Chapter 1 General introduction

1.1 Structural investigations of biological systems with NMR spectroscopy

A mechanistic understanding of biological function requires the determination of the three-dimensional molecular structures of biological macromolecules. Nuclear magnetic resonance (NMR) spectroscopy is a unique technique for measuring structural and dynamic properties of complex biomolecules under near-physiological conditions. High-resolution solution NMR has become a standard method for structure determination of soluble proteins and high-resolution spectra are a result of fast isotropic molecular tumbling. In many cases, however, the most natural condition to study molecular structure and dynamics of macromolecules is in a membrane-integrated form.

A leading technique for investigating biological systems is x-ray crystallography. However, well-ordered 3D single crystals are the major requirement for attaining high-resolution structures of biomolecules of any size, and growing a crystal that diffracts beyond 3 Å can take a long time. Membrane proteins, for example, can not be easily crystallized and as a result, to date only few high-resolution structures of membrane proteins have been solved. Studying the structure of membrane proteins remains a serious challenge, considering their importance. They play a crucial role in the cell, being involved in many vital cellular processes, acting as channels, pumps, receptors and enzymes. Membrane proteins are estimated to constitute a third of the genomes of mammalians and some of them, the G-protein coupled receptors, represent primary receptor targets for drug discovery[1]. Over the past two decades, solid-state NMR has emerged as an effective method for structural studies of quasi-immobilized biomolecules, such as membrane proteins and amyloid systems. In most

solids, fast molecular tumbling is absent and anisotropic interactions like chemical shift anisotropy (CSA), dipolar interactions and, for spins > 1/2, quadrupolar couplings, lead to resonance broadening. To overcome this problem, two general approaches for obtaining high-resolution solid-state NMR spectra have been developed. The first approach relies on oriented samples such as membranes layered on glass slides[2,3]. Orientational restraints are derived from dipolar couplings and chemical shift interactions, whose values depend on how the molecule is aligned relative to the external magnetic field. An example of a solid-state NMR experiment on oriented samples is the so-called PISEMA experiment, which is used to study the orientation of secondary structure elements[4]. The second approach for obtaining high-resolution spectra is the magic-angle spinning (MAS) technique, where the spectral resolution is improved by mechanically rotating the sample at the "magic" angle, that is around an axis tilted of 54.7° relative to the external magnetic field[5,6]. In this work, we have focused on MAS methods for structural studies, with the advantage that MAS can be applied to randomly-oriented molecules.

MAS applied to oriented and non-oriented samples produces sufficiently resolved spectra and allows the detection of the isotropic chemical shifts. This has important advantages for structural investigations by solid-sate MAS NMR. First, well-established strategies for resonance assignment known from solution NMR can be implemented in solid-state MAS NMR, and chemical shift databases compiled from solution NMR studies can be accessed to identify amino-acids by means of their characteristic side-chain correlation patterns[7,8] (see Chapter 2). Second, the solid-state NMR assignment can be exploited to obtain information about secondary structure motifs, as is common practice in solution NMR[9] (see Chapter 6). Moreover, analysis of chemical shift differences in solution and in solid-state may provide structural information about the contact interfaces or, in the case of ligand-receptor binding studies, to probe the binding area[8,10] (see Chapter 7).

1.2 Solid-state magic-angle spinning NMR

The anisotropic nuclear interactions that are of interest in solid-state NMR spectroscopy can be described by second-rank tensors which couple the nuclear magnetic moment to the pertinent field and together form the nuclear spin Hamiltonian H

$$H = H_Z + H_{RF} + H_{CS} + H_D + H_J + H_Q.$$

H_Z is the Zeeman term, which describes the interaction between a nuclear spin **I** and the external field $\mathbf{B}_0$

$$H_Z = \mathbf{I}\, Z\, \mathbf{B}_0,$$

where **I** and $\mathbf{B}_0$ are represented by vectors and Z is a second-ranked tensor.

Transitions between the Zeeman states are induced by an oscillating field $\mathbf{B}_1$ and are described by the H_{RF} term

$$H_{RF} = \mathbf{I}\, Z\, \mathbf{B}_1,$$

where the $\mathbf{B}_1$ field is generally thousand times smaller than the $\mathbf{B}_0$ field.

The remaining terms of the Hamiltonian give rise to the characteristic features of an NMR spectrum. These are the chemical shift hamiltonian H_{CS}, the dipolar hamiltonian H_D, the J-coupling hamiltonian H_J and the quadrupolar coupling hamiltonian H_Q.

The chemical shift hamiltonian H_{CS} can be written as

$$H_{CS} = \gamma\, \mathbf{I}\, \sigma\, \mathbf{B}_0,$$

where σ is the shielding tensor and describes the effect of the electron distribution around the nuclear spin. Because the electron distribution is not uniform, the chemical shift interaction depends on the orientation of the nucleus with respect to $\mathbf{B}_0$. In a first-order perturbation theory, the truncated chemical shift hamiltonian H_{CS} for a single spin becomes

$$H_{CS} = \gamma\, I_z\, \sigma_{zz}^{LF}\, \mathbf{B}_0.$$

The index LF indicates that σ_{zz}^{LF} is the zz element of the σ matrix in its laboratory-frame (LF) representation.

