Structure refinement of photosynthetic components with multidimensional MAS NMR dipolar correlation spectroscopy

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Chapter
1

General introduction

1.1 Introduction

Magic-angle spinning nuclear magnetic resonance (MAS NMR) dipolar correlation spectroscopy is rapidly forthcoming as a versatile technique for *de novo* structure determination of microscopically ordered systems without long-range translation symmetry [1]. It has been shown that *de novo* structure determination is possible with ^{13}C homonuclear MAS NMR dipolar correlation spectroscopy when multiple labeling is used [1-4]. While ^{13}C homonuclear dipolar correlation spectroscopy is now being used routinely in assignment and structure refinement studies of organic solids, ^{1}H MAS NMR has not yet found a widespread application as a tool for structure determination. Due to the combination of the strong homonuclear dipolar interactions between the abundant protons in the solid state and the small proton chemical shift dispersion, the proton resolution is often very limited which makes ^{1}H NMR in solids difficult.

The scope of the research described in this thesis is to demonstrate that protons can be used in structure refinement studies of biological systems in the solid state using cross-polarization (CP) MAS NMR dipolar correlation spectroscopy. To this end, first a set of NMR techniques should be developed to suppress the ^{1}H homonuclear dipolar interactions under MAS conditions. In a second step, the versatility of protons for structure determination with solid-state NMR should be demonstrated by exploiting the novel NMR techniques in a structural investigation of various biological systems.

The contents in the thesis are divided into two main parts. Chapters 2-5 focus on the development of the solid-state NMR. Chapter 2 provides a short theoretical background, while Chapters 3-5 are concerned with the development of the novel solid-state NMR spectroscopy techniques that enable the use of protons in multidimensional heteronuclear dipolar correlation spectroscopy. The second part of the thesis, Chapters 6-9, is dedicated to the application of these and other solid-state NMR techniques to study systems in the field of photosynthetic research, like native chlorosome antennae, synthetic antenna model systems and photosynthetic bacterial reaction centers.

1.2 Photosynthesis

Photosynthesis is the collection of life-sustaining processes that convert (solar) light-energy into chemical energy, which is stored in energy-rich organic material, collectively called biomass. Photosynthetic organisms can be divided into two groups. Plants, algae and cyanobacteria belong to the first group that is capable of oxygenic photosynthesis, in which the light-energy is used in a reductive fixation of carbon dioxide into carbohydrates under oxidization of water. In this process oxygen is produced. The second group of photosynthetic organisms comprises the anoxygenic photosynthetic bacteria, that use molecules other than water as an electron donor, for instance H_2S or organic acids. In the anoxygenic photosynthetic energy conversion no oxygen is produced. The photosynthetic organelles that are investigated in this thesis are extracted from bacteria of this second group.

Photosynthesis is a multistep process with a high degree of compartimentalization. It takes place in a set of complex molecules located in or attached to the photosynthetic membrane. The two major steps that can be distinguished in the primary processes of the photosynthetic energy conversion are:

I: The harvesting of light and transfer of the light-energy to the reaction center (RC)

II: Charge separation and subsequent electron transport in the RC

The absorption of light, the conversion of the light-energy into electronic excitation-energy and the subsequent migration of the excitation-energy to the RC in step I, take place in the photosynthetic antenna system. In higher plants, green algae, heliobacteria and some purple bacteria, the antenna system is embedded within the photosynthetic membrane. These antennae consists of chlorophyll-carotenoid-protein complexes. In other photosynthetic bacteria the antenna is situated in extra-membranous organelles, connected to the photosynthetic membrane. For instance, in green sulfur bacteria (*Chlorobiaceae*) and green filamentous bacteria (*Chloroflexaceae*), the major light-harvesting apparatus is formed by chlorosomes, which are oblong bodies that are attached to the cytoplasmic side of the cell membrane [5-7]. In contrast to plant antenna systems, the chlorosomes are nearly protein-free and their structure and function rely on the self-aggregation of the antenna pigments not mediated by a protein [8,9]. In this respect the chlorosomes form a unique class of photosynthetic antennae. Chlorosomes can be separated from the photosynthetic apparatus and have been the subject of many studies (for a recent review of the experimental work, see Olson, 1998 [10]).

The RC is a transmembrane protein complex. It plays a key-role in the photosynthetic energy conversion, since here the actual charge separation and photochemistry take place. RCs of purple photosynthetic bacteria, for instance *Rhodobacter* (*Rb.*) *sphaeroides*, have been extensively investigated in the past few years. Nowadays efficient procedures exist for growing strains of this species, for the isolation of the RC complexes in the native membrane, and for the preparation and manipulation of samples for a wide variety of investigations [11]. For the purple bacteria *Rhodopseudomonas viridis* and *Rb. sphaeroides*, RCs have been crystallized and studied with X-ray diffraction, from which a detailed knowledge about their structure was obtained [12-16]. The RCs of the purple bacteria *Rb. sphaeroides* R26 consist of three polypeptide subunits supporting nine cofactors: four bacteriochlorophylls (B), two bacteriopheophytins (Φ), two quinones (Q) and one non-heme Fe^{2+}. Two bacteriochlorophyll molecules form the special pair P. The cofactors are arranged in two branches, designated A and B, with a nearly two-fold symmetry (Fig. 1.1).

Upon illumination, the special pair is photo-oxidized and an electron is transferred along the A-branch across the membrane, first to the primary quinone Q_A. Q_A is tightly and permanently bound to the protein and serves as a one-electron gate. It temporarily accepts a single electron, which is subsequently transferred to the secondary quinone Q_B. Following a second excitation, Q_B is doubly reduced and doubly protonated and leaves the RC as a diquinol. Subsequently, the empty Q_B binding pocket is occupied by a new ubiquinone-10 (UQ_{10}) molecule which is taken up from the quinone-pool (for a review, see *e.g.* [17]). During these electron transfer processes, a cytochrome at the periplasmic side of the RC regenerates the oxidized special pair P^+ to P. After replacement with a fresh UQ_{10} molecule, the RC has returned to its original state and the reaction is cyclic.

Under physiological conditions, electrons have a strong preference to travel along the A-branch. Thus, despite the apparent two-fold symmetry of the A- and B-branch (cf. Fig. 1.1), the molecular mechanisms of the function of the RC are highly asymmetric [18]. The question why nature has chosen for this functional symmetry-breaking has been an important issue in the photosynthesis research field during the past decade.

Unraveling the molecular mechanisms of photosynthesis and gaining fundamental knowledge about one of the most important processes in living nature already provide important motives for studying the concepts of photosynthesis. Second, a thorough understanding of the molecular mechanisms of photosynthetic energy conversion will potentially be of help for the development and improvement of artificial photosynthesis devices, which can become an important source of renewable energy in the foreseeable future [19].

1.3 MAS NMR spectroscopy and photosynthesis

In recent years, progress has been made in forging pathways for obtaining MAS NMR access to membrane proteins in general and to photosynthetic components in particular [20]. These studies rely on the use of labeling schemes and ^{13}C CP/MAS NMR. The natural abundance of ^{13}C is low, ~ 1 %, and in order to enhance the sensitivity, the use of ^{13}C enrichment is a prerequisite. For instance, using labeled spheroidene obtained by total synthesis, the configuration of the 15-15’ bond of the carotenoid reconstituted into R26 RCs was established [21]. The electronic ground-state of the one-electron gate Q_A in the RC has been probed with MAS NMR and isotope labeling [22]. An advantage of selective labeling is a direct and straightforward chemical shift assignment of the response from the nucleus of interest. However, selective labeling is most often difficult to realize. In particular for chlorophyll, to arrive at a complete set of specifically labeled molecules at every individual position will take many years of organic synthesis work.

Recently, different routes for obtaining information from multiply enriched samples were explored. The details of the electronic ground-state structure of the RC protein complex of *Rb. sphaeroides* have been investigated for tyrosine side chains labeled at the 4’-position, with particular focus on M210 and L162, that are of importance for the efficiency of the charge separation and re-reduction processes in the RC [23-25]. In these studies, all tyrosines in the RC were selectively labeled, and the assignment of the response from the two specific residues, M210 and L162, was achieved by illumination or mutation [23-25]. In another approach, a novel example of photochemically-induced dynamic nuclear polarization (photo-CIDNP) was discovered, yielding strong emissive signals for Q_A depleted, or pre-reduced uniformly ^{15}N-labeled RCs [26]. Finally, it was found that the large chemical shift dispersion of the ^{13}C response of ~ 200 ppm can be exploited for high-resolution dipolar correlation spectroscopy of ^{13}C nuclei in multiply enriched samples, due to a truncation of the homonuclear dipolar interactions by the chemical shift dispersion in high magnetic fields. This yields remarkably narrow lines in the 2-dimensional (2-D) MAS NMR ^{13}C homonuclear dipolar correlation spectra of uniformly ^{13}C-enriched ([U-^{13}C]) chlorophylls [2]. It was used recently to refine the structure at the molecular level of an uniformly labeled intact chlorosome photosynthetic antenna system that is inaccessible to X-ray or solution NMR approaches [2-4,27].

1.4 Heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy

An important aim of this thesis is to demonstrate that protons can be utilized in MAS NMR for assignment strategies, structure determination and structure-function studies of microscopically ordered systems without long-range translation symmetry in general, and photosynthetic components in particular. Thus far, the dipolar correlation spectroscopy has not found a widespread application to study hydrogens in large biological preparations like membrane proteins in the solid state. Protons play a very important role in the structure and function of proteins, since they are involved in the formation of hydrogen bonds that determine the secondary structure of a protein. In addition, protons take part in the binding of cofactors to a protein and stabilize the self-assembly of pigments in *e.g.* chlorosome antenna systems. However, the dipolar line-broadening in the solid state generally results in a proton resolution that is insufficient for structural research.

In the current opinion an improved resolution can only be obtained by taking advantage of the large ^{13}C chemical shift dispersion in heteronuclear (^{1}H-^{13}C) correlation spectroscopy. First, in **Chapter 2** a simple theoretic framework is provided to describe the effect of off-resonance radio-frequency irradiation of the protons during ^{1}H evolution and CP. Next, in **Chapter 3** it is shown that increasingly high magnetic fields are essential to improve the spectral resolution in multidimensional spectroscopy with MAS. It is demonstrated that straightforward high-speed MAS heteronuclear (^{1}H-^{13}C) CP wide-line separation (CP/WISE) spectroscopy [28] performed at a high magnetic field and without any homonuclear decoupling scheme during the proton evolution, already yields resolved ^{1}H-^{13}C correlations. Proton chemical shifts can be obtained directly from such spectra [29].

It is demonstrated in **Chapter 4** that well-resolved MAS NMR heteronuclear (^{1}H-^{13}C) dipolar correlation spectra of multispin ^{13}C clusters can be acquired at high spinning speeds when frequency-switched Lee-Goldburg irradiation is applied during the ^{1}H evolution [30-33]. The assignment of solid-state proton chemical shifts from the heteronuclear correlation spectra can provide information about the electronic structure of a densely packed solid. Non-bonding interactions can be quite strong in the solid state and even small shifts can be significant, if the shift effects are correlated in the sense that they follow a pattern or that they are extended over a region of the molecule [3,4]. In addition, the range of the coherent spin-diffusion in the solid state is intrinsically much larger than in solution [34]. It is shown in Chapter 4 that Lee-Goldburg CP [35,36] in combination with heteronuclear dipolar correlation spectroscopy can be exploited to detect ^{1}H-^{13}C heteronuclear intermolecular correlations and to provide unambiguous structural restraints [37].

In **Chapter 5**, a method is presented that can be applied in uniformly ^{13}C-enriched compounds to extract ^{1}H-^{13}C heteronuclear distances with good precision from CP build-up curves, which are recorded at high MAS rates under simultaneous suppression of the ^{1}H homonuclear dipolar interactions. The Fourier transform of the time-oscillatory magnetization build-up curves provides direct access to heteronuclear (^{1}H-^{13}C) dipolar coupling strengths. An empirical relation between the heteronuclear distance and the dipolar coupling strength is constructed from a series of simulations. It is demonstrated for a [U-^{13}C] tyrosine·HCl model compound that this relationship can be useful in the translation of experimental coupling strengths into distances between the coupled spins. The experimentally determined internuclear distances compare very well with the distance information extracted from the neutron diffraction structure of tyrosine·HCl [38].

A concept for structure determination using ^{13}C homonuclear and ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy is presented in **Chapter 6** [1,4]. The concept is applied in a 3-dimensional (3-D) structure determination study of aggregates of [U-^{13}C] chlorophyll *a* / H_2O. Chlorophyll *a* (Chl *a*) is the green pigment involved in photosynthetic harvesting of light and subsequent conversion of light-energy into chemical energy by higher plants and related species, like algae and cyanobacteria. It forms aggregates when exposed to water [39]. The aggregated Chl *a* is thought to represent a paradigm for a system that is potentially important for artificial-photosynthesis research [39]. It is shown in Chapter 6 that knowledge about the electronic structure deduced from the solid-state proton assignment from intramolecular heteronuclear (^{1}H-^{13}C) correlations, as well as distance constraints obtained from the observation of several intermolecular heteronuclear correlations, provide information that can be interpreted consistently into a 3-D structural model of the self-assembled Chl *a* / H_2O.

