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APPLICATIONS OF SOLID STATE NMR TO CARDIOLIPIN AND ELASTIN

By

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M.S., Saratov State University, 2003
Ph.D., Saratov State University, 2006

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Doctor of Philosophy

Department of Chemistry
University of Louisville
Louisville, KY

May 2012

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A Dissertation Approved on

May 7, 2012

by the following Dissertation Committee:

Dissertation Director Dr. Richard J. Wittebort

To My Parents
and
Denis

Knowing what
thou knowest not
is in a sense
omniscience

Piet Hein

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ABSTRACT

APPLICATIONS OF SOLID STATE NMR TO CARDIOLIPIN AND ELASTIN

Tatiana Victorovna Krivokhizhina

May 7, 2012

This dissertation has five chapters. Following an introduction, chapter two describes an investigation of the hypothesis that cardiolipin has a proton storage function in mitochondrial membranes. Chapters three and four present a novel NMR study of the elastic mechanism in elastin. Directions for the future research are given in the last chapter.

To investigate the hypothesis that cardiolipin has a proton storage function, we have used ^{31}P , ^1H and ^{13}C NMR. A single axially symmetric ^{31}P pattern with reversed anisotropy compared to the static pattern is observed from pH 4 to 9 for tetraoleoyl cardiolipin (TOCL). This confirms that (a) the samples studied are in the biologically relevant liquid crystalline phase, (b) the phosphatidic moieties are equivalent, and (c) TOCL molecules reorient axially about the bilayer normal. Well resolved ^{13}C and ^{31}P MAS NMR spectra show no pH dependent chemical shifts. Based on a large number of ^1H , ^{13}C

and ^{31}P NMR experiments, we conclude that there is no evidence that lamellar multibilayers of TOCL possess a titratable group with a pK_a in the range of 4 to 9. We also discuss why the proton storage hypothesis is not energetically realistic.

The mechanism of elastin's recoil is addressed in chapters three and four. The conventional view based on conformational entropy is not supported by preliminary ^{13}C NMR studies, which show no observable increase in backbone ordering with stretch.

To investigate the alternative hypothesis that the hydrophobic effect drives elastic recoil, novel ^2H double quantum NMR experiments were implemented. NMR pulse sequences, phase cycles, and methods of analysis were devised to determine the amount of ordered water in elastin and how it changes with stretch, temperature and hydration. Based on the data, which shows a significant increase in the amount of ordered water in all three cases, we conclude that the hydrophobic effect has a significant role in the mechanism of elastin's reversible recoil. Consequently, elastic recoil exploits the increase of the solvent ordering on the hydrophobic surface of the protein when it is stretched, and recoil is driven by a favorable increase of the solvent entropy.

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CHAPTER 1

INTRODUCTION

Solid-state nuclear magnetic resonance (SSNMR) spectroscopy is a diverse and powerful method for investigation of structure and dynamics of biological molecules and it has been successfully applied for studying globular [1-7] and fibrous proteins [8-12], as well as membrane proteins [13-18] and lipids [19-24]. SSNMR is particularly beneficial in cases when conventional solution NMR and X-ray crystallography can not be used. These include membrane proteins and phospholipids for which retaining the intact environment is essential for studying structure and function. SSNMR is also an effective method for investigation of insoluble systems with no long-range order which can not be crystallized.

The present work is dedicated to the development and application of SSNMR methods (1) for investigating a structural hypothesis about the biological role of an important phospholipid of the inner mitochondrial membrane, cardiolipin, and (2) for determining the molecular mechanism of elastic recoil in nature's predominant elastomer, elastin. Our interest in the molecular ordering and dynamics of soft solids (membranes and elastic materials) is the common

objective. The following is a review of key background information relevant to our studies of cardiolipin and elastin. List of symbols and abbreviations used in the text is given in Appendix D.

1.1. Cardiolipin

Cardiolipin (CL) or diphosphatidylglycerol is a phospholipid found primarily in the inner mitochondrial membrane [25] of mammals, invertebrates, yeast and algae. It is also present in plasma membranes of Gram-negative and Gram-positive bacteria and this is the basis for the hypothesis about the bacterial origin of mitochondria in eukaryotes [26-27]. CL was first isolated from the beef heart tissue by Mary Pangborn in 1942 [28] and it was named after chemical characterization [29] showed that it is a polyglycero-phospholipid. CL's covalent structure was determined more than 20 years later, in 1964, by LeCocq and Ballou [30].

The unique structural feature that distinguishes CL from other phospholipids is the presence of two (as opposed to one) phosphatidic acid (PA) moieties per molecule. This is reflected in the name, diphosphatidylglycerol, i.e. CL is a dimer of PA's bridged by a glycerol. Consequently CL has four fatty acids and their composition in CL is highly conserved with mono and diunsaturated 16 and 18 carbon fatty acids being the most abundant [31] in mitochondrial CL. Bacterial CL is less restricted to fatty acid composition and it favors saturated and monounsaturated 14 to 19 carbon fatty acids [32].

Biosynthesis of CL

In prokaryotes, CL is synthesized from two molecules of phosphatidylglycerol, in eukaryotes – from one molecule of phosphatidylglycerol and one molecule of cytidinediphosphate-diacylglycerol. As opposed to most phospholipids, which are synthesized in the cytosol adjacent to endoplasmic reticulum, CL is synthesized in mitochondrion. A complete description of the process with all enzymes involved in the synthesis is reviewed in [25]. Interestingly, biosynthesis of CL is followed by acyl chain remodeling. During a remodeling stage, fatty acid composition is altered to obtain CL enriched with acyl chains specific to the particular organism and tissue. For example, mitochondrial CL in human tissues are enriched with oleic (18:1n-9) or linoleic (18:2n-6) acid. Malfunction of proteins involved in biosynthesis and modification of acyl chain composition of CL are responsible for several diseases [33]. In Barth syndrome, diminution of tetralinoleoyl (18:2n-6) CL and accumulation of monolysocardiolipin cause cardiomyopathy and skeletal weakness. Compositional abnormalities related to increased content of shorter and saturated acyl chains impair mitochondrial function and were reported in brain and stem cell tumors [34].

Polymorphism

Lipids, in general, are thermotropic and able to reorient their hydrophilic and hydrophobic moieties to take on a wide variety of structures (lipid polymorphism). Organization of CL's molecules in vivo is very sensitive to the characteristics of the local environment such as ionic strength and pH. Although

most lipids in membranes are in lamellar bilayer phases, some of the lipid molecules exist in non-bilayer phases. Transitions between different phases can be induced by changes in environmental conditions (temperature, pH, presence of ions in the media, etc.) and phase “signatures” can be recognized by SSNMR [35]. CL exists in either inverted hexagonal or lamellar, liquid crystalline phase. The hexagonal phase is favored at low pH and high ionic strength, while the lamellar phase is stabilized by interaction with other proteins [31]. In addition to environmental factors, the phase of CL is, to some extent, determined by the fatty acid composition. Longer and more unsaturated acyl chains favor a hexagonal phase [31]. Polymorphism of lipids is an important feature that allows them to perform a variety of biologically important functions. For example, in the contacts between inner and outer mitochondrial membranes, CL present in the hexagonal phase performs transmembrane protein transfer or membrane fusion. However most of the CL within the membrane exists in the lamellar, liquid crystalline phase.

Processes involving CL

Despite extensive research, CL’s biological function is still not clear. On the other hand, many processes which involve CL are now recognized. The most prominent characteristic of CL is the ability to react with proteins via non-covalent bonding. Proteins of electron transport chain work more efficiently upon CL binding which activates and stabilizes their structure. However, the presence of CL is not essentially required and these proteins are functional without it. Moreover, CL stabilizes not only separate proteins, but protein complexes of the

respiratory chain and facilitates electron transfer [36-37]. CL also mediates the apoptosis by activation of caspase-8 [38]. CL increases the mitochondrial membrane potential, thereby making ATP synthesis and protein transfer more efficient.

1.2. Elastin

Elastin is an essential fibrous protein that imparts elasticity to tissues and organs that must sustain large amplitude extensions and reversible recoil. It is found in elastic fibers of connective tissue (where it amounts to nearly 90% of the elastic fiber mass), in vascular tissue and ligaments (50-60 %), in lungs and skin (5-7 %) [39]. Dry elastin is an insoluble, brittle, pale yellow polymer and it acquires elasticity only when swollen in solvent. The first studies of elastin were published nearly a century ago. In 1914, McCartney studied elastin's composition and thermoelastic behavior and these experiments involved separation of elastin from collagen by digestion of collagen with pepsin [40]. Due to its insolubility, the initial studies of elastin employed chemical degradation procedures for the evaluation of amino acid composition. Since the study of soluble material is accessible by a variety of techniques, early research effort was focused on obtaining elastin's soluble counterparts. It was found that elastin is solubilized by oxalic acid (α -elastin) or potassium hydroxide (κ -elastin) [41]. Later it was found that, the natural precursor of elastin, tropoelastin, is also soluble. Tropoelastin was initially hard to isolate because once formed and transferred to the extracellular environment, it is converted into insoluble elastin.

Elastin has been identified in most vertebrates (except primitive jawless fishes) and has not been found in invertebrates [42]. Types of elastin that are actively studied include mammalian (baboon, cat, cow, dog, human, pig, mouse), avian (chicken), teleost (zebrafish) and amphibian organisms (frog) [33-34]. Interestingly, there are significant variations in the amino acid sequence of elastins, even in closely related species. The research group of Dr. John Parkinson (Toronto, Canada) created an online data base, ElastoDB <http://www.compsysbio.org/elastin/>, that summarizes all known elastin sequences (28 sequences). Sequence length varies between species and the average length is about 700 amino acids. It is hypothesized [42-44] that the variations in the sequence allow elastin to adapt to different environments such as encountered by cold-blooded versus warm-blooded animals.

Elastogenesis

Elastogenesis begins with the secretion of tropoelastin by smooth muscle cells and fibroblasts and transfer onto the cell surface by elastin binding protein [39, 45-49]. Elastin fiber growth includes coacervation of tropoelastin followed by the deposition of tropoelastin aggregates onto fibrillin microfibrils and their subsequent cross-linking. During coacervation, tropoelastin monomers of about 60-70 kDa self-assemble and form spherical clusters with radius of about 1-3 μm [49]. The fact that optimal conditions for coacervation coincide with physiological conditions (37° C, pH 7-8, 150 mM NaCl) suggests that extracellular coacervation occurs rapidly [39]. The process continues by deposition of these clusters onto fibrillin microfibrils present in the extracellular matrix. Fibrillin

serves as a scaffold for the growing elastin fiber. Finally, elastin fiber structure is stabilized by intra- and intermolecular cross-linking of tropoelastin. Cross-linking is accomplished by an enzyme that oxidizes lysine sidechains. Unique desmosine and isodesmosine cross-links render elastin fibers insoluble and very durable. In the context of robustness, it is noteworthy that the majority of elastin in mammals is formed during the development and post-natal growth. In mature organisms, the production ceases and only occurs in response to injury [50-51]. The turnover rate of cross-linked elastin is very slow with a half-life of about 70 years [52].

Secondary and tertiary structure

Tropoelastin has a modular organization with alternating hydrophobic and cross-linking domains of ~30 and ~20 residues, respectively. Strictly speaking, the presence of alternating elastomeric and cross-linking domains is a common feature of all elastomers [53]. Tropoelastin's hydrophobic domains are rich in the non-polar amino acids glycine, alanine, valine and proline. Interaction of hydrophobic domains is responsible for self-assembly of tropoelastins [39]. In addition, two highly conserved cysteine residues from the C-terminus of tropoelastin are important for self-assembly. Their removal greatly reduces the self-assembly rate [54]. Proline in hydrophobic domains is important for preserving elastin-like fibrous structure, since its depletion or substitution with glycine causes formation of amyloid-like structures [55-57]. Cross-linking domains are rich in alanine and lysine. Desmosine cross-links are formed by the lysines in the cross-linking domains. Study of cooperativity between domain

types shows that they are not independent. In particular, substitutions in cross-linking domains change structure and dynamics in hydrophobic domains [58].

Proper assembly and cross-linking of elastin is essential for health. There are severe diseases such as cutis laxa (inelastic skin), SVAS (supravalvular aortic stenosis, narrowing of the opening of aortic valve) [59], elastoderma (laxity of skin) [60] which are induced by mutations in the gene encoding elastin.

Circular dichroism studies of soluble, chemically modified elastins by Gotte [61] in the 1970's supported a twisted rope organization of elastin fibers with α -helical and β -turn conformations. Later, in 1980, Aaron and Gosline demonstrated that single water-swollen elastin fibers are optically isotropic suggesting a random coil network. The same result was indicated in isotope labeling structure studies by Perry et al. [62]. Based on the results of ^{13}C and ^2H SSNMR studies, Pometun et al. concluded that the backbone in hydrated elastin is highly disordered [63]. Current knowledge about elastin's structure is based on experiments that show conformationally flexible random-coil backbone [62-69].

Elasticity mechanisms

Since physical properties of hydrated elastin are similar to those of rubber, i.e. it is highly flexible and extensible, the similarity in their structural organization and elasticity mechanisms was assumed. From the perspective of the network organization, elastin is similar to rubber, which is composed of highly mobile (kinetically free) cross-linked polyisoprene chains, which are

similar to hydrophobic domains within cross-linked tropoelastin chains. With stretch, the polymer has fewer conformations. Therefore recoil is driven by increase in entropy of the polymer. That said, the fact that elastin is only elastic when swollen in solvent requires consideration of the role of solvent in elasticity, i.e. a mechanism that is different from rubber elasticity.

Thus, the mechanism of elasticity is still a subject of debate. At present, there are two main theories. The rubber elasticity mechanism based on configurational entropy of the polymer and a mechanism based on the hydrophobic effect, i.e. a decrease in solvent entropy.

1.3. Goals and methods

Cardiolipin

In chapter two, we examine the hypothesis that formation of strong H-bonds within the headgroup of CL significantly raises headgroup pK_a 's from the value characteristic of a phosphodiester group. 1H , ^{31}P and ^{13}C NMR titrations are used. Protons stabilized in H-bonds have distinct downfield shifts and in some cases can be observed in 1H NMR spectra. Intramolecular H-bonds with headgroup phosphates should also change ^{31}P and ^{13}C chemical shifts. Therefore ^{31}P and ^{13}C spectra at different pH are useful for the determination of this putative structural change. For these studies, CL samples were prepared in the biologically relevant lamellar, liquid crystalline phase. This was confirmed by ^{31}P NMR. A detailed investigation of putative structural change in CL's headgroup is important for understanding the biological role of CL at the molecular level.

Elastin

To investigate the mechanism of reversible elasticity in elastin, we test the two qualitatively distinct hypotheses for elastin's mechanism of recoil. In both hypotheses, elastic recoil is an entropy driven order to disorder process, however, the molecular sources of entropy are different, either the protein backbone or the solvent. To experimentally determine the relative importance of these two mechanisms in the function of elastin, we measure changes in ordering of the backbone, using ^{13}C NMR, and the solvent, using ^2H MQ NMR, as a function of stretch, hydration and temperature. These studies are fundamental for the design of biocompatible elastomeric materials for wound repair, construction of artificial blood vessels [51] and other applications of elastin in tissue engineering.

CHAPTER 2

SSNMR INVESTIGATION OF STRUCTURAL HYPOTHESIS FOR CARDIOLIPIN

2.1. Introduction

The involvement of anionic phospholipids in proton conduction in the membranes of energy producing organelles was hypothesized in the early 1980's [70]. Michel et al. observed lateral relocation of protons along the membranes of halobacteria [71]. Haines suggested a mechanism for this process [70]. According to his hypothesis, the headgroups of anionic lipids in the membrane serve as proton carriers. This is in contrast to the previous notion about proton transfer via bulk aqueous phase. Protons in such membranes are transferred laterally via dynamic formation of acid-anion pairs, in which two anions share a single proton via a hydrogen bond. Later, in the early 1990's, a similar mechanism of proton stabilization was hypothesized for cardiolipin (CL) [72]. An unusual buffering capacity of CL in KCl was observed during a potentiometric titration. The titration curve indicated a pK_a value above 7 [72]. Haines and coworkers suggested the formation of a cyclic structure within the headgroup that stabilizes the proton by hydrogen bonding thereby increasing a higher pK_a .

Our goal is to test the structural hypothesis and identify possible titratable groups by performing a CL titration monitored by the pH dependence of ^{13}C and ^{31}P chemical shifts. To address this hypothesis with a physiologically relevant sample, we prepared unsonicated, lamellar CL multibilayers from which we obtained high resolution magic angle spinning (MAS) ^1H , ^{13}C and ^{31}P NMR spectra.

NMR titration

To understand the effects of titration on the NMR spectrum, let us consider the following. Conversion of protonated species A (acid) into deprotonated B (conjugate base) results in different chemical shifts δ_A and δ_B for A and B, respectively. With the assumption of fast chemical exchange, a single chemical shift is observed, δ_{obs} , that is a weighted average of the chemical shifts of A and B:

$$\delta_{\text{obs}} = \delta_A f_A + \delta_B f_B = \delta_A \frac{A}{A+B} + \delta_B \frac{B}{A+B},$$

where f_A and f_B are mole fractions of A and B respectively. With the equilibrium expression for the acid dissociation

$$K_a = \frac{H^+ B}{A},$$

we obtain

$$\frac{A}{A+B} = \frac{A}{A + \frac{K_a A}{H^+}} = \frac{1}{1 + \frac{K_a}{H^+}} = \frac{H^+}{H^+ + K_a}$$

and

$$\frac{B}{A+B} = \frac{B}{\frac{H^+B}{K_a} + B} = \frac{1}{\frac{H^+}{K_a} + 1} = \frac{K_a}{H^+ + K_a}$$

Substituting into the expression for δ_{obs} , we obtain

$$\delta_{obs} = \frac{\delta_A H^+}{H^+ + K_a} + \frac{\delta_B K_a}{H^+ + K_a}$$

which this is rearranged to give,

$$H^+(\delta_{obs} - \delta_A) = K_a(\delta_B - \delta_{obs})$$

After taking logarithms of both sides, we obtain

$$pH = pK_a + \log \frac{(\delta_{obs} - \delta_A)}{(\delta_B - \delta_{obs})}$$

This is the the Henderson-Hasselbalch equation:

$$pH = pK_a + \log \frac{B}{A}$$

in a form that can be used to determine a titration curve obtained by plotting chemical shifts against pH. This provides pK_a .