The dipolar hamiltonian H_D describes the through-space coupling between two nuclear spins $\mathbf{I}^I$ and $\mathbf{I}^J$

$$H_D = \mathbf{I}^I\, D\, \mathbf{I}^J,$$

where D is the dipolar coupling tensor, which describes how the field due to the spin I varies with the orientation of the I-J internuclear vector in the applied field (θ_{IJ}). The dipolar coupling has also an r^{-3} distance dependence, which is source of distance restraints in NMR. The truncated dipolar hamiltonian is

$$H_D = -\frac{\mu_0}{4\pi}\hbar \sum_I \sum_J \frac{\gamma^I \gamma^J}{r_{IJ}^3}\frac{1}{2}(3\cos^2(\theta_{IJ}) - 1) 2 I_z^I I_z^J .$$

J-couplings are dipolar couplings that are mediated through bonds by the electrons. In the solid-state J-couplings are much smaller than the dipolar interactions, nevertheless solid-state NMR experiments have recently been presented, which utilize J-couplings to establish homonuclear[11,12] and heteronuclear[13,14] correlations.

The quadrupolar coupling hamiltonian H_Q is non-zero only for nuclei with spins greater than ½ and is proportional to the electric-field gradient tensor V.

The chemical shift tensor σ, the dipolar interaction tensor D and the electric-field gradient tensor V contain all a $(3\cos^2\theta - 1)$ orientation dependence factor. Consequently, in a MAS NMR experiment, chemical shift, dipolar and quadrupolar interactions can be averaged by mechanically spinning the sample about an axis aligned at 54.7º (the magic-angle) with respect to the external magnetic field, as depicted in Fig. 1.1. When the sample is at the magic-angle, the anisotropic factor vanishes and under fast rotation the anisotropic broadening is removed. The isotropic shift interactions and the isotropic J-couplings are left and high-resolution NMR spectra can be recorded, as in solution NMR.

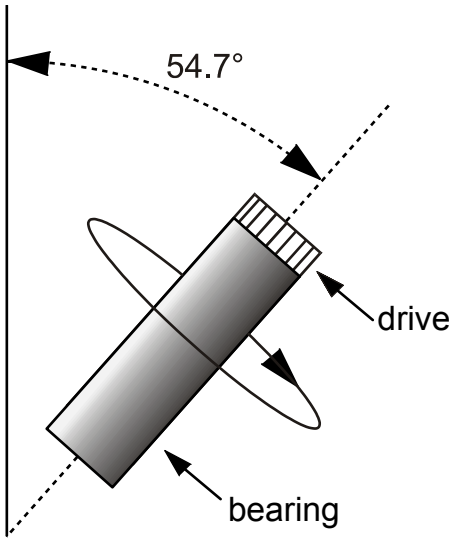

Fig. 1.1 Schematic representation of the magic-angle spinning (MAS) technique. The rotor, that contains the sample, is tilted at 54.7º respect to the external magnetic field. By the combined application of bearing and driving pressures, the sample rotates around itself, at the desired frequency.

Using the terminology of Maricq and Waugh[15], anisotropic interactions can be divided in homogeneous and inhomogeneous; inhomogeneous interactions include the chemical shift, the first-order quadrupolar interaction and dipolar coupling between unlike spins, while dipolar interactions between like spins are homogeneous. The effect of MAS on ^{1}H and ^{13}C spectra relates to the dominating interactions. In a ^{13}C spectrum, the isotropic and anisotropic chemical shift interactions override the homonuclear dipolar interaction. The effect of MAS on a inhomogeneously broadened line consists of producing a set of side-bands, even if the rotation frequency is much lower than the inhomogeneous interaction. The result is a narrow resonance at the isotropic chemical shift, which is flanked by sets of rotational side-bands

spaced by the MAS frequency. In Fig. 1.2, the broad powder line shape due to a CSA interaction of a carboxyl carbon, obtained in static conditions, is shown on the top. Upon rotating the sample, the static line breaks up into a centre-band and spinning side-bands. As the MAS frequency is increased, the intensities of the side-bands is transferred to the centre-band. When the MAS frequency exceeds the CSA of the resonance, only the centre band remains. The number and intensity of the spinning side-bands vary with the magnitude of the CSA and can be used to calculate the chemical shift tensor[16]. A different situation is usually encountered in ^{1}H solid-state NMR, where the homonuclear dipolar interaction is the major anisotropic interaction. For a homogeneous interaction, the broadened line does not split up in a set of spinning side-bands for spinning speed lower than the homogeneous broadening, in contrast to inhomogeneous interactions[15]. In a homogeneously broadened spectrum, the rotation rate must exceed the line-width to obtain substantial narrowing, and even at the highest MAS frequency of ~ 50 kHz all residual dipolar broadening cannot be removed.