In **Chapter 7**, the arrangement of [U-^{13}C] bacteriochlorophyll (BChl) *c* in intact chlorosome antenna systems is studied using 2-D (^{1}H-^{13}C) and 3-D (^{1}H-^{13}C-^{13}C) heteronuclear dipolar correlation spectroscopy. Since 3-D crystals of the chlorosome antenna were not yet obtained, the system is not amenable to high-resolution diffraction techniques. BChl *c* is the major chromophore of the chlorosomes of the bacterium *Chlorobium tepidum*, and is known to form aggregates [40-42]. BChl *c* is related to Chl *a* in the sense that both pigments have three unsaturated pyrrole rings, unlike *e.g.* BChl *a* and BChl *b*, that only have two unsaturated rings. There is growing evidence that the internal structure in the chlorosomes is based on the self-organization of BChl *c* not directly mediated by proteins [8,9]. In particular, from previous NMR studies using ^{13}C homonuclear dipolar correlation spectroscopy it was concluded that the stacking of BChl *c* in the chlorosomes and in artificial aggregates is highly similar, which provides convincing evidence that indeed the self-organization of the chromophore is the main mechanism to support the structure of the chlorosomes [3]. In Chapter 7 the proton assignment from the heteronuclear correlation spectroscopy is used to refine the model for the arrangement of the BChl *c* in the chlorosomes.

In **Chapter 8**, the binding of ground-state Q_A and Q_B in the RC protein complex of *Rb. sphaeroides* and the formation of hydrogen bonds to the surrounding protein is investigated. Knowledge about the hydrogen-bonding interactions of the quinones to the protein can help to understand the different electrochemical function and binding properties of Q_A and Q_B in the RC in the ground state (Fig. 1.1). To this end, RCs reconstituted with [1-^{13}C] UQ_{10} [43] for Q_A or Q_B are studied with heteronuclear dipolar correlation spectroscopy and CP build-up curves of the label signal are recorded. In this way, the proton(s) that interact with the 1-^{13}C=O of Q_A or Q_B are characterized in terms of their chemical shift and the distance to the 1-^{13}C of the quinones. Strong evidence is provided by the NMR for a strong hydrogen-bonding interaction in ground-state RCs of both 1-^{13}C=O Q_A and Q_B with the surrounding protein. This contrasts with Fourier transform infrared spectroscopy, which suggested an essentially free or weakly bound 1-^{13}C=O Q_A [44-46].

Finally, in **Chapter 9**, the protein environment of the tyrosine residue L162 that is of importance for the efficiency of the charge separation and re-reduction processes in the RC of *Rb. sphaeroides* is investigated. To this end, details of the electronic ground-state structure are studied for L162YL mutant RCs, *i.e.*, with leucine substituted for tyrosine L162, in which all tyrosines were selectively labeled at the 4'-position [25]. In this study an unambiguous assignment of the solid-state NMR response from L162 is achieved by comparing the data of [4'-^{13}C] Tyr enriched L162YL mutant RCs with [4'-^{13}C] Tyr enriched RCs of *Rhodobacter sphaeroides* R26 and of the [4'-^{13}C] Tyr enriched mutant M210YW [23-25]. M210YW and L162YL are the first two mutant RCs that have been studied to atomic resolution with solid-state NMR [24,25].

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Chapter 2

Theoretical background: frequency-switched Lee-Goldburg irradiation and Lee-Goldburg cross-polarization

2.1 Introduction

Many solid-state NMR experiments rely on the application of radio frequency (RF) pulses to manipulate the nuclear spin Hamiltonian. In most experiments, the RF field $H_{\mathrm{RF}}(t) = 2H_1 \cos(\omega_{\mathrm{RF}} t + \psi_{\mathrm{RF}})$ is applied on or nearly on-resonance. In this case $\omega_{\mathrm{RF}} \cong \gamma H_0$, which is positive by definition [1]. Here γ is the gyromagnetic ratio of the spin and H_0 is the applied static magnetic field (Fig 2.1A). Alternatively, the design of an NMR technique can be based on the application of an off-resonance RF field. In that case an effective field $\mathbf{H}_{\mathrm{eff}}$ arises, inclined at an angle $\theta \neq \pi/2$ with respect to the rotating frame *z*-axis (Fig 2.1B).

In this thesis, the focus will be on techniques that enable the use of protons in solid-state NMR. In solids, the protons, or *I* spins, are mutually coupled via strong homonuclear dipolar interactions. For a static sample, the homonuclear dipolar interaction between the *I* spins in the spin-pair approximation can be expanded into five terms $\mathcal{H}_{\mathrm{II}}^{M}$, with $M = -2, \ldots, +2$ [2]. Only $\mathcal{H}_{\mathrm{II}}^{0}$ is secular, which means that it commutes with the static-field Zeeman interaction. In a high magnetic field, this is the term that remains and the truncated Hamiltonian for the homonuclear dipolar interaction has the form [2]

$$\mathcal{H}_{\mathrm{II}}^{0} = \sum_{i<j} a_{ij} (3 I_{zi} I_{zj} - \mathbf{I}_i \cdot \mathbf{I}_j) \quad , \tag{2.1}$$

with

$$a_{ij} = -\left(\frac{\mu_0}{4\pi}\right)\frac{\gamma_{\mathrm{I}}^2\hbar^2}{2}\frac{(3\cos^2\theta_{ij}-1)}{r_{ij}^3} \quad , \tag{2.2}$$

where r_{ij} is the size of the distance vector $\mathbf{r}_{ij}$ between the spins *i* and *j*, and θ_{ij} is the angle between $\mathbf{r}_{ij}$ and the *z*-axis. For off-resonance RF irradiation, $\mathcal{H}_{\mathrm{II}}^0$ can be transformed to a tilted frame, with the new $\tilde{z}$-axis along the effective field $\mathbf{H}_{\mathrm{eff}} = H_1\mathbf{e}_x + [H_0 - (\omega_{\mathrm{RF}}/\gamma_{\mathrm{I}})]\mathbf{e}_z$ (Fig. 2.1B). This yields the tilted Hamiltonian [3]

$$\widetilde{\mathcal{H}}_{\mathrm{II}} = \tfrac{1}{2}(3\cos^2\theta - 1)\cdot\sum_{i<j} a_{ij}(3\tilde{I}_{zi}\tilde{I}_{zj} - \tilde{\mathbf{I}}_i\cdot\tilde{\mathbf{I}}_j) + \mathcal{H}_{\text{non secular}} \quad . \tag{2.3}$$

The non-secular part of this Hamiltonian contains terms that do not commute with $\sum_i \tilde{I}_{zi}$.

In Section 2.2, Lee-Goldburg (LG) irradiation is discussed, which can be used to suppress the strong homonuclear dipolar coupling between the *I* spins [3]. During LG irradiation, the RF frequency is chosen off-resonance in such a way that the effective field $\mathbf{H}_{\mathrm{eff}}$ is inclined at the magic angle θ_{m} = $\tan^{-1}(\sqrt{2})$ = 54.7° with respect to the static magnetic field $\mathbf{H}_0$ along the *z*-axis. As a result, the secular part of the homonuclear dipolar interaction vanishes due to the $\tfrac{1}{2}(3\cos^2\theta - 1)$ dependence in Eq. (2.3) [3]. With $\Delta\mathrm{LG} = \omega_{\mathrm{RF}} - \gamma_{\mathrm{I}}H_0$ and $|\omega_{1\mathrm{I}}| = \gamma_{\mathrm{I}}H_{1\mathrm{I}}$, a LG condition can be defined according to $\tan^{-1}(|\omega_{1\mathrm{I}}|/\Delta\mathrm{LG}) = \theta_{\mathrm{m}}$.

In Section 2.3, cross-polarization (CP) between an ^{1}H spin *I* and a ^{13}C spin *S* with suppression of the ^{1}H homonuclear dipolar interactions using LG irradiation is discussed. During CP, the two spin species are irradiated simultaneously on-resonance and $\theta = \pi/2$ for both (Fig 2.1A), yielding $\tfrac{1}{2}(3\cos^2\theta - 1) = -\tfrac{1}{2}$ in Eq. (2.3). In the doubly-rotating frame the spin Hamiltonian for a single spin *S* coupled to a set of interacting spins *I* during CP has the form [4]

$$\mathcal{H} = \omega_{1\mathrm{I}}\sum_i I_{xi} + \omega_{1\mathrm{S}}S_x - \tfrac{1}{2}\sum_{i<j} a_{ij}(3I_{xi}I_{xj} - \mathbf{I}_i\cdot\mathbf{I}_j) + \sum_i b_i I_{zi}S_z \tag{2.4}$$

with $\omega_{1\mathrm{I}} = -\gamma_{\mathrm{I}}H_{1\mathrm{I}}$ and $\omega_{1\mathrm{S}} = -\gamma_{\mathrm{S}}H_{1\mathrm{S}}$, which are negative for positive γ in a right-handed coordinate system [1]. This Hamiltonian is again truncated and contains only the part of the homonuclear dipolar interaction that commutes with the RF irradiation term along the *x*-axis. The last term represents the heteronuclear dipolar interaction $\mathcal{H}_{\mathrm{IS}}$ between the *S* spin and the *I* spins, with

$$b_i = -\left(\frac{\mu_0}{4\pi}\right)\frac{\gamma_{\mathrm{I}}\gamma_{\mathrm{S}}\hbar^2}{2}\frac{(3\cos^2\theta_i-1)}{r_i^3} \quad , \tag{2.5}$$

where r_i is the distance between S and I_i, and θ_i is the angle between the distance vector $\mathbf{r}_i$ and the z-axis. If the CP experiment is performed in such a way that the I spins are irradiated off-resonance at a Lee-Goldburg condition, the third term in Eq. (2.4) due to the homonuclear dipolar interaction between the I spins vanishes.

During magic-angle spinning (MAS) NMR, the sample is rotated with a spinning speed $\omega_\mathrm{r}/2\pi$ around an axis that is inclined at the magic angle θ_m with respect to the z-axis. With MAS, the form of the spin operators in the Hamiltonians in Eqs. (2.3) and (2.4) remains the same, while the coefficients of the homonuclear and heteronuclear dipolar interaction $a_{ij}(t)$ and $b_i(t)$ contain terms that vary periodically in time with frequencies $k\omega_\mathrm{r}$, with $k = -2, -1, +1, +2$ [5].

In the terminology of Maricq and Waugh, the homonuclear dipolar interactions between like spins in a rotating sample are homogeneous, which means that the corresponding Hamiltonian does not commute with itself at different times [6]. In practice, the spinning speed $\omega_\mathrm{r}/2\pi$ is often in the slow MAS range $(\omega_\mathrm{r}/2\pi)^2 << |\,\mathsf{H}_\mathrm{II}^0\,|^2$, and the homonuclear dipolar couplings lead to a homogeneous broadening of the NMR response. In contrast, the dipolar interactions between unlike spins are inhomogeneous, since the interaction terms commute with themselves at all times. In absence of homonuclear dipolar couplings, the total spin Hamiltonian comprising chemical shift and heteronuclear dipolar coupling terms is also inhomogeneous. During MAS, the spectrum associated with such a inhomogeneous Hamiltonian breaks up in a pattern of spinning side-bands, with the relative intensities of the spinning side-bands determined by both the dipolar interaction and the chemical shift [6,7]. At high MAS rates >10 kHz the heteronuclear dipolar interaction and the chemical shift anisotropy are substantially reduced by the sample spinning. During CP, the RF irradiation of the two spin species renders the total spin Hamiltonian homogeneous, even in absence of homonuclear dipolar interactions. This effectively leads to a recoupling of the IS interactions that are otherwise averaged by the MAS and the relevant dynamics will involve the homogeneous part of the spin Hamiltonian.

2.2 Lee-Goldburg irradiation

In Chapter 4 it will be demonstrated that the use of frequency- and phase-switched Lee-Goldburg irradiation (FSLG) during the ^{1}H evolution of 2-D heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy strongly enhances the proton resolution. In this section the effect of off-resonance RF irradiation on the spin Hamiltonian of strongly coupled I spins will be discussed.

For a static sample in high magnetic field, the secular term $\mathcal{H}_{\mathrm{II}}^{0}$ in Eq. (2.1) comprises the major source of line-broadening [8] and it needs to be attenuated for high-resolution spectroscopy. The application of an off-resonance RF field $H_{\mathrm{RF}}(t) = 2H_1\cos(\omega_{\mathrm{RF}}t + \psi_{\mathrm{RF}})$ to the *I* spins in the off-resonance rotating frame, rotating with angular frequency $\omega_{\mathrm{frame}} = -\omega_{\mathrm{RF}}$ along the *z*-axis of the laboratory frame, yields a truncated Hamiltonian

$$\mathcal{H} = \sum_i \{\delta_i I_{zi} - \gamma_{\mathrm{I}}(H_0 - \omega_{\mathrm{RF}}/\gamma_{\mathrm{I}})I_{zi} - \gamma_{\mathrm{I}}H_1 I_{xi}\} + \mathcal{H}_{\mathrm{II}}^{0} \quad , \tag{2.6}$$

with the chemical shift dispersion $\sum_i \delta_i I_{zi}$ included explicitly. When the RF power is high, $(\gamma H_{\mathrm{eff}})^2 >> |\mathcal{H}_{\mathrm{II}}^{0}|^2$, $\mathcal{H}$ can be transformed to a tilted rotating frame, defined by the transformation $\prod_i \exp\{-i\theta_{\mathrm{m}} I_{yi}\}$ [9], with the tilted $\tilde{z}$-axis along the direction of the effective field $\mathbf{H}_{\mathrm{eff}} = H_1\mathbf{e}_x + (H_0 - \omega_{\mathrm{RF}}/\gamma_{\mathrm{I}})\mathbf{e}_z$. In this tilted frame, $\mathcal{H}_{\mathrm{II}}^{0}$ transforms to five terms $\tilde{\mathcal{H}}_{\mathrm{II}}^{M}$, which yields, with $\omega_{\mathrm{eff}} = -\gamma_{\mathrm{I}}H_{\mathrm{eff}}$

$$\tilde{\mathcal{H}} = \sum_i \left\{(\delta_i\cos\theta + \omega_{\mathrm{eff}})\tilde{I}_{zi} - (\delta_i\sin\theta)\tilde{I}_{xi}\right\} + \sum_{M=-2}^{2}\lambda_M(\theta)\,\tilde{\mathcal{H}}_{\mathrm{II}}^{M} \quad , \tag{2.7}$$

where

$$\begin{cases} \lambda_0(\theta) = \tfrac{1}{2}(3\cos^2\theta - 1) \\ \lambda_{\pm1}(\theta) = -\tfrac{3}{2}\sin\theta\cos\theta \\ \lambda_{\pm2}(\theta) = \tfrac{3}{4}\sin^2\theta \end{cases} , \tag{2.8}$$

$$\begin{cases} \tilde{\mathcal{H}}_{\mathrm{II}}^{0} = \sum_{i<j} a_{ij}(3\tilde{I}_{zi}\tilde{I}_{zj} - \tilde{\mathbf{I}}_i\cdot\tilde{\mathbf{I}}_j) \\ \tilde{\mathcal{H}}_{\mathrm{II}}^{1} = \left(\tilde{\mathcal{H}}_{\mathrm{II}}^{-1}\right)^{\dagger} = \sum_{i<j} a_{ij}(\tilde{I}_{+i}\tilde{I}_{zj} + \tilde{I}_{zi}\tilde{I}_{+j}) \\ \tilde{\mathcal{H}}_{\mathrm{II}}^{2} = \left(\tilde{\mathcal{H}}_{\mathrm{II}}^{-2}\right)^{\dagger} = \sum_{i<j} a_{ij}\tilde{I}_{+i}\tilde{I}_{+j} \end{cases} . \tag{2.9}$$

