placement within the rotor cycle, the π pulses (usually 4 or 6 per cycle) prevent the CSA from being averaged by sample rotation, and the resulting 2D-NMR spectrum correlates the effective CSA with the isotropic chemical shift for each carbon site. Although simpler than some other methods, this approach has not yet been applied to lipid membranes.

Many of these types of experiment can also be performed on static lipid samples which have been either mechanically or magnetically oriented [51,61,62]. Here, it should be noted that the recent discovery that detergent/PC mixtures in the presence of 1-5% of several lanthanide ions/PC (mol/mol), spontaneously orient with the bilayer normal (director) parallel to the magnetic field [63], suggests a simple method to carry out related PDLF experiments in membranes.

Moreover, the values of the extracted order parameters can then be combined with the cross-polarization rates ($1/T_{\varphi}$) of the corresponding ^{13}C sites to obtain dynamical information [28,64], since:

$$1/T_{\varphi} \propto S_{ch}^2 J_x(\Delta\omega_e)$$

where $J_x(\Delta\omega_e)$ is the cross-polarization spectral density at a frequency ($\Delta\omega_e$) equal to the difference between the effective resonance frequencies of the I and S spins in the rotating frame, which is near zero under normal CP conditions.

Taken together, these results indicate that it is now possible to obtain from natural abundance ^{13}C NMR spectra much of the detailed information previously only available from ^{2}H NMR with selective labeling, an important development which should extend the range of biological and model membrane systems amenable to detailed static and dynamic structural analysis. For example, in earlier work with ^{2}H NMR, it was shown to be possible to build up a detailed structural model for the sugar headgroup in a cerebroside [65] based on ^{2}H order parameter information. Given similar order parameters for resolved ^{13}C sites, it should likewise be possible to develop structural models for these and other lipid headgroups as well, possibly even including those in biological membranes such as myelin, where many lipid sites have been resolved and assigned [66].

4. Membrane peptides and proteins

Although most specific membrane functions, such as active or facilitated transport, signal recognition and scalar or vectorial enzymatic activities, are carried out by proteins, little is known about the structure and function of these fundamental cellular components. As indicated in the Introduction, the reason for this limited knowledge is that membrane proteins and membrane-active peptides are much more difficult to study by the methods developed for soluble proteins, including multidimensional NMR and x-ray crystallography. Meeting this challenge are new solid state NMR methods which do not require highly crystalline samples, and at least from the standpoint of relaxation have no limit with respect to the molecular weight of the components involved. These new methods are providing the first structural details of proteins and peptides embedded in lipid membranes. Two general approaches have proven particularly successful. The first involves the use of samples in which the membrane planes are mechanically or magnetically oriented with respect to the external magnetic field, allowing the measurement of several magnetic interactions for each of the structural units of the polypeptide backbone, and the precise determination of molecular structures with atomic resolution [67-71]. In the second approach, unoriented samples with specific isotopic labeling are used to determine both homo- and heteronuclear dipolar interactions, from which precise internuclear distances can be obtained, using pulsed NMR techniques which allow the recovery of these interactions under MAS conditions [68,70,72,73]. We will describe in some detail both approaches, and provide illustrative examples.

4.1 Conformational constraints from NMR of oriented samples

The use of oriented samples for lipid and protein structure determination has had many early applications, e.g. for spin-label ESR, ^{2}H NMR of nematic and lyotropic liquid crystals [74] and also, ^{14}N NMR [75], and ^{2}H NMR of labeled proteins [76], in which the anisotropies in the magnetic susceptibilities of both diamagnetic and paramagnetic substances were used for alignment purposes, along with purely mechanical methods. Over the past 10 years, interest has focused on determination of the structures of peptides and small proteins in mechanically oriented systems, although quite recently lanthanide-doped "bicelles" have also shown considerable promise as an orientable platform for structure determination [77].

To date, most results have been obtained by depositing lipid/protein samples onto thin or ultra-thin microscope cover-slips. Geometries are typically a stack of ~30 slips, ~7 mm x 20 mm, with ~2-10 mg peptide, and up to ~300 mg lipid. Great care needs to be exercised to prevent dehydration (due to high rf decoupling powers), and the square geometries decrease rf homogeneity. In the case of bacteriorhodopsin, ^{2}H-labeled samples have been dried down directly onto glass plates, but the best alignments are with fluid lipid bilayers, where mosaic spreads of $\leq 0.3^{\circ}$ can be obtained.