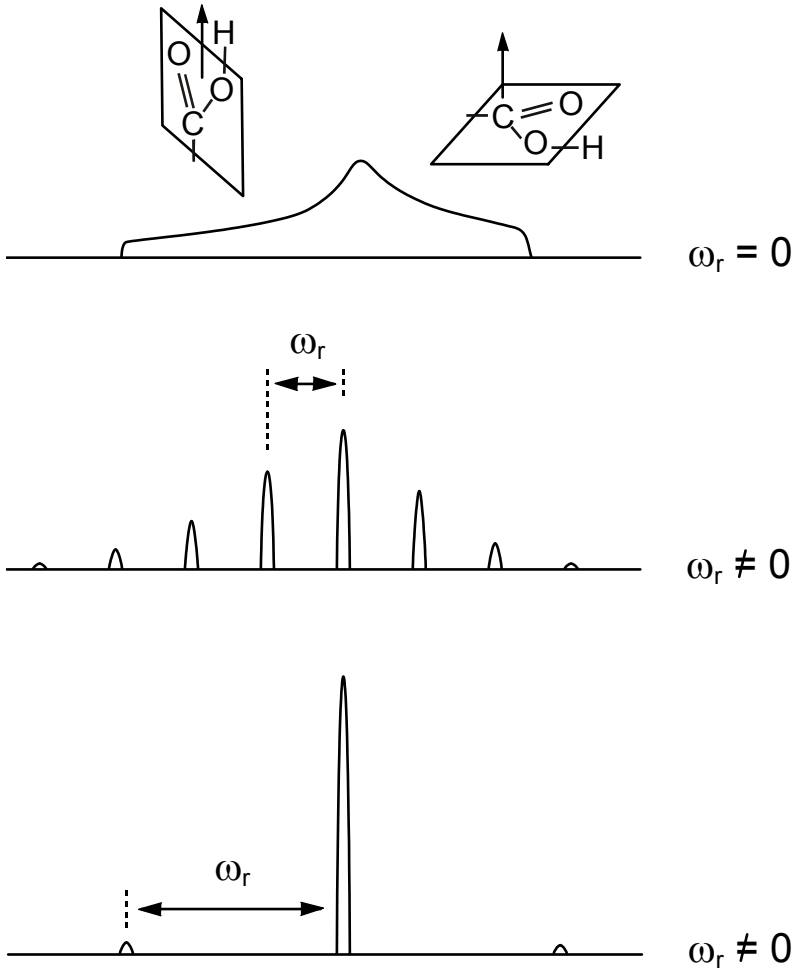

Fig. 1.2 Simulated spectra, showing the effect of MAS on the anisotropic lineshape due to the CSA interaction of a ^{13}C nucleus in a carboxyl group.

1.3 Basic tools in solid-state MAS NMR

In solution NMR, the dipolar and *J*-couplings between ^{1}H spins in the molecule are used to define the structure. Low-γ nuclear spins, such as ^{13}C and ^{15}N are employed to increase resolution of overlapping ^{1}H resonances, using multidimensional techniques. Protons not only have a high gyromagnetic ratio but also high natural abundance. In an NMR experiment, the sensitivity, i.e. the signal-to-noise ratio (S/N), depends on both these parameters and, of all naturally occurring nuclei, the proton has the best sensitivity. In MAS NMR, structural details are primarily obtained from low-γ and dilute I=1/2 spins, i.e. ^{13}C and ^{15}N. Protons are generally not the observed nuclei because they form a strongly dipolarly coupled network of spins.

In MAS solid-state NMR experiments, the detection of low abundance ^{13}C and ^{15}N nuclei usually requires isotope labelling for sensitivity enhancement. To further increase the sensitivity and resolution of solid-state spectra, MAS is combined with high-power proton decoupling[17] and cross-polarization (CP)[18,19]. Fig. 1.3 shows the effect of these different solid-state methods on 1D ^{13}C spectra of the α-spectrin SH3 domain. All the spectra were recorded with a total acquisition time of 10 min. The MAS spectrum in (a) is obtained by simply applying a 90º pulse on the ^{13}C spins and then acquiring the carbon signal. The spectrum shows both low sensitivity and low resolution. By the introduction of ^{1}H high-power decoupling during acquisition (Fig. 1.3 b), the resolution of the spectrum sensibly improves. ^{1}H-decoupling is necessary to eliminate residual ^{1}H-^{13}C dipolar couplings under MAS and ^{1}H-^{13}C residual *J*-couplings. Decoupling field strengths of 80-100 kHz are now commonly used. The simplest decoupling method involves continuous irradiation with *rf* pulse of fixed phase during the acquisition of the FID and is termed continuous wave (CW)[17]decoupling. Recently, more sophisticated methods based on rapid phase switching, such as the TPPM (two-pulse phase modulation) technique, have been introduced, which improve significally the decoupling efficiency[20].

In ^{13}C or ^{15}N MAS spectra, CP[18,19] between protons and low-γ nuclear spins can be used to greatly enhance the signal intensity. In Fig. 1.3 c, a 1D ^{13}C CP MAS spectrum is shown. In a CP experiment, the field strengths in the rotating frame are set to the Hartmann-Hahn condition, as depicted in Fig. 1.4. The standard pulse program of a CP experiment starts with a ^{1}H excitation 90º-pulse and then magnetization is transferred from protons to the low frequency channel (e.g. ^{13}C) by the matched pair of CP pulses. In the presence of molecular

motion or with increasing spinning speed, problems in establishing and maintaining the Hartmann–Hahn matching condition can be encountered.