From Eq. (2.9) the following commutation relations are obtained

$$\left[\sum_i \tilde{I}_{zi}, \tilde{\mathcal{H}}_{\mathrm{II}}^{M}\right] = M\,\tilde{\mathcal{H}}_{\mathrm{II}}^{M} \quad , \tag{2.10}$$

hence $\tilde{\mathcal{H}}_{\mathrm{II}}^{0}$ is the only secular term in Eq. (2.7) [3].

During FSLG irradiation, the RF field $H_{\mathrm{RF}}(t)$ is frequency switched between $\omega_{\pm\Delta\mathrm{LG}} = \gamma_{\mathrm{I}}H_0 \pm \Delta\mathrm{LG}$ with $\Delta\mathrm{LG} = \tfrac{1}{2}\sqrt{2}|\omega_{1\mathrm{I}}|$, and phase switched between $\psi_{\pm\Delta\mathrm{LG}}$, with $|\psi_{+\Delta\mathrm{LG}} - \psi_{-\Delta\mathrm{LG}}| = \pi$, after successive periods $\tau_{\mathrm{LG}} = \sqrt{2/3}\cdot(2\pi/|\omega_{1\mathrm{I}}|)$ [10,11]. As a result, ω_{eff} toggles between $\Delta\mathrm{LG}/\cos\theta$ and $-\Delta\mathrm{LG}/\cos\theta$. Subsequent transformation to the frame rotating with ω_{eff} along the $\tilde{z}$-axis is performed using the propagator

$$U_{\mathrm{eff}}(t)=\prod_i \exp(-i\omega_{\mathrm{eff}}t\tilde{I}_{zi})\ , \tag{2.11}$$

and leads to the two time-dependent Hamiltonians

$$\tilde{\mathsf{H}}^{\pm\Delta\mathrm{LG}}(t)=U_{\mathrm{eff}}(t)\,\tilde{\mathsf{H}}U_{\mathrm{eff}}^{-1}(t)-\omega_{\mathrm{eff}}\sum\nolimits_i\tilde{I}_{zi} \tag{2.12}$$

for $\omega_{\mathrm{eff}}=\pm\Delta\mathrm{LG}/\cos\theta$ during the successive periods τ_{LG} and

$$\begin{aligned}\tilde{\mathsf{H}}^{\pm\Delta\mathrm{LG}}(t)= &\sum_i\left\{(\delta_i\cos\theta)\tilde{I}_{zi}-\delta_i\sin\theta(\tilde{I}_{xi}\cos\omega_{\mathrm{eff}}t\mp\tilde{I}_{yi}\sin\omega_{\mathrm{eff}}t)\right\}\\ &+\lambda_0(\theta)\,\tilde{\mathsf{H}}_{\mathrm{II}}^{0}+\sum_{\substack{M=-2\\M\neq 0}}^{2}\exp(\pm M\omega_{\mathrm{eff}}t)\lambda_M(\theta)\,\tilde{\mathsf{H}}_{\mathrm{II}}^{M}\ .\end{aligned} \tag{2.13}$$

When the off-resonance frequency is set to a LG condition, the $\tilde{\mathsf{H}}_{\mathrm{II}}^{0}$ term vanishes due to the $\lambda_0(\theta)=\frac{1}{2}(3\cos^2\theta-1)$ dependence [3]. The remaining linewidth originates from the non-secular terms $\tilde{\mathsf{H}}_{\mathrm{II}}^{\pm1}$ and $\tilde{\mathsf{H}}_{\mathrm{II}}^{\pm2}$ [3]. These non-secular terms will be truncated when the tilted effective field is large, *i.e.*, $(\gamma H_{\mathrm{eff}})^2>>|\,\tilde{\mathsf{H}}_{\mathrm{II}}^{M}\,|^2$. The truncation will be most effective for the $\tilde{\mathsf{H}}_{\mathrm{II}}^{\pm2}$ terms which have a $2\omega_{\mathrm{eff}}$ time-dependence.

It is important to realize that all five terms $\tilde{\mathsf{H}}_{\mathrm{II}}^{M}$ in the tilted rotating frame originate from the single term $\mathsf{H}_{\mathrm{II}}^{0}$ in the rotating frame. This implies an additional advantage when $\mathsf{H}_{\mathrm{II}}^{0}$ is attenuated by a high static field, since in that case the terms $\tilde{\mathsf{H}}_{\mathrm{II}}^{M}$ should also be reduced. An increased proton chemical shift dispersion in high fields effectively attenuates the truncated homonuclear dipolar couplings and high magnetic fields have a line-narrowing effect on the MAS proton response. An experimental verification of this phenomenon is presented in Chapter 3.

The factor $\cos\theta=1/\sqrt{3}$ in Eq. (2.13) scales the chemical shift dispersion. The time-dependent transverse part will be refocused due to the sign reversal of ω_{eff} between the two successive periods. By setting $\omega_{\mathrm{eff}}\tau_{\mathrm{LG}}=2\pi$, the two frames rotating with $\pm\omega_{\mathrm{eff}}$ along the $\tilde{z}$-axis will coincide with the tilted rotating frame at the beginning and at the end of each period τ_{LG}. Hence the evolution of the spin system can be monitored from the $\pm\omega_{\mathrm{eff}}$ rotating frames, in which it evolves under the two Hamiltonians according to Eq. (2.13), and the proton magnetization will effectively evolve in the plane perpendicular to the tilted $\tilde{z}$-axis.

For a spinning sample, the coefficient a_{ij} of the homonuclear dipolar interaction in Eq. (2.1) is time-dependent. Since we assumed above that the RF power is high in the sense $(\gamma H_{\mathrm{eff}})^2>>|\,\mathsf{H}_{\mathrm{II}}^{0}\,|^2$, the secular part of the Hamiltonian (2.13) will be suppressed due to the $\lambda_0(\theta)=\frac{1}{2}(3\cos^2\theta-1)$ dependence. On the other hand, the non-secular terms with $M\neq 0$, in Eq. (2.13) will be suppressed over a full FSLG cycle $2\cdot\tau_{\mathrm{LG}}$, provided that the cycle time is short compared to the period of the sample rotation. In theory, this implies that the RF power should be sufficiently high, since this will both lead to a short $2\cdot\tau_{\mathrm{LG}}$ and to a more complete truncation of the non-secular part of the Hamiltonian. In practice, however, it turns out that in high magnetic fields a moderately high RF power corresponding with an ^{1}H nutation frequency of ~ 60 kHz is already sufficient to achieve good resolution with FSLG irradiation at MAS rates up to 15 kHz, as will be shown in Chapter 4.

2.3 Lee-Goldburg cross-polarization

In Chapter 5 the time-evolution of the signal intensity of a ^{13}C spin *S* during CP/MAS with Lee-Goldburg irradiation applied to a small finite number of *I* spins (^{1}H) is analyzed numerically. In this section, we present a first-order analytical theoretical description of a two-spin system that consists of a spin *S* coupled to a single spin *I*, rotating with a MAS rate $\omega_{\mathrm{r}}/2\pi$.

The spin Hamiltonian in the doubly rotating frame for the two-spin system during the mixing time of the Lee-Goldburg CP (LG-CP) experiment can be represented as

$$\mathcal{H} = \omega_{1\mathrm{I}} I_x + \omega_{1\mathrm{S}} S_x - \Delta\mathrm{LG} I_z + b(t) I_z S_z \quad , \tag{2.14}$$

where $\Delta\mathrm{LG} = \omega_{\mathrm{RF}} - |\omega_{0\mathrm{I}}|$, with $\omega_{0\mathrm{I}} = -\gamma_{\mathrm{I}} H_{0\mathrm{I}}$, the frequency offset for the RF irradiation applied to the *I* spin [3,12,13]. The last term in Eq. (2.14) represents the heteronuclear dipolar interaction $\mathcal{H}_{\mathrm{IS}}$ between the two spins, with [14]

$$\begin{gathered} b(t) = 2\omega_{\mathrm{d}}\left[G_1\cos(\omega_{\mathrm{r}}t+\phi) + G_2\cos(2\omega_{\mathrm{r}}t+2\phi)\right] \quad , \\ \omega_{\mathrm{d}} = -\frac{\mu_0}{4\pi}\frac{\gamma_{\mathrm{I}}\gamma_{\mathrm{S}}\hbar^2}{r_{\mathrm{IS}}^3} \quad . \end{gathered} \tag{2.15}$$

In the tilted frame, defined by the transformation $\exp\{-i\theta_{\mathrm{m}} I_y\}\exp\{-i(\pi/2)S_y\}$, the tilted spin Hamiltonian can be written as $\tilde{\mathcal{H}} = \tilde{\mathcal{H}}_0 + \tilde{\mathcal{H}}_1(t)$, where

$$\begin{gathered} \tilde{\mathcal{H}}_0 = \omega_{\mathrm{eff,I}}\tilde{I}_z + \omega_{1\mathrm{S}}\tilde{S}_z \quad , \\ \tilde{\mathcal{H}}_1(t) = b(t)\left(\sin(\theta_{\mathrm{m}})\tilde{I}_x\tilde{S}_x - \cos(\theta_{\mathrm{m}})\tilde{I}_z\tilde{S}_x\right) \quad , \end{gathered} \tag{2.16}$$

with the effective field $\omega_{\mathrm{eff,I}} = -(\omega_{1\mathrm{I}}^2 + \Delta\mathrm{LG}^2)^{1/2}$. The time-independent part $\tilde{\mathcal{H}}_0$ can be removed from the Hamiltonian by transformation to the interaction frame, according to $\tilde{\mathcal{H}}_1^* = \exp\{i\tilde{\mathcal{H}}_0 t\}\tilde{\mathcal{H}}_1\exp\{-i\tilde{\mathcal{H}}_0 t\}$. For the *n* = ±1 Hartmann-Hahn (HH) matching condition,

$\omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}} = \pm\omega_{\mathrm{r}}$, the flip-flop part representing the heteronuclear dipolar interaction has the form

$$\begin{aligned}\tilde{\mathcal{H}}^* = \frac{\omega_{\mathrm{d}}}{4} G_1 \sin\theta_{\mathrm{m}} \cdot \Big[\tilde{I}_+\tilde{S}_-\big\{&\exp(i(\omega_{\mathrm{r}} - \omega_{\mathrm{eff,I}} + \omega_{\mathrm{1S}})t + i\phi) + \exp(-i(\omega_{\mathrm{r}} + \omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}})t - i\phi)\big\} \\ + \tilde{I}_-\tilde{S}_+\big\{&\exp(i(\omega_{\mathrm{r}} + \omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}})t + i\phi) + \exp(-i(\omega_{\mathrm{r}} - \omega_{\mathrm{eff,I}} + \omega_{\mathrm{1S}})t - i\phi)\big\}\end{aligned} \tag{2.17}$$

which leads to a time-independent Hamiltonian

$$\tilde{\mathcal{H}}^* = \begin{cases} \dfrac{\omega_{\mathrm{d}}}{4} G_1 \sin\theta_{\mathrm{m}} \cdot \Big[\tilde{I}_+\tilde{S}_- \exp(i\phi) + \tilde{I}_-\tilde{S}_+ \exp(-i\phi)\Big] & \text{for } \ \omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}} = +\omega_{\mathrm{r}} \\ \dfrac{\omega_{\mathrm{d}}}{4} G_1 \sin\theta_{\mathrm{m}} \cdot \Big[\tilde{I}_+\tilde{S}_- \exp(-i\phi) + \tilde{I}_-\tilde{S}_+ \exp(i\phi)\Big] & \text{for } \ \omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}} = -\omega_{\mathrm{r}} \end{cases} . \tag{2.18}$$