2.2. Materials and methods

The choice of a cardiolipin is dictated by two conditions – the lipid has to be in the lamellar, liquid crystalline phase at room temperature and it has to be common in eukaryotes. It has been found that mono- and diunsaturated chains composed of 16 and 18 carbons occur more often in eukaryotic CL [31]. To choose a CL convenient for studying the liquid crystalline L_α phase, we have compared the more complete data on the temperatures of gel to liquid crystalline

phase transitions of glycerophospholipids to the more limited data for CL. Phosphatidylcholine, phosphatidylglycerol and phosphatidylserine containing myristyl (C_{14} , saturated) moieties undergo gel to liquid crystalline phase transitions at 23, 23 and 35°C respectively [76]. These transition temperatures decrease systematically when myristic acid is replaced by oleic acid (C_{18} , unsaturated) to -20 , -18 and -11°C , respectively [76]. Based on this, and the fact that the tetramyristoyl CL gel to liquid crystalline phase transition occurs at 40°C [77] we chose tetraoleoyl 18:1 CL, since at room temperature this lipid is reasonably expected to be in the lamellar, liquid crystalline phase.

Natural abundance (unlabelled) 1,1',2,2'-tetraoleoyl cardiolipin (TOCL) in lyophilized powder form was purchased from Avanti Polar Lipids Inc. and used without further purification. In this synthetic cardiolipin, all four fatty acids are monounsaturated oleic acid (18:1n-9).

For experiments at pH values from 2.2 to 5.5, the buffer was phosphate/citrate prepared from 100 mM citric acid and 200 mM Na_2HPO_4 . For pH values from 6.0 to 9.0 100 mM Na phosphate buffer was prepared by mixing appropriate volumes of 200 mM NaH_2PO_4 and 200 mM Na_2HPO_4 and adding an equal volume of distilled water. Sample preparation included equilibration of solid cardiolipin in excess buffer followed by separation of the lipid and buffer phases by centrifugation. The pH of supernatant was measured after repeating of equilibration process at least three times to ensure pH of cardiolipin. Simple vortexing was used to mix lipid and buffer. Then the lipid phase was transferred to the MAS rotor. Once ^{13}C , ^{31}P spectra were obtained, the same sample was

used for another round of buffer equilibration and spectra accumulation. With this procedure, the same lipid sample was used for obtaining data at different pH values.

Ultrasonication, which alters the physical state of the membrane [78], is unnecessary to obtain high spectral resolution. No sonication was used in this study. Samples were concentrated by several rounds of centrifugation at 10,000xg.

For HSQC solution NMR experiments, used to make resonance assignments, TOCL was dissolved in deuterated chloroform (CDCl_3).

^{31}P static and MAS spectra were recorded on a homebuilt NMR spectrometer (11.7 Tesla), and ^{13}C and ^1H MAS and solution NMR spectra were obtained on a Varian Inova-800 NMR spectrometer (18.8 Tesla). Spectra were recorded several times with different samples and all spectra were found to be reproducible.

2.3. Results and discussion

Phase state assignment by ^{31}P SSNMR: lamellar phase

Phase behavior of phospholipids can be evaluated by ^{31}P SSNMR, since various phase states have unique ^{31}P static powder patterns. Possible lipid phases include hexagonal or inverted hexagonal phase (H_{II}), micellar hexagonal phase (H_{I}), liquid crystalline phase (L_{α}) and gel phase (L_{β}). The lamellar, liquid crystalline phase is a “pile” of L_{α} lipid bilayers with a thin layer of water solution between lamellae. The lamellar phase is also denoted as L_{α} and it is the

biologically relevant phase for membrane studies. Figure 2.2 illustrates ^{31}P static powder patterns of TOCL aqueous dispersions in two phase states. The hexagonal phase has a static powder pattern with positive span while the lamellar phase has an axially symmetric pattern and the span, $s = \sigma_{33} - \sigma_{11}$, has the opposite sign. In sonicated lipid dispersions, lipids are contained in spherical, unilamellar vesicles so chemical shift anisotropy (CSA) is averaged to a relatively narrow line due to isotropic reorientation of the vesicles. The gel phase has larger span than L_α and a non-zero asymmetry because of restricted mobility.

The static ^{31}P NMR spectra of TOCL (Figure 2.3) have ^{31}P shielding anisotropy of 33.6 ppm with negative span. This is consistent with the spectrum of L_α phase TOCL reported in [79] and shown in Figure 2.2. Therefore, the samples of TOCL studied in sodium phosphate buffer pH from pH 4 to pH 9 are in the L_α , lamellar, multibilayers phase.

Figure 2.2. Static ^{31}P powder patterns of aqueous dispersions of TOCL and corresponding phase states.

Implications of static and MAS ^{31}P spectra

Static and MAS ^{31}P spectra of lamellar CL multibilayers at different pH are given in Figure 2.3. The sharp peak in the center of the powder pattern corresponds to the buffer phosphate and its chemical shift changes with titration. In effect, it serves as an internal “pH-meter”. The same shift of the buffer peak is also seen in the fast MAS spectra. Static ^{31}P powder patterns are consistent with the slow (1 kHz) MAS spectra, i.e. MAS spectra spinning sideband peaks can be calculated from the powder pattern. The MAS spectra provide improved resolution of isotropic chemical shifts.

At least two conclusions can be drawn from the data in Figure 2.3. First, static spectra indicate that the residual shielding anisotropy is axially symmetric. Since the static shielding is non-axial, this requires that TOCL molecules undergo axially symmetric motion about the bilayer normal. Second, the fact that a single shielding tensor in powder patterns and a single lipid peak in fast (6.5 kHz) MAS spectra are observed shows equivalency of the two phosphates in the headgroup of TOCL.

Figure 2.3. Static and MAS ^{31}P NMR spectra of TOCL dispersions in phosphate/citrate phosphate buffer at various pH.

³¹P CSA and mobility of the headgroup

The anisotropy of the ³¹P shielding tensor depends on the structure and motion of the headgroup. Therefore, conformational changes and/or restrictions of mobility of the headgroup lead to observable changes in ³¹P CSA. It is known that the headgroup of phosphatidylcholine (PC) is flexible and it reorients around the bilayer normal. Compared to the ³¹P anisotropy of liquid crystalline phase PC, 45 ppm [80], spectra of lamellar TOCL, Figure 2.3, show smaller ³¹P anisotropy, about 34 ppm. Therefore, the headgroup of CL is somewhat more flexible than that of PC. Moreover, whereas some lipids have pH dependent anisotropy [81], TOCL shows no significant change in CSA over the pH range from 4 to 9.

¹H Search for a protonated phosphate

Haines and co-workers suggested [72] that their observation of an unusually high pK_a is a consequence of the formation of hydrogen bond between the central glycerol hydroxyl group and a protonated phosphate from a PA moiety. Acid protons stabilized in a hydrogen bond have characteristic chemical shifts (>10 ppm) that are well downfield of “regular” proton shifts. In principle, such protons can be observed with ¹H NMR due to their unusual chemical shift. For example, formation of strong hydrogen bonds in the active sites of serine proteases has been suggested [82] based on downfield chemical shifts in ¹H NMR. Figure 2.4 shows an example of a proton trapped in the intramolecular hydrogen bond in sodium hydrogen maleate (NaHM). The lower ¹H spectrum is NaHM trihydrate dissolved in DMSO and the top spectrum was obtained after a

small amount of water was added. The peaks at ~ 2.5 , 3.5 and ~ 6 ppm are from residual solvent, water and maleate, respectively. The acid-anion trapped proton is at ~ 20 ppm. It, however disappears when a small amount of water is added, presumably due to rapid exchange with solvent water (~ 3.7 ppm). We conclude that in aqueous samples, trapped protons are not necessarily observable by NMR.

Figure 2.5 shows ^1H spectra of TOCL at pH 6.5 and 9.0. The only peak observed in the downfield region below 20 ppm corresponds to the spinning sideband of the lipid peak. This was confirmed by changing the spinning speed (ν_{rot}). In addition, ^1H lipid chemical shifts are the same at pH 6.5 and 9.0. These results provide negative evidence for the formation of the proposed CL cyclic, acid-anion structure within the headgroup. However, as seen in hydrogen maleate example, the downfield peak of the stabilized proton can disappear due to fast exchange. Therefore, we next used ^{13}C NMR to search for evidence of ionizable groups in CL with physiologically interesting pK_a 's. ^{13}C spectroscopy is a sensitive monitor of local ionizable groups when charge changes upon titration.

Figure 2.4. ¹H spectra of sodium hydrogen + 50 µl H₂O maleate.

Figure 2.5. ^1H MAS spectra of TOCL at pH 6.5 ($\nu_{\text{rot}} = 10$ kHz) and 9.0 ($\nu_{\text{rot}} = 10.3$ kHz). Spectra are broken into 2 sections: the lipid region (0 to 4 ppm) and the downfield region where the acid-anion proton is expected (>10 ppm). The signals below 20 ppm are spinning sidebands of the lipid. The downfield sections are vertically expanded 5-fold compared to the upfield sections.

Assignment of ^{13}C and ^1H NMR spectra

^{13}C MAS NMR spectra with good S/N are obtained for TOCL over the pH range from 4.5 to 9.0. The ^{13}C spectrum (Figure 2.6) has distinct regions as labeled. For our purpose, the most interesting region of the spectrum is the glycerol region with five resolved peaks. If the cyclic structure with trapped proton forms as suggested by Haines [72], the headgroup glycerol carbons are expected to have pH-dependent chemical shifts.

A schematic of the TOCL structure with nomenclature used for the assignment is shown on Figure 2.7. G_1 , G_2 and G_3 are C atoms at position 1, 2 and 3 of the side PA “glycerols”; G'_1 are at equivalent positions 1, 3 and G'_2 is at position 2 of the central “glycerol”. Atoms, carbons and protons, from 1 to 18 are from the fatty acyl chains with the double bonds at the indicated positions.

Figure 2.6. ^{13}C MAS NMR spectrum of TOCL in phosphate buffer at pH 7.0.

Figure 2.7. Schematic structure of TOCL and nomenclature used for the assignment of ^{13}C and ^1H NMR spectra.

Figure 2.8. HSQC spectrum of TOCL in CDCl_3 .

The strategy used to assign the closely spaced glycerol region of spectra in Figure 2.9 and Figure 2.10 is to correlate ^{13}C and ^1H shifts using the HSQC experiment (Figure 2.8). This approach is possible since chemical shifts observed in MAS spectra of the L_α phase CL dispersions in phosphate buffer are essentially the same as when the lipid is dissolved in deuterated chloroform. In the HSQC spectrum, negative (purple) peaks for methine (G_2 and G'_2) carbons at 70.3 ppm and 71.4 ppm are readily distinguished from the positive (yellow) methylene signals (G_1 , G_3 and G'_1). On the basis of signal intensity, the smaller methine peak at 70.3 ppm is assigned to G'_2 (1 carbon) and the ~ 2 -fold larger signal at 71.4 ppm to G_2 (2 carbons). Acylation at glycerol methylene carbons induces inequivalency of the attached protons, consequently the ^1H split signals with a ^{13}C shift of 63.7 ppm are assigned to G_1 . The remaining methylene signals, 64.4 ppm and 67.2 ppm, are assigned to G_3 (2 carbons) and G'_1 (2 carbons). Based on ^{13}C shifts observed in lecithins [78] the 64.4 ppm signal is tentatively assigned to G_3 .

Remaining signals are due to the acyl chains. The most downfield signals are from fatty acid carbonyl groups, 173.51 ppm (in CDCl_3), and the unsaturated carbons (C_9 and C_{10}) of the fatty acid chains at 129.60 ppm. Assignments in the aliphatic region were based on the known assignments of DMPC [83] and fatty acid esters. Complete listing of these results is given in Figure 2.9, Figure 2.10 and in the Table 2.1.

Figure 2.9. ^{13}C MAS NMR spectrum of TOCL in phosphate buffer.

Figure 2.10. ^1H MAS NMR spectrum of TOCL in phosphate buffer.

Table 2.1. Assignments of ^1H and ^{13}C NMR spectra of TOCL.

Atom position	^{13}C chemical shift		^1H chemical shift	
	CDCl_3	100 mM Na	CDCl_3	100 mM Na
		phosphate buffer, pH 7.0		phosphate buffer, pH 7.0
1	173.51	174.4	-	-
2	34.06	34.8	2.22	2.3
3	24.86	25.8	1.51	1.6
9, 10	129.60	130.2	5.26	5.3
8, 11	27.22	28.1	1.93	2.0
4-7, 12-15	29.50	30.4	1.24	1.3
16	31.89	32.8	1.19	1.3
17	22.65	23.5	1.20	1.3
18	14.10	14.7	0.90	0.8
G_1	62.85	63.7	4.12, 4.34	4.2, 4.5
G'_1	67.23	67.2	3.91	3.9-4.0
G_2	70.51	71.4	5.17	5.2
G'_2	69.41	70.3	3.87	3.9-4.0
G_3	63.40	64.4	3.87	3.9-4.0

NMR titration: ^{13}C and ^{31}P chemical shifts vs. pH

^{13}C chemical shifts of TOCL from pH 4.5 to 9.0 are presented in Figure 2.11. We observe that chemical shifts are constant within experimental error, $\pm 0.1\text{ppm}$, over this physiologically important range.

Figure 2.11. ^{13}C MAS NMR spectra of TOCL in phosphate buffer (a) and site specific chemical shifts (b).

Similarly, Figure 2.12 and Figure 2.13 show that ^{31}P chemical shifts of TOCL are also independent of pH. Note that in these spectra, the buffer signal moves downfield with increased pH.

Figure 2.12. ^{31}P MAS NMR spectra of TOCL.

Figure 2.13. ^{31}P chemical shifts of TOCL (●) and phosphate/citrate phosphate buffer (×) as function of pH.

It is instructive to compare the behavior of phosphoric acid when it is titrated and observed by NMR with the behavior of Phosphodiester, such as CL. This comparison is appropriate since both phosphoric acid and phosphodiester are neutral and have similar pK_a values (Figure 2.14). The results of an NMR titration of phosphoric acid are shown in Figure 2.15.

Figure 2.14. Analogy between phosphoric acid and phosphodiester.

Figure 2.15. NMR titration curve for H_3PO_4 . pK_a values are from [84].

The change in chemical shift upon deprotonation of phosphoric acid, H_3PO_4 , is 0.32 ppm and we expect a similarly small change for deprotonation of

phosphodiester like CL. While ^{31}P is not a particularly sensitive probe for this, our experiments have enough resolution to see such small changes and we did not observe any change in ^{31}P shifts with titration.

2.4. Discussion and conclusions

Well resolved ^{13}C and ^{31}P MAS NMR spectra of TOCL show pH-independent chemical shifts and indicate no titration over the physiologically relevant pH range tested. Moreover, ^1H MAS NMR spectra show no peak for an acid proton stabilized by a hydrogen bond. Thus, we conclude that the structural hypothesis for the CL's headgroup in which phosphates form a tight bicyclic structure H-bonding to the hydroxyl group of the connecting glycerol and trap a proton from the environment [72] is incorrect.

To appreciate this conclusion, we estimate the H-bond energy required to raise a phosphodiester pK_a from a normal value, $\text{pK}_a \sim 1.5$, to a value that would make it a proton storage trap at physiological pH values, $\text{pK}_a \sim 7.5$. To do this, consider the thermodynamic cycle (Figure 2.16) in which the singly protonated cardiolipin, CLH, can either lose a proton directly with pK_{a1} or form a stabilizing bond, CLH---B, with free energy change $\Delta G_{\text{stab}} < 0$.

Figure 2.16. Model for the estimation of energy of stabilizing bond.

Subsequently, CLH $\rightleftharpoons$ B, can lose its proton with $pK_{a2} > pK_{a1}$. This cycle shows that the pK_a 's and ΔG_{stab} are related by

$$\Delta G_{stab} + \Delta G_2 = \Delta G_1$$

or

$$\Delta pK_a = pK_{a2} - pK_{a1} = -\Delta G_{stab}/(2.303RT) \sim -\Delta G_{stab}/(5.8 \text{ kJ/mol}).$$

If the stabilizing interaction is a hydrogen bond, as suggested in [72], then $|\Delta G_{stab}|$ is not more than 12 kJ/mol and, consequently, ΔpK_a is not more than 2. To stabilize a phosphodiester, $pK_{a1} \sim 1.5$, so that its pK_{a2} is increased to 7.5, would require stabilization in excess of 35 kJ/mol. We view this as unrealistic. We conclude that there is no evidence or chemical basis that lamellar CL possesses a titratable group with a pK_a in the range of 4 to 9.

Figure 2.17. Model for the axially symmetric reorientation of CL within the bilayer.

Our results also show that the ^{31}P CSA of the TOCL headgroup is axially symmetric and the anisotropy is less than that of PC. We conclude that it is less ordered than the flexible headgroup of PC and suggest the following model for lamellar cardiolipin membranes (Figure 2.17). Headgroups within a CL molecule are flexibly connected and each CL molecule reorients with axially symmetric motion within the membrane bilayer.

CHAPTER 3

^2H MULTIPLE QUANTUM NMR

3.1. Introduction

The advent of two-dimensional Fourier transform spectroscopy [85-86] triggered the development of new NMR fields: homo- and heteronuclear correlation spectroscopy and multiple quantum (MQ) spectroscopy [86-87]. The availability of the second dimension is essential for MQ spectroscopy, since coherent MQ oscillations of spins do not produce magnetic dipole radiation [88], i.e. they induce no signal and can only be detected indirectly after conversion to observable single quantum (1Q) coherence. At the initial stage of development, MQ spectroscopy dealt with improvement of spectral resolution via elimination of quadrupolar and field inhomogeneity broadening [86-87, 89], with relaxation time measurements [90-92], and with detection of couplings [93-94]. The spectrum of applications continues and is now used in measurements of dipolar couplings [95-97], detection of transient ordering in gases [98] and fluids [99-100], magnetic resonance imaging [101-105], and MQ diffusion studies [106-107] etc.