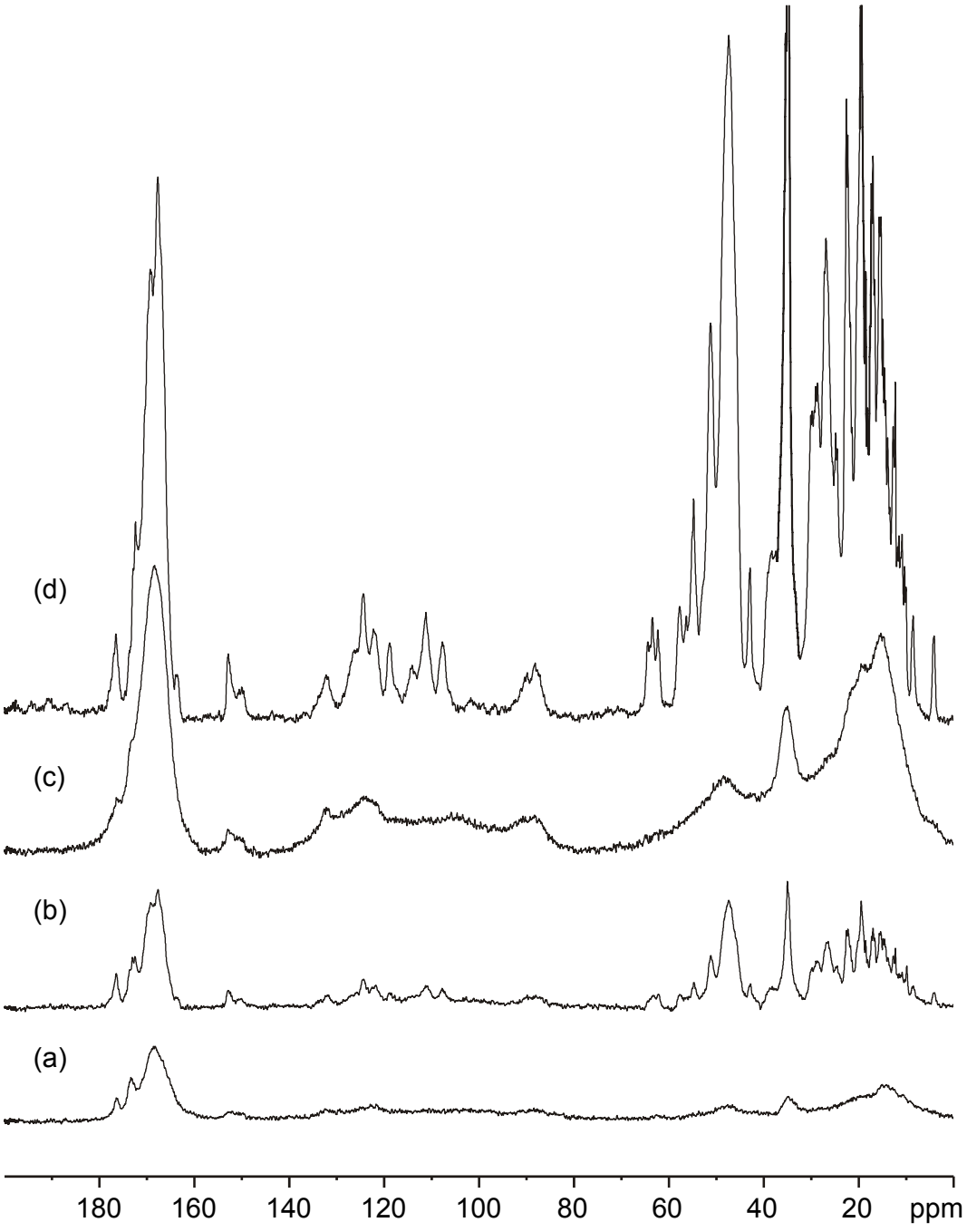

Fig. 1.3 Solid-state ^{13}C MAS NMR spectra of the α-spectrin SH3 domain, acquired with various experimental settings, at a field of 9.4 T, with a spinning frequency $\omega_R/2\pi$ = 8.0 kHz and at a temperature of 298 K. All spectra are recorded using a total acquisition time of 10 min. The spectrum in (a) is obtained by applying a single 90º pulse on the ^{13}C magnetization. In (b), additional TPPM decoupling is employed during acquisition to improve resolution. The spectrum in (c) is recorded using the ramp-CP method, with the effect of an improved signal to noise ratio respect to (a). The combination of ramp-CP and ^{1}H TPPM decoupling produces the spectrum shown in (d).

Recently, it has been shown that a ramped pulse on one of the channels improves signal stability, compensates for B_1 inhomogeneity and increases sensitivity[21,22]. The signal enhancement resulting from CP is due to two factors. First, the larger gyromagnetic ratio of protons (e.g. γ_H is 4 times bigger than γ_C) creates a larger ^{1}H polarization which is transferred to the low-γ nucleus. Second, the repetition time of the experiment is determined by the shorter ^{1}H relaxation time relative to low-γ spins and hence the experiment repetition rate can be increased. For typical ^{1}H-^{13}C CP experiment, these two factors can easily result in a 10-fold increase in sensitivity, as demonstrated by comparing the spectra in a and c. Finally, the spectrum in d shows the combined effect of CP and proton decoupling that produces sharp and intense peaks.

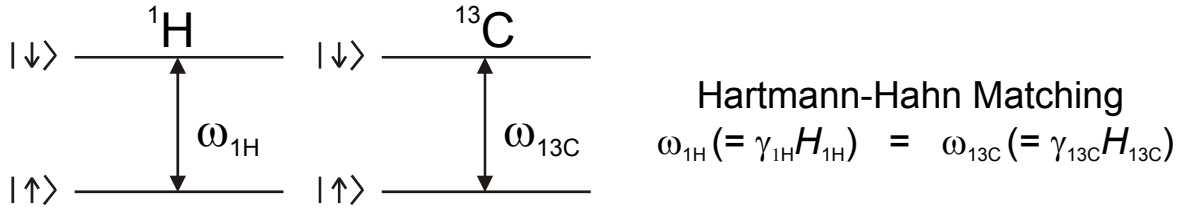

Fig. 1.4 The classic Hartmann-Hahn matching condition for a non-spinning sample, that allows transfer of polarization from ^{1}H to low-γ nuclei, such as ^{13}C. For a spinning sample, the MAS frequency has to be taken into account and the matching condition becomes: ω_{1H}-$\omega_{13C} = \pm n\ \omega_r$.