Likewise, the $n = \pm 2$ HH matching conditions, $\omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}} = \pm 2\omega_{\mathrm{r}}$, yield a Hamiltonian of the form

$$\begin{aligned}\tilde{\mathcal{H}}^* = \frac{\omega_{\mathrm{d}}}{4} G_2 \sin\theta_{\mathrm{m}} \cdot \Big[\tilde{I}_+\tilde{S}_-\big\{&\exp(i(2\omega_{\mathrm{r}} - \omega_{\mathrm{eff,I}} + \omega_{\mathrm{1S}})t + 2i\phi) + \exp(-i(2\omega_{\mathrm{r}} + \omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}})t - 2i\phi)\big\} \\ + \tilde{I}_-\tilde{S}_+\big\{&\exp(i(2\omega_{\mathrm{r}} + \omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}})t + 2i\phi) + \exp(-i(2\omega_{\mathrm{r}} - \omega_{\mathrm{eff,I}} + \omega_{\mathrm{1S}})t - 2i\phi)\big\}\end{aligned} \tag{2.19}$$

with time-independent terms

$$\tilde{\mathcal{H}}^* = \begin{cases} \dfrac{\omega_{\mathrm{d}}}{4} G_2 \sin\theta_{\mathrm{m}} \cdot \Big[\tilde{I}_+\tilde{S}_- \exp(2i\phi) + \tilde{I}_-\tilde{S}_+ \exp(-2i\phi)\Big] & \text{for } \ \omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}} = +2\omega_{\mathrm{r}} \\ \dfrac{\omega_{\mathrm{d}}}{4} G_2 \sin\theta_{\mathrm{m}} \cdot \Big[\tilde{I}_+\tilde{S}_- \exp(-2i\phi) + \tilde{I}_-\tilde{S}_+ \exp(2i\phi)\Big] & \text{for } \ \omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}} = -2\omega_{\mathrm{r}} \end{cases} . \tag{2.20}$$

The factor $\sin\theta_{\mathrm{m}}$ in these expressions for $\tilde{\mathcal{H}}^*$ scales the heteronuclear dipolar interaction. Only the flip-flop term is relevant for polarization transfer. The single quantum term $-b(t)\cos(\theta_{\mathrm{m}})\tilde{I}_z\tilde{S}_x$ in Eq. (2.16) does not lead to polarization transfer and has been discarded. In addition, double quantum terms of the form $\tilde{I}_+\tilde{S}_+$ and $\tilde{I}_-\tilde{S}_-$ have been left out in Eqs. (2.17)-(2.20), since for the $n = \pm 1$ and ± 2 HH matching conditions, these terms connect diagonal elements in the product basis $|m_{\mathrm{I}}m_{\mathrm{S}}\rangle$ that have a large difference in energy and can be discarded according to perturbation theory. Finally, rapidly oscillating terms of the form $\cos\omega_{\mathrm{r}}t,\ \cos 2\omega_{\mathrm{r}}t,\ etc.$, are neglected, since they should be small on average compared to the time-independent term. This is justified as long $\omega_{\mathrm{r}} >> |\tfrac{1}{4}G_{|n|}\omega_{\mathrm{d}}\sin\theta_{\mathrm{m}}|$.

As an example, we will now study the time-dependent *S* spin magnetization build-up during LG-CP, adjusted for the $\omega_{\mathrm{eff,I}} - \omega_{\mathrm{1S}} = +\omega_{\mathrm{r}}$ HH matching condition. The derivation of the expressions for the other matching conditions proceeds in a fully analogous way. We will write the Hamiltonian for the heteronuclear dipolar coupling as

$$\tilde{\mathcal{H}}^* = \frac{\delta}{4}\left[\tilde{I}_+\tilde{S}_- \exp(i\phi) + \tilde{I}_-\tilde{S}_+ \exp(-i\phi)\right] \ , \tag{2.21}$$

with $\delta \equiv G_1\omega_\mathrm{d} \sin\theta_\mathrm{m}$. Following excitation of the I spins, the density operator at the beginning of the LG-CP period in the interaction frame can be written as $\rho_0 = -Z^{-1}\beta_\mathrm{L}\omega_{0\mathrm{I}}\tilde{I}_z$, with $\beta_\mathrm{L} = 1/k_\mathrm{B}T$. The time-evolution of ρ under $\tilde{\mathcal{H}}^*$ can be evaluated using $\rho(t) = \exp(-i\,\tilde{\mathcal{H}}^* t)\rho_0 \exp(i\,\tilde{\mathcal{H}}^* t)$, which leads to the following expression, in matrix-notation in the product basis $\left|m_\mathrm{I} m_\mathrm{S}\right\rangle$,

$$\rho(t) = -\tfrac{1}{4}Z^{-1}\beta_\mathrm{L}\omega_{0\mathrm{I}} \cdot \begin{array}{c} \begin{pmatrix} \frac{1}{2} & 0 & 0 & 0 \\ 0 & -\frac{1}{2} & 0 & 0 \\ 0 & 0 & \frac{1}{2}\cos\frac{1}{2}\delta t & \frac{i}{2}\exp(i\phi)\sin\frac{1}{2}\delta t \\ 0 & 0 & -\frac{i}{2}\exp(-i\phi)\sin\frac{1}{2}\delta t & -\frac{1}{2}\cos\frac{1}{2}\delta t \end{pmatrix} \end{array} \begin{array}{l} \left|++\right\rangle \\ \left|--\right\rangle \\ \left|+-\right\rangle \\ \left|-+\right\rangle \end{array} \tag{2.22}$$

The S-spin signal $S(t)$ can be calculated by evaluation of the expectation value of $\tilde{S}_z$, according to $<\tilde{S}_z(t)> = \mathrm{Tr}(\rho(t)\tilde{S}_z)$, which leads to

$$S(t) = -\tfrac{1}{4}Z^{-1}\beta_\mathrm{L}\omega_{0\mathrm{I}}\left(1-\cos\tfrac{1}{2}\delta t\right) = -\tfrac{1}{4}Z^{-1}\beta_\mathrm{L}\omega_{0\mathrm{I}}(1-\tfrac{1}{2}\exp(+\tfrac{i}{2}\delta t)-\tfrac{1}{2}\exp(-\tfrac{i}{2}\delta t)) \ . \tag{2.23}$$

Hence, during the LG-CP, the S-spin signal oscillates with angular frequency $\frac{1}{2}\delta$ around the average value $-\frac{1}{4}Z^{-1}\beta_\mathrm{L}\omega_{0\mathrm{I}}$, and Fourier transformation of $S(t) + \frac{1}{4}Z^{-1}\beta_\mathrm{L}\omega_{0\mathrm{I}}$ results in a spectrum with two singularities at frequencies $\omega = \pm\frac{1}{2}\delta = \pm\frac{1}{2}G_1\omega_\mathrm{d}\sin\theta_\mathrm{m}$.

The expectation value for $<\tilde{S}_z(t)>$ has been evaluated for a single crystallite. For a powder sample, it should be integrated over all crystallites. With $G_1 = \frac{3}{4}\sin(2\theta_\mathrm{m})\sin(2\theta_{ij})$ [14], and θ_{ij} the angle that the internuclear vector connecting spins i and j makes with the rotor axis, we can write $\omega(\theta_{ij}) = \frac{1}{2}\delta_0 \sin(2\theta_{ij})$, where we defined $\delta_0 \equiv \frac{3}{4}\omega_\mathrm{d} \sin(\theta_\mathrm{m})\sin(2\theta_\mathrm{m}) = \omega_\mathrm{d} \cos(\theta_\mathrm{m})$. The powder average can be evaluated using $S(\omega) = P(\theta_{ij}) \Big/ \left| d\theta_{ij}(\omega)/d\omega \right|$, with $P(\theta_{ij})$ the angular distribution function [15]. Since the interaction is axially symmetric, we simply have $P(\theta_{ij}) = \sin\theta_{ij}$ [15], which leads to

$$S(\omega) = \frac{\sqrt{\frac{1}{2} - \sqrt{\frac{1}{4} - (\omega/\delta_0)^2}} + \sqrt{\frac{1}{2} + \sqrt{\frac{1}{4} - (\omega/\delta_0)^2}}}{2\sqrt{\frac{1}{4} - (\omega/\delta_0)^2}} \quad , \tag{2.24}$$

with $-\delta_0/2 \le \omega \le \delta_0/2$. This powder spectrum is plotted in Fig. 2.2. It represents the LG-CP *S*-spin spectrum for the *IS* spin-pair, which is obtained after Fourier transformation of the time-oscillating *S*-spin signal build-up [16]. The shape resembles a static Pake-pattern [17], although the characteristic high-frequency 'ears' are missing. The frequency splitting between the two maxima equals δ_0, which is related to the heteronuclear distance r_IS via ω_d in Eq. (2.15).

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Chapter 3

High magnetic field and high-speed MAS for enhanced proton resolution in heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy

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3.1 Introduction

It is generally accepted that ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy in solids requires the application of multiple-pulse techniques to suppress the strong homonuclear dipolar interactions between the abundant protons in order to achieve a sufficient line-narrowing in the ^{1}H dimension. In this chapter, it is shown that a high magnetic field strength, in combination with fast magic-angle spinning (MAS), can already improve the proton resolution to such an extent that ^{1}H-^{13}C correlations and proton chemical shifts can be directly obtained from 2-dimensional (2-D) spectra, collected without the application of ^{1}H homonuclear decoupling schemes during the proton evolution. In addition, it will be shown that in favourable cases, heteronuclear correlations between molecules, *e.g.* intermolecular hydrogen bonds, can be obtained with the simplest possible pulse schemes.

To demonstrate this, the most straightforward and very simple 'cross-polarization wideline-separation' (CP/WISE) technique is used [1] (Fig. 3.1A) to study a small model compound, labeled tyrosine (Fig. 3.1B). The CP/WISE pulse scheme does not apply any proton line-narrowing method. Following a $\pi/2$ pulse on the protons, a time increment t_1 before the CP allows for the observation of the proton evolution with detection via the carbons. Since the protons are allowed to evolve freely during t_1, the technique enables a comparison of the ^{1}H homonuclear dipolar line-broadening under various experimental conditions. The purpose of this chapter is to demonstrate the effectiveness of increasingly higher fields in combination with rapid spinning for enhancing the proton resolution in heteronuclear correlation spectroscopy. We recognize that the ^{1}H resolution in the correlation spectra presented below is still limited and that additional improvement can be achieved by implementation of multiple-pulse line-narrowing techniques to suppress the strong ^{1}H homonuclear dipolar couplings. However, the point that we would like to make here is that almost every pulse scheme involving direct or indirect detection of protons can, and in fact should, benefit from the additional resolution enhancement provided by a strong magnetic field and a high MAS rate. Hence, we feel that in the design of new pulse schemes, their applicability at high magnetic field strengths and at high MAS speeds should be an important consideration. In Chapter 4, a more sophisticated technique exploiting frequency-switched Lee-Goldburg irradiation during the ^{1}H evolution will be presented, providing excellent proton resolution in 2-D correlation spectra recorded at high fields and with fast MAS [2].

3.2 Experimental

The CP/WISE correlation spectra have been recorded using MSL-400 (9.4 T) and DMX-600 (14.1 T) spectrometers, equipped with 4mm triple- and double-resonance CP/MAS probes, respectively (Bruker, Karlsruhe, Germany). A home-built spinning-speed controller was used to keep the spinning speed $\omega_r/2\pi$ constant to within a few Hz [3]. A ramped-amplitude CP sequence (RAMP-CP) was implemented to restore a broader Hartmann-Hahn matching profile at high MAS frequencies [4]. To avoid homonuclear coherence transfer processes in both proton and uniformly ^{13}C-enriched carbon spin reservoirs during CP and to guarantee that each carbon will effectively receive its magnetization only from the neighbouring protons, the RAMP-CP mixing times were kept short, typically <500 µs. In Chapters 4 and 5

Fig. 3.1: CP/WISE pulse scheme used for heteronuclear (^1H-^{13}C) dipolar correlation experiments (A) and chemical structure with IUPAC numbering scheme for tyrosine (B).

a different route will be followed, where heteronuclear polarization transfer is established under simultaneous ^{1}H homonuclear decoupling. This allows for the use of longer CP transfer times without loss of selectivity in the correlation experiment. At the highest field strength, the protons are decoupled from the carbons during the acquisition time t_2 by use of the two-pulse phase-modulation (TPPM) decoupling scheme [5]. The phase-modulation angle and pulse length for the TPPM decoupling are 20 degrees and 8 μs, respectively. Finally, phase-sensitive detection in the ^{1}H dimension has been simulated by varying the proton preparation pulse in a TPPI scheme [6].

Fig. 3.2: Contour plots of 2-D heteronuclear dipolar correlation spectra obtained from [U-^{13}C] L-tyrosine·HCl with the pulse sequence of Fig. 3.1A, at magnetic fields of 9.4 T (A) and 14.1 T (B). The arrow in (B) indicates intermolecular polarization transfer from a hydrogen-bonded proton.

The t_1 acquisition time for both 2-D spectra is 1.066 ms. Prior to Fourier transformation, a sine-squared apodization in the proton dimension was applied in the t_1 domain, phase-shifted by $\pi/5$. A Lorentz-Gauss window with the maximum at 0.1 of the acquisition time and a broadening of 50 Hz was applied in the t_2 dimension.

3.3 Results and discussion

Fig. 3.2 shows 2-D heteronuclear (^{1}H-^{13}C) dipolar correlation spectra from a preparation of uniformly ^{13}C-enriched ([U-^{13}C]) L-tyrosine·HCl (Cambridge Isotopes), recorded at higher magnetic field strengths of 9.4 T (Fig. 3.2A) and 14.1 T (Fig. 3.2B). The spinning speed for the measurement at 9.4 T is 14.5 kHz, while the 14.1 T dataset has been obtained with a MAS rate $\omega_r/2\pi$ = 15.0 kHz. The RAMP-CP mixing time for the experiments was fixed at 100 μs. This short CP mixing time ensures that predominantly the protons in the immediate vicinity of a particular carbon contribute to the signal build-up. It is clear from the two datasets shown in Fig. 3.2 that the resolution in both dimensions, which is already quite good at the moderately high field of 9.4 T, is considerably improved when the field strength is increased to 14.1 T. In particular the downfield ^{1}H signals correlated with 1-^{13}C at 172.2 ppm and with 4'-^{13}C at 151.6 ppm are much better resolved at the highest field strength. The improved resolution in the ^{13}C dimension in Fig. 3.2B is due to a combination of the higher field and the good performance of the TPPM decoupling. From Fig. 3.2B a complete assignment of the proton chemical shifts is readily obtained. This assignment is listed in Table 3.1.