In spite of the diversity of applications, the pulse sequences employed in these experiments underwent only modest changes. The initial three-pulse experiment was supplemented with refocusing pulses and with 16-step phase

cycles for signal filtration. Even the most recent studies exploit relatively simple pulse sequences and phase cycles. In some cases it suffices, but in others it leads to partial cancellation of the desired signals that have opposite phases. This leads to dispersive signals and poor S/N in general.

In this study, we have designed a more general pulse sequence and phase cycle. The ultimate goal is to address the mechanism of elasticity in elastin by observing the solvent part of an elastin sample. The solvent is potentially ordered on the surface of the elastin fiber, and our task is to detect the amount of ordered solvent, while suppressing the signal from bulk, isotropically reorienting solvent. This is relevant to an alternative hypothesis about the mechanism of elasticity that is based on the hydrophobic effect. MQ spectroscopy is useful in this case because it allows one to distinguish ordered, anisotropically reorienting water, from the bulk, isotropic water. MQ NMR has been used for solvent ordering measurements in a variety of systems, including purple membrane suspensions [99], polymer membranes like Nafion [100], biological samples such as blood vessels [105], cartilage [104], spinal disk tissue [108-109], collagen [103, 110-111], and elastin [112-113]. We have improved on the pulse sequence commonly used, and devised a method for analysis of the results that allows us to estimate the amount of ordered water in the sample and the strength of the residual quadrupolar coupling, i.e., the degree to which the water is ordered at the protein surface.

In this chapter, I present background information on MQ spectroscopy, a description of the development of a new pulse sequence and phase cycle,

experimental verification of the sequence, and the method for analysis of the results. Methods described in this theoretical chapter are then used for the study of the mechanism of elastin's elasticity, described in the following chapter. The approach is general and can be used to study solvent ordering in other systems.

3.2. MQ spectroscopy background

Density operator

The state of a quantum system is described by the wavefunction, ψ , that can be expressed as a linear combination of eigenstates:

$$\psi = |\psi\rangle = a_1 |\phi_1\rangle + a_2 |\phi_2\rangle + \dots = \sum_i a_i |\phi_i\rangle$$

with coefficients $a_i = \langle \phi_i | \psi \rangle$. The basis functions, $|\phi_i\rangle$, are orthogonal $\langle \phi_i | \phi_j \rangle = 0$ and normalized $\langle \phi_i | \phi_i \rangle = 1$, and the wavefunction, ψ , is a vector:

$$\psi = \begin{pmatrix} a_1 \\ a_2 \\ \vdots \end{pmatrix}.$$

Evolution of the wavefunction is governed by the time-dependent Schrödinger equation,

$$H|\psi\rangle = i\hbar \frac{\partial |\psi\rangle}{\partial t},$$

where $\hbar$ is set to 1, and H is the interaction Hamiltonian. In the case of a time-independent Hamiltonian, the solution is

$$|\psi(t)\rangle = e^{-iHt} |\psi(0)\rangle.$$

To make H time-independent, we use rotating frame Hamiltonians that remove the high frequency Larmor precession, ω_0 , resulting from the large Zeeman interaction. With this, peak positions have frequency $\omega_0 - \omega_{RF}$ that are defined relative to the center of the spectrum, ω_{RF} . For our purposes, there are three relevant spin Hamiltonians. Two of these, offset and quadrupolar, are internal, H_{os} and H_q , and are the energies of the nuclear spin interacting with the surrounding electrons. The third Hamiltonian, H_{RF} , is for the applied resonant RF field that the spectroscopist uses to manipulate the spin system.

$$H_{os} = -\Delta\omega_{os} I_z,$$

$$H_q = \frac{1}{2I+1} \omega_q (3I_z^2 - I(I+1)),$$

$$H_{RF} = -\omega_1 I_k,$$

where I_k ($k = x, y, z$) is the k -component of spin angular momentum, I is a spin quantum number, $\Delta\omega_{os} = \omega_0 - \omega_{RF}$ is an offset frequency, ω_q is a quadrupolar coupling, and (as we shall see) an RF pulse of duration t and strength γB_1 rotates 1Q coherence through an angle $\theta = \omega_1 t$ about the k -axis. Since the Zeeman and chemical shielding interactions depend in the same way on spin variables, $\Delta\omega_{os}$ contains the effect of chemical shielding and the small difference between a spin's precession frequency and the rotating frame frequency. In solids and semisolids, chemical shielding is spatially anisotropic, so $\Delta\omega_{os}$ depends on molecular orientation.

In quantum mechanics, the expectation value $\langle \Omega \rangle$ of an observable, Ω , is given by

$$\langle \Omega \rangle = \langle \psi | \Omega | \psi \rangle = \sum_{i,j} a_i^* a_j \langle \phi_i | \Omega | \phi_j \rangle = \sum_{i,j} a_i^* a_j \Omega_{ij}.$$

The matrix elements $a_i^* a_j$ are tabulated in the form of a Hermitian $(2l+1) \times (2l+1)$ matrix, ρ_{ij} , which is known as the density matrix or density operator ρ . Therefore,

$$\langle \Omega \rangle = \sum_{i,j} \Omega_{ij} \rho_{ij} = \text{Tr}(\Omega \rho).$$

Comparison of the above shows that the density operator is the direct product of the wave vector with itself,

$$\rho = |\psi\rangle\langle\psi| = \begin{pmatrix} a_1^* a_1 & a_1^* a_2 & \cdots \\ a_2^* a_1 & a_2^* a_2 & \cdots \\ \vdots & \vdots & \ddots \end{pmatrix}.$$

The density operator contains all information about the dynamics of the spin system. Elements of the density matrix have clear physical meaning: the diagonal element ρ_{ii} represents the population of energy level i , while the off-diagonal element ρ_{ij} corresponds to the transition between $|i\rangle$ and $|j\rangle$ states.

Time evolution of the density operator is then given by

$$\begin{aligned} \frac{\partial \rho}{\partial t} &= \frac{\partial |\psi\rangle\langle\psi|}{\partial t} = \frac{\partial |\psi\rangle}{\partial t} \langle\psi| + |\psi\rangle \frac{\partial \langle\psi|}{\partial t} \\ &= -iH|\psi\rangle\langle\psi| + i|\psi\rangle\langle\psi|H = -iH\rho + i\rho H \\ &= -i[H, \rho], \end{aligned}$$

and is known as the Liouville-von Neumann equation. The solution of this equation, in the case of time-independent, rotating frame Hamiltonians is

$$\rho(t) = e^{-iHt}\rho(0)e^{iHt},$$

where $\rho(0)$ describes the spin system at $t = 0$.

Single transition operators and coherences

A logical extension of the analogy between the wavefunction and the density operator is to formulate the density operator as a linear combination of orthogonal and normalized basis operators, O_i :

$$\rho = \sum_i a_i O_i.$$

Insofar as the basis operators cover the space, their choice is arbitrary. It is convenient to choose operators with clear physical meaning. With trace orthogonality, the expectation value of each of the basis operators is equal to the corresponding coefficient in the linear combination, $\langle O_i \rangle = a_i$.

For ^2H , $I=1$, there are $2I+1=3$ possible spin states and the density operator has $3 \times 3 = 9$ elements (we refer the reader to Appendix A for the similar description of ^{17}O). Therefore, to represent the density operator as a linear combination of basis operators, a 9 operator basis set is required. We use operators related to the “x, y and z components” of the 3 transitions. These “single transition operators”, I_x^{ij} , I_y^{ij} and I_z^{ij} , describe selective excitation of transitions between states $|i\rangle$ and $|j\rangle$. These operators are defined as follows [114]:

$$\langle i | I_x^{rs} | j \rangle = \frac{1}{2} (\delta_{ir} \delta_{js} + \delta_{is} \delta_{jr})$$

$$\langle i | I_y^{rs} | j \rangle = \frac{i}{2} (-\delta_{ir} \delta_{js} + \delta_{is} \delta_{jr})$$

$$\langle i | I_z^{rs} | j \rangle = \frac{1}{2} (\delta_{ir} \delta_{jr} - \delta_{is} \delta_{js}).$$

The z-component operators, $I_z^{1,0}$, $I_z^{0,1}$ and $I_z^{1,1}$, correspond to level populations and $I_z^{1,0} + I_z^{0,1} = I_z^{1,1}$ (I_z^{ij} are not linearly independent). The “transverse” components, I_k^{ij} (where $k = x, y$), are known as coherences and are closely related to the transverse magnetizations of the allowed transitions, $I_y = I_y^{1,0} + I_y^{0,1}$ and $I_x = I_x^{1,0} + I_x^{0,1}$. A coherence between two eigenstates $|i\rangle$ and $|j\rangle$ is non-zero when $\rho_{ij}(t) = a_i^* a_j$ does not vanish [115]. Each eigenstate is characterized by magnetic quantum number m . The change in magnetic quantum number, Δm , is called the order of the coherence. Single quantum coherences ($\Delta m = \pm 1$) are the observable transverse magnetizations, and multiple quantum coherences ($\Delta m \neq \pm 1$) for $I = 1$ are $I_x^{1,1}$ and $I_y^{1,1}$. Explicit 3 x 3 matrices for the 9 basis operators of 2H are given in Table 3.1 and the Octave script for their calculation is given in Appendix B.

Table 3.1. Matrix representations of single transition operators, I_k^{ij} , for $l = 1$.

$i \rightarrow j$	$k = x$	$k = y$	$k = z$
$-1 \rightarrow 0$	$\begin{pmatrix} 0 & 1/2 & 0 \\ 1/2 & 0 & 0 \\ 0 & 0 & 0 \end{pmatrix}$	$\begin{pmatrix} 0 & -i/2 & 0 \\ i/2 & 0 & 0 \\ 0 & 0 & 0 \end{pmatrix}$	$\begin{pmatrix} 1/2 & 0 & 0 \\ 0 & -1/2 & 0 \\ 0 & 0 & 0 \end{pmatrix}$
$0 \rightarrow 1$	$\begin{pmatrix} 0 & 0 & 0 \\ 0 & 0 & 1/2 \\ 0 & 1/2 & 0 \end{pmatrix}$	$\begin{pmatrix} 0 & 0 & 0 \\ 0 & 0 & -i/2 \\ 0 & i/2 & 0 \end{pmatrix}$	$\begin{pmatrix} 0 & 0 & 0 \\ 0 & 1/2 & 0 \\ 0 & 0 & -1/2 \end{pmatrix}$
$-1 \rightarrow 1$	$\begin{pmatrix} 0 & 0 & 1/2 \\ 0 & 0 & 0 \\ 1/2 & 0 & 0 \end{pmatrix}$	$\begin{pmatrix} 0 & 0 & -i/2 \\ 0 & 0 & 0 \\ i/2 & 0 & 0 \end{pmatrix}$	$\begin{pmatrix} 1/2 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & -1/2 \end{pmatrix}$

Other useful operators are:

$$I_+ = \begin{pmatrix} 0 & 0 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \end{pmatrix} \quad I_- = \begin{pmatrix} 0 & 1 & 0 \\ 0 & 0 & 1 \\ 0 & 0 & 0 \end{pmatrix} \quad I_z = \begin{pmatrix} -1 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 1 \end{pmatrix}$$

$$I_x = \frac{1}{2}(I_+ + I_-) = I_x^{-1,0} + I_x^{0,1} = \begin{pmatrix} 0 & 1/2 & 0 \\ 1/2 & 0 & 1/2 \\ 0 & 1/2 & 0 \end{pmatrix}$$

$$I_y = \frac{i}{2}(I_- - I_+) = I_y^{-1,0} + I_y^{0,1} = \begin{pmatrix} 0 & -i/2 & 0 \\ i/2 & 0 & -i/2 \\ 0 & i/2 & 0 \end{pmatrix}$$

$$Q = 3I_z^2 - 2 = \begin{pmatrix} 1 & 0 & 0 \\ 0 & -2 & 0 \\ 0 & 0 & 1 \end{pmatrix}$$

We note that these 9 operators (Table 3.1) are a straightforward generalization of Pauli spin matrices, i.e., matrix representations of I_x , I_y and I_z ,

for $I = 1/2$. Moreover, these matrices transform under rotations in the same way as the classical magnetization vectors in Larmor's theorem.

A convenient way to trace orthogonalize ($\text{Tr} O_i O_j = \delta_{ij}$) the basis operators introduced above, is to replace the z components, $I_z^{-1,0}$, $I_z^{0,1}$ and $I_z^{-1,1}$, with I_z , H_q and 1 (respectively, $I_z^{-1,0} + I_z^{0,1} + I_z^{-1,1}$, Q related to the quadrupolar Hamiltonian and the identity matrix). The expectation value of I_z corresponds to the net z-magnetization or Zeeman order and the expectation value of H_q is similarly referred to as quadrupolar order. With the 6 single transition coherences, $I_k^{i,j}$ ($k = x, y$), these operators span the 9-dimensional space of $I = 1$. Since $\rho = \sum_i a_i O_i$, multiplication of both sides by O_j gives: $O_j \rho = \sum_i a_i O_j O_i$. Then, $\text{Tr}(O_j \rho) = \sum_i a_i \text{Tr}(O_j O_i)$, and orthogonality ($\text{Tr} O_i O_j = \delta_{ij}$) confirms that $\langle O_i \rangle = a_i$, i.e. the expansion coefficient a_i is the expectation value $\langle O_i \rangle$. For example, equilibration in a magnetic field generates population difference, but no net transverse magnetization, i.e. $\rho_{\text{eq}} = 1 + c I_z$ where c is the Curie law constant.

Density operator manipulations: RF pulses and quadrupolar coupling

Evolution of the density matrix has the well-known form of a matrix transformation, $R^\dagger \rho R$, where “ $\dagger$ ” notes Hermitian conjugate and R is a propagator. Application of an RF pulse, transforms the density operator by $R = \exp(i\omega_1 I_k t)$, ($k = x, y$). This is the form of a rotation, i.e. an RF pulse acts as a rotation about the “k-axis” through an angle $\theta = \omega_1 t$. Consequently, application of

a 90° pulse ($\theta = \omega_1 t$) with phase ϕ to the equilibrium density operator can be summarized by the following

$$I_z \xrightarrow{90_0} \frac{1}{\sqrt{2}}(I_y^{-1,0} + I_y^{0,1}).$$

The identity operator and the Curie constant have been dropped since they are not affected by the unitary transformations of quantum mechanics. Simply stated, the 90° pulse converts a population difference into transverse single quantum coherences of both transitions. If the 90° pulse is followed by an evolution period in which the spins evolve under chemical shielding alone, i.e., as in solution NMR, then the density operator is subject to the transformation

$$R = \exp(i\Delta\omega_{os}I_z t),$$

and we obtain

$$\frac{1}{\sqrt{2}}(I_y^{-1,0} + I_y^{0,1}) \xrightarrow{H_{os}, t} \frac{1}{\sqrt{2}}(I_y^{-1,0} + I_y^{0,1}) \cos(\Delta\omega_{os}t) - \frac{1}{\sqrt{2}}(I_x^{-1,0} + I_x^{0,1}) \sin(\Delta\omega_{os}t).$$

In geometrical terms, both 1Q coherences rotate about z-axis at the same frequency, $\Delta\omega_{os}$, so they are detected as a single line. Alternatively, in a solid, evolution after the pulse is dominated by the quadrupolar coupling. The transformed density operator is easily calculated by first rewriting H_q using single transition operators:

$$H_q = \omega_q (I_z^{-1,0} - I_z^{0,1}),$$

$$\frac{1}{\sqrt{2}}(I_y^{-1,0} + I_y^{0,1}) \xrightarrow{H_q, t} \frac{1}{\sqrt{2}}(I_y^{-1,0} + I_y^{0,1}) \cos(\omega_q t) + \frac{1}{\sqrt{2}}(I_x^{-1,0} + I_x^{0,1}) \sin(\omega_q t).$$

The two coherences counter rotate at the same frequency, ω_q , but with different sense and are observed in the spectrum as a doublet with lines at $\pm\omega_q$. The solid and solution results differ due to the quadratic dependence of H_q on the angular momentum operator, I_z .

MQ coherence preparation, evolution and detection

Recall that the goal is to detect the signal from transiently ordered water molecules and suppress the background signal from bulk water, which is, potentially in large excess. The non-zero quadrupolar coupling is the essential feature of the ordered water molecules that can be used for filtration of their signal from the bulk water signal. To implement this, consider the following two-pulse train in Figure 3.1 originally described by Ernst and Wokaun [116].

$$90_x - \tau - 90_x$$

Figure 3.1. "Pulse sequence" for the preparation of MQ coherence.

For simplicity, a small frequency offset is neglected during the short period, $\tau = \pi / 2\omega_Q$, and the transformations are:

$$I_z \xrightarrow{90_0} \frac{1}{\sqrt{2}}(I_y^{-1,0} + I_y^{0,1}) \xrightarrow{H_q, \tau = \pi/2\omega_q} \frac{1}{\sqrt{2}}(I_x^{-1,0} - I_x^{0,1}) \xrightarrow{90_0} -I_y^{-1,1}.$$

The net effect is that I_z is completely converted into double quantum (2Q) coherence. In practice, this sequence is a general method to prepare 2Q coherence, i.e. it is analogous to a 90° pulse for the preparation of 1Q coherence. Note, that if $\omega_q = 0$, no 2Q coherence is created.

Figure 3.2. Energy level scheme for nuclei with $I = 1$.

Another unique feature of MQ coherence is their dependence on the offset frequency. The observed offset for an n^{th} -order coherence is $n\Delta\omega_{os}$. Consider 2Q coherence example. The energy level scheme in Figure 3.2 shows two familiar cases. In isotropic fluids, the quadrupolar coupling is zero and the allowed transitions appear as a single line at the chemical shift frequency, while in a solid a doublet centered at $\Delta\omega_{os}$ with splitting $2\omega_q$ is observed. As anticipated, 2Q coherence evolves at twice the offset frequency $2\Delta\omega_{os}$ and is independent of the quadrupolar coupling. Note, no independence on ω_q drove the initial interest in the detection of MQ transitions, since the corresponding spectral lines have no quadrupolar broadening and thus increases resolution.