The anisotropic interactions present in the solid-state are averaged out by rapid MAS. On the other hand, these interactions contain valuable structural and dynamic information. This information can be brought back by employing so-called "recoupling methods" to recover the anisotropic interactions lost during MAS. Different techniques have been developed for recoupling dipolar interactions, which function by the application of *rf* pulses to counterbalance the effect of the sample rotation. These techniques can be separated in two general categories; techniques that recouple dipolar couplings between like spins include RFDR[23], RIL[24], MELODRAMA[25], DRAWS[26], C7[27], post-C7[28], CMR7[29], SPC-5[30]. Techniques that measure dipolar couplings between unlike spins are for example REDOR[31] and TEDOR[32]. Recoupling through *rf* pulses is illustrated schematically in Fig. 1.5. Without pulses the time average of the dipole hamiltonian vanishes during MAS (Fig. 1.5a). However, application of a rotor-synchronized pulse train (Fig. 1.5b) yields a non-zero time-average of the dipolar coupling, where the amount of efficiency of the recoupling is scaled by a factor S, that depends on the details of the pulse train.

The application of homonuclear dipolar correlation spectroscopy on uniformly ^{13}C-labelled systems is important for assignment strategies in solid-state NMR. ^{13}C-homonuclear correlation experiments are used for the identification of amino-acid side-chains.

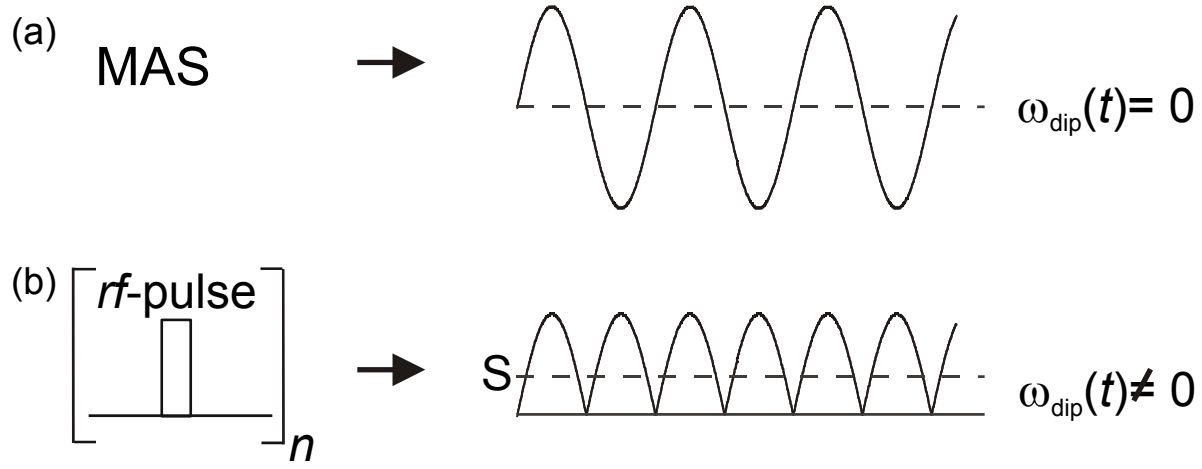

Fig. 1.5 Schematic illustration of the idea behind recoupling methods. (a): MAS produces an oscillatory behaviour of the dipolar coupling which yields $\omega_{dip}(t) = 0$. (b): application of rotor synchronized π pulses yields a non zero $\omega_{dip}(t)$.

Spectral assignment requires also heteronuclear transfer steps that direct polarization from one spin to the next in the polypeptide chain, to obtain the sequential assignment. For heteronuclear transfer in polypeptides, the conventional CP approach has been modified to direct polarization transfer between two low-γ spins in a chemical shift selective manner[33]. Selective magnetization transfer between the amide N and the C^{α} and CO backbone nuclei can be established using specific ^{15}N-^{13}C cross-polarization (specific-CP)[33], where off-resonance low-power *rf* fields are applied to spin lock the ^{15}N and ^{13}C spins. The spin-locking has to fulfil the specific-CP matching condition

$$\sqrt{\Omega_C^{\,2} + \omega_C^{\,2}} - \sqrt{\Omega_N^{\,2} + \omega_N^{\,2}} = n\omega_r,$$

where ω_r denotes the sample spinning frequency, Ω the frequency offsets and ω the *rf* field strengths for the ^{15}N and ^{13}C spins. The low *rf* powers ensure that the CP is band-selective, while the off-resonance components can be adjusted to fulfil the specific-CP condition for any two bands in the ^{15}N and ^{13}C spectra. The application of homonuclear and heteronuclear techniques for assignment purposes is described in more detail in Chapter 2.