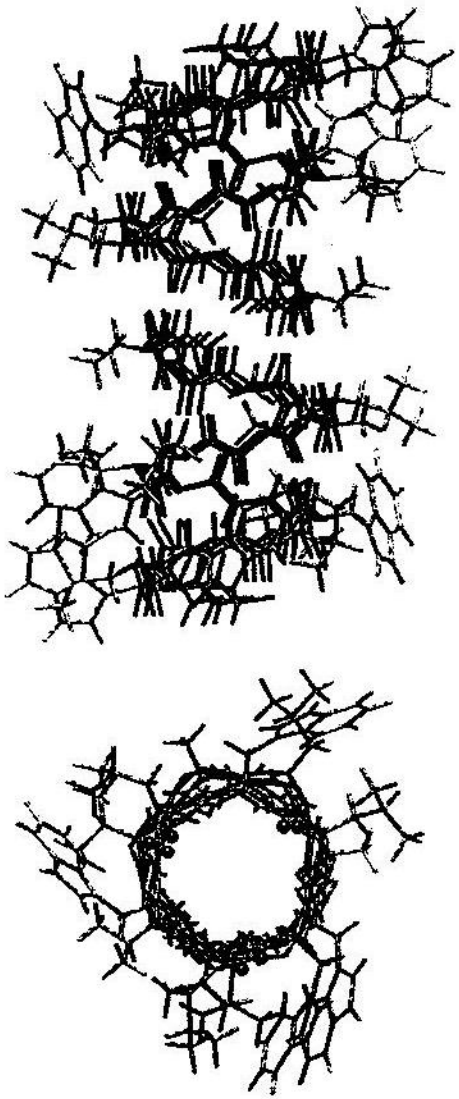

Figure 5. Complete structure of gramicidin A in a lipid bilayer determined solely from solid-state NMR-derived orientational constraints and the amino acid sequence. Taken from [71], with permission.

There have been two basic types of experiment. In one case, interest has focused on the peptide backbone structure, then sidechain structural information is added. In the other, the structure and orientation of a chromophore, the retinylidene Schiff base in bacteriorhodopsin, has been of primary interest. Here, we focus on the more general topic of backbone and sidechain structure determination in membrane peptides and proteins. At least two different spectroscopic measurements are required for each structural unit of the polypeptide chain, such as the planar peptide planes, and ^{13}C-^{1}H, ^{15}N-^{1}H and ^{13}C-^{15}N dipolar interactions, as well as ^{13}C and ^{15}N resonance frequencies, which depend on the

orientation of the internuclear vector or CSA tensor with respect to the external magnetic field, have been used extensively for structure determination. The first complete high resolution structure of a peptide in its native lipid environment using this methodology was that of gramicidin A [68,78]. Here, it was necessary to carry out an extensive series of syntheses in which backbone ^{15}N sites were specifically labeled, as were the H^{α} and aliphatic and aromatic sidechains. Knowledge of the ^{15}N tensor orientation, and measurement of ^{15}N shifts, N-H dipolar couplings and ^{2}H quadrupolar splittings, permitted over the years the gradual build-up of specific pieces of structural information, which culminated in the first full 3D structure of a membrane associated peptide. This structure has been further refined by including dynamical information derived from the averaging of powder patterns [69,79], and the complete structure of both the backbone and sidechains, again derived solely from NMR-derived orientational constraints, has recently been published [71], as shown in Figure 5. The accuracy of the structure is exemplified by the fact that the rms deviation of i to i+6 hydrogen bonds from the ideal β-strand values is approximately 0.5 Å.

When the secondary structure of a polypeptide is suspected, for example by multidimensional NMR studies in micelles, the frequency of the NMR signal from a single labeled amide site in an oriented sample of the peptide in a bilayer has been used in combination with hydropathy plots and molecular dynamics calculations to propose the orientation of the peptide with respect to the bilayer plane [67,68,80], as shown in Figure 6.