The signal induced in the NMR coil is proportional to $I_y = I_y^{-1,0} + I_y^{0,1}$ and $I_x = I_x^{-1,0} + I_x^{0,1}$, which are orthogonal to $I_k^{-1,1}$, ($k = x, y$). Thus, 2Q coherence cannot be directly detected. To circumvent this problem, an indirect dimension, the evolution dimension, is introduced during which the 2Q coherence evolves as a function of time. Then 2Q coherence is transferred to single quantum coherence that is then detected as a function of t_2 yielding a 2D array of free induction

decays (FID's), $S(t_1, t_2)$. It is important to understand, that coherence transfer in itself, does not provide the desired 2Q selectivity, and it is essential for the spins to undergo a coherence specific manipulation, such as the phase shift of the transmitter and/or the offset dependence, to give coherence specific filtration. In other words, the coherences (1Q coherence or transverse magnetization) are subjected to their characteristic response to shifts of the transmitter phase and/or offset frequency in such a way that 1Q coherence is either filtered out or, in a 2D experiment, separated in frequency from MQ coherences.

The evolution of 2Q coherence under H_q and H_{os} is given by

$$-I_y^{-1,1} \xrightarrow{H_q + H_{os}, t_1} -I_y^{-1,1} \cos(2\Delta\omega_{os}t_1).$$

Conversion of 2Q coherence to single quantum coherence is affected by a 90° pulse:

$$-I_y^{-1,1} \cos(2\Delta\omega_{os}t_1) \xrightarrow{90^\circ} -\frac{1}{4} \cos(2\Delta\omega_{os}t_1) (I_y^{-1,0} + I_y^{0,1}).$$

Double Fourier transformation of $S(t_1, t_2)$ yields a two-dimensional spectrum with an indirect frequency axis, F_1 , and a direct or detection axis, F_2 . The 2Q transition is observed as a signal at twice the offset frequency in the indirect dimension, F_1 , and only ordered water deuterons with non-zero quadrupolar coupling give signals at $F_1 = 2\Delta\omega_{os}$.

3.3. Pulse sequence and phase cycle development

Pulse sequence

The formalism described above is readily implemented by numerical methods for simulating any pulse sequence. We simulate pulse sequences and phase cycles using a scripted language, Octave [117]. As a starting point we have used the pulse sequence shown in Figure 3.3 [84-87, 93-96].

Figure 3.3. Simple pulse sequence for MQ NMR.

To summarize, note that after the 1st 90° pulse, transverse magnetization from the two 1Q coherences evolve under the quadrupolar coupling and fan out at the angular frequencies $\pm\omega_q$. The 180° pulse refocuses the offset interaction so that it has no net effect during the preparation period, and the accumulated phase difference of the 1Q coherences is $2\omega_q t_1$. With $\omega_q t_1 = 45^\circ$ ($t_1 = 1/(4\nu_q)$), the 1Q coherences are “antiparallel” and the state is pure 1Q anti-phase coherence, $I_x^{-1,0} - I_x^{0,1}$. The 2nd 90° pulse converts anti-phase coherence into 2Q coherence. The 2Q coherence (unobservable) precesses at twice the offset frequency during the evolution period. Since coherent evolution of spins is unitary, the final 90° pulse converts 2Q coherence back to observable anti-phase 1Q coherence, and a doublet with signals of opposite phase are detected. An obvious problem with this sequence is that if the quadrupolar coupling is comparable to the linewidth,

the two 1Q coherences that are observed have opposite phase and signal cancellation occurs.

To overcome this, we have implemented the usual density operator formalism to account for both coherent evolution and relaxation of the spin system, and designed the pulse sequence shown in Figure 3.4.

Figure 3.4. Advanced pulse sequence for the detection of MQ coherence.

2Q preparation

The preparation period is equivalent to that described above and the overall transformation in this period is

$$I_z \xrightarrow{90_{\alpha} - \frac{t_1}{2} - 180_{\alpha+90} - \frac{t_1}{2} - 90_{\alpha}} -\cos(\omega_q t_1) I_z + 2 \sin(\omega_q t_1) \left[I_y^{-1,1} \cos(2\alpha) - I_x^{-1,1} \sin(2\alpha) \right].$$

For example, advancing the transmitter phase α from 0° to 90° , leaves the polarization unchanged and inverts 2Q coherence. In general, if rotating the transmitter phase by α rotates the 1Q by α , then the 2Q coherence is rotated in the same sense by 2α .

Constant time evolution

Typically, 1Q signals are eliminated in 2Q-filtered experiments by phase cycling, i.e., 2Q filtration. However, in cases where the 1Q and 2Q signals overlap and the former is potentially much larger, the demands on filtration are very high, so it is useful to also separate signals by the order of their coherence,

i.e., their different dependence on the offset frequency. We use a constant time evolution period so that relaxation effects during the evolution period are independent of the evolution time, t_1 , and depend only on the length of the evolution period, T , which should be kept to a minimum for purposes of good signal to noise. Finally, given an efficient probe, ν_{os} can be made sufficiently large so that T is short compared to T_2^{2Q} (transverse relaxation time of 2Q signal). In this way, there is negligible signal loss due to relaxation during evolution.

Since I_z commutes with H_q and H_{os} , it is stationary and only 2Q coherence evolves during the evolution period. If $t_1 = 0$, it is readily shown that 2Q coherence is refocused. Thus, aside from a phase shift and relaxation, the density operator is unchanged in the evolution period for arbitrary τ .

Straightforward calculation shows that for non-zero t_1 , 2Q coherence rotates clockwise in the rotating frame at frequency $2\omega_{os}$ as follows:

$$\rho(\alpha, t_1, t_2) = \left\{ \begin{array}{l} I_z \cos(\omega_q t_1) - 2 \cos(2\alpha) \cos(4\beta) \sin(\omega_q t_1) \\ \quad \times [I_y^{1,1} \cos(2\omega_{os} t_2) + I_x^{1,1} \sin(2\omega_{os} t_2)], \alpha = 0^\circ, 90^\circ \dots \\ I_z \cos(\omega_q t_1) - 2 \cos(2(\alpha - 45^\circ)) \cos(4\beta) \sin(\omega_q t_1) \\ \quad \times [I_x^{1,1} \cos(2\omega_{os} t_2) - I_y^{1,1} \sin(2\omega_{os} t_2)], \alpha = 45^\circ, 135^\circ \dots \end{array} \right\},$$

i.e., the density operator contains three terms: I_z , $I_y^{1,1}$ and $I_x^{1,1}$.

Conversion

The transformations of quantum mechanics are unitary so the conversion of 2Q coherence to observable 1Q coherence is analogous to the preparation period. With $\gamma = 0^\circ$ and $t_1 = 1/4v_q$ the transformations are

$$\begin{aligned}
 I_z &\xrightarrow{90_0} I_y \xrightarrow{\frac{t_1}{2} - (180)_{90} - \frac{t_1}{2}} \sqrt{2} (I_x^{-1,0} - I_x^{0,1}), \\
 I_x^{-1,1} &\xrightarrow{90_0} \frac{1}{2} (I_x^{-1,1} - \frac{1}{6}Q) \xrightarrow{\frac{t_1}{2} - (180)_{90} - \frac{t_1}{2}} \frac{1}{2} (I_x^{-1,1} - \frac{1}{6}Q), \\
 I_y^{-1,1} &\xrightarrow{90_0} -\frac{1}{\sqrt{2}} (I_x^{-1,1} - I_x^{0,1}) \xrightarrow{\frac{t_1}{2} - (180)_{90} - \frac{t_1}{2}} I_y.
 \end{aligned}$$

Alignment and purge

The I_z term in the evolution period evolves to an observable signal, which is unwanted for 2Q filtration and eliminated by a suitable phase cycle. Whereas $I_y^{-1,1}$ is completely converted into an observable signal by pulse phases that are integral multiples of 90° , $I_x^{-1,1}$ is similarly converted only if the phase is an odd-integral multiple of 45° . Consequently, conversion of 2Q coherence selects either $I_y^{-1,1}$ or $I_x^{-1,1}$. In other words, the signal originating from 2Q coherence is amplitude rather than phase modulated in t_2 .

Observable 1Q coherences created in this way are in anti-phase and are aligned by the 180° pulse with delay $t_1 = 1/4v_q$. Unoriented samples, for example, have a distribution of couplings so the anti-phase coherences cannot,

in general, be “refocused”, i.e., realigned independent of v_q . For arbitrary t_1 and $\gamma = 0^\circ, 90^\circ, 180^\circ$ and 270° the result is

$$I_y^{1,1} \xrightarrow{90_\gamma - \frac{t_1}{2} - 180_{\gamma+90} - \frac{t_1}{2}} \left\{ \begin{array}{l} \pm \frac{1}{2} (I_x^{0,1} - I_x^{-1,0}) \cos \omega_q t_1 \pm I_y \sin \omega_q t_1, \quad \text{for } \gamma = 0^\circ \text{ or } 180^\circ \\ \pm \frac{1}{2} (I_y^{-1,0} - I_y^{0,1}) \cos \omega_q t_1 \pm I_x \sin \omega_q t_1, \quad \text{for } \gamma = 90^\circ \text{ or } 270^\circ \end{array} \right\}.$$

From the above, it is apparent that phases of undesired anti-phase and desired in-phase coherence shift in the same way with the transmitter phase. Therefore a phase cycle cannot be used to cancel the anti-phase components while preserving the in-phase components. An alternative, used here, is a purge: two 90° pulses separated by a delay, τ' , such that $T_2^{2Q} \ll \tau' \ll T_1$ (T_1 is longitudinal relaxation time). The initial pulse converts the anti-phase component into rapidly decaying 2Q coherence and stores the in-phase component as long lasting I_z . Following the delay, I_z is reconverted to the in-phase signal. Effectively, the purge period removes the anti-phase component from the signal.

The consequences of eliminating the alignment of 1Q coherences and not purging are shown in Figure 3.5, for two cases, when the quadrupolar doublet is larger than or comparable to the 1Q linewidth. The latter is of particular importance, for example, when a solvent interacts with a surface and is only weakly ordered. In this case, there is severe signal loss from cancellation of the two coherences of the opposite phase. Superficially, the absorption and dispersion signals appear interchanged and the latter is typically reported with the characteristic feature that the wings of the signal drop below the baseline. With refocusing, there is no signal cancellation and the absorption mode signal is

observed with greater sensitivity. With the purge, peak intensities are independent of the linewidth and proportional to $\sin^2 \omega_q t_1$.

A recent study of elastin [112-113] by 2Q NMR reports dispersion signal which hampers quantitative analysis. The pulse sequence designed here successfully circumvents these problems due to addition of alignment and purging and allows observation of an absorption mode signals.

Figure 3.5. Simulated 2Q spectra as a function of the preparation/conversion time, t_1 (in boxes as a fraction of $1/4\nu_q$). Spectra on the left have resolved quadrupolar splittings and on the right the splitting, while non-zero, is obscured by the linewidth. In the top row, the complete sequence described herein is used and signal intensities are $\propto \sin^2 \omega_q t_1$. In the middle row, purge is eliminated and the signal intensity in the weakly coupled case depends also on the linewidth. In the bottom row, alignment and purge are eliminated. Here, the 1Q coherences are observed in anti-phase with nearly complete cancellation of the absorption mode signal in the weak coupling case. The more easily recognized dispersion signal is shown.

Phase cycle

It is important to note, that the pulse sequence alone does not provide the necessary elimination of the 1Q signal, and the role of the phase cycle should not be underestimated. The phase cycle is responsible for the elimination of unwanted coherences and consequently determines what is detected during t_3 .

The signal arising from the I_z component of the density operator at the end of the preparation period is removed by cycling the preparation phase. For example, shifting α by 90° inverts the 2Q coherence and leaves the signal originating from I_z unchanged, so subtracting the resulting signals removes the latter. The signal arising from the spins with zero quadrupolar coupling is also eliminated by the phase cycle.

Table 3.2 summarizes a 16-step phase cycle that, in principle, suffices to accomplish the goal of complete 1Q cancellation. In practice, to cancel also artifacts originating from mis-set pulse widths and hardware, we use a 64-step phase cycle. The phase of the refocusing pulse in the evolution period is cycled 45° , 135° , 225° and 315° , for each of the 16 steps in Table 3.2. Overall, the sequence has accumulated 64 steps. Consequently, the number of transients in the experiment should be a multiple of 64 in order to cycle through all the phases equally.

Table 3.2. Phase cycle to cancel 1Q signal.

step	α	β	γ	start	prep, 2Q	evol, 2Q*	conv, 2Q	prep, 1Q	evol, 1Q	conv, 1Q	rcvr
1	0	45	0	I_z	$+aI_y^{-1,1}$	$+a'I_y^{-1,1}$	$-a''I_y$	$-bI_z$	$+bI_z$	$-2bI_y$	0
2	0	45	90	"	$+aI_y^{-1,1}$	$+a'I_y^{-1,1}$	$-a''I_y$	$-bI_z$	$+bI_z$	$+2bI_x$	90
3	0	45	180	"	$+aI_y^{-1,1}$	$+a'I_y^{-1,1}$	$+a''I_y$	$-bI_z$	$+bI_z$	$+2bI_y$	180
4	0	45	270	"	$+aI_y^{-1,1}$	$+a'I_y^{-1,1}$	$+a''I_y$	$-bI_z$	$+bI_z$	$-2bI_x$	270
5	90	45	0	"	$-aI_y^{-1,1}$	$-a'I_y^{-1,1}$	$+a''I_y$	$-bI_z$	$+bI_z$	$-2bI_y$	180
6	90	45	90	"	$-aI_y^{-1,1}$	$-a'I_y^{-1,1}$	$+a''I_y$	$-bI_z$	$+bI_z$	$+2bI_x$	270
7	90	45	180	"	$-aI_y^{-1,1}$	$-a'I_y^{-1,1}$	$-a''I_y$	$-bI_z$	$+bI_z$	$+2bI_y$	0
8	90	45	270	"	$-aI_y^{-1,1}$	$-a'I_y^{-1,1}$	$-a''I_y$	$-bI_z$	$+bI_z$	$-2bI_x$	90
9	180	45	0	"	$+aI_y^{-1,1}$	$+a'I_y^{-1,1}$	$-a''I_y$	$-bI_z$	$+bI_z$	$-2bI_y$	0
10	180	45	90	"	$+aI_y^{-1,1}$	$+a'I_y^{-1,1}$	$-a''I_y$	$-bI_z$	$+bI_z$	$+2bI_x$	90
11	180	45	180	"	$+aI_y^{-1,1}$	$+a'I_y^{-1,1}$	$+a''I_y$	$-bI_z$	$+bI_z$	$+2bI_y$	180
12	180	45	270	"	$+aI_y^{-1,1}$	$+a'I_y^{-1,1}$	$+a''I_y$	$-bI_z$	$+bI_z$	$-2bI_x$	270
13	270	45	0	"	$-aI_y^{-1,1}$	$-a'I_y^{-1,1}$	$+a''I_y$	$-bI_z$	$+bI_z$	$-2bI_y$	180
14	270	45	90	"	$-aI_y^{-1,1}$	$-a'I_y^{-1,1}$	$+a''I_y$	$-bI_z$	$+bI_z$	$+2bI_x$	270
15	270	45	180	"	$-aI_y^{-1,1}$	$-a'I_y^{-1,1}$	$-a''I_y$	$-bI_z$	$+bI_z$	$+2bI_y$	0
16	270	45	270	"	$-aI_y^{-1,1}$	$-a'I_y^{-1,1}$	$-a''I_y$	$-bI_z$	$+bI_z$	$-2bI_x$	90
*Sum							$-16a''I_y$			0	

$$a = 2\sin(\omega_q t_1), a' = a\cos(2\omega_{os} t_2), a'' = a'\sin^2(\omega_q t_1), b = \cos(\omega_q t_1)$$

*The term containing $I_x^{-1,1}$ is neglected since it is not converted into an observable.

+ Signals are rotated according to the receiver phase before summing.

3.4. Types of experiments

Depending on the experimental goal, it is possible to construct different types of experiments by slight variations of the pulse sequence on Figure 3.4.

Preparation curve

To determine the quadrupolar coupling, the evolution time τ is fixed and t_1 values for the preparation/conversion times are incremented. Fourier transform of $S(t_1, t_3)$ with respect to t_3 yields Figure 3.6, obtained from an elastin sample.

Figure 3.6. Stack plot of preparation spectra, obtained from elastin sample. Only 100 of spectra are shown (490 were taken). The peak in each spectrum corresponds to the point of the preparation curve.

The ridge plotted as a function of t_1 is hereafter referred to as a preparation curve. In practice, small oscillations of the ridge plot at the offset frequency are observed. This results from small errors in 90° pulse widths and transmitter phases. These have been removed by the simple expedient of using an offset frequency that is large compared to the rate at which 2Q coherence

evolves, i.e. $\nu_{OS} \gg \nu_q$. After Fourier transformation of the preparation curve, offset signals are removed by zeroing the wings of the spectrum. Inverse Fourier transformation returns the undistorted preparation curve.

Evolution planes

The constant time evolution period allows separation of coherences by order because of their different dependence on the offset frequency: 1Q coherence precesses at the offset frequency, and 2Q coherence precesses at double the offset frequency. When the preparation time is kept constant and the length of the evolution period varies, we obtain a 2D spectrum composed of the evolution planes (Figure 3.7).

To perform this experiment efficiently, a minimal number, 2^n , of t_2 increments can be recorded without causing truncation artifacts if the acquisition time, $2^n d_2$ (d_2 is the dwell time (d) in the indirect dimension) is an integral multiple of the offset period, $1/\nu_{OS}$. In practice, we collect data for two offset periods with 8 points, $d_2 = 1/4\nu_{OS}$, and apply a complex Fourier transform in t_2 . This gives 8 F_1 slices at integral multiples of the offset frequency, i.e. $F_1 = -3\nu_{OS} \dots 2\nu_{OS} \dots 4\nu_{OS}$ and the amplitude modulated 2Q signal appears at $F_1 = \pm 2\nu_{OS}$.

Figure 3.7. Example of evolution plane, obtained from elastin sample using 16-step phase cycle (a) and 64-step phase cycle (b).

In the 2D evolution spectra, Figure 3.7, the direct dimension is F_3 (detection) and the 2Q frequencies appear in the indirect dimension, F_2 . Figure 3.7 (a) shows incomplete 1Q cancellation with a 16-step phase cycle. This 1Q “signal” would contribute to the signal, for example, in the preparation experiment, spoiling the result. Efficient 1Q silencing (Figure 3.7 (b)) is, however, verified for the 64-step phase cycle discussed above.