Even though ^{1}H NMR is currently not of central importance in the solid-state, high-resolution ^{1}H solid-state NMR methods have recently been developed, raising expectations that the significance of protons in MAS NMR may increase in the next future. A method to

improve proton resolution is based on the Lee-Goldburg (LG) technique. The basic idea of the Lee and Goldburg experiment consists in irradiation of the proton spins in a continuous fashion with a field that is off-resonance, in such a way that the total effective field H_{eff} is inclined at the magic angle θ_m = 54.7° with respect to the positive or negative z-axis, as depicted in Fig. 1.6. The LG conditions can be written as

$$\Omega_{\pm\Delta LG} = \omega_{\pm\Delta LG} - \gamma H_0 = \pm\frac{1}{2}\sqrt{2}\left|\omega_1\right|.$$

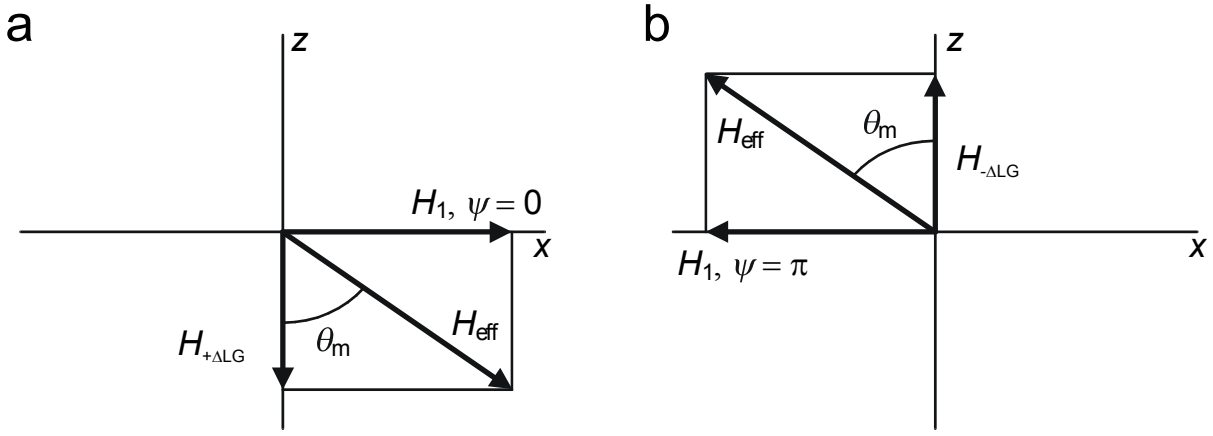

Fig. 1.6 Application of off-resonance fields ($H_{+\Delta LG}$ and $H_{+\Delta LG}$) in such a way that the effective field H_{eff} is inclined at θ_m with respect to the negative (a) or positive (b) z-axis. In the LG experiment, θ_m is set to be the magic-angle (54.7°).

A further improvement of the LG decoupling is achieved with the frequency- and phase-switched Lee-Goldburg (FSLG) experiment suggested by Levitt and co-workers[34]. In this experiment the *rf* field is frequency-switched between $\omega_{+\Delta LG}$ and $\omega_{-\Delta LG}$, and the phase is switched between $\Psi_{+\Delta LG}$ and $\Psi_{-\Delta LG}$, with $\left|\Psi_{+\Delta LG} - \Psi_{-\Delta LG}\right| = \pi$. A different approach was proposed by Vega and co-workers, with the introduction of the phase-modulated Lee-Goldburg (PMLG)[35]. The basic idea of the PMLG experiment is to mimic the off-resonance *rf* irradiation using an on-resonance pulse with continuously changing phase. Both these methods were employed in the proton assignment of the α-spectrin SH3 domain, as described in Section 2.3.

1.4 Overview of the thesis

In this thesis, a methodology for structure determination of proteins by solid-state MAS NMR is presented. The potential of the method is demonstrated with the calculation of the structure

of the 62 residue α-spectrin SH3 domain, used as a model system. This method should be widely applicable for structural investigation of those proteins which do not easily form crystals or are not accessible to solution NMR studies, such as membrane proteins and amyloid systems.

The initial step in a structural study by NMR is the resonance assignment. In **Chapter 2**, the solid-state assignment strategy of ^{13}C and ^{15}N signals is described, which involves two main steps: first the identification of the single residues by homonuclear dipolar correlation spectroscopy, followed by the sequential assignment, achieved by heteronuclear correlation experiments. The assignment of the ^{13}C and ^{15}N resonances of the SH3 domain is shown as an example. On the basis of the ^{13}C and ^{15}N assignment, we have obtained the proton resonance assignment, by multi-dimensional NMR spectroscopy, as reported in Section 2.3. In particular, the resonances of non-exchangeable protons are assigned by 3D ^{1}H-^{13}C-^{13}C correlation spectroscopy. For the amide proton resonance assignment, a novel 3D (^{1}H-^{15}N-^{13}C) heteronuclear correlation experiment is used.

In **Chapter 3**, our approach for determining the structure of proteins by solid-state MAS NMR is introduced. From exploring all potential short distances in proteins, and considering the limited resolution of the proton spectrum in the solid-state, it is evident the carbon-carbon distances are of prime importance in defining a 3D structure. Our method is based on the collection of a large number of low-accuracy ^{13}C-^{13}C structure-defining distances from multiply-enriched samples. In a fully ^{13}C-enriched sample, dipolar truncation effects and spin-diffusion mechanisms do not allow detection of long-range restraints. To overcome this problem, dilution of the labelling is used, in combination with a broad-band recoupling method.