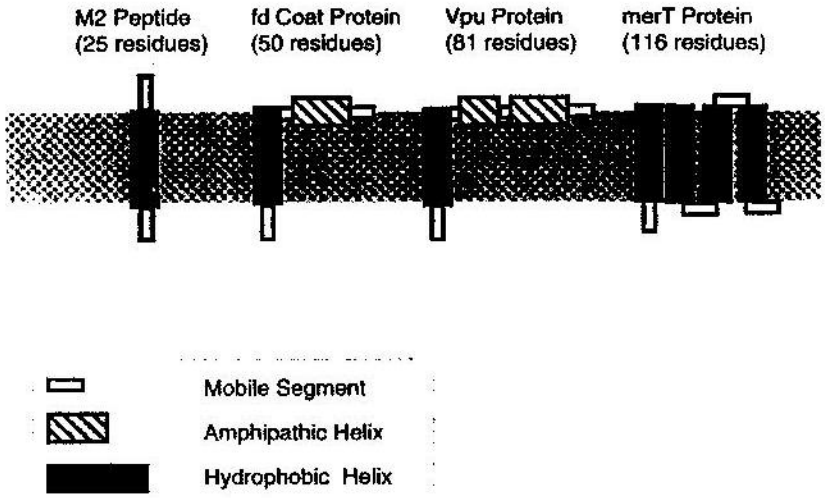

Figure 6. The architectures of four membrane proteins as predicted from their primary sequences using a combination of hydropathy plots and molecular dynamics simulations [80]. There are considerable experimental data indicating that the predicted architectures are correct for the M2 peptide [67] and fd coat protein [81] in membrane environments. The limited amount of experimental data available at the present time about Vpu and merT proteins is supportive of the predicted architectures in membrane environments. The 25 residue M2 peptide has the sequence of the M2 pore forming segment of the acetylcholine receptor protein. fd coat protein has 50 residues and resides in the cell membrane prior to assembly into virus particles. Vpu is an 81 residue accessory protein from HIV-1 found in the membrane of infected cells. MerT has 116 residues and is the transport protein from the bacterial mercury detoxification system. All four of these protein can be prepared with uniform isotopic labeling in the relatively large amounts needed for NMR studies.

A second route to sample orientation involves the use of magnetically orientable lipid bilayers or "bicelles" (which are though to be basically pieces of smectic liquid crystal bilayer containing a surfactant "rim"), and mixtures of long-chain phosphatidylcholines with detergents such as CHAPSO or dihexanoylphosphatidylcholine [51,61-63] have been used to study bilayer surface associated or integral membrane peptides and proteins. In the original methodology, the bicelles orient with their director perpendicular to the external magnetic field [82,83], which can lead to powder patterns if the axial rotation of the membrane associated protein is not fast. Fortunately, the addition of lanthanide ions, e.g. Th^{3+}, Er^{3+}, "flips" the bicelle director to an orientation parallel to the external field, by switching the orientation of the bicelles' susceptibility tensor[63]. This permits high resolution of the peptide resonances to be obtained [77] and is analogous to the change in myoglobin microcrystal orientation observed on a high → low spin state transition in ^{2}H-labeled myoglobin [76]. Figure 7 shows a comparison of the 1D ^{15}N NMR spectrum of ^{15}N-labeled fd coat protein in unoriented and magnetically oriented bilayers, with and without paramagnetic lanthanide ions [77]:

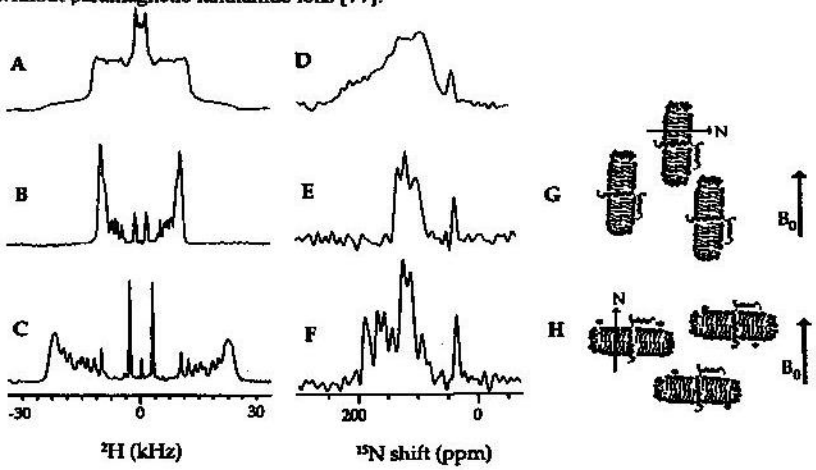

Figure 7. 8.45 Tesla ^{2}H (A-C) and ^{15}N (D-F) spectra of (^{2}H) DMPC-protein (^{15}N -fd phage) systems. Random powder spectra are shown in A (^{2}H) and D (^{15}N), while spectra of perpendicular director orientations (G) are shown in B (^{2}H) and E (^{15}N), and parallel (H) orientations in C(^{2}H) and F (^{15}N). Bicelle orientations are varied by addition of lanthanide ions. Taken from Howard and Opella [77], with permission.