3.5. Analysis of experimental data – preparation curve fitting

During the preparation (or conversion sequence) of length t_1 , single quantum coherence evolves from (or to) I_z into (or from) 2Q coherence with transfer efficiency proportional to $\sin \omega_q t_1$. Thus, the overall efficiency of the sequence for the detection of 2Q coherence is $\sin^2 \omega_q t_1$. In addition to coherent evolution, there is relaxation that, in accord with Redfield theory, results in exponential decay, $e^{-t_1/T_2^{\text{surf}}}$, where T_2^{surf} is the decay time for the 1Q coherences

that contribute to the 2Q signal. Consequently, the overall transfer efficiency of the sequence for the fixed evolution time is

$$S_{2Q}(t_1) = A_{2Q} \exp(-2t_1/T_2^{\text{surf}}) \sin^2(\omega_q t_1),$$

where A_{2Q} is a constant proportional to the number of spins with $\omega_q \neq 0$.

For the randomly oriented samples, like elastin fibers, the presence of a non-zero quadrupolar coupling necessarily implies a distribution of quadrupolar frequencies, ω_q , since there is a random orientation of the coupling tensor with respect to the magnetic field. The expression for $S_{2Q}(t_1)$ can be generalized for this by explicitly including the orientation dependence in the quadrupolar frequency, $\omega_q(\theta)$, and integrating over the orientations, θ ,

$$S_{2Q}(t_1) = A_{2Q} \exp(-2t_1/T_2^{\text{surf}}) \int \sin^2(\omega_q(\theta) t_1) d\theta,$$

or by integrating over quadrupolar frequencies with an appropriate weighting factor, $P(\omega_q)$, that accounts for a randomly oriented sample

$$S_{2Q}(t_1) = A_{2Q} \exp(-2t_1/T_2^{\text{surf}}) \int \sin^2(\omega_q t_1) P(\omega_q) d\omega_q \quad \text{eq. 1}$$

To implement either, we assume that the residual coupling is axially symmetric (Figure 3.8), so $\omega_q(0) = \omega_q(3\cos^2\theta - 1)/2$. With the substitution, $x = \cos\theta$, the first case is then

$$S_{2Q}(t_1) = A_{2Q} \exp(-2t_1/T_2^{\text{surf}}) \int_0^1 \sin^2\left(\frac{1}{2}\omega_q(3x^2 - 1)t_1\right) dx \quad \text{eq. 2}$$

The function $P(\omega_q)$ has the shape of the usual powder pattern for a single transition, shown in Figure 3.8 and calculated numerically for two different linewidths.

Figure 3.8. Calculated $P(\omega_q)$ for an axially symmetric residual quadrupolar coupling, $\omega_q = 200$ Hz and linewidths of 1 Hz and 50 Hz ($T_2^{\text{surf}} = 1/\pi\Delta\nu = 318$ ms and 6.4 ms, respectively).

S_{2Q} in the case of a single coupling is shown in Figure 3.9 (top), both in the absence of relaxation and with $T_2^{\text{surf}} \approx 6$ ms (linewidth = 50 Hz). Simulations of this type have been used before, but agreement with experimental results is only approximate for short times ($t_1 < 5$ ms) and, for longer times fails since the oscillations are not observed.

Simulations based on numerical integrations of eq. 1 and eq. 2 are shown in Figure 3.9 (bottom). In the absence of relaxation (black solid curve, calculations based on eq. 1 and circles for calculations based on eq. 2), the transfer efficiency builds up at a rate that increases with ω_q and then oscillates. However, unlike the single frequency case (top, no relaxation, black line), a non-zero limit of $1/2$ is approached. Calculations based on eq. 1 (red solid curve) and eq. 2 with 1 Hz broadening in $P(\omega_q)$ (red circles) are essentially equivalent. With the addition of relaxation, $T_2^{\text{surf}} = 6.36$ ms, the transfer efficiency decays rapidly as expected. Matching the linewidth of $P(\omega_q)$ to the decay rate shows a smooth, monotonic decay (solid purple line).

Figure 3.9. Illustration of the effect of relaxation and distribution of quadrupolar couplings on preparation curves. Top, no distribution of couplings: black curve, no relaxation, blue curve, $T_2^{\text{surf}} = 6.36$ ms. Bottom, distribution of quadrupolar couplings, with ν_q 200 Hz: black curve, no relaxation and eq. 1, circles, no relaxation and eq. 2; with relaxation $T_2^{\text{surf}} = 6.36$ ms, and linewidth $\Delta\nu = 1$ Hz, eq. 1 – red solid curve, eq. 2 – red circles; purple solid curve – assumption of axially symmetric coupling distribution and linewidth $\Delta\nu = 50$ Hz) on the simulated preparation curve.

Figure 3.10. An experimental 2Q preparation curve obtained from native, hydrated elastin fibers. Each data point (red circle) is the 2Q filtered signal intensity at the indicated preparation/conversion time, t_1 . The blue solid curve is a least squares fit of the data to the fitting function S_{2Q} , eq. 2.

An example of an experimental preparation curve obtained from the native, hydrated elastin fibers, is shown in Figure 3.10. Fitting the preparation curve with eq. 2 requires three parameters: the 2Q signal amplitude, A_{2Q} , the residual quadrupolar frequency, $\omega_q = 2\pi\nu_q$, and T_2^{surf} , the 1Q transverse relaxation time. It is observed that the fit is not unique unless T_2^{surf} is determined independently.

3.6. Conclusions

Based on numerical simulations, a new pulse sequence and phase cycle were designed. Experimental tests of the 2Q filter show excellent 1Q silencing. This required a 64-step phase cycle that overcomes limitations such as imprecise transmitter phase shifts or pulse widths. Other improvements are also implemented. The constant time evolution removes relaxation effects during this period and allows clean separation of coherences by order. This was used to confirm 2Q filter efficiency. Alignment and purge increase S/N due to the detection of absorptive signal rather than dispersive signals resulting in significant enhancement of S/N and reduction of acquisition time.

A new analysis of preparation experiments was developed that accounts for the distribution of quadrupolar couplings. Fitting the preparation curve allows quantitative determination of the size of the 2Q signal (the amount of ordered water), and the residual coupling (a measure of the degree to which the surface water is ordered).

CHAPTER 4

MECHANISM OF ELASTICITY IN ELASTIN

4.1. Introduction

The mechanism of elasticity in materials like rubber is based on the conformational entropy of the polymer [118-120]. It is also generally assumed that the driving force for reversible recoil in elastin is an increase in entropy associated with contraction. This is consistent with the fact that the fiber contracts if temperature is increased. However, dry elastin is brittle and inelastic and the presence of solvent is crucial for elasticity. In this heterogeneous system of protein and solvent, the entropy sources are the protein, the solvent or both. The canonical view is that elastin is like rubber, i.e. stretch reduces the protein's conformational entropy. The alternative view [121-124] is based on the hydrophobic effect and proposes that stretch increases water exposed hydrophobic surface area thereby increasing solvent order and lowering solvent entropy. This is the basis of the hydrophobic effect which is generally regarded as the dominant thermodynamic driving force in protein folding [125]. While somewhat counterintuitive, the latter view is consistent with both molecular dynamics simulations [124, 126] and calorimetric experiments on elastin [122]

that show that the large amount of heat released upon stretch is consistent with the known thermodynamic properties of the hydrophobic effect.

To evaluate the relative contributions of backbone and solvent ordering to elastin's recoil mechanism, we use a combination of ^{13}C and 2Q filtered ^2H SSNMR of stretched and unstretched elastic fibers. This approach depends on the unique capability of SSNMR to accurately determine the averaging effect of molecular reorientation on anisotropic nuclear spin interactions; specifically ^{13}C chemical shielding and ^2H nuclear quadrupolar coupling.

NMR can be used to evaluate molecular ordering by the degree to which molecular motions decrease the observable anisotropy, known as the inhomogeneous broadening, that results from anisotropic chemical shielding or quadrupolar coupling. In the first approximation, the ratio of the observed broadening, $\Delta\sigma^{\text{obs}}$, in the soft solid to the broadening observed in a rigid solid (or the soft solid under appropriate conditions such as low temperature), $\Delta\sigma^{\text{static}}$, is the order parameter, $S^{(2)}$. Values of $|S^{(2)}|$ vary from 0 (for isotropic reorientation) to 1 (for a rigid solid). The lower limit that order parameters can be determined in weakly ordered systems is the ratio of the homogeneous linewidth, i.e., the linewidth in the absence of anisotropic interactions to the magnitude of the static anisotropy. Magnitudes of shielding and quadrupolar coupling anisotropies in rigid solids are summarized in Table 4.1.

Table 4.1. Chemical shielding and quadrupolar coupling anisotropies for different nuclei.

Spin	CS anisotropy (11.7 T)	Q anisotropy
^1H	10 kHz	0
^{13}C	15 kHz	0
^{15}N	8 kHz	0
^2H	1.5 kHz	200 kHz
^{17}O	10 kHz	6 MHz

Shielding anisotropy of ^{13}C and ^{15}N are useful for studying backbone (polymer) ordering and the large quadrupolar coupling anisotropies of ^2H and ^{17}O are useful for detecting very weakly ordered water molecules. In this dissertation, the results using ^{13}C and ^2H spectroscopy are presented. Applications of ^{15}N require isotopically labeled material and ^{17}O NMR introduce additional technical complications. Thus, ^{15}N and ^{17}O experiments are indicated as future directions. To evaluate the hydrophobic effect based mechanism of elastin's recoil, the signal from water that does not interact with the protein (water that reorients isotropically and has no residual quadrupolar coupling) must be separated from the signal from water at the protein surface (water that is weakly and transiently ordered). To do this, we use the special properties of multiple quantum ^2H NMR, chapter 3.

To explore the mechanism of elasticity by NMR, we also consider quantitative aspects of how the molecular properties observed in the NMR experiments are related to elastin's mechanical function. To make this

connection, we use thermodynamics and establish the relation between NMR observables and thermodynamic entropy. Connecting the NMR experiment to Boltzmann entropies, S_B [127], has been discussed by several groups [128-131] and we adapt that procedure to our experiments in section 4.2.

These studies not only allow us to relate spectroscopic NMR parameters to macroscopic, mechanical function, but are also fundamental in more general contexts. First, the hydrophobic effect is the dominant thermodynamic force for protein folding [132] and it remains poorly understood at the molecular level. Insofar as elastin is the most hydrophobic protein known and its hydrophobicity can be altered by mechanical manipulation, it is an excellent system for studying the hydrophobic effect at the molecular level. Transient interaction of solvent at the surface and diffusional exchange with surrounding solvent are inherent features of the hydrophobic effect [132] and a unique advantage of NMR compared to other experimental methods in this context is that time-dependent processes can be studied while the system is at equilibrium. Second, magnetic resonance imaging (MRI) based on MQ NMR is an emerging technique [97, 104, 110-112] and the underlying physical chemistry of how water interacts with a soft tissue and can be used to efficiently generate MQ coherence is fundamental to obtaining image contrast.

The experiments described below are unique since we investigate both solvent and protein in native material (fully hydrated, elastic protein).

4.2. Theory

To connect what is measured in the NMR experiments (residual anisotropies) with thermodynamic entropy, we adapt the approach used by the groups of Wand, Kay and Palmer [128-131] that is based on the generalized order parameter, S^2 , [129] determined from relaxation experiments. This line of reasoning is readily adapted to the task here where a somewhat different order parameter, $S^{(2)}$, is determined. $S^{(2)}$ and the rotational contribution to Boltzmann entropy, S_B , are related to the normalized orientational distribution function, $P^{eq}(\Omega)$, by the following expressions,

$$S^{(2)} = \frac{v_q}{v_q^{static}} = \int P_2(\cos\theta) P^{eq}(\theta) \sin\theta d\theta$$

and

$$S_B = -nR \int_0^\pi P^{eq}(\theta) \ln P^{eq}(\theta) \sin\theta d\theta.$$

In the above, v_q is the observed coupling, v_q^{static} is the static quadrupolar coupling (for 2H $v_q^{static} = 216$ kHz in ice [133]) and $P_2(\cos\theta) = \frac{1}{2}(3\cos^2\theta - 1)$ is the second Legendre polynomial. For simplicity, we have assumed solvent dynamics are axially symmetric and thus a function only of the polar angle θ . Expanding $P^{eq}(\Omega)$ in a complete set of orthogonal functions, $P_l(\cos\theta)$ [134],

$$P^{eq}(\theta) = \sum S^{(l)} P_l(\cos\theta) \text{ and } S^{(l)} = \int_0^\pi P_l(\cos\theta) P^{eq}(\theta) \sin\theta d\theta,$$

indicates the following problem: to determine entropy, all $S^{(l)}$ are required, but only $S^{(2)}$ is available from the NMR experiment. It is possible to overcome this problem by assuming a model distribution function that depends on a single parameter. Several groups [128-129, 134-136] have assumed the following model: The “bond vector” (here the unique axis of the quadrupolar coupling tensor) rotationally diffuses in a cone of semiangle θ_0 ,

$$P^{eq}(\theta) = \begin{cases} (1 - \cos\theta_0)^{-1}, & 0 \leq \theta \leq \theta_0 \\ 0, & \theta > \theta_0 \end{cases}.$$

This is the “diffusion in a cone” model [136] illustrated in (Figure 4.1) using a $^2\text{H}_2\text{O}$ molecule. Two limiting cases are a well ordered solid, $\theta_0 = 0$, and an isotropic fluid, $\theta_0 = \pi$. This model has been justified [129] by molecular dynamics simulations for $\theta_0 > 15^\circ$ and is expected to provide an upper limit for S_B [128]. With this distribution function, the order parameter and entropy are,

$$S^{(2)} = \frac{\cos\theta_0}{2}(1 + \cos\theta_0) \text{ and } S_B = nR \ln(1 - \cos\theta_0),$$

where n is the number of moles of the solvent and R is the gas constant. The assumption is that it is equally probable for the “bond vector” to explore any orientation within the shaded area (Figure 4.1). For the case of interest here, the solvent is weakly ordered, i.e., θ_0 approaches π . Therefore it is convenient to define the small angle $\theta_1 = \pi - \theta_0$. The “bond vector” in this case can occupy any orientation on the sphere except within the cone of semiangle θ_1 .

Figure 4.1. The diffusion in a cone model for a water molecule. The D-O bond explores orientations within the shaded area. The narrow cone of semiangle θ_0 is for well ordered water and the sphere excluding the cone of semiangle $\theta_1 = \theta_0 - \pi$ is for weakly ordered water.

With Taylor series expansion in θ_1 , the following expressions are obtained for the order parameter and entropy:

$$S^{(2)} = -\frac{1}{4}\theta_1^2 \text{ and } S_B = nR \ln 2 - \frac{1}{4}nR\theta_1^2,$$

where the first term of S_B describes an isotropic water with order parameter $S^{(2)} = 0$. Combining the above and taking into account the cancellation of the $R \ln 2$ factor (since the total amount of water in the sample does not change with stretch) gives a simple expression for estimating the entropy change from the NMR data:

$$\Delta S_B = R \left(n_{l2} S_{l2}^{(2)} - n_{l1} S_{l1}^{(2)} \right).$$

4.3. Materials and methods

Elastin fibers from bovine neck ligament, prepared by the neutral extraction method [41], were obtained from Elastin Products Co., Owensville, Missouri.

Static ^{13}C NMR spectra of relaxed and $\sim 50\%$ stretched elastin fiber were used to evaluate polymer residual shielding anisotropies. Spin-lattice relaxation (longitudinal relaxation) times, T_1 , of different carbon types (backbone, C' and C_α , and side-chain methyl groups, CH_3) were also measured using the standard inversion recovery experiment with sequence $180^\circ - \tau - 90^\circ - \text{FID}$. In this experiment, equilibrium longitudinal magnetization is first inverted (from $+z$ to $-z$) by a 180° pulse and magnetization evolves back due to spin-lattice relaxation during the recovery time, τ . Subsequently, a 90° pulse converts the longitudinal magnetization into detected transverse magnetization and the experiment is repeated with different τ values. The exponential rise of peak intensity, S , as a function of recovery time was least squares fit to the function $S = S_0[1 - (1+a)\exp(-\tau/T_1)]$ to determine T_1 .

The fraction of water in the elastin sample that is ordered and the degree to which that water is ordered are determined with the MQ preparation experiment described in chapter 3 (Figure 3.4) in combination with Hahn echo experiments. Regarding the former, a constant time, $t_2 = 40\ \mu\text{s} \ll T_2^{2Q} \approx 10\ \text{ms}$, was used to minimize attenuation of 2Q signal through relaxation during the 2Q evolution period. Absorption mode spectra were obtained with a purge time, $\tau' = 15\ \text{ms}$, which was found to be sufficient to eliminate antiphase

coherence. The 2D preparation experiments were analyzed using the following function introduced in chapter 3,

$$S_{2Q}(t_1) = A_{2Q} \exp(-2t_1/T_2^{\text{surf}}) \int_0^\pi \sin^2\left(\frac{1}{2}\omega_q(3\cos^2\theta - 1)t_1\right) \sin\theta d\theta.$$

This function has three parameters: A_{2Q} , T_2^{surf} and ω_q that are fit to experiment.

A_{2Q} is proportional to the ordered water fraction in the sample, ω_q is the residual quadrupolar coupling of the ordered water and T_2^{surf} accounts for spin relaxation during the preparation and conversion periods of length t_1 . This experiment is shown schematically in Figure 4.2, (b) and compared with a simple 1Q experiment, Figure 4.2, (a).

Figure 4.2. Parameters for the evaluation of the fraction of ordered water in the sample and the residual quadrupolar coupling from 1Q (a) and 2Q (b) experiments. The signal from all water in the sample, A_{1Q} , is the peak intensity of the 1Q signal evaluated from a Hahn echo ($\tau \ll T_2$) spectrum, (a). In the 2D preparation experiment, (b), peak intensities are plotted as a function of preparation/conversion time, t_1 .