In order to compare the resolution in the proton dimension for the two different field strengths in more detail, vertical slices representing the proton signals correlated with separate carbons were extracted from the two 2-D spectra (Fig. 3.3). For an objective comparison of the linewidths, the data have been processed without apodization in t_1 and were plotted on a Hz scale. Fig. 3.3A shows the proton responses correlated with the 1-^{13}C at 172.2 ppm, which mainly represent the 1-OO^{1}H signals, Fig. 3.3B the 2-^{1}H resonances correlated with 2-^{13}C at 56.3 ppm, and Fig. 3.3C the signals from the 5'-^{1}H observed via the aromatic 5'-^{13}C at 118.0 ppm. The ^{1}H slices obtained from the spectrum recorded at 9.4 T are represented with dashed lines, while the solid lines label the data at a field of 14.1 T. As can be verified from the slices, the proton resolution is enhanced for the 2-D correlation spectrum recorded at the strongest field of 14.1 T. In particular, the broad foot in the spectra recorded at a field of 9.4 T appears not to be present in the high-field data.

The linewidths for the proton resonances at the two fields have been determined and are listed in Table 3.1. For all protons the linewidth in Hz is smaller at a field of 14.1 T than at 9.4 T. However, the effective gain in proton resolution is different for the various protons. For instance, the resolution enhancement expressed in terms of the linewidth in ppm for the downfield shifted 1-OO^{1}H, 4’-O^{1}H and 6’-^{1}H is the highest and exceeds a factor two. In particular the linewidth of 1.7 ppm for the carboxylic hydrogen at the magnetic field of

Table 3.1: Solid-state NMR proton shifts σ_i and linewidths of L-tyrosine·HCl

		Linewidth (Hz) [a]			Linewidth (ppm) [a]		
		9.4 T	14.1 T	11.8 T [c]	9.4 T	14.1 T	11.8 T [c]
Position	σ_i (ppm) [b]	14.5 kHz	15.0 kHz	25.0 kHz	14.5 kHz	15.0 kHz	25.0 kHz
1-OO^{1}H	13.0	1450	1000	700	3.6	1.7	1.4
2-^{1}H	4.1	2740	2200	1650	6.9	3.7	3.3
3-^{1}H$_2$	1	~8600	~7900	~6600	~21.5	~13	~13
2’-^{1}H	5.3	3130	2840	2040	7.8	4.7	4.1
3’-^{1}H	6.7	3710	3400	2380	9.3	5.7	4.8
4’-O^{1}H	10.3	2500	1580	1060	6.3	2.6	2.1
5’-^{1}H	4.8	2730	2080	1550	6.8	3.5	3.1
6’-^{1}H	7.9	3900	2750	2250	9.8	4.6	4.5

[a] Full width at half height (FWHH), from spectra processed without apodization in t_1. [b] At a magnetic field of 14.1 T. [c] Data supplied by and used with kind permission of Bruker [8].

14.1 T compares well with the typical widths achieved with more elaborate line-narrowing techniques. The improvement for the other protons is within the range 1.7-1.9, with exception of the 3’-^{1}H resonance for which a factor 1.6 is found. The enhancement of the overall proton resolution is obviously more than the factor 1.5 due to the increase in the chemical shift dispersion alone. Since the field strength is the only parameter that was varied, the reduction of the linewidth can readily be related to an effect of the increased magnetic field. It can thus be concluded that the high-field resolution enhancement is non-linear, since apart from the increased proton chemical shift dispersion, an additional narrowing of the proton resonances is observed, induced by the increased magnetic field strength itself.

The ^{1}H shift of 13 ppm for the 1-OOH correlated with 1-^{13}C is in the characteristic downfield range of 8-15 ppm for hydrogen-bonded protons. From the neutron diffraction structure of tyrosine·HCl, it is known that the carboxylic proton forms a hydrogen bond to the phenolic oxygen of the 4’ hydroxyl group [7]. The importance of the hydrogen-bonded proton in the initial stages of the CP transfer is evident from the observation of a weak intermolecular correlation of the 4’-^{13}C with the carboxylic proton around 13 ppm in the dataset obtained at 14.1 T, indicated with an arrow in Fig. 3.2B. Since the 2-D dataset was recorded with a short CP contact time of only 100 μs, the occurrence of the intermolecular correlation between the 4’-^{13}C and the 1-OO^{1}H resonance at 13 ppm means that the CP polarization transfer from the carboxylic proton along the hydrogen bond to the 4’-^{13}C in its phenolic hydrogen-bonding partner must be quite efficient. A more elaborate study of the initial CP transfer events responsible for the 4'-^{13}C signal build-up will be provided in Chapter 5.

In order to study the effect of ultra-high MAS speeds on the ^{1}H response, proton linewidths obtained from an ^{1}H-^{13}C heteronuclear CP/WISE spectrum recorded with a spinning speed $\omega_r/2\pi$ = 25.0 kHz at a field of 11.8 T [8] have been included in Table 3.1. The high MAS rate has a pronounced narrowing effect on the proton resonances. The lines are better resolved than for the spectrum recorded with a lower MAS speed of 15.0 kHz at 14.1 T. Expressed in terms of the linewidth in ppm, the resolution enhancement is 1.1-1.2, almost uniform for the various proton resonances.

In the description of the NMR response of strongly dipolar-coupled protons in solids, the small ^{1}H chemical shift dispersion is usually neglected. This is definitely correct for lower magnetic field strengths and for moderately high spinning speeds. In the strong dipolar limit, the proton energy levels are nearly degenerate and the T_2-type relaxation responsible for the large proton linewidth easily proceeds through the rigid and strongly coupled dipolar-coupling lattice. It is obvious that this approach must break down at some point when the magnetic field becomes sufficiently strong or the MAS rate sufficiently high. It has been shown for small spin clusters that the dipolar line-broadening reduces if the chemical shift difference increases [9]. It has been demonstrated for a two-proton system, that the FWHH of the ^{1}H powder lineshape varies inversely proportional to the chemical shift difference [9]. Hence an increase of the external magnetic field should have the largest effect on the linewidth of protons whose resonance frequency is well separated from the main response of the spectrum. The observed high-field narrowing of the downfield shifted 1-OO^{1}H, 4'-O^{1}H and 6'-^{1}H tyrosine resonances is fully in line with these considerations. On the other hand, the magnetic field will have less effect on the linewidth of protons in the aliphatic region of the spectrum, where the resonances are nearly degenerate. For instance, the 3-^{1}H$_2$ signal correlated with 3-^{13}C at 36.6 ppm is quite broad even at the highest field of 14.1 T. This is the only methylene moiety in the tyrosine molecule, that has two strongly coupled protons with only slightly differing chemical shifts [10].

Concerning the MAS rate, it has been calculated in a theoretical approach that the energy spread of the levels due to the ^{1}H homonuclear interactions reduces for increasing spinning speed. This will effectively decouple the protons and will produce a narrowing effect on the proton response [9,11]. The CP/WISE spectrum from [U-^{13}C] L-tyrosine·HCl recorded with a high MAS frequency of 25.0 kHz at a field of 11.8 T [8] is well resolved and provides the best proton resolution without the use of line-narrowing techniques (cf. Table 3.1). MAS rates up to 35 kHz are achievable today, and the effective decoupling of protons with ultra-high speed MAS offers promising possibilities for future solid-state NMR research. However, in the MAS NMR research of biological preparations, the use of ultra-high spinning rates may still be somewhat limited, since considerable centripetal forces will be experienced which can impose deformation or degradation of the sample.

A final line-narrowing mechanism we want to draw attention to is connected with the density or proximity of the coupled protons. Since the dipolar interactions rapidly decrease with the distance, the ^{1}H homonuclear dipolar line-broadening will be attenuated in systems that are effectively dilute in protons. For example, in Chapter 7 it is demonstrated that for uniformly ^{13}C-enriched chlorophyll systems with relatively few protons attached to the macro-aromatic ring, CP/WISE heteronuclear spectroscopy allows an unambiguous assignment of almost all ring protons. Additional ^{1}H dilution can be accomplished by random substitution of a part of the protons with deuterons and it has been shown recently that this can lead also to a considerable improvement of proton resolution in 2-D heteronuclear dipolar correlation spectroscopy [12].

3.4 Conclusions

In this chapter it is demonstrated for a preparation of uniformly ^{13}C-enriched L-tyrosine·HCl salt that the resolution enhancement in the dimension of the protons in 2-D heteronuclear dipolar correlation spectra obtained with higher magnetic field strengths and fast MAS is sufficient to determine the proton chemical shifts directly from the 2-D experiment. In addition, intermolecular heteronuclear correlations and hydrogen-bonding characteristics in the solid state can already be determined with very simple pulse schemes. It is anticipated that further improvement of the proton resolution can be obtained with increasingly higher magnetic field strengths. This will be advantageous for heteronuclear dipolar correlation spectroscopy exploiting multiple-pulse proton decoupling techniques to achieve additional attenuation of the proton linewidths, and will be particularly useful for the development of future proton assignment strategies and structure determination.

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Figure captions:

Fig. 3.1: CP/WISE pulse scheme used for heteronuclear (^1H-^{13}C) dipolar correlation experiments (A) and chemical structure with IUPAC numbering scheme for tyrosine (B)

Fig. 3.2: Contour plots of 2-D heteronuclear dipolar correlation spectra obtained from [U-^{13}C] L-tyrosine·HCl with the pulse sequence of Fig. 3.1A, in magnetic fields of 9.4 T (A) and 14.1 T (B). The arrow in (B) indicates intermolecular polarization transfer from a hydrogen bonded proton.

Fig. 3.3: Proton slices extracted from 2-D ^1H-^{13}C heteronuclear dipolar correlation spectra recorded from [U-^{13}C] L-tyrosine·HCl, processed without apodization in the t_1 domain, for 1-OOH (A), (B), (C). The spectra are acquired in fields of 9.4 T (dashed lines) and 14.1 T (dashed lines).

Chapter 4

Heteronuclear dipolar correlation spectroscopy with frequency-switched Lee-Goldburg homonuclear decoupling

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4.1 Introduction

Heteronuclear dipolar correlation spectroscopy is an important tool for resolution enhancement in multidimensional NMR spectroscopy. For solids, line narrowing in the proton dimension is generally achieved by application of 'combination of rotation and multiple-pulse spectroscopy' (CRAMPS) techniques to suppress the strong homonuclear dipolar interactions between the abundant protons [1-11]. However, in high-speed MAS NMR research of, for instance, biological systems, the use of multiple-pulse techniques to study the proton chemical shift dispersion is limited for several reasons. First, the application of multiple-pulse techniques usually requires cycle times that are short compared to the rotor period. This effectively puts a restriction on the spinning speeds that can be used and the efficacy of the MAS averaging. It also limits the possibilities for application of CRAMPS techniques in high-field MAS, where high spinning speeds are required to suppress the chemical shift anisotropy and to obtain sufficient resolution in the multidimensional spectra. For instance, WAHUHA-4 [6], which has the shortest cycle time, performs well only at moderately high spinning rates [12]. More elaborate CRAMPS sequences like MREV-8 [8] or BR-24 [10] require longer cycle times, and the possibilities for application in high-speed MAS will be even less favourable. In addition, biological MAS NMR research often has to be performed at low temperatures, which makes the use of 'tuned-up' multiple-pulse sequences impractical.

In the previous chapter it was demonstrated that from straightforward 2-dimensional (2-D) high-speed MAS heteronuclear (^{1}H-^{13}C) CP/WISE spectra collected at a high magnetic field and without any homonuclear decoupling scheme during the proton evolution, ^{1}H-^{13}C correlations and proton chemical shifts in the solid state can be obtained directly. At high field the homonuclear dipolar line-broadening is reduced by the increased chemical shift dispersion. This leads to an enhanced resolution for protons with NMR signals that are considerably shifted, *e.g.* carboxylic or hydroxylic protons. On the other hand, the resolution in the upfield region is not optimal, due to a combination of strong homonuclear dipolar couplings and small shift dispersion for most aliphatic protons. To obtain a good proton resolution over the entire spectrum, the application of a technique to suppress the homonuclear dipolar interactions between the abundant protons is necessary.