At present, however, the best results have been obtained by using mechanically oriented samples. Here, the recent developments of Opella, Ramamoorthy and coworkers [54,84-88] are of particular note. In model systems, 1,2,3 and 4-dimensional experiments have been reported, using the PISEMA technique, with flip-flop Lee-Goldburg decoupling, and dilute spin ^{15}N-^{15}N exchange. By focusing on the ^{15}N nucleus, the large ^{15}N CSA can be used as a spreading parameter, as can the anisotropic ^{1}H shift and the ^{15}N-^{1}H dipolar interaction, with derivation of ^{15}N-^{15}N connectivities also being possible in model systems, and likely in proteins, at high magnetic field strength. These techniques have enabled considerable resolution in 2D and 3D spectra of several membrane peptides and proteins [85]. Since excellent resolution is obtained in the oriented fd phage coat protein in a lipid bilayer at only 400 MHz, there is considerable reason to be optimistic that even larger systems may be tractable, at higher magnetic field strengths, using the resolution gains afforded by multidimensional NMR. And, as noted above, in the solid state there are no relaxation problems associated with increasing molecular weight, unlike the situation in solution NMR spectroscopy.

At present, related ^{13}C NMR results have not been reported. For ^{13}C, sensitivity can be expected to exceed ^{15}N, based purely on gyromagnetic ratio considerations, but there will generally be more complex ^{13}C-^{13}C and ^{13}C-^{1}H dipolar interactions, and a smaller CSA

(even for ^{13}C'). Nevertheless, ^{13}C is likely to play a role in future structure determinations in rigid systems, and the ability to evaluate most carbon shielding tensors using ab initio quantum chemical methods may play a useful role in structure determination and refinement [89-91].

Solid state NMR methods have also be used to study cofactors and other molecules tightly bound to membrane proteins. For example, oriented purple membrane samples from *Halobacterium salinarium* having specifically ^{2}H labeled retinals have been used to obtain the orientation of the individual methyl groups of the chromophore embedded in this proton-pumping protein, and the structural re-alignment of retinal through the photocycle [91,92]. The mosaic spread of bacteriorhodopsin in this system is quite large, and the observed quadrupole splittings were ambiguous with respect to orientation. This ambiguity was however cleverly resolved by use of tilted samples, enabling a detailed picture of the retinal chromophore to be developed.

4.2 Internuclear distances in membrane proteins

One of the more powerful capabilities of modern solid state NMR methods is the possibility of recovering internuclear dipolar interactions from high resolution CP/MAS NMR spectra of unoriented solid samples, from which precise interatomic distances can be derived. Both heteronuclear and homonuclear distances can be measured, and the combination of these methods with current molecular biology and chemical synthesis procedures to selectively introduce isotopic labels in membrane proteins and peptides has permitted structural studies in these systems with unprecedented detail [68,70,72,73]. There are two basic approaches.

4.2.1 Heteronuclear spin pairs

One of the most frequently used methods to measure dipolar interactions in isolated heteronuclear spin pairs (I-S, such as ^{13}C-^{15}N, ^{13}C-^{31}P) in rotating solids is the rotational-echo double-resonance (REDOR) technique of Schaefer et al. [94,95]. In this technique the averaging effect of MAS is defeated by the placement of rotor-synchronized π pulses on one of the interacting nuclei (e.g.: I), which changes the sign of the local dipolar field experienced by the S spin. As a consequence of this, the magnitude of the rotational echo of the S spins is reduced when compared with the same procedure in the absence of the I spin π-pulses. The experimental measurement is then a difference 1D spectrum between the normal signal (S_o, in the absence of π pulses), and the reduced signal. This is generally reported as $\Delta S/S_o$ or in the form of a 2D spectrum (where the train of I spin π pulses is applied during the evolution period). From this the internuclear distance can in principle be calculated rather accurately [72]. Such experiments are generally performed with selective isotopic enrichment of both the I and S spins of interest. However, the intensity of the S-spin rotational echo can also be affected by natural abundance I spins, and by T_2 relaxation, and corrections related to these two effects are among the more important problems associated with this method, particularly in biological macromolecules. Despite these limiting factors, REDOR has been successfully used in the determination of structural parameters in crystalline helical polypeptides [96,97], and in gramicidin A incorporated in unoriented DMPC membranes [98].