In **Chapter 4,** the preparation of biosynthetically site-directed ^{13}C-labelled samples is described. The selective and extensive labelling is important for the detection of long-range restraints for structure determination in solid-state MAS NMR. Spin dilution is obtained by using selectively labelled glycerol in the protein expression medium. The ^{13}C labelling pattern obtained with this method, is estimated from solution NMR data.

The first structure calculation of the SH3 domain by solid-state NMR is obtained by combing dilution of ^{13}C spins with the broad-band proton-driven spin diffusion (PDSD) method, as presented in **Chapter 5**. Within this chapter, methods are described for the conversion of the ^{13}C-^{13}C long-range restraints from 2D PDSD spectra into 4 different

distance classes. In addition, a description is given for how we could distinguish between intra- and inter-molecular correlations. The first structure of the SH3 is obtained only from 2D spectroscopy.

In **Chapter 6**, a refinement of the structure is achieved by 3D ^{15}N-^{13}C-^{13}C dipolar correlation spectroscopy and chemical shift analysis. Through the addition of a ^{15}N dimension, it becomes possible to identify new backbone carbon-carbon restraints, that were not accessible in 2D experiments. Additional restraints are obtained by using the information contained in the chemical shifts, which provided backbone torsion angle restraints.

Differences in the chemical shift values obtained from solution and solid-state spectra of the same protein, can be used to dissect structural variations of the protein under these two conditions. In **Chapter 7**, we analyze the chemical shift differences in the case of our SH3 sample, in solution and in a microcrystalline state. Similar studies may be instrumental in ligand-receptor binding investigations, where perturbations of the ligand resonances upon binding can be used to acquire structural insights of the binding interface.

Finally, in **Chapter 8** the mechanism of magnetization transfer between low-γ nuclei via a proton-driven spin diffusion process is interpreted. ^{15}N-^{15}N PDSD spectra are recorded on a ^{15}N-labelled SH3 sample, to analyze the relation between build-up curves of cross-peak intensities and ^{15}N-^{15}N distances.

References

1. Torres, J., Stevens, T. J., & Samso, M. (2003). Membrane proteins: the 'Wild West' of structural biology (vol 28, pg 137, 2003). *Trends Biochem. Sci.* **28**, 174.

2. Marassi, F. M. & Opella, S. J. (1998). NMR structural studies of membrane proteins. *Curr. Opin. Struct. Biol.* **8**, 640-648.

3. Marassi, F. M. & Opella, S. J. (2000). A solid-state NMR index of helical membrane protein structure and topology. *J. Magn Reson.* **144**, 150-155.

4. Wu, C. H., Ramamoorthy, A., & Opella, S. J. (1994). High resolution heteronuclear dipolar solid-state NMR spectroscopy. *J. Magn. Reson. A* **109**, 270-272.

5. Andrew, E. R., Bradbury, A., & Eades, R. G. (1959). Removal of Dipolar Broadening of Nuclear Magnetic Resonance Spectra of Solids by Specimen Rotation. *Nature* **183**, 1802-1803.

6. Lowe, I. J. (1959). Free Induction Decays of Rotating Solids. *Phys. Rev. Lett.* **2**, 285-287.

7. Pauli, J., van Rossum, B., Forster, H., de Groot, H. J. M., & Oschkinat, H. (2000). Sample optimization and identification of signal patterns of amino acid side chains in 2D RFDR spectra of the alpha-spectrin SH3 domain. *J. Magn. Reson.* **143**, 411-416.

8. McDermott, A., Polenova, T., Bockmann, A., Zilm, K. W., Paulson, E. K., Martin, R. W., Montelione, G. T., & Paulsen, E. K. (2000). Partial NMR assignments for uniformly (13C, 15N)-enriched BPTI in the solid state. *J. Biomol. NMR* **16**, 209-219.

9. Cornilescu, G., Delaglio, F., & Bax, A. (1999). Protein backbone angle restraints from searching a database for chemical shift and sequence homology. *J. Biomol. NMR* **13**, 289-302.

10. Williamson, P. T., Bains, S., Chung, C., Cooke, R., & Watts, A. (2002). Probing the environment of neurotensin whilst bound to the neurotensin receptor by solid state NMR. *FEBS Lett.* **518**, 111-115.

11. Brown, S. P., Perez-Torralba, M., Sanz, D., Claramunt, R. M., & Emsley, L. (2002). The direct detection of a hydrogen bond in the solid state by NMR through the observation of a hydrogen-bond mediated N-15-N-15 J coupling. *J. Am. Chem. Soc.* **124**, 1152-1153.

12. Lesage, A., Auger, C., Caldarelli, S., & Emsley, L. (1997). Determination of through-bond carbon-carbon connectivities in solid-state NMR using the INADEQUATE experiment. *J. Am. Chem. Soc.* **119**, 7867-7868.

13. Lesage, A., Steuernagel, S., & Emsley, L. (1998). Carbon-13 spectral editing in solid-state NMR using heteronuclear scalar couplings. *J. Am. Chem. Soc.* **120**, 7095-7100.

14. Lesage, A. & Emsley, L. (2001). Through-bond heteronuclear single-quantum correlation spectroscopy in solid-state NMR, and comparison to other through-bond and through-space experiments. *J. Magn. Reson.* **148**, 449-454.