4.2 Frequency-switched Lee-Goldburg irradiation

The application of an RF field $H_{\mathrm{RF}}(t) = 2H_1\cos(\omega_{\pm\Delta\mathrm{LG}}t + \psi_{\pm\Delta\mathrm{LG}})$ off-resonance according to one of the Lee-Goldburg (LG) conditions $\pm\Delta\mathrm{LG} = \omega_{\pm\Delta\mathrm{LG}} - \gamma H_0 = \pm\frac{1}{2}\sqrt{2}|\omega_1|$, with $\omega_1 = -\gamma H_1$, produces in the rotating frame an effective field $\mathbf{H}_{\mathrm{eff}}$ that is inclined at the magic angle $\theta_{\mathrm{m}} = \tan^{-1}(\sqrt{2})$ to the static field $\mathbf{H}_0$ [13]. It was shown by Lee and Goldburg that the rapid spin precession around this magic-angle axis effectively results in a cancellation of the secular part of the homonuclear dipolar interactions in the tilted rotating frame [13]. It has been demonstrated that when the RF field is both frequency switched between $\omega_{\pm\Delta\mathrm{LG}}$ and phase switched between $\psi_{\pm\Delta\mathrm{LG}}$, with $|\psi_{+\Delta\mathrm{LG}} - \psi_{-\Delta\mathrm{LG}}| = \pi$ (FSLG, Fig. 4.1) after successive periods $\tau_{\mathrm{LG}} = \sqrt{2/3}\cdot(2\pi/|\omega_1|)$, corresponding with the time to complete a 2π rotation around the tilted axis, the efficiency of the homonuclear decoupling is significantly improved [14,15]. In this thesis, the off-resonance frequency $\omega_{+\Delta\mathrm{LG}}$ for the positive offset +ΔLG corresponds to a increased RF-carrier frequency. For spins with a positive γ, pulses appear static in a frame rotating with $\omega_{\mathrm{frame}} = -\omega_{\pm\Delta\mathrm{LG}}$, with $\omega_{\pm\Delta\mathrm{LG}}$ positive by definition [16]. Thus for the offset +ΔLG, ω_{frame} becomes more negative, and viewed from this frame the spins precess in a positive sense, *i.e.* anticlockwise, which should be represented by a field along the *z*-axis in the negative direction (Fig. 4.1A). Likewise, the offset -ΔLG corresponds to a field in the positive *z*-direction (Fig. 4.1B). A short theoretical description of the FSLG irradiation is provided in Chapter 2.

FSLG can be used for homonuclear (^{1}H-^{1}H) decoupling in a heteronuclear correlation experiment, since it is not necessary to observe the protons directly [17]. The LG cycle time τ_{LG} equals the inverse of the nutation frequency $\nu_{\mathrm{eff}} = \gamma H_{\mathrm{eff}} / 2\pi$ along the tilted axis. For moderately high RF power, τ_{LG} is typically 13 μs, which is short compared to the MAS period, even for high spinning speeds. Modern NMR spectrometers enable rapid coherent shifting of both the RF frequency and phase during a pulse, and the FSLG sequence is relatively straightforward to implement. Since the only parameters that have to be adjusted are the offset frequencies $\omega_{\pm\Delta\mathrm{LG}}$ and the duration of the successive frequency intervals τ_{LG}, the sequence is robust and can easily be readjusted if necessary.

4.3 Heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy

4.3.1 Preparation and evolution

The pulse sequence to collect 2-D heteronuclear (^{1}H-^{13}C) dipolar correlation spectra with FSLG ^{1}H homonuclear decoupling is depicted in Fig. 4.2. The sequence starts with a proton $\pi/2$ *x*-pulse [18] or, alternatively, with a $(\pi/2 + \theta_{\mathrm{m}})$ *y*-pulse [17], directly followed by a train of frequency- and phase-switched LG pulses in the *xz*-plane. Both preparation pulses place the ^{1}H polarization perpendicular to the direction of the effective field during the FSLG. However, the $\pi/2$ *x*-pulse improves the elimination of ring-down effects via phase cycling and is preferred. During the FSLG irradiation the protons are allowed to evolve for a time t_1 under continuous homonuclear decoupling. At the end of the evolution period, components of the ^{1}H signal in the plane of precession that is perpendicular to the LG-axis can be selected and tilted back into the *xy*-plane by the magic-angle *y*-pulse (Fig. 4.3). In addition, the magic-angle pulse will rotate any spin-locked component along the LG-axis to the *z*-axis (Fig. 4.3). The phases of the preparation pulse, the entire FSLG sequence and the magic-angle pulse are varied in a TPPI scheme to simulate phase-sensitive detection [19].

4.3.2 The mixing period

The mixing period forms an important element of the pulse sequence for heteronuclear dipolar correlation spectroscopy. During the mixing, proton magnetization is transferred to the carbons and correlations build up. For correlation spectroscopy it is relevant that this transfer proceeds without loss of selectivity. This means that each carbon nucleus should only correlate with its adjacent protons, or less stringent, that a correlation with a nearby proton is more intense than one with a remote proton. In the solid state, the interactions between the abundant protons are much stronger than the proton-carbon couplings. Consequently, the protons can also exchange magnetization during the CP, and care must be taken to maintain the selectivity during the mixing period in the correlation experiment.

To achieve selective magnetization transfer, two routes can be followed. In the first approach the mixing period consists of a normal CP sequence with a short contact time, typically 100-500 μs. A second approach relies on the application of a CP pulse scheme that locks the ^{1}H and ^{13}C spins, and simultaneously suppresses the strong dipolar interactions between the abundant protons.

4.3.3 Short CP contact times

At high spinning speeds in the range 10-15 kHz and at high magnetic fields, the carbon signal intensity shows an oscillatory behaviour as a function of the CP contact time. For instance, for a CH or CH_2 moiety, the maximum signal intensity can already be obtained with a mixing time of ~ 75 μs and a short CP contact is sufficient to build up strong heteronuclear correlations. This is illustrated in Fig. 4.4, that shows the signal intensity during CP of the 3’-^{13}C (A) and the 3-^{13}C (B) resonances of [U-^{13}C] tyrosine·HCl (Fig. 4.5A, inset), as representatives of CH and CH_2 moieties, respectively. On the other hand, in the previous chapter it was shown that the ^{1}H homonuclear dipolar interactions are reduced by the high spinning speed and high magnetic field, which will slow down the polarization exchange among the protons. This, in combination with a short CP contact time, will prevent extensive ^{1}H homonuclear magnetization exchange during CP and a selective heteronuclear correlation spectrum will result. In addition, the spin-locking itself leads to a reduction of the homonuclear dipolar coupling strengths between the protons, reflected by the factor $\frac{1}{2}$ in Eq. 2.4, which will also benefit the selectivity.

The method is illustrated in Fig. 4.5A, which shows a contour plot and projections of a heteronuclear dipolar correlation spectrum with FSLG ^{1}H homonuclear decoupling, recorded at a moderately high field of 9.4 T from a preparation of [U-^{13}C] L-tyrosine·HCl salt (inset). The data were obtained with a DMX-400 NMR spectrometer equipped with a 4mm double-resonance MAS probe (Bruker, Karlsruhe, Germany). The spinning speed was set to 13.0 kHz and a CP contact time of 500 μs was applied. The RF power used for the FSLG

decoupling corresponds with a proton nutation frequency of ~ 90 kHz.

The proton-carbon correlations are well resolved, also in the aliphatic region of the spectrum. The overall sensitivity is good, due to the high spinning speed used for the experiment. The proton lines are partially resolved, also in the F1 projection, which underlines the good performance of the FSLG decoupling during the proton evolution. The strongest correlations in Fig. 4.5A are between carbons and directly bonded protons, although both intramolecular and through-space intermolecular correlations with distant protons occur at lower levels of cross-peak intensity, which will be extensively discussed in Chapter 5. Mutual correlations between 1-^{13}C, 2-^{13}C and 3-^{13}C are observed around 8.0 ppm in the ^{1}H dimension that originate from the NH_3^+ moiety. The enhanced overall resolution allows a refinement of the assignments of the proton chemical shifts, which are listed in Table 4.1.

An example of the performance of the technique at lower RF power is illustrated with Fig. 4.5B, which shows a contour plot of a 2-D FSLG-decoupled heteronuclear dipolar correlation spectrum recorded from a preparation of natural abundance ^{13}C valine·HCl (inset). The data were obtained at a high field of 14.1 T using a DMX-600 NMR spectrometer equipped with a 4mm double-resonance MAS probe (Bruker, Karlsruhe, Germany). A moderate RF power corresponding with an ^{1}H nutation frequency of ~ 60 kHz was used for the FSLG decoupling. A spinning speed of 13.0 kHz and a CP contact time of 250 μs were applied. The correlations in the spectrum are well resolved, and it can be concluded that the FSLG performs well even at a moderate RF power. This makes the technique suitable for spectrometers that are not equipped with high-power RF transmitters. The proton assignment for valine·HCl is included in Table 4.1.

Table 4.1: Solid-state proton chemical shifts σ_i (ppm) for L-tyrosine·HCl and valine·HCl, obtained from MAS NMR 2-D ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy. The error is estimated at ~ 0.2 ppm.

Tyrosine		Valine	
Proton	σ_i (ppm)	Proton	σ_i (ppm)
1-OOH	13.1	1-COOH	13.0
2-H	4.4	2-H	4.5
2-NH_3^+	8.0	2-NH_3^+	9.2
3-H_2	2.1	3-H	2.6
2’-H	5.3	4-H_3	1.4
3’-H	6.7	4’-H_3	1.4
4’-COH	10.3		
5’-H	5.1		
6’-H	7.5		

4.3.4 Lee-Goldburg CP

In some cases a short CP contact time may not be appropriate for the experiment, for instance, if one is interested to detect intermolecular heteronuclear (^{1}H-^{13}C) correlations. Intermolecular correlations are generally remote, hence the polarization has to be transferred over relatively long distances. Therefore, a sufficiently long CP contact time >1 ms is usually required to create detectable correlations. To preserve the selectivity during long CP contact times, a pulse technique is required to decouple the protons in order to slow down or block the ^{1}H spin-diffusion processes. It was found that the Lee-Goldburg CP (LG-CP) [13,20,21] can be applied successfully at high MAS rates up to 15 kHz to achieve a selective polarization transfer [22]. During the LG-CP, an off-resonance ^{1}H RF field is applied in such a way that in the rotating frame the effective field is inclined at the magic angle with respect to the static field in the z-direction, while simultaneously the ^{13}C spins are irradiated on-resonance. The part of the ^{1}H magnetization that is parallel to the effective field will be spin-locked and the carbon spins can be locked in the xy-plane. In this way, heteronuclear spin-locking with simultaneous suppression of the ^{1}H homonuclear dipolar couplings is achieved and polarization can be transferred selectively from the protons to the carbon nuclei.

Fig. 4.6 shows a series of proton slices extracted from 2-D heteronuclear [^{1}H-^{13}C] dipolar correlation spectra of [U-^{13}C] L-tyrosine·HCl, recorded with a 'normal' CP (left) and Lee-Goldburg CP (right) contact time of 4.0 ms. The slices show correlations with 1-^{13}C (A), 2-^{13}C (B), 3-^{13}C (C), 4'-^{13}C (D) and 5'-^{13}C (E). For the normal CP all the proton slices correlated with the different carbon nuclei are virtually identical. On the other hand, the spectrum recorded with the LG-CP (right) is very selective, even for mixing times as long as 4.0 ms. The strongest correlations for the carbon nuclei are with their attached protons, while remote correlations are less intense. In addition, the 4'-^{13}C (Fig. 4.6D) has a strong correlation with the nearby 4'-O^{1}H at 10.3 ppm, weak intramolecular correlations with 3'-^{1}H (6.7 ppm) and 5'-^{1}H (5.1 ppm), and a weak intermolecular correlation with the remote 1-OO^{1}H (13.1 ppm). In Chapter 6 it is demonstrated that the detection of heteronuclear (^{1}H-^{13}C) intermolecular correlations can provide structural restraints that are instrumental for structure refinement studies.

4.3.5 Multidimensional correlation spectroscopy

Due to the simplicity of the FSLG sequence, the number of dimensions in the heteronuclear correlation experiment can easily be extended, albeit at the expense of a prolonged data collection time, and implementation in 3-dimensional (3-D) heteronuclear (^{1}H-^{13}C-^{13}C) dipolar correlation spectroscopy is straightforward. For example, 2-D FSLG-decoupled ^{1}H-^{13}C heteronuclear correlation spectroscopy can be combined with 2-D radio frequency-driven dipolar recoupling (RFDR) [23,24] ^{13}C homonuclear dipolar correlation spectroscopy to acquire well-resolved 3-D spectra. This is illustrated with Fig. 4.7A, which shows a 3-D dipolar correlation spectrum recorded from [U-^{13}C] L-tyrosine·HCl at a field of 14.1 T on a DMX-600 spectrometer, with the pulse program depicted in Fig. 4.7B.

4.3.6 Conclusions

In this section it is demonstrated that heteronuclear dipolar correlation spectra can be recorded at high MAS rates with FSLG ^{1}H homonuclear decoupling during proton evolution. The resolution enhancement obtained in this way makes it possible to incorporate protons in concepts for assignment strategies and structure determination of uniformly ^{13}C-enriched spin clusters in biological systems, using 2-D or 3-D dipolar correlation spectroscopy. In Chapter 6 it is illustrated how such a concept can be exploited by presenting the results of a structural investigation of aggregates of uniformly ^{13}C-enriched chlorophyll *a* / H_2O.

4.4 The Lee-Goldburg scaling factor

In two successive FSLG periods, the chemical shift dispersion is scaled by the theoretical factor $\cos\theta_{\mathrm{m}} = 1/\sqrt{3} = 0.577$ (cf. Eq. 2.13). In practice, this factor may turn out slightly different. Since knowledge about the exact scaling factor is vital for this type of experiment, it is important to determine it experimentally.

The standard method to determine the scaling factor is by application of additional frequency shifts Δ_{offset} to the LG frequencies $\omega_{\pm\Delta\mathrm{LG}}$. The slope of a plot of the observed frequency ω_{obs} of a proton signal as function of Δ_{offset} provides the scaling factor. This is illustrated with Fig. 4.8A, for several proton resonances of [U-^{13}C] L-tyrosine·HCl. Since the FSLG decoupling is windowless, it can not be used for direct proton observation in 1-D NMR, and the ω_{obs} are obtained indirectly from a series of 2-D heteronuclear (^{1}H-^{13}C) FSLG-decoupled correlation spectra. As can be verified from Fig. 4.8A, ω_{obs} varies linearly with Δ_{offset} for the three protons, but with slightly different slopes. This implies that the observed proton dispersion varies with Δ_{offset}, *i.e.*, that the scaling factor depends on the frequency offset [25].