One particularly elegant approach to eliminate the effects of the natural-abundance I-spin background is the so-called transfer-echo double resonance (TEDOR) experiment [99], in which I-spin magnetization from a specific labeled site is transferred to S spins as a consequence of the recovery of the I-S interaction by the action of rotor-synchronized π pulses (generally two per cycle). The TEDOR signal is a complicated function of the magnitude of the dipolar interaction, the rotation period, and the number of π-pulses applied, requiring a 2D experiment for quantitation. TEDOR can, however, be used as a selectivity filter for REDOR experiments, in order to eliminate the natural abundance background. Figure 8 shows an example of such an experiment with a helical polypeptide in the solid state, which allowed the measurement of an 8 Å distance between specifically labeled ^{13}C and ^{19}F sites [100]:

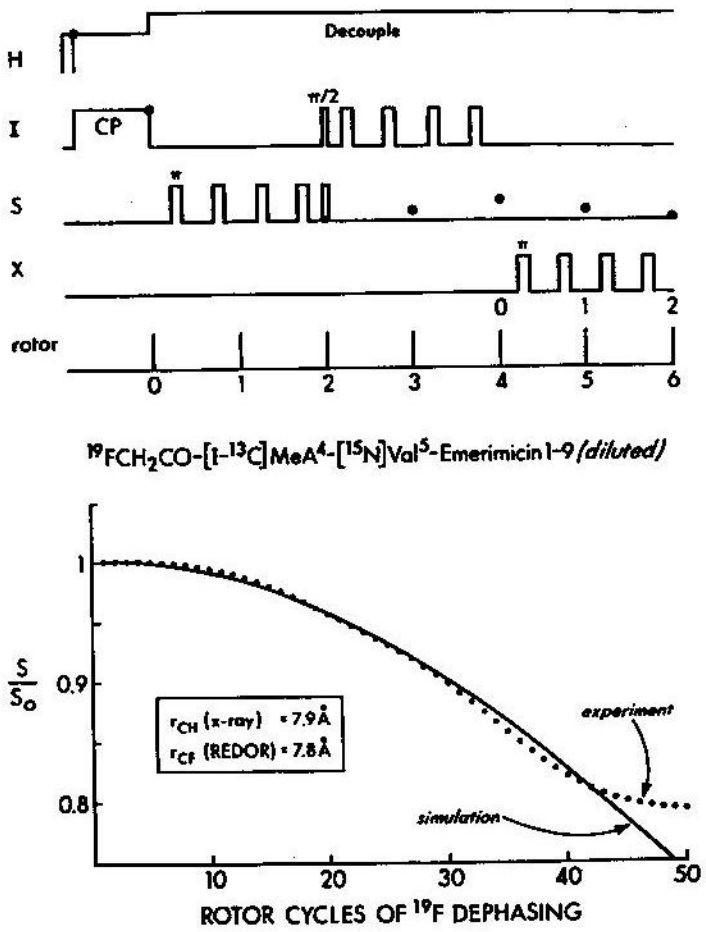

Figure 8. TOP: Pulse sequence for TEDOR-REDOR. Following cross-polarization transfer from abundant protons to generate initial magnetization, the coupling from protons is removed by resonant decoupling. The first (TEDOR) part of the experiment selects S magnetization by coherence transfer from I spins while the second (REDOR) part measures dipolar coupling to a third rare spin, X. BOTTOM: TEDOR-REDOR ^{13}C NMR data for a ^{19}F, ^{15}N, ^{13}C -triple labeled peptide fragment from the antibiotic emerimicin diluted 10-fold with natural abundance peptide. The ^{15}N selected ^{13}C signal is sampled once each rotor period with (S) or without (S_0) ^{19}F REDOR dephasing π pulses. The experimental ratios agree with a three-spin simulation. with a best-fit r_{CF} of 7.8 Å. Taken from [100], with permission.