The 1Q signal intensity, A_{1Q} , is proportional to the total amount of water in the sample and A_{2Q} is proportional to the number of ordered waters, i.e., spins with non-zero quadrupolar coupling. The proportionality constant is a property of the NMR instrument. Thus, it is the same in both cases so the ratio A_{2Q}/A_{1Q} is the fraction of waters in the sample that have non-zero quadrupolar coupling. While A_{1Q} is directly available from the 1Q spectrum (a Bloch decay or a Hahn echo), extraction of A_{2Q} from the 2Q experiment is more difficult. This is because the 1Q transverse relaxation rate is larger than the quadrupolar coupling, $1/T_2 \geq \nu_q$, i.e., the quadrupolar splitting is unresolved. Moreover, preparation/conversion times, t_1 , are also comparable to ν_q . This is why S_{2Q} , Figure 4.2, (b), depends on both relaxation and quadrupolar coupling. The upper dotted curve shows the diminution of signal from relaxation and the lower curves (experiment, red, and fit, blue) show the combined effects of relaxation and coherent evolution under the quadrupolar interaction. The parameters ω_q , A_{2Q} and T_2^{surf} are highly correlated and, even in cases when the data has high S/N, unique values of all three parameters are not determined by this data alone. Consequently, to obtain unique parameter values, the fit is constrained to the value of T_2^{surf} measured independently.

Three different T_2 relaxation times were measured, T_2^{surf} , T_2^{bulk} and, for comparison, the relaxation time of pure solvent, T_2^{pure} . T_2^{bulk} is the composite transverse relaxation time for all observed water in the elastin sample ("bulk"

designates all the solvent). This and T_2^{pure} were measured with a standard Hahn echo experiment, $90^\circ - \tau/2 - 180^\circ - \tau/2 - \text{FID}$, [137]. T_2^{surf} is the relaxation time of the solvent that interacts with the protein surface ("surf" designates solvent interacting with the protein surface). T_2^{surf} was measured with a 2Q filtered version of Hahn echo sequence, i.e., replacing the usual 90° pulse in the Hahn echo with the 2Q filtration sequence described in chapter 3. Values of T_2^{surf} measured in hydrated elastin at different hydration levels and with added polyethylene glycol (PEG, $\langle \text{MW} \rangle = 6$ kDa) are shown in Table 4.2. In most cases, the assumption of transverse relaxation characterized by a single exponential time-constant, T_2^{surf} , is verified. The exceptions are for pure aqueous solvent where the relaxation rate is more rapid at longer times, $t_1 > 10$ ms. Enhanced, non-exponential relaxation is a well-known consequence of diffusion or spin exchange and likely represents the fact that solvent is only transiently ordered at the protein surface [138]. That said, for times $t_1 < 10$ ms, the effects of relaxation on the MQ experiment are quantitatively consistent with single exponential relaxation, i.e., eq. 1, and our model operationally accounts for this.

Table 4.2. T_2^{surf} relaxation times as a function of PEG and the amount of solvent.

78

0%
PEG

5%
PEG

10%
PEG

Table 4.3. Dependence of ^2H 2Q signal size of elastin fiber on the amount of solvent in the sample at constant PEG (5 % PEG) and constant stretch (39 % stretch).

The data in Table 4.2 also shows that results are dependent on the % water in the sample, defined as

$$\% w_{\text{sol}} / w_{\text{pro}} = \frac{\text{weight of hydrated sample} - \text{weight of dry sample}}{\text{weight of dry fiber}} \times 100\%.$$

Table 4.3. The above results show, that in this two-phase system, maintaining the solvent/protein mass (weight) ratio constant is essential for valid comparison of the samples when another variable such as temperature or stretch is varied. Results presented in this dissertation were obtained at constant sample hydration level of $\sim 300 \% w_{\text{sol}}/w_{\text{pro}}$.

To systematically vary the thermodynamic activity of water while holding $w_{\text{sol}}/w_{\text{pro}}$ constant, we varied the amount of PEG in the solvent [139]. The choice of PEG with $\langle \text{MW} \rangle = 6 \text{ kDa}$ was dictated by having $\langle \text{MW} \rangle > 1 \text{ kDa}$ to prevent interactions of PEG with intrafibrillar water. It has been shown that a mixed PEG/H₂O solvent does not significantly alter elastin's microscopic structure [139]. Adding PEG decreases the amount of water available for hydrating elastin without decreasing the solvent volume.

Samples for stretch dependent experiments used single, weighed elastin fibers ($\sim 1 \text{ mm} \times 10 \text{ mm}$ and $\sim 5\text{-}10 \text{ mg}$) glued to plastic (G10) rods, equilibrated against solvent to a known mass and then sealed in a 3 mm NMR tube as shown in Figure 4.3. Fibers were stretched by moving the rods and securing their positions with either parafilm or epoxy glue.

Figure 4.3. Elastin samples at a different stretch.

Figure 4.4. Experimental apparatus (a) Probe with Dewar for temperature controlled experiments. (b) Double resonance $^1\text{H}/^2\text{H}$ or $^1\text{H}/^{13}\text{C}$ static sample NMR probe. (c) Hydrated elastin sample in a 3 mm NMR tube.

Spectra were obtained with a home built 11.7 T instrument and the $^1\text{H}/^2\text{H}$ probe, which can also be used as $^1\text{H}/^{13}\text{C}$ probe after soldering appropriate capacitors mounted on the probe bulkhead [140]. The probe has a ^2H 90° pulse width of $2\ \mu\text{s}$ (~ 150 watts) and a ^{13}C 90° pulse width of $3\ \mu\text{s}$ (~ 110 watts). The probe circuit is shown in Figure 4.5.

Figure 4.5. Double resonance circuit.

The sample coil, L_S , is tuned to both the ^1H and X (^{13}C or ^2H) frequencies. To assure optimum power transfer between the probe and the transmitter or receiver, the input impedances of the circuit (Figure 4.5) are matched to $50\ \Omega$. C_1 and C_2 are the matching and tuning capacitors on the low frequency channel, X, while C_3 , L_G and L_{MH} are the tuning capacitor, the ground inductance and the matching inductance on the high frequency channel, ^1H . The approximate tuning and matching conditions are given in Table 4.4.

Table 4.4. Tuning and matching conditions for the double resonance probe.

Channel	Tuning	Matching
X	$\omega_X = \frac{1}{\sqrt{(C_1 + C_2)L}}$	$\frac{1}{Q\omega_X^3 C_1^2 L} = Z_0$
^1H	$\omega_{^1\text{H}} = \frac{1}{\sqrt{C_3^{\text{II}}(L' + L_{MH})}}$	$\frac{Q'\omega_{^1\text{H}} L_{MH}^2}{L'} = Z_0$

Note: Q and Q' are the quality factors for X and ^1H channels respectively; $L = L_S + L_G$ and $L' = L_S L_G / (L_S + L_G)$; $Z_0 = 50\ \Omega$. Two approximations were used: (1) losses of L and L' ($R = \omega_X L / Q$ and $R' = \omega_{^1\text{H}} L' / Q'$ respectively) are small for high Q sample coil and (2) stray capacitance and inductance can be ignored.

To determine any possible contribution of amide deuterons to the observed 2Q “signals”, we used experiments (see results) in which amide-solvent exchange was either quenched (pH 3, 5°C) or made rapid (pH 7, 37°C). The arrangement for doing this is shown in Figure 4.6 and the buffers used were as follows: pH 7 and pH 3 100 mM Na phosphate in $^2\text{H}_2\text{O}$, and pH 3, 100 mM Na phosphate in H_2O .

Figure 4.6. Setup for the H-D exchange experiment: (a) heat block with elastin samples; (b) close up for the elastin sample exchanging with the solvent in the Eppendorf tube at 37°C.

4.4. Results: ^{13}C spectroscopy of the polymer

The hypothesis that recoil is driven by conformational entropy of the stretched polymer, as in rubbers, implies that the residual shielding anisotropy in ^{13}C NMR spectrum increases with stretch.

Residual shielding

To investigate this assumption, we obtained the natural abundance, static ^{13}C spectra of fully hydrated elastin in relaxed and stretched states (Figure 4.7). These spectra are a refinement of published result [63]. Spectral resolution,

given that the spectra are obtained without magic angle spinning, is remarkable. This is because residual shielding anisotropies are small and approach that of isotropic fluids. Although the static shielding anisotropy, $\Delta\sigma^{\text{static}}$, of a typical peptide carbonyl is 150 ppm [63], the breadth of the carbonyl peak, $\Delta\sigma^{\text{obs}}$, in hydrated elastin, as seen from the results in Figure 4.7, is no more than 10 ppm.

Figure 4.7. Static ^{13}C NMR spectra of hydrated elastin.

Comparison of the ^{13}C spectra for stretched and relaxed elastin shows no significant change in residual shielding anisotropies with stretch (the lineshapes are almost identical in both cases). Evaluation of stretch induced changes in

$\Delta\sigma^{\text{obs}}$ is subject to two limitations here. At the small residual anisotropies observed, the contributions from homogeneous broadening originating from relaxation and a range of isotropic chemical shifts are not negligible. Thus, while we cannot exclude the possibility of stretch induced ordering of the polymer chain, our results show no evidence for it. Obtaining accurate shielding anisotropies will require experimental refinements, like using model elastin peptides with simpler sequences and/or labeling of individual atomic sites. For example, one can label the nitrogen atoms with ^{15}N , which has a smaller range of isotropic chemical shifts, and use 2D heteronuclear correlation experiments to better resolve carbon and nitrogen signals.

T_1 Studies

Longitudinal relaxation times, T_1 , were also determined from inversion recovery experiments for stretched and relaxed elastin fiber (Figure 4.7). Each spectrum in Figure 4.7 is taken at certain recovery time τ (indicated on the time axis). Determined T_1 values are summarized in Table 4.5.

Table 4.5. Longitudinal relaxation time T_1 for stretched and relaxed elastin fiber.

Site	Stretched T_1 , sec.	Relaxed T_1 , sec.
C'	5.30±0.80	4.10±0.40
C $_{\alpha}$	1.29±.22	1.20±.14
CH $_3$	0.53±0.04	0.52±0.03

The new result from these studies is the observation of little or no significant change (within the given standard errors) in T_1 for backbone (C' and C $_{\alpha}$) and side-chain methyl groups.

4.5. Results: MQ NMR studies of the solvent

If solvent is involved in the entropy change that drives the reversible recoil of elastin, then it must interact with the protein and, consequently, is at least transiently ordered on the protein surface. To evaluate the possible contribution of solvent to the mechanism of recoil, the amount of ordered water in the sample (the 2Q signal intensity, A_{2Q}) and the strength of solvent ordering (the residual quadrupolar coupling, ν_q) were measured in response to stretch, hydration and temperature.

Source of the ^2H NMR signals

Before presenting these results, we confirm that the observed NMR signal originates from the solvent and not from exchanged labelled amides. This is important since equilibrating elastin in $^2\text{H}_2\text{O}$, exchange labels both the solvent and the protein at amide groups. 1D, 2Q filtered spectra shown in Figure 4.8 verify that the contribution of protein amides to the 2Q signal is negligible. To do this, we have taken advantage of the capability of acquiring 2Q filtered spectra in a time (~ 1 minute) that is short compared to the exchange time ($t_{1/2} \sim 20$ minutes) of protein amides at pH 3 and 5°C [141].

The three spectra in Figure 4.8 were obtained from a sample following: (a) exhaustive exchange against $^2\text{H}_2\text{O}$ with equilibration at pH 3, (b) back exchange for 6 minutes against $^1\text{H}_2\text{O}$ buffer at pH 3, 5°C and (c) re-exchange against $^2\text{H}_2\text{O}$.

Since spectrum (b) was obtained in a time insufficient to significantly exchange the amide deuterons, the 200-fold attenuation of the signal in spectrum (b) relative to spectrum (a) confirms that the 2Q signal in (a) arises exclusively from rapidly exchangeable solvent. Spectrum (c) confirms that solvent back exchange is rapid.

Figure 4.8. Ordered water signal after exchange against $^2\text{H}_2\text{O}$ (a), $^1\text{H}_2\text{O}$ (b) and $^2\text{H}_2\text{O}$ (c).

Therefore, we have confirmed that contribution of the protein amides to the 2Q signal is negligible and the use of 2Q filtered ^2H NMR (Figure 3.4) is legitimate for the detection of anisotropic solvent.

Effect of stretch and hydration on relaxation times

T_2^{pure} and T_2^{bulk} were measured with stepwise increases in the fiber length by as much as 50% and at three PEG levels, 0%, 5% and 10%.

For comparison with the elastin samples, T_2 for the deuterated solvent alone, T_2^{pure} , was measured at room temperature without and with the addition of PEG, Table 4.6. The effect of PEG on pure solvent is simply to increase solution viscosity and the expected decrease in T_2^{pure} is observed.

Table 4.6. T_2^{pure} relaxation time of pure solvent as a function of PEG content.

T_2^{pure} values (Table 4.6) yield rotational correlation times (see Appendix C), τ_c , of 4.6 ps for pure $^2\text{H}_2\text{O}$ and 5.3 ps in 10 % w/v PEG. These were obtained using standard expressions, Appendix B, for quadrupolar relaxation and the known ^2H quadrupolar coupling constant for liquid $^2\text{H}_2\text{O}$ at room temperature, $\nu_q = 245$ kHz [142]. These values are a small overestimation of the expected value, $\tau_c = 2.4$ ps [143], because samples were not degassed and dissolved paramagnetic oxygen causes efficient relaxation. Increasing the viscosity by addition of PEG leads to slower molecular reorientation and increased correlation times since the correlation time is proportional to the reciprocal of the diffusion constant and the Stokes-Einstein relation has a reciprocal dependence of the diffusion constant on viscosity. Overall, the correlation time is directly proportional to the viscosity.

Experimental values of T_2^{bulk} as a function of fiber stretch and PEG level are shown in Table 4.7. The data are well described by single exponential relaxation time with the indicated error limits.

Table 4.7. T_2^{bulk} relaxation time as a function of stretch and PEG.

Compared to pure solvent value, $T_2^{\text{pure}} \sim 200$ ms, $T_2^{\text{bulk}} \sim 10$ ms indicates a significant slowing of the effective water reorientational rate. Important trends in the data are (a) increasing stretch decreases T_2^{bulk} , (b) addition of PEG at fixed stretch leads to an increase in T_2^{bulk} (opposite to the effect in pure solvent) and (c) signal intensity at zero echo delay does not change with stretch or PEG concentration if the fiber masses (0 % PEG (11 mg), 5 % PEG (9 mg) and 10 % PEG (5 mg)) are taken into account. Observations (a) and (c) are discussed below in the context of T_2^{surf} data and (c) shows that the fraction of the total sample water that is observed in this NMR experiment does not vary significantly with either stretch or with the addition of PEG. We conclude that the observed bulk water signal likely encompasses all of the water in the sample.

The 2Q filtered data from which values of T_2^{surf} were determined as a function of stretch and PEG content are given in Table 4.8. The experiment was made at the optimal preparation/conversion time, separately determined in the 2D preparation experiment.

Table 4.8. T_2^{surf} relaxation time as a function of stretch and PEG.

In contrast to the unfiltered experiment (Table 4.7) in which all of the water in the sample is observed, the size of the 2Q signal increases substantially, ~ 10 -fold, with stretch (Table 4.8). Thus, a larger fraction of the total water in the sample passes through the 2Q filter when the elastin is stretched, i.e., the size of the ordered water “pool” increases with stretch. This, and the fact that T_2^{bulk} and T_2^{surf} values are somewhat different, shows that bulk and ordered water do not fully exchange on the time scale of this experiment. To put this conclusion in perspective, consider the distance water diffuses during the MQ experiment in relation to the known microstructural features of elastin fibrils. The root mean square diffusional distance, $\sqrt{2Dt} \approx 0.7 \mu\text{m}$ (using the self-diffusion constant for water, $D \approx 2.23 \times 10^{-9} \text{ m}^2/\text{s}$ [144] and a typical preparation time, $t = 10 \text{ ms}$) is not larger than a typical fibril diameter, $1\text{-}3 \mu\text{m}$ [145], or the interfibrillar spaces, $1\text{-}10 \mu\text{m}$ [145]. Consistent with the increase in the ordered water signal intensity with stretch, addition of PEG decreases sample elasticity and significantly decreases the 2Q filtered signal intensity. Thus, the amount of interfibrillar water contributing to the 2Q signal decreases with PEG and T_2^{bulk} increases as observed. An important observation is that the signal size in the 2Q filtered experiment is far more sensitive monitor of the changes in the sample with stretch and hydration (PEG) than are changes in either T_2^{bulk} or T_2^{surf} .

Variation of the ordered solvent fraction and residual coupling with stretch

The change in 2Q signal with stretch observed in the measurements of T_2^{surf} , are confirmed in the 2D preparation experiments, Table 4.9. In combination

with the relaxation results (above), two important parameters are also determined: the fraction of water that is ordered in the sample, A_{2Q}/A_{1Q} , and the residual quadrupolar coupling, ν_q . On the time scale of the 2D preparation experiment, ~ 10 ms, we reasonably assume that there is diffusional exchange between the two water pools. Consequently, fits of the 2Q signal intensities to the parameters A_{2Q}/A_{1Q} and ν_q are constrained to the range of T_2 values from T_2^{surf} to T_2^{bulk} . Parameter ranges determined in this way are significantly larger than parameter standard errors resulting from random errors such as finite S/N. Consequently, parameter ranges but not standard errors are listed. The A_{1Q} values used are signal intensities from the $\tau = 0$ spectrum of the data set used to determine T_2^{bulk} .

For 0 % w/v PEG, the fraction of water in the sample that is ordered increases with stretch from 6 % to 73 % and the residual coupling varies only by a small amount, 24-42 Hz. Since T_2^{surf} decreases with stretch, the effect of relaxation is to decrease the 2Q signal intensity. Thus, the observed ~ 10 -fold increase in 2Q signal can only be explained by an increase in the amount of ordered water. As mentioned earlier, increasing PEG stepwise from 0% to 10% w/v is known to reduce elasticity. Thus reduced elasticity also correlates with a decrease in the 2Q signal, T_2^{surf} and T_2^{bulk} . Slight increase in quadrupolar coupling with PEG indicates stronger interaction of the solvent with the protein.

Table 4.9. ^2H 2Q signal and parameters from preparation curve experiment as a function of stretch and PEG.

* with T_2^{surf} , $A_{2Q}/A_{1Q} > 1$ and neglected.