The determination of the scaling factor from a series of 2-D correlation experiments is time consuming. In addition, due to the Δ_{offset}-dependency of the scaling factor, the interpretation of a plot like Fig. 4.8A is difficult. An alternative way to determine the scaling factor is by plotting scaled tyrosine proton chemical shifts obtained from a FSLG-decoupled heteronuclear correlation spectrum versus the corresponding non-scaled proton chemical shifts obtained from a correlation spectrum recorded with the CP/WISE technique that was discussed in the previous chapter. This method is illustrated with Fig. 4.8B. The slope again provides the scaling factor. The advantage of this procedure is that only a single 2-D FSLG-decoupled experiment needs to be recorded as a reference, which will take less than 10 minutes spectrometer time. In addition, the reference spectrum can be recorded with the same frequency offset as will be used for the final experiment, which removes complications that arise from the Δ_{offset}-dependency of the chemical shift scaling factor.

4.5 Frequency-switched LG and sample rotation

In general, multiple-pulse homonuclear decoupling techniques can only be implemented successfully if the cycle time is short compared to the period associated with MAS. Since irradiation at one of the Lee-Goldburg conditions leads to a suppression of the secular part of the tilted homonuclear dipolar spin Hamiltonian (Chapter 2), regardless of the time dependence of the dipolar coefficients imposed by the sample rotation, part of the Lee-

Goldburg irradiation will still be effective at high spinning speeds. On the other hand, higher-order terms arising from the non-secular part of the spin Hamiltonian will only be suppressed over a full FSLG cycle. Due to a reorientation of the spatial coordinates before completion of the full cycle, interference is to be expected of the MAS with the FSLG.

For the FSLG, the total cycle time corresponds to $2\cdot\tau_{\mathrm{LG}}$, which is the time required to complete two 2π rotations back and forth around the tilted axes for the successive LG offsets. For a moderate RF power corresponding with an ^{1}H nutation frequency of 63 kHz, the cycle time amounts ~26 μs, corresponding to a repetition rate of 39 kHz. At a MAS rate $\omega_r/2\pi = 13$ kHz, the rotor period of 77 μs is only 3 times longer than the cycle time. Fig. 4.9 shows the proton projections of a series of 2-D FSLG-decoupled heteronuclear (^{1}H-^{13}C) dipolar correlation spectra, recorded from [U-^{13}C] L-tyrosine·HCl at a field of 14.1 T. The MAS rate is varied from 12.5 kHz to 13.0 kHz, in steps of 100 Hz. An RF power corresponding with a proton nutation frequency of 63 kHz is used for the FSLG irradiation. In the projections, an additional signal is observed, marked with the asterisk. The position of the extra signal changes linearly as function of the spinning speed (cf. Fig. 4.9A-F), but remains unaffected by changing the offset frequency (data not shown). The extra signal behaves like a 'rotor line' reported by Roberts *et al.* [1]. A change of 100 Hz of the MAS rate imposes a non-scaled shift of the signal position of 300 Hz. This suggests interference of the FSLG with a multiple 3 of the spinning speed, in line with the fact that three times the MAS rate matches the repetition rate of the FSLG cycle.

The appearance of the rotor lines is an annoying feature of multiple-pulse MAS experiments in general [1]. By mixing with the actual spectrum, the rotor lines can complicate the proton assignment, in particular when they are not recognized as artefacts. In addition, interference of the rotor lines with the spectrum can actually alter the peak position of the proton signals, as can be verified from Figs. 4.9D and E, where the rotor line is near the proton resonance of the 4'-O^{1}H at 10.3 ppm. The 4'-O^{1}H proton resonance appears split, and its true spectral position is obscured by the interference.

To avoid these unwanted effects, it is very desirable to 'remove' the rotor lines from the spectral region of interest. Since the position of the proton spectrum shifts with the offset frequency and that of the rotor lines with the MAS rate, in practice this can be achieved by an appropriate choice of the spinning speed and frequency offset.

4.6 Experimental details

All spectra were recorded using a ramped-amplitude CP sequence on the carbons (RAMP-CP) [26] to restore a broader Hartmann-Hahn matching at high spinning speeds and to reduce the sensitivity to RF-power instabilities at high MAS frequencies, with exception of the CP build-up curves shown in Fig. 4.4. During the carbon acquisition, the protons were decoupled from the carbons by using a two-pulse phase modulation (TPPM) decoupling scheme with phase-modulation angles set to 15.0 (DMX-400 spectrum) or 20.0 degrees (DMX-600 spectra) [27]. The sample volumes of the [U-^{13}C] tyrosine·HCl and valine·HCl preparations were restricted to the middle of the rotor to improve the RF homogeneity.

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Figure captions

Fig. 4.1: Schematic representation of a negative (A) and positive (B) Lee-Goldburg frequency offset, viewed from the $-|\omega^{-\Delta LG}|$ *(A) and* $-|\omega^{+\Delta LG}|$ *(B) off-resonance rotating frames.*

Fig. 4.2: Pulse sequence for heteronuclear dipolar correlation spectroscopy with frequency switched Lee-Goldburg irradiation during the proton evolution.

Fig. 4.3: Schematic representation of the effect of the magic angle y-pulse. The plane perpendicular to the tilted axis (A) is brought back into the „normal“ xy-plane (B). A spin-locked component along the effective field is turned along the z-axis.

Fig. 4.5: 2-D heteronuclear dipolar correlation spectra recorded at a MAS rate of 13.0 kHz from [U-^{13}*C] L-tyrosine·HCl, in a field of 9.4 T (A) and from [U-*^{13}*C] valine·HCl, in a field of 14.1 T (B). The* ^{1}*H homonuclear interactions were suppressed with FSLG irradiation during proton evolution, applying an RF power corresponding with a nutation frequency of 90 kHz (A) or 63 kHz (B).*

Fig. 4.6: Proton slices correlated with 1-^{13}*C (A), 2-*^{13}*C (B), 3-*^{13}*C (C), 4'-*^{13}*C (D) and 5'-*^{13}*C (E), extracted from 2-D FSLG decoupled heteronuclear (*^{1}*H-*^{13}*C) dipolar correlation spectra, recorded from [U-*^{13}*C] L-tyrosine·HCl in a field of 14.1 T, using a MAS rate of 12.0 kHz. A 'normal' CP (left) or LG-CP (right) contact of 4 ms was applied.*

Fig. 4.7: 3-D heteronuclear (^{1}*H-*^{13}*C-*^{13}*C) dipolar correlation spectrum of [U-*^{13}*C] L-tyrosine·HCl. The data were acquired in a field of 14.1 T, with the pulse-program depicted in (B).*

Fig. 4.8: Plot of observed frequency versus offset frequency for the 2'-^{1}*H, 4'-O*^{1}*H and 1-OO*^{1}*H proton resonance, extracted from a series of 2-D heteronuclear FSLG decoupled correlation spectra from [U-*^{13}*C] L-tyrosine·HCl, recorded in a field of 14.1 T and using a MAS rate of 11.0 kHz (A), and: plot of scaled chemical shifts obtained from a heteronuclear FSLG decoupled correlation spectrum versus corresponding non-scaled chemical shifts from a CP/WISE correlation spectrum, recorded in a field of 14.1 T (B). The numbers in the boxes indicate the slopes of the curves.*

Fig. 4.9: Proton projections of 2-D heteronuclear FSLG decoupled dipolar correlation spectra recorded from [U-^{13}*C] L-tyrosine·HCl in a magnetic field of 14.1 T, at MAS rates of 12.5 kHz (A), 12.6 kHz (B), 12.7 kHz (C), 12.8 kHz (D), 12.9 kHz (E) and 13.0 kHz (F). The asterix indicates the position of the rotor frequency-induced artefact.*

Chapter 5

A method for measuring heteronuclear (^{1}H-^{13}C) distances in high-speed MAS NMR

This chapter has been submitted to *J. Am. Chem. Soc.*

5.1 Introduction

Magic-angle spinning nuclear magnetic resonance (MAS NMR) techniques for structure determination of uniformly enriched compounds or multispin clusters in solid-type biological systems without translation symmetry are rapidly developing [1]. The measurement of heteronuclear (^{1}H-^{13}C) intermolecular distances is potentially an important tool in structure determination of solids, since it can provide restraints that can be used for structure refinement by modeling. Information about the internuclear distance between two spins can be deduced from the strength of the heteronuclear (^{1}H-^{13}C) dipolar interaction. For a static sample, the dipolar interaction between the two spins of a heteronuclear spin-pair yields a symmetric Pake doublet [2], in which the separation between the two maxima provides a direct measure for the heteronuclear dipolar coupling strength. MAS, however, averages the heteronuclear dipolar interactions and gives rise to pattern of spinning side-bands, with the relative intensities of the spinning side-bands determined by both the dipolar interaction and the chemical shift [3]. For small shift anisotropies, the envelope of the side-band intensities reflects the static Pake pattern at low spinning speeds < 3 kHz, while at high MAS rates > 10 kHz the dipolar interaction is substantially reduced by the sample spinning, like the chemical shift anisotropy (CSA).

One way to determine heteronuclear dipolar couplings with MAS is to perform separated local-field spectroscopy. In this technique the dipolar interaction is separated from the chemical shift interaction, resulting in 2-dimensional (2-D) spectra with the chemical shift of each nucleus in one dimension correlated with its dipolar coupling to neighbouring spins in the second dimension [3-11]. However, this high-resolution method requires slow MAS to ensure sufficient side-band intensity to obtain the strength of the dipolar interactions [3,5]. On the other hand, to achieve a good resolution in the chemical shift dimension for multispin clusters at high magnetic field strengths, fast MAS is required to suppress the CSA.

In this chapter a method is presented that can be applied to extract heteronuclear distances r_{CH} in uniformly ^{13}C-enriched ([U-^{13}C]) compounds with good precision from cross-polarization (CP) build-up curves, recorded at high MAS rates $\omega_{\mathrm{r}}/2\pi = 10-15$ kHz under simultaneous suppression of the ^{1}H homonuclear dipolar interactions. The Fourier transform of the time-oscillatory magnetization build-up curves provides dipolar-broadened response profiles reflecting the heteronuclear (^{1}H-^{13}C) dipolar coupling strengths that can be related to internuclear distances between the coupled spin-pairs.

5.2 Experimental

The solid-state CP/MAS spectra were recorded with a DMX-600 and a DMX-750 spectrometer, equipped with 4mm double-resonance CP/MAS probes (Bruker, Karlsruhe, Germany). For the experiments with the DMX-600 instrument, a home-built spinning-speed controller was used to stabilize the spinning speed [12]. The pulse sequences for the 2-D and 3-dimensional (3-D) heteronuclear polarization transfer experiments are shown in Figs. 5.1A and B, respectively. The 2-D sequence starts with a magic-angle preparation pulse that puts the ^{1}H polarization at the magic angle with respect to the *z*-axis and parallel to the direction of the effective field in the rotating frame during the Lee-Goldburg CP (LG-CP) [10,13-15]. Alternatively, a 90° proton preparation pulse can be applied, which improves elimination of probe ring-down via phase-cycling, at the expense of a ~ 20 % reduction of the signal. The 2-D dataset presented in this chapter was recorded with a magic-angle preparation pulse, while the 3-D experiment discussed below effectively makes use of the variant with a 90° preparation pulse. Following ^{1}H excitation in the 2-D experiment, LG-CP is applied to transfer the magnetization from the protons to the carbons during a variable time t_1. The LG-CP spin-locks the ^{1}H signal along the effective field, while the ^{13}C spins can be locked on-resonance in the *xy*-plane. In this way, heteronuclear spin-locking is achieved, while simultaneously the ^{1}H homonuclear dipolar interactions are significantly suppressed. Fourier transformation with respect to t_1 provides the ^{13}C heteronuclear dipolar interaction dimension of the correlation experiment. A moderate proton RF power corresponding with an ^{1}H nutation frequency of 60 kHz was used for the LG-CP. The ^{13}C free induction decays (FIDs) were recorded in the t_2 domain, using proton decoupling with the two-pulse phase modulation (TPPM) scheme [16]. The Hartmann-Hahn (HH) matching profile during MAS was mapped by sweeping the ^{13}C RF power while following the ^{13}C signal intensity in t_2. In this way, the $n = \pm 1$ HH matching conditions for the LG-CP can be determined experimentally.

The pulse program for the 3-D experiment in Fig. 5.1B combines frequency-switched Lee-Goldburg (FSLG) decoupled ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy in t_2 and t_3 with the ^{1}H excitation and LG-CP evolution in t_1 of the pulse program shown in Fig. 5.1A. After ^{1}H excitation, the protons are allowed to evolve for a time t_2 with FSLG irradiation to suppress the ^{1}H homonuclear dipolar couplings [1,17,18]. The plane of precession is perpendicular to an axis that is inclined at the magic angle with respect to the z-axis, and the magic-angle y-pulse at the end of t_2 rotates components of the ^{1}H signal into the xy-plane. The phases of the proton preparation pulse, the FSLG sequence and the magic-angle pulse are varied according to a time-proportional phase-incrementation scheme to simulate phase-sensitive detection in t_2 [19]. The LG-CP selects and spin-locks the component of the ^{1}H signal that is parallel to the effective field. Following the heteronuclear polarization transfer during t_1, the ^{13}C FIDs are recorded in t_3 with TPPM decoupling. The resulting 3-D dataset can be represented as a series of heteronuclear correlation spectra recorded with different LG-CP contact times in t_1. The contributions of the various protons in the vicinity of a ^{13}C to the magnetization exchange can be studied separately in the ^{1}H frequency domain after Fourier transformation in t_1.