4.2.2 Homonuclear spin pairs

The flip-flop term of the homonuclear dipolar interaction $(I_{+1}I_{-2} + I_{-1}I_{+2})$ for two nuclei having chemical shifts δ_1 and δ_2 is time dependent with a frequency $\Delta=\delta_1-\delta_2$. If the sample is spinning, the angular part of the interaction is also oscillatory, and the combined time dependence then has two time dependent contributions of the form $\cos(\Delta-\omega_r)$ and $\cos(\Delta-2\omega_r)$, where ω_r is the sample rotation frequency. In general, this oscillatory behavior will average the flip-flop term to zero, but if the condition $\Delta = n\omega_r$ is satisfied,

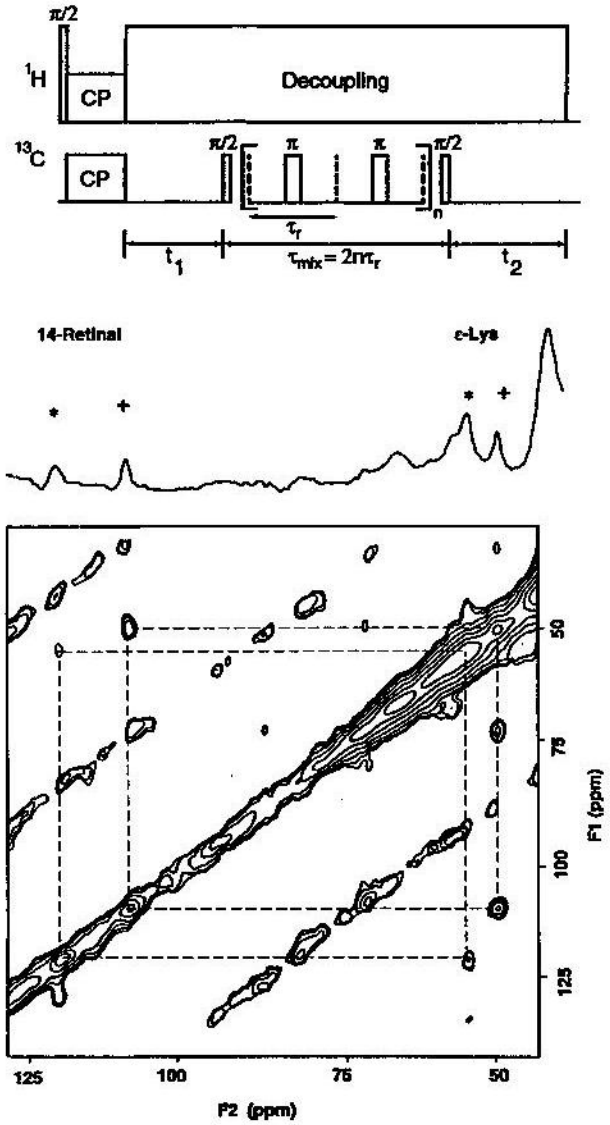

Figure 9. TOP: 2D RFDR pulse sequence. Initial ^{13}C magnetization is generated by cross-polarization from protons, followed by an evolution period with decoupling (t_1); a single non-selective $\pi/2$ pulse generates longitudinal magnetization and rotor-synchronized π pulses recouple ^{13}C nuclei which can then exchange magnetization during the mixing period (t_{mix}). Finally, a second $\pi/2$ pulse generates observable transverse magnetization detected with proton decoupling during t_2. BOTTOM: 2D-RFDR spectrum of [14-^{13}C]retinal, [ε-^{13}C, ^{15}N]-bacteriorhodopsin (bR). Signals at 48 and 53 ppm for [ε-^{13}C]-bacteriorhodopsin and 110 and 122 for [14-^{13}C]retinal correspond to bR_{555} (+) and bR_{568} (*). Cross-peaks identify dipolar coupled nuclei. Taken from [116], with permission.