Effect of temperature on solvent ordering

Increasing order with temperature is a signature of hydrophobic forces. In all elastomers, the effect of stretch is force generation and it is generally agreed that elastin is an entropic spring, i.e., entropy decreases with stretch, $(\partial S/\partial l)_T < 0$. Consequently, the Maxwell relation, $-(\partial S/\partial l)_T = (\partial F/\partial T)_l$, predicts that increasing temperature increases the recoil force at fixed length, l . If, the hydrophobic effect is involved in this force, it follows that increased temperature must also increase water ordering. This expectation, contrary to what is expected from thermalization of the water ordering, is confirmed in Figure 4.9.

Figure 4.9. Water ordering as function of temperature.

At 20% and 40% stretch, the ordered water 2Q signal increases by approximately 200% and 50%, respectively, as the temperature is increased from 13°C to 40°C. Interestingly, the onset of the effect, from 30°C to 40°C, coincides with the transition temperature for tropoelastin coacervation, 37°C [146]. Whether or not this is coincidental is a subject of future investigations.

4.6. Discussion and conclusions

The results from ^{13}C NMR reported here show no observable increase in shielding anisotropy of elastin protein backbone carbons upon stretch, i.e. no evidence for ordering of the polymer is seen in this experiment. As noted, small changes in anisotropy are masked by finite linewidths and dispersion in isotropic shifts. Thus, while we cannot exclude the possibility of stretch-induced ordering of the polymer, our results show no support for a recoil mechanism based primarily on polymer entropy. This is consistent with the calorimetric results [122] of Gosline and MD calculations [124, 126] from Daggett, et al., both of which indicate that the hydrophobic effect is the predominant mechanism of recoil. A more recent report based on ^{13}C relaxation (T_1 and $T_{1\rho}$) studies [113], however, argues in favor of a polymer configurational entropy based mechanism. In our view, these authors have made four incorrect assumptions in interpreting their results. First, stretch-induced changes in relaxation times were not significantly larger than the indicated error limits. Second, the correlation times determined from the relaxation data made ad hoc assumptions about the size of dipolar couplings rather than using accepted spin coupling parameters (^{13}C and ^1H gyromagnetic ratios and C-H distances). Third, the stretch-induced decrease in polymer correlation times so obtained were interpreted as the reciprocal oscillator frequency of an undamped, harmonic oscillator. Undamped harmonic motions are far too high in frequency to cause spin relaxation and are a poor model for large amplitude reorientational motions about chemical bonds [147] which are better described as diffusive or jump-like [148]. Finally, interpreting a decrease in

correlation times, i.e., an increase in dynamical rates, as an increase in entropy is not necessarily valid since entropy is an equilibrium property and depends only on the equilibrium distribution of orientations and not on the rates at which different orientations or configurations are sampled. In contrast, the properties emphasized in this study, residual shielding anisotropy and quadrupolar coupling, are functions of the equilibrium distribution of orientations, $P^{\text{eq}}(\Omega)$, and independent of dynamical rates.

That elastomers can operate on the basis of interfacial forces was first suggested by Weis-Fogh and Andersen in 1970 [121]. Consider also that the dominant driving force of elastin's recoil is closely related to the dominant force of protein folding. More specifically, elastic recoil from stretched to relaxed state is similar to the folding of a protein from a fully extended polypeptide to the native, folded state; experiments that can now be performed using pulling experiments based on atomic force microscopy. These experiments show that only a modest portion (~ 20 kT) of the total work (~ 170 kT) done by a single polyubiquitin protein on folding from a fully extended polymer is due to the protein's configurational entropy [149]. From the heterogeneity of folding pathways, these authors [149] concluded the existence of an additional driving force of the protein folding. MD simulations indicated that the "remainder of the work, ~ 150 kT, must be mainly due to hydrophobic interactions". Insofar as, extension of elastin fibers by ~ 50 % is a much smaller conformational change than complete unfolding of a protein, we reasonably expect that elastin's configuration entropy change is only

a modest contribution to the recoil force. This finding supports our hypothesis that elastin's recoil has a significant contribution from hydrophobic forces.

In the context of interfacial forces between a polymer and solvent, we note that the experimental results reported here show large stretch induced changes compared to, for example, changes in shielding anisotropy or relaxation rates observed for the polymer. It is also instructive to compare the $^2\text{H}_2\text{O}$ swollen elastin experiments reported here to seminal ^2H NMR experiments of Samulski and coworkers on a rubber, polyisoprene, swollen with a hydrophobic, deuterated solvent (CDCl_3 , C_6D_6 , etc.) [150-152]. These experiments are the basis of stretched gel based NMR experiments for determining residual dipole couplings. In the rubber and stretched gel experiments, no residual coupling is observed in the absence of stretch, however, splitting appears and increases as the sample is stretched and the signal intensity remains constant. In elastin/ $^2\text{H}_2\text{O}$, relaxed material has a non-zero coupling, the coupling is approximately constant with stretch and the fraction of solvent ordered increases with stretch. These differences make a clear distinction between a hydrophobic solvent in a rubber matrix and a hydrophobic protein, elastin, in water.

The rubber and gel experiments were interpreted by Samulski and coworkers as uniaxial ordering of the polymer network along the stretch axis. The solvent (deuteriochloroform, for example) senses the local ordering and an exchange averaged coupling with fixed intensity is observed since the rates of solvent translational diffusion and exchange at the polymer surface are rapid on the NMR timescale. In elastin, we observe a residual coupling in unstretched

material from either single fibers or randomly oriented bundles of fibers. We conclude that the solvent ordering is the result of a transient interaction with the randomly oriented protein matrix. Since the fraction of water that has nonzero quadrupolar coupling is small compared to 1 in unstretched material, complete exchange averaging does not occur. With stretch, the fraction ordered increases but change in ordering is less significant. Insofar as the water is a probe also of local ordering of the protein matrix, this does not occur with elastin stretch.

Qualitatively, the results presented here show that water in elastin is ordered upon fiber stretch. An important quantitative question follows: Is the entropy change from this solvent ordering a significant part of the thermodynamic driving force to account for elastin's mechanical recoil? To answer this, we obtain an estimate of the contribution of the water to the free energy change using the simple theory described in section 4.2,

$$\Delta S_B = R \left(n_{l_2} S_{l_2}^{(2)} - n_{l_1} S_{l_1}^{(2)} \right),$$

and the experimental results presented above. In the above, l_1 and l_2 are relaxed and stretched fiber lengths and n is the number of moles of ordered water obtained from the NMR data in Table 4.9. For 10 mg elastin fiber hydrated with ~ 24 mg we have $n_{l_2} \approx .88$ mmole and $n_{l_1} \approx .08$ mmole. When the fiber is stretched from $l_1 = 1$ cm to $l_2 = 1.5$ cm, the ratio A_{2Q}/A_{1Q} increases from ~ .065 to ~ .730 and the coupling is essentially constant at ~ 40 Hz. Thus, with $\nu_q^{\text{static}} = 216$ kHz, we calculate $T\Delta S_B \approx 0.74$ mJ ($T = 300$ K) for the stretch. For comparison, this would be equivalent to the total work (free energy change) if the

fiber is reversibly stretched the same amount by applying the gravitational force from a 15 g mass. We anticipate that this comparison is reasonable and indicates that the entropic contribution from water ordering is a significant part of the driving force for recoil.

To conclude, we suggest a model elastin recoil that is consistent with the results presented above. Unstretched fibers are rich in convolutions that are formed as a result of a hydrophobic collapse favored by the very hydrophobic protein. This expels water and reduces solvent entropy. Stretching the fiber opens the convolutions, increases solvent exposed surface area and orders water (Figure 4.10).

Figure 4.10. Model for elastin's reversible recoil.

Increase in the amount of transiently immobilized water implies decrease of the solvent entropy with stretch. Favorable increase in the solvent entropy drives the recoil of the protein. This model is consistent with all spectroscopic results presented here, including the decrease of T_2^{surf} relaxation times of surface and bulk water T_2^{bulk} with stretch.

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

FOR FUTURE RESEARCH

Based on the NMR titrations of lamellar TOCL monitored by the pH dependence of ^{13}C , ^{31}P and ^1H chemical shifts, we conclude that there is no evidence for the existence of a titratable group with a pK_a in the range of 4 to 9. The estimation of the stabilization energy required for raising the pK_a value of a phosphodiester by approximately 5 units is also in line with this conclusion. In our view, the likely source of contradiction of results from our NMR studies and potentiometric titrations is a difference in phase. Unfortunately, the authors of the structural hypothesis [72] did not provide any details on the phase of CL in their experiments. Insofar as the lamellar phase is the most interesting for investigation of the biological role of CL, performing potentiometric titration on lamellar liquid crystalline phase CL is suggested.

To refine the tentative assignments of the two peaks in the glycerol region of the HSQC spectrum and distinguish G_1' and G_3 peaks explicitly we suggest to use the Heteronuclear Multiple Bond Correlation (HMBC) experiment. $^1\text{H}/^{13}\text{C}$

HMBC will allow correlation of carbon chemical shifts with hydroxyl proton (in case of G_1') and methine proton (for G_3).

The novel ^2H MQ methods devised in this work includes several considerable improvements compared to the generally used methods for surface ordering studies. Inclusion of alignment and purge significantly enhances S/N due to the detection of absorptive signal and therefore reduces the time required for the experiments. Spectra with excellent S/N can be obtained in experiments that last about one minute using small, single fiber samples. This allows us to perform experiments involving H/D exchange and single fiber samples which are ideal for evaluations of mechanical properties.

The approach for the design of the 2Q pulse sequence and phase cycles for MQ filtration that was successfully applied for ^2H can be adapted to any other quadrupolar nucleus. ^{17}O is particularly beneficial in aqueous solvent studies. Transition frequencies and basis operators for $I=5/2$ spins are provided in Appendix A. The large number of possible coherences and their interference make the development of the phase cycle for the selection of certain coherences the primary obstacle to overcome. New pulse sequences for ^{17}O studies will be required.

Comparisons between rubber and elastin for the amount of stretch induced changes in ordered solvent and the degree of ordering [152], clearly indicate important differences in their elastic mechanisms. The increase of the amount of ordered solvent with approximately constant quadrupolar coupling upon stretch are distinct from the results for rubber and indicate a significant role

of the solvent in the elastic mechanism of elastin. Moreover, the increase in solvent ordering with increased temperature, demonstrates the importance of the hydrophobic effect in elastin recoil. From this perspective, it would be interesting, to look at the variations in the amount of ordered solvent and strength of recoil when the hydrophobicity of the polymer is systematically varied. Analogous to proteins, we anticipate that in elastin the thermodynamic propensity for folding/recoil will increase [153] if the protein's hydrophobicity is increased.

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APPENDIX A

¹⁷O MULTIPLE QUANTUM NMR

¹⁷O is potentially useful for characterization of weakly ordered water since it is not exchangeable with sites on the polymer, i.e. the signal originates only from the solvent. Moreover, the larger quadrupolar coupling implies more accurate determination of small order parameters, as in case of weakly ordered water. Implementation of MQ filtering approach to ¹⁷O requires more sophisticated than for ²H schemes for studying particular coherences. The pulse sequence and phase cycle devised in Chapter 3 for spins with $I=1$, require modification for $I=5/2$ (¹⁷O) because the number of transitions is larger and additional actions are required to eliminate multiple quantum interference effects.

A.1. Transition frequencies and operators

Transition frequencies

The transition frequencies at which different MQ coherences are evolving are calculated from the general formula

$$\omega^{mm'} = E^m - E^{m'}/\hbar,$$

for transition between states m and m' . Energy levels are

$$E^m = E_{os}^m + E_q^m,$$

where E_{os}^m and E_q^m are the diagonal matrix elements

$$E_{os}^m = \langle m | H_{os} | m \rangle ,$$

$$E_q^m = \langle m | H_q | m \rangle .$$

Hamiltonians H_{os} and H_q are given in chapter 3 (page 38). For $I = 5/2$, possible spin states are $m = 5/2, 3/2, 1/2, -1/2, -3/2, -5/2$ and the quadrupolar coupling constant is $\omega_q = 3eqQ^2/40\hbar$. To 1st order, only the part of H_q that commutes with the Zeeman Hamiltonian, H^0 , is required,

$$H_q = \omega_q (I_z^2 - 35/12) .$$

In total, there are 15 possible transitions between 6 states as follows: 5 single quantum transitions (1Q), 4 double quantum transitions (2Q), 3 triple quantum transitions (3Q), 2 quadruple quantum transitions (4Q) and one pentuple quantum transition (5Q). The 1Q frequencies are

$$\omega_{12} = E^{-5/2} - E^{-3/2} = \Delta\omega_{os} + 4\omega_q , \quad \omega_{23} = E^{-3/2} - E^{-1/2} = \Delta\omega_{os} + 2\omega_q ,$$

$$\omega_{56} = E^{3/2} - E^{5/2} = \Delta\omega_{os} - 4\omega_q , \quad \omega_{45} = E^{1/2} - E^{3/2} = \Delta\omega_{os} - 2\omega_q \text{ and}$$

$$\omega_{34} = E^{-5/2} - E^{-3/2} = \Delta\omega_{os} ,$$

with satellite splitting of 0, $4\omega_q$ and $8\omega_q$. 2Q frequencies are

$$\omega_{13} = E^{-5/2} - E^{-1/2} = 2\Delta\omega_{os} + 6\omega_q , \quad \omega_{24} = E^{-3/2} - E^{1/2} = 2\Delta\omega_{os} + 2\omega_q ,$$

$$\omega_{35} = E^{-1/2} - E^{3/2} = 2\Delta\omega_{os} - 6\omega_q \text{ and } \omega_{46} = E^{1/2} - E^{5/2} = 2\Delta\omega_{os} - 2\omega_q ,$$

with satellite splitting $4\omega_q$ and $12\omega_q$. Three 3Q frequencies with satellite splitting 0 and $12\omega_q$ are

$$\omega_{14} = E^{-5/2} - E^{1/2} = 3\Delta\omega_{os} + 6\omega_q, \quad \omega_{25} = E^{-3/2} - E^{3/2} = 3\Delta\omega_{os} \text{ and}$$

$$\omega_{36} = E^{-1/2} - E^{5/2} = 3\Delta\omega_{os} - 6\omega_q.$$

The two 4Q transitions are

$$\omega_{15} = E^{-5/2} - E^{3/2} = 4\Delta\omega_{os} + 4\omega_q \text{ and } \omega_{26} = E^{-3/2} - E^{5/2} = 4\Delta\omega_{os} - 4\omega_q,$$

and a single 5Q transition frequency is

$$\omega_{16} = E^{-5/2} - E^{5/2} = 5\Delta\omega_{os}.$$

From above, it is clear that only two MQ coherencies are independent of the quadrupolar coupling. These are the 5Q and the central 3Q transition, which evolve at $5\Delta\omega_{os}$ and $3\Delta\omega_{os}$, respectively. Detection of these transitions is favorable since there is no quadrupolar broadening due to their independence on ω_q .

Basis operators

For ^{17}O , $I = 5/2$, there are $2I+1 = 6$ spin states and the density operator has $6 \times 6 = 36$ elements. Therefore, to represent the density operator as a linear combination of basis operators, the basis set has 36 operators. We use, as in the $I = 1$ case, a set obtained from single transition operators, I_k^{rs} . In addition to the identity operator, these are the coherences, I_x^{rs} and I_y^{rs} (for the 15 rs values), and 5 operators $I_z^{r,r+1}$ that are orthogonalized by the Gram-Schmidt procedure as follows:

$$O_z^{12} = I_z^{12}, \quad O_z^{23} = I_z^{23} - \frac{\text{Tr}(O_z^{12} I_z^{23})}{\text{Tr}(O_z^{12} O_z^{12})} O_z^{12},$$

$$O_z^{34} = I_z^{34} - \frac{\text{Tr}(O_z^{23} I_z^{34})}{\text{Tr}(O_z^{23} O_z^{23})} O_z^{23} - \frac{\text{Tr}(O_z^{12} I_z^{34})}{\text{Tr}(O_z^{12} O_z^{12})} O_z^{12},$$

$$O_z^{45} = I_z^{45} - \frac{\text{Tr}(O_z^{34} I_z^{45})}{\text{Tr}(O_z^{34} O_z^{34})} O_z^{34} - \frac{\text{Tr}(O_z^{23} I_z^{45})}{\text{Tr}(O_z^{23} O_z^{23})} O_z^{23} - \frac{\text{Tr}(O_z^{12} I_z^{45})}{\text{Tr}(O_z^{12} O_z^{12})} O_z^{12},$$

$$O_z^{56} = I_z^{56} - \frac{\text{Tr}(O_z^{45} I_z^{56})}{\text{Tr}(O_z^{45} O_z^{45})} O_z^{45} - \frac{\text{Tr}(O_z^{34} I_z^{56})}{\text{Tr}(O_z^{34} O_z^{34})} O_z^{34} - \frac{\text{Tr}(O_z^{23} I_z^{56})}{\text{Tr}(O_z^{23} O_z^{23})} O_z^{23} - \frac{\text{Tr}(O_z^{12} I_z^{56})}{\text{Tr}(O_z^{12} O_z^{12})} O_z^{12}.$$

Since I_x^{rs} and I_y^{rs} are normalized to $1/2$, $O_z^{r,r+1}$ are normalized also to $1/2$. These operators are given in Table A.1.

Table A.1. Matrix representations of orthogonalized operators for $l = 5/2$.