15. Maricq, M. M. & Waugh, J. S. (1979). Nmr in Rotating Solids. *J. Chem. Phys.* **70**, 3300-3316.

16. Herzfeld, J. & Berger, A. E. (1980). Sideband intensities in NMR spectra of samples spinning at the magic angle. *J. Chem. Phys.* **73**, 6021-6030.

17. Bloch, F. (1958). Theory of Line Narrowing by Double-Frequency Irradiation. *Physical Review* **111**, 841-853.

18. Pines, A., Waugh, J. S., & Gibby, M. G. (1972). Proton-Enhanced Nuclear Induction Spectroscopy - Method for High-Resolution Nmr of Dilute Spins in Solids. *J. Chem. Phys.* **56**, 1776-&.

19. Schaefer, J. & Stejskal, E. O. (1976). C-13 Nuclear Magnetic-Resonance of Polymers Spinning at Magic Angle. *J. Am. Chem. Soc.* **98**, 1031-1032.

20. Bennett, A. E., Rienstra, C. M., Auger, M., Lakshmi, K. V., & Griffin, R. G. (1995). Heteronuclear decoupling in rotating solids. *J. Chem. Phys.* **103**, 6951-6958.

21. Hediger, S., Meier, B. H., Kurur, N. D., Bodenhausen, G., & Ernst, R. R. (1994). Nmr Cross-Polarization by Adiabatic Passage Through the Hartmann-Hahn Condition (Aphh). *Chem. Phys. Lett.* **223**, 283-288.

22. Metz, G., Wu, X. L., & Smith, S. O. (1994). Ramped-Amplitude Cross-Polarization in Magic-Angle-Spinning Nmr. *J. Magn. Reson. A* **110**, 219-227.

23. Bennett, A. E., Ok, J. H., Griffin, R. G., & Vega, S. (1992). Chemical-Shift Correlation Spectroscopy in Rotating Solids - Radio Frequency-Driven Dipolar Recoupling and Longitudinal Exchange. *J. Chem. Phys.* **96**, 8624-8627.

24. Baldus, M., Tomaselli, M., Meier, B. H., & Ernst, R. R. (1994). Broad-Band Polarization-Transfer Experiments for Rotating Solids. *Chem. Phys. Lett.* **230**, 329-336.

25. Sun, B. Q., Costa, P. R., Kocisko, D., Lansbury, P. T., & Griffin, R. G. (1995). Internuclear Distance Measurements in Solid-State Nuclear-Magnetic-Resonance - Dipolar Recoupling Via Rotor Synchronized Spin Locking. *J. Chem. Phys.* **102**, 702-707.

26. Gregory, D. M., Mitchell, D. J., Stringer, J. A., Kiihne, S., Shiels, J. C., Callahan, J., Mehta, M. A., & Drobny, G. P. (1995). Windowless Dipolar Recoupling - the Detection of Weak Dipolar Couplings Between Spin-1/2 Nuclei with Large Chemical-Shift Anisotropies. *Chem. Phys. Lett.* **246**, 654-663.

27. Lee, Y. K., Kurur, N. D., Helmle, M., Johannessen, O. G., Nielsen, N. C., & Levitt, M. H. (1995). Efficient Dipolar Recoupling in the Nmr of Rotating Solids - A Sevenfold Symmetrical Radiofrequency Pulse Sequence. *Chem. Phys. Lett.* **242**, 304-309.

28. Hohwy, M., Jakobsen, H. J., Eden, M., Levitt, M. H., & Nielsen, N. C. (1998). Broadband dipolar recoupling in the nuclear magnetic resonance of rotating solids: A compensated C7 pulse sequence. *J. Chem. Phys.* **108**, 2686-2694.

29. Rienstra, C. M., Hatcher, M. E., Mueller, L. J., Sun, B. Q., Fesik, S. W., & Griffin, R. G. (1998). Efficient multispin homonuclear double-quantum recoupling for magic-angle spinning NMR: C-13-C-13 correlation spectroscopy of U-C-13-erythromycin A. *J. Am. Chem. Soc.* **120**, 10602-10612.

30. Hohwy, M., Rienstra, C. M., Jaroniec, C. P., & Griffin, R. G. (1999). Fivefold symmetric homonuclear dipolar recoupling in rotating solids: Application to double quantum spectroscopy. *J. Chem. Phys.* **110**, 7983-7992.

31. Gullion, T. & Schaefer, J. (1989). Rotational-Echo Double-Resonance Nmr. *J. Magn. Reson.* **81**, 196-200.

32. Hing, A. W., Vega, S., & Schaefer, J. (1992). Transferred-Echo Double-Resonance Nmr. *J. Magn. Reson.* **96**, 205-209.

33. Baldus, M., Petkova, A. T., Herzfeld, J., & Griffin, R. G. (1998). Cross polarization in the tilted frame: assignment and spectral simplification in heteronuclear spin systems. *Mol. Phys.* **95**, 1197-1207.

34. Bielecki, A., Kolbert, A. C., & Levitt, M. H. (1989). Frequency-Switched Pulse Sequences - Homonuclear Decoupling and Dilute Spin Nmr in Solids. *Chem. Phys. Lett.* **155**, 341-346.

35. Vinogradov, E., Madhu, P. K., & Vega, S. (1999). High-resolution proton solid-state NMR spectroscopy by phase-modulated Lee-Goldburg experiment. *Chem. Phys. Lett.* **314**, 443-450.