where n is an integer, this term becomes time independent, and reintroduces the I-I dipolar interaction. This is the physical principle of the rotational resonance (R^2) method for determining homonuclear dipolar interactions from high resolution NMR spectra of solids [101,102]. In practice, the magnetization transfer which ensues between the recoupled spins depends on the magnitude of the dipolar interaction (including motional averaging effects), the CSA of both spins (and their relative orientations with respect to the dipolar principal axis system for n>1) and T_{zq}, the zero quantum relaxation time. This last effect is more difficult to evaluate since it arises from incomplete proton decoupling, and nearby natural abundance ^{13}C nuclei which do not satisfy the R^2 condition. Despite these complexities, the method has been shown to provide very accurate measurements of internuclear distances - up to ca. 7 Å [103], and has been used in a series of now classical studies on the conformation of the retinal chromophore in bacteriorhodopsin [104], and in its interactions with the protein [105,106]. The rotational resonance technique has also proven to be very useful in the study of the structure and orientation of the transmembrane domain of glycophorin A, an abundant intrinsic protein from human erythrocytes, in lipid bilayers [107], as well as in the determination of intramolecular (dipalmitoyl-PC) and intermolecular (lipid-lipid and lipid-glycophorin) distances [108]. In the latter studies, however, it was necessary to keep the temperature at -50°C to quench motional averaging, and enhance the otherwise relatively weak dipolar couplings. More recently, studies in model systems have shown that lineshape analyses at the rotational resonance condition can provide accurate estimates of internuclear distances (up to ~4.4 Å) without the requirement of lengthy 2D spin exchange experiments [109].

Despite its technical simplicity, R^2 suffers from the limitation that it is highly frequency selective, and very small deviations (ca. 0.1% of Δ) of the spinning rate from the resonance condition lead to detectable changes in lineshapes and magnetization transfer rates [72,73]. Other procedures allow the reintroduction of the dipolar interactions across a broader range of chemical shifts and MAS rates. Among these broadband dipolar recoupling schemes: DRAMA (Dipolar Recovery At the Magic Angle) uses rotor-synchronized π/2 pulses (two pulses per cycle) to produce an average Hamiltonian of the form $D(I_{z1}I_{z2}-I_{y1}I_{y2})$, where D depends on the placement of the RF pulses in the rotor cycle, and is generally nonzero [110,111]; and SEDRA (Simple Dephasing of Rotational Amplitudes) uses a train of rotor-synchronized π pulses, which in the case of two spins having differing isotropic chemical shifts, leads to a net dipolar evolution of the spin system due to a flip-flop $(I_{+1}I_{-2} + I_{-1}I_{+2})$ term [112,113]. Another important approach is RFDR (RF-driven recoupling), which combines rotor-synchronized π pulses with a longitudinal spin mixing scheme. This is particularly suitable for 2D homonuclear correlation spectroscopy [114,115]. Figure 9 illustrates the pulse sequence and a 2D dipolar correlation spectrum of bacteriorhodopsin labeled with ^{13}C at Lys_{216} and [14-^{13}C]retinal, showing the connectivity of the dipolar coupled nuclei; from the magnitudes of the cross-peaks, or 1D-RFDR exchange spectra, quantitative estimates of the internuclear distances can be made [116].

To date there are approximately one dozen dipolar mixing schemes being used in solid-state NMR studies of amino-acids, peptides and proteins. However, highly resolved spectra of uniformly labeled proteins have not yet been obtained. For selected distance determinations, such methods do, however, have great utility.

5. Conclusions and Perspectives

During the past 5-10 years, there have been remarkable developments in the area of solid-state NMR of membranes. For lipids, a variety of techniques now permit a detailed examination of ordering at specific molecular sites, a feat only achievable previously with specific ^2H-labeling, and in the near future it should be possible to apply these methods to real biological membranes. However, it is clearly the peptide and protein NMR in model systems area which is expanding most rapidly. The first complete structure of a ion channel, gramicidin in DMPC, has been reported, and exciting new multidimensional techniques are now permitting structural investigations of even larger systems - genuine membrane proteins. For such rigid solids, there are no penalties to be paid in terms of linewidths and relaxation as molecular weights increase, and indeed, motion in many cases

actually causes problems, by decreasing T_2 or by interfering with the efficiency of dipolar decoupling. How big can one get? An open question, but given the fact that selective solid-state distance determinations have already been made in a protein having a molecular weight of 80,000 [117], that about one in three proteins are membrane-associated (and probably cannot be readily crystallized), and that GHz spectrometers are (hopefully) not too far off, is clear that there will be many exciting developments in the next few years in the area of solid-state NMR studies of membrane structure.

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