$O_z^{12} = \begin{pmatrix} a & 0 & 0 & 0 & 0 & 0 \\ 0 & -a & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \end{pmatrix}, \quad a = f(1)$	$O_z^{23} = \begin{pmatrix} b & 0 & 0 & 0 & 0 & 0 \\ 0 & b & 0 & 0 & 0 & 0 \\ 0 & 0 & -2b & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \end{pmatrix}, \quad b = f(2)$
$O_z^{34} = \begin{pmatrix} c & 0 & 0 & 0 & 0 & 0 \\ 0 & c & 0 & 0 & 0 & 0 \\ 0 & 0 & c & 0 & 0 & 0 \\ 0 & 0 & 0 & -3c & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \end{pmatrix}, \quad c = f(3)$	$O_z^{45} = \begin{pmatrix} d & 0 & 0 & 0 & 0 & 0 \\ 0 & d & 0 & 0 & 0 & 0 \\ 0 & 0 & d & 0 & 0 & 0 \\ 0 & 0 & 0 & d & 0 & 0 \\ 0 & 0 & 0 & 0 & -4d & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \end{pmatrix}, \quad d = f(4)$
$O_z^{56} = \begin{pmatrix} e & 0 & 0 & 0 & 0 & 0 \\ 0 & e & 0 & 0 & 0 & 0 \\ 0 & 0 & e & 0 & 0 & 0 \\ 0 & 0 & 0 & e & 0 & 0 \\ 0 & 0 & 0 & 0 & e & 0 \\ 0 & 0 & 0 & 0 & 0 & -5e \end{pmatrix}, \quad e = f(5)$	$f(r) = [2r(r+1)]^{-1/2}$

A.2. Scripts

Octave density matrix scripts allow numerical simulation of the evolution of the spin system during times and under the interactions determined by the pulse sequence. In addition, phases of the pulses are set separately and allow developing of a phase cycle. In this way, each coherence can be plotted as a function of preparation time and these graphs are used to determine optimal preparation times (maximum signal) for a given quadrupolar coupling. The “#” character indicates comment lines.

The script “mq_O17” simulates pulse sequence and phase cycle. An illustration of the settings used in the script is shown in Figure A.1. Main script “mq_O17” requires additional scripts for creation of relevant operators (“ops6levels”), simulation of RF pulses (“evol_ThPh”) and evolution periods (“evol_Q_CS”). At any time current density operator can be decomposed into linear combination of basis operators using script “decomp”.

Figure A.1. Settings for the pulse sequence script, “mq_O17”.

mq_O17

```
clear
l=5/2
# initiate the sequence by obtaining the necessary spin operators and by
# setting values of the quadrupolar coupling, vq, and offset (vos)
# frequencies (in Hz)
# delays
# 2*time(1) = evolution time in preparation and conversion, time(2)=echo delay
# for refocusing 2Q
# evolution, time(3)=2Q evolution following refocusing.
# Total time between 1Q to 2Q preparation and 2Q to 1Q conversion periods is
# 2*time(2)+time(3)
time=[.125,.10,0]

# pulses widths (deg) for all pulses
# in the order that they appear in the sequence
theta=[90,180,90,180,90,180]

# pulse phases phase(jcycle,kpulse) with jcycle=cycle number
# and kpulse=pulse number
phase= [0,90,90,0,90,0;
        0,180,90,0,90,0;
        0,0,90,90,0,0;
        0,0,90,90,90,0;
        90,0,180,90,0,0;
        0,0,90,0,90,0;
        0,0,0,270,0,0;
        0,0,0,315,0,0;
        180,180,180,0,0,0;
        180,180,180,45,0,0;
        180,180,180,90,0,0;
        180,180,180,135,0,0;
        180,180,180,180,0,0;
        180,180,180,225,0,0;
        180,180,180,270,0,0;
        180,180,180,315,0,0]

# set vos and vq
vq=1
vos=0
# Initialize sequence by creating relevant operators
ops6levels;
for jcycle=1:1:2;
# set the initial density operator to Iz
rho=Iz;
```

```

# step 1-5 of sequence, MQ preparation
Th=theta(1);
Ph=phase(jcycle,1);
evol_ThPh;
'coherences after initial 90'
decomp
t_evol=time(1);
evol_Q_CS;
Th=theta(2);
Ph=phase(jcycle,2);
evol_ThPh;
t_evol=time(1);
evol_Q_CS;
'coherences prior to 2nd 90 in preparation'
decomp
Th=theta(3);
Ph=phase(jcycle,3);
evol_ThPh;
'coherences after 2Q preparation is complete'
decomp

# steps 6-8, DQ echo and evolution
t_evol=time(2);
evol_Q_CS;
Th=theta(4);
Ph=phase(jcycle,4);
evol_ThPh;
t_evol=time(2);
evol_Q_CS;
'mq refocusing complete'
decomp
# MQ evolution following refocusing
t_evol=time(3);
evol_Q_CS;
'evolution complete, before conversion pulse'
decomp

# step 9 detection
Th=theta(5);
Ph=phase(jcycle,5);
evol_ThPh;
'coherences after conversion pulse'
decomp
endfor
*****

```

The script “ops6levels” creates basis operators for $I = 5/2$.

ops6levels

```
# Prepares the 30 6x6 matrix representations of spin 5/2 single transition
#coherences, lx(r,s)=l(1,r,s,,:) and ly(2,r,s,,:) ; r=1->5, s=r+1->6,
# 5 orthogonal polarization operators,l(3,r,s,,:); r=1->5, s=r+1,
#lx, ly, lz.
#and the raising and lowering operators, l+=lp; l-=lm
dum=zeros(6);
for r=1:1:5;
    for s=r+1:1:6;
        l(1,r,s,,:)=dum(:,,:);
        l(1,r,s,r,s)=1/2;
        l(1,r,s,s,r)=1/2;
        l(2,r,s,,:)=dum(:,,:);
        l(2,r,s,r,s)=-i/2;
        l(2,r,s,s,r)=i/2;
    endfor;
endfor;
# check coherences
    for r=1:1:5
        for s=r+1:1:6
            r
            s
            x=dum;
            x(:,,:)=l(1,r,s,,:);
            y(:,,:)=l(2,r,s,,:);
        endfor;
    endfor;
    x
    y
# create the 5 orthogonalized single quantum polarizations,
# l(2,r,s,,:) r=1->5, s=r+1
for r=1:1:5
    s=r+1;
    l(3,r,s,,:)=zeros(6);
endfor;
l(3,1,2,1,1)=1/2;
l(3,1,2,2,2)=-1/2;
a=sqrt(3/4);
l(3,2,3,1,1)=a/3;
l(3,2,3,2,2)=a/3;
l(3,2,3,3,3)=-2*a/3;
a=sqrt(2/3);
l(3,3,4,1,1)=a/4;
```

```

l(3,3,4,2,2)=a/4;
l(3,3,4,3,3)=a/4;
l(3,3,4,4,4)=-3*a/4;
a=sqrt(5/8);
l(3,4,5,1,1)=a/5;
l(3,4,5,2,2)=a/5;
l(3,4,5,3,3)=a/5;
l(3,4,5,4,4)=a/5;
l(3,4,5,5,5)=-4*a/5;
a=sqrt(3/5);
l(3,5,6,1,1)=a/6;
l(3,5,6,2,2)=a/6;
l(3,5,6,3,3)=a/6;
l(3,5,6,4,4)=a/6;
l(3,5,6,5,5)=a/6;
l(3,5,6,6,6)=-5*a/6;
# check polarization operators
for r=1:1:5
    r
    s=r+1
    z=dum;
    z(:,:)=l(3,r,s,,:,:)
endfor
# check orthogonality
for j=1:1:5;
    k=j+1;
    for j2=j+1:1:5;
        k2=j2+1;
        j
        k
        j2
        k2
        z1(:,:)=l(3,j,k,,:);
        z2(:,:)=l(3,j2,k2,,:);
        prod=trace(z1*z2)
    endfor
endfor
# set nuclear spin quantum number j (ex. j=5/2)
# matrix for the z-component of angular momentum
# general case of spin j, Hilbert space has 2j+1 dimensions
lz
j=5/2;
lz=diag([j:-1:-j]);
# matrix for the x-component of angular momentum
lx
lx=zeros(2*j+1);

```

```

n=1;
while (n<2*j+1)
lx(n,n+1)=0.5*sqrt(n*(2*j+1-n));
lx(n+1,n)=0.5*sqrt(n*(2*j+1-n));
n++;
endwhile
lx
# matrix for the y-component of angular momentum
# lz and lx are defined, so we just use commutator [lz,lx]=ily to find ly
ly=(1/i)*(lz*lx-lx*lz);
ck=i*(ly*lx-lx*ly)
lp=lx+i*ly
*****

```

The script “evol_ThPh” calculates the effect of an RF pulse with flip angle Th and phase Ph on the current density operator, rho.

evol_ThPh

```

# evolution of the current density operator, rho, subject to a hard RF
# pulse with flip angle Th (deg) and phase Ph (deg)
phir=pi*Ph/180;
thetar=pi*Th/180;
op=(cos(phir)*lx)+(sin(phir)*ly);
prop=expm(-i*(thetar)*op);
r=prop'*rho*prop;
rho=r
*****

```

The script “evol_Q_CS” calculates the effect of evolution under quadrupolar coupling and chemical shift on the current density operator, rho.

evol_Q_CS

```

wq=2*pi*vq;
wos=2*pi*vos;
ld=((sp*(sp+1))/3)*eye(2*sp+1);
Hq=wq*((lz*lz)-ld);
Hcs=wos*lz;
dum=i*(Hcs+Hq)*t_evol;
prop=expm(-dum);
r=prop'*rho*prop;
rho=r
*****

```

The script “decomp” takes the current density operator, rho, and decomposes it into a linear combination of basis operators.

decomp

```
# decompose the density operator, rho, into single transition operators
# and store in the complex vector st(q). For elements q=1-->15, real(st) and
# imag(st) are lx(rs), ly(rs), respectively, in the order (r,s) = (1,2), (1,3),.....(1,6),
# (2,3)...(5,6). Orthogonalized lz(r,s) coefficients (real) are q=16-->20 in the order
# (r,s) = (1,2), (2,3),.....(5,6). Finally, coefficients for lx and ly are real(st(21) and
# imag(st(21)), i.e., st(21)=trace(l+*rho), and lz=s(22).
q=0;
for r=1:1:5;
    for s=r+1:1:6;
        q=q+1;
        dum=2*real(rho(r,s));
        st(q)=dum;
        dum=-2*imag(rho(r,s));
        st(q)=st(q)+i*dum;
    endfor;
endfor;
l=15;
for r=1:1:5;
    s=r+1;
    l=l+1;
    dum=0;
    for jk=1,6;
        dum=dum+(l(3,r,s,jk,jk)*rho(jk,jk));
    endfor;
    st(l)=dum;
endfor
st(21)=trace(lp*rho);
st(22)=trace(lz*rho);
v1q=[st(1),st(6),st(10),st(13),st(15)]
v2q=[st(2),st(7),st(11),st(14)]
v3q=[st(3),st(8),st(12)]
v4q=[st(4),st(9)]
v5q=[st(5)]
*****
```

APPENDIX B

^2H SCRIPT

Operators for $I = 1$ are given in chapter 3 (pages 41-42). All scripts in appendix A can be used for $I = 1$ (^2H) simulations by substituting “ops6levels” with the following script, “ops3levels”, that creates basis operators for $I = 1$.

ops3levels

```
# Prepares the 6 3x3 matrix representations of  $I = 1$  single transition
# coherences,  $l_x(r,s)=l(1,r,s,,:)$  and  $l_y(2,r,s,,:)$  ;  $r=1 \rightarrow 2$ ,  $s=r+1 \rightarrow 3$ ,
# 2 orthogonal polarization operators,  $l_z$ ,  $Q$ 
#  $l_x$ ,  $l_y$  and the raising operator,  $l+=l_p$ 
dum=zeros(3);
for r=1:1:2;
    for s=r+1:1:3;
        l(1,r,s,,:)=dum(:,,:);
        l(1,r,s,r,s)=1/2;
        l(1,r,s,s,r)=1/2;
        l(2,r,s,,:)=dum(:,,:);
        l(2,r,s,r,s)=-i/2;
        l(2,r,s,s,r)=i/2;
    endfor;
endfor;
# check coherences
for r=1:1:2
    for s=r+1:1:3
        r
        s
        x=dum;
        x(:,,:)=l(1,r,s,,:)
        y(:,,:)=l(2,r,s,,:)
    endfor;
endfor;
lz
```

```

j=1;
lz=diag([j:-1:-j]);
# Q=quadrupolar order=lz*lz-l(l+1)
Q=(lz*lz)-(2*eye(3));
# matrix for the x-component of angular momentum
# lx
lx=zeros(2*j+1);
n=1;
while (n<2*j+1)
    lx(n,n+1)=0.5*sqrt(n*(2*j+1-n));
    lx(n+1,n)=0.5*sqrt(n*(2*j+1-n));
    n++;
endwhile
# matrix for the y-component of angular momentum
# lz and lx are defined, so we just use commutator [lz,lx]=ily to find ly
ly=(1/i)*(lz*lx-lx*lz);
#ck=i*(ly*lx-lx*ly)
lp=lx+i*ly
*****

```


APPENDIX C

²H SPIN RELAXATION RATES

Deuterium, ²H, is a quadrupolar nucleus and the dominant mechanism of spin relaxation is due to reorientation of the nuclear electric quadrupole moment relative to the applied field. For spin 1 nuclei, the rates are [138]

$$R_1 = \frac{1}{T_1} = C \left(1 + \frac{\eta^2}{3} \right) (J(\omega_0) + 4J(2\omega_0))$$

and

$$R_2 = \frac{1}{T_2} = \frac{1}{6} C \left(1 + \frac{\eta^2}{3} \right) (9J(0) + 15J(\omega_0) + 6J(2\omega_0)),$$

with

$$C = \frac{3}{10} \pi^2 \left(\frac{e^2 q_{zz} Q}{h} \right)^2 = \frac{3}{10} \pi^2 v_q^2.$$

For diffusional motion in isotropic fluids,

$$J(\omega_0) = \frac{\tau_c}{1 + (\omega_0 \tau_c)^2}.$$

The function $J(\omega_0)$ is the spectral density (Fourier transform of the autocorrelation function) of the fluctuating field caused by diffusive reorientation. Relaxation is efficient when molecular motions, characterized by the correlation

time τ_c , have the same time scale as the reciprocal magnetic resonance frequency, $\tau_c \sim \omega_0$. In the above $\eta = (q_{yy} - q_{xx})/q_{zz}$ is the asymmetry parameter of the quadrupolar coupling with $|q_{xx}| \leq |q_{yy}| \leq |q_{zz}|$, ν_q is the quadrupolar coupling constant in Hz, e is the electron charge, Q is the nuclear quadrupolar moment, and eq_{zz} is the principal z-component of the electric field gradient tensor.

Graphical representations of these equations are shown in Figure C.1. Short correlation times correspond to fast motion whereas slow molecular motion, as in viscous liquids, implies long correlation times. When correlation times are short (known as the extreme narrowing limit), $\omega_0\tau_c \ll 1$, as T_1 and T_2 are equal. T_1 reaches a minimum at $\tau_c \approx 1/\omega_0$, and T_1 and T_2 diverge in the slow motion or long correlation time limit wherein T_1 increases and T_2 decreases.

Figure C.1. Dependence of T_1 (dashed line) and T_2 (solid line) relaxation times on the correlation time τ_c for intramolecular quadrupolar relaxation (logarithmical axes are used).

For ^2H , $\eta \leq 0.2$ [142] and the asymmetry can be neglected to a good approximation. In the extreme narrowing limit, $J(0) = J(\omega_0) = J(2\omega_0) = \tau_c$, and equations for the relaxation rates further reduce to

$$R_1 = R_2 = \frac{1}{T_1} = \frac{1}{T_2} = 5C\tau_c$$

from which the correlation time can be calculated if either relaxation time and the quadrupolar coupling are known. For convenience, a plot and simple regression curve are shown in Figure C.2. We have used the constant $C = 1.78 \times 10^{11} (\text{Hz}^2)$, based on the known ^2H quadrupolar coupling constant at room temperature (liquid $^2\text{H}_2\text{O}$), $\nu_q = 245 \text{ kHz}$ [142].

Figure C.2. Linear approximation (solid line) of the dependence of T_2 (red circles) on the correlation time τ_c in the range of relevant T_2 values.

APPENDIX D
LIST OF SYMBOLS AND ABBREVIATIONS

φ – basis function

ρ – density operator

σ – chemical shielding tensor with principal components σ_{11} , σ_{22} , σ_{33}

τ_c – rotational correlation time

ω_0 – Larmor precession frequency

ω_q – quadrupolar coupling frequency, $\omega_q = 2\pi\nu_q$

Ω – observable

ψ – wavefunction

1D and 2D spectra – one- and two-dimensional spectra

1Q, 2Q, 3Q, 4Q and 5Q – single, double, triple, quadruple and pentuple quantum

A_{2Q} – 2Q signal intensity

$CDCl_3$ – deuterated chloroform

CL, TOCL – cardiolipin, tetraoleoyl cardiolipin

CSA – Chemical Shift Anisotropy

D – self-diffusion constant

DMSO – dimethyl sulfoxide

F_1 , F_2 and F_3 – direct, indirect and detection dimensions

FID – Free Induction Decay

$J(\omega_0)$ – spectral density

H – interaction Hamiltonian

H_3PO_4 – phosphoric acid

H_{II} – inverted hexagonal phase

HSQC – Heteronuclear Single-Quantum Correlation spectroscopy

I – spin quantum number

L_α – lamellar, liquid crystalline phase

L_β – gel phase

L_G , L_{MH} and L_S – ground, matching and sample coil inductances

MAS – Magic Angle Spinning

MD – Molecular Dynamics

MQ – Multiple Quantum

MRI – Magnetic Resonance Imaging

NaHM – sodium hydrogen maleate

PA – phosphatidic acid

PC – phosphatidylcholine

PEG – polyethylene glycol

$P^{eq}(\Omega)$ – normalized orientational distribution function

RF pulse – radiofrequency pulse

pK_a – acid dissociation constant

s – span, $s = \sigma_{33} - \sigma_{11}$

$\mathcal{S}^2$ – generalized order parameter

$S^{(2)}$ – order parameter

S_B – Boltzmann entropy

S/N – signal to noise ratio

SSNMR – Solid-State Nuclear Magnetic Resonance spectroscopy

T_1 – longitudinal relaxation time

T_2^{2Q} – transverse relaxation time of the 2Q signal

T_2^{pure} – transverse relaxation time of the solvent alone, sample is “pure” of protein

T_2^{bulk} – transverse relaxation time of the bulk solvent, no 2Q filtration

T_2^{surf} – transverse relaxation time of the solvent ordered at the protein surface,

2Q filtered signal

Tr – trace

v_{rot} – spinning speed of the rotor

$w_{\text{sol}}/w_{\text{pro}}$ – solvent/protein mass (weight) ratio

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