Fig. 5.1C shows the pulse program for 2-D ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy. The protons evolve during t_1 under suppression of the ^{1}H homonuclear dipolar interactions with the FSLG. LG-CP was used to establish selective heteronuclear polarization transfer during a long CP mixing time of 1.5 ms [15], while a ramped CP spin-lock pulse on the carbon nuclei was applied to broaden the CP matching profile at high MAS rates [20].

In a convenient way to determine the scaling factor for a 2-D ^{1}H-^{13}C heteronuclear dipolar correlation spectrum, the slope was determined of a plot of scaled L-tyrosine·HCl proton chemical shifts obtained from a FSLG experiment against the corresponding non-scaled proton chemical shifts obtained from a correlation spectrum recorded with the CP/WISE technique in a high magnetic field and with fast MAS [21,22].

Simulations of LG-CP MAS ^{13}C signals are performed using an IBM SP2 computer following the method described by Ray *et al.* [23]. Coordinates for carbon and hydrogen atoms in L-tyrosine·HCl (Fig. 5.2) were taken from the high-resolution neutron diffraction structure [24].

5.3 Results

The aim of this chapter is to demonstrate that the time-oscillatory build-up of the carbon signal during CP for fast MAS directly provides the heteronuclear (^{1}H-^{13}C) dipolar coupling strength, which can be related to a distance r_{CH}. To this end, interactions other than the heteronuclear dipolar coupling should be removed from the spin Hamiltonian. The locking of the ^{1}H and ^{13}C spins during CP suppresses the chemical shift evolution of both spin types, while the ^{13}C homonuclear dipolar couplings average to zero at high MAS rates. Therefore, the two major remaining interactions that determine the time-evolution of the spin system are the ^{1}H homonuclear and the ^{1}H-^{13}C heteronuclear dipolar couplings, ignoring the weaker *J*-couplings. The ^{1}H homonuclear dipolar interactions can be removed successfully from the spin Hamiltonian by application of the LG-CP. This will prevent ^{1}H homonuclear spin diffusion during CP, while ^{1}H-^{13}C polarization exchange can proceed mediated by the heteronuclear dipolar interactions.

Fig. 5.3 shows a 2-D experiment recorded with the pulse sequence in Fig. 5.1A from [U-^{13}C] L-tyrosine·HCl with an ^{1}H frequency of 600 MHz using a MAS rate $\omega_r/2\pi$ = 12 kHz. The LG-CP was adjusted for the $n = -1$ HH matching condition. An experiment using the $n = +1$ matching condition yielded virtually identical results (data not shown). The $n = 0$ condition should not be chosen, since this results in a reduction of the effective heteronuclear dipolar interactions, which will lead to a much less efficient polarization transfer [25].

The data in Fig. 5.3 were processed with a Fourier transform in the ^{13}C dimension only. In this series of ^{13}C spectra the isotropic carbon chemical shifts are correlated with the variation of the signal intensity during the LG-CP mixing time. In this way the LG-CP build-up curves are separated according to their carbon shifts. Fig. 5.4 shows the F1 slices correlated with the 2-^{13}C response at 56.3 ppm (Fig. 5.4A) and with the 3-^{13}C signal at 36.6 ppm (Fig. 5.4B). The CP signal builds up in time in an oscillatory manner [26]. Since the ^{1}H homonuclear dipolar interactions and the chemical shift evolution of both spin types are suppressed during the LG-CP, the oscillations reflect evolution of the ^{13}C magnetization and coherent transfer of ^{1}H polarization [10,14].

The Fourier transforms of the LG-CP build-up curves of the 2-^{13}C (Fig. 5.4C) and the 3-^{13}C (Fig. 5.4D) carbons yield effective carbon responses in the frequency domain. Prior to Fourier transformation, the data were corrected for the vertical baseline offset. The LG-CP carbon spectrum for the CH moiety in Fig. 5.4C is very similar to the theoretically predicted response in Fig. 2.2 (Chapter 2) for an isolated CH spin-pair, and is modulated with spectral intensities at integral multiples of the rotor speed $\omega_r/2\pi$ = 12 kHz. It appears that the major contribution to this spectrum originates from dipolar transfer within the CH spin-pair, while the lower frequency intensities can be attributed to transfer from weakly coupled remote protons. From the peak-to-peak distance $\Delta\omega/2\pi$ between the maxima, a LG-CP dipolar coupling frequency $\Delta\omega/2\pi$ = 12,070 Hz is estimated for the CH pair. The LG-CP carbon spectrum for the CH_2 group is more complicated and exhibits a doublet structure that gives rise to a total of four maxima (Fig. 5.4D). The splitting for the interior pair of maxima equals to a frequency $\Delta\omega/2\pi$ = 12,040 Hz.

In the virtual environment of the computer, an ideal LG decoupling is easily mimicked by omitting the ^{1}H homonuclear dipolar interactions in the simulations. In addition, the ^{1}H-^{13}C heteronuclear dipolar interaction is scaled in the simulations by a factor $\sin(\theta_{\mathrm{m}}) = 0.817$. To follow the experiment, the simulations were performed for the $n = -1$ HH matching condition. The simulations for the CH moiety were done for a two-spin cluster that consisted of a ^{13}C spin coupled to a directly bonded proton and for a six-spin cluster of a ^{13}C spin coupled to a total of five protons, taking into account the coordinates of the five nearest protons according to the neutron diffraction structure [24]. Likewise, for the CH_2 group the simulations were performed for a ^{13}C spin with its two covalently bonded protons, and for a spin cluster containing three additional remote protons. Powder spectra were constructed by adding the spectral intensities of a total of 1154 different crystal orientations.

Simulations of the LG-CP carbon spectra for the 2-CH and 3-CH_2 moieties are shown in Figs. 5.4E and F, respectively. The LG-CP carbon spectra for the spin systems comprising five protons are indicated with dotted lines. Since all relaxation processes were ignored in the simulations, the simulated spectra have been artificially broadened in order to allow for a straight comparison with the experimental data. From the envelope of the experimental magnetization build-up curve of the 2-^{13}C in Fig. 5.4A, a pseudo transverse relaxation time T_2' of ~ 0.4 ms was estimated. From this, a Lorentzian linewidth $\Gamma \approx 800$ Hz was derived according to $\Gamma = 1/\pi T_2'$, which was used in an Lorentzian apodization of the simulated build-up curves in the time domain.

The overall similarity between experimental and simulated responses is encouraging and many essential features of the LG-CP carbon spectra in Figs. 5.4C and D can be recognized in the MAS Floquet simulations of the heteronuclear spin clusters spinning at a rate of 12 kHz (Figs. 5.4E and F). For the CH moiety, the characteristic powder pattern envelope in Fig. 2.2 transpires (Fig. 5.4E). Additional intensity is present in the form of narrow lines at integral multiples of the MAS rate. The simulations confirm that the main broad contribution to the spectrum arises from the dipolar interactions with the directly bonded proton, while a weaker and narrower response in the center around 0 Hz is associated with dipolar transfer from the remote protons. It was observed during the course of the simulations that the dipolar couplings to remote protons do have a minor effect on the shape of the response from the strongly coupled CH spin-pair. Adding an increasing number of distant protons in the simulation leads to a gradual broadening of the LG-CP response and to a shift of the maxima to higher frequencies. The LG-CP dipolar frequencies $\Delta\omega/2\pi$ for the spin clusters with one and five protons in Fig. 5.4E are 12,780 Hz and 12,920 Hz, respectively. These values compare well with the splitting $\delta_0 = \omega_\mathrm{d}\cos(\theta_\mathrm{m})$ predicted from the theory in Chapter 2. This value equals to δ_0 = 13,100 Hz, for the same distance r_CH = 1.1 Å that was used in the simulations.

The major contribution to the doublet structure in the simulation of the LG-CP response for the CH_2 group in Fig. 5.4F can be attributed to polarization exchange within the CH_2 moiety. Just like for the CH, the remote protons contribute mainly to the central part of the spectrum, and they have a small broadening effect on the CH_2 shape. For a simulation of an isolated CH_2 pair, the LG-CP dipolar frequency $\Delta\omega/2\pi$ = 13,240 Hz is defined as the separation between the maxima. For a simulation of a spin system containing five protons this increases to $\Delta\omega/2\pi$ = 13,310 Hz. In the simulations the interior doublet is more intense than the exterior part. This is in good qualitative agreement with the experimental data. Other features are also qualitatively reproduced, for example the intensity around ±16 kHz, which was not observed for the CH moiety, and high-frequency shoulders at ±7300 Hz. The agreement between the experimental and simulated splittings $\Delta\omega/2\pi$ in Fig. 5.4 will be discussed in the next section.

Fig. 5.5 shows the polarization build-up for the 4’-^{13}C and its Fourier transform. The 4’-^{13}C is a quaternary carbon and therefore only weakly dipolar coupled to protons (Fig. 5.5A). In the Fourier transform of the slow oscillations in the time domain signal in Fig.

5.5B, three frequency components appear, which can be assessed most accurately by taking the second derivative of the data. This is illustrated in Fig. 5.5C, from which the splittings $\Delta\omega/2\pi$ = 2100 ± 25 Hz and $\Delta\omega/2\pi$ = 1150 ± 100 Hz are obtained. A relatively large uncertainty in $\Delta\omega/2\pi$ of ~ 100 Hz is estimated for the lower frequency component, since the value depends on the baseline correction during the data processing.

According to the neutron-diffraction data, there are four protons in the vicinity of the 4'-C [24]. The shortest intramolecular distances are to the 4'-OH phenolic proton (1.982 Å), and the two aromatic protons 3'-H (2.150 Å) and 5'-H (2.148 Å). In addition, the oxygen of the 4' hydroxyl group forms an intermolecular hydrogen bond to the 1-OOH of a neighbouring molecule in the crystal, with the hydrogen-bonding proton located at a distance of 2.521 Å from the 4'-C (Fig. 5.6).

Interestingly, we have observed that the 4'-^{13}C predominantly receives its LG-CP polarization through intramolecular transfer from the 4'-O^{1}H proton and intermolecular transfer from the 1-OO^{1}H proton, while the 3'-^{1}H and 5'-^{1}H predominantly exchange polarization with 3'-^{13}C and 5'-^{13}C and appear to contribute little to the initial 4'-^{13}C signal build-up. Fig. 5.7 shows a contour plot of a heteronuclear dipolar correlation spectrum recorded with the pulse scheme in Fig. 5.1C from [U-^{13}C] L-tyrosine·HCl. The spectrum was obtained with FSLG ^{1}H homonuclear dipolar decoupling during the proton evolution [17,18] at a MAS rate $\omega_r/2\pi$ = 15 kHz and using a high magnetic field strength of 17.6 T corresponding with an ^{1}H NMR frequency of 750 MHz. Selective magnetization transfer was achieved by applying a LG-CP contact time of 1.5 ms [14]. Using the procedure described in the experimental Section 5.3, a scaling factor of 0.50 was found for the FSLG-decoupled heteronuclear correlation experiment. This is somewhat lower than the theoretical factor $\cos\theta_{\mathrm{m}} = 1/\sqrt{3} = 0.577$ [13], and improvement of the resolution may be reached by fine-tuning of the LG offset frequencies. However, we felt no need for additional parameter optimization, for the reason that the lines are already fully resolved in the proton dimension in this dataset.

As can be observed from the 2-D spectrum, the 4'-^{13}C response has a strong heteronuclear correlation with the 4'-O^{1}H proton and a weaker correlation with the 1-OO^{1}H proton, indicated with a small arrow in the plot in Fig. 5.7. The correlation with the 1-OO^{1}H provides unambiguous evidence for intermolecular magnetization transfer, from the hydrogen-bonded proton (Fig. 5.6). In contrast, the dipolar correlations with the nearby aromatic 3'-^{1}H and 5'-^{1}H protons are very weak, beyond the limit set by the contours in Fig. 5.7.

Since predominantly the 4'-O^{1}H and the 1-OO^{1}H appear to contribute to the initial polarization build-up of the 4’-^{13}C signal, we performed a MAS Floquet simulation for a spin system that consisted of the 4’-^{13}C, the 4’-O^{1}H and the 1-OO^{1}H (Fig. 5.5D). The simulated LG-CP carbon spectrum was broadened using a Lorentzian apodization of 800 Hz, the same value that was used in the calculations of the CH and CH$_2$ responses in Fig. 5.4. The higher frequency part of the experimental spectrum in Fig. 5.5B is closely reproduced in the simulated response in Fig. 5.5D, which corroborates our findings that mainly two protons appear to be involved in the polarization transfer to the 4’-^{13}C. The shape of the higher frequency part of the LG-CP carbon spectrum is similar to the one in Fig. 5.5B. On the other hand, there are also differences, like the presence of a narrow component centered around 0 Hz in the experimental response, which is absent in Fig. 5.5D. In addition, the experimental response has wings extending to higher frequencies. This may reflect the interactions of the

4’-^{13}C with the relatively close 3’-^{1}H and 5’-^{1}H, which may also take part in, for instance, relayed transfer processes of the form 3’-^{1}H → 3’-^{13}C → 3’-^{1}H → 4’-^{13}C.

To disentangle the contributions from the various protons involved in each heteronuclear dipolar transfer process, a 3-D spectrum was recorded with the pulse program of Fig. 5.1B at 600 MHz ^{1}H frequency, using a MAS rate $\omega_r/2\pi$ = 12 kHz. The combination of 2-D heteronuclear (^{1}H-^{13}C) FSLG-decoupled correlation spectroscopy with variable LG-CP times in a third dimension allows for a separation of the polarization transfer events in the carbon and proton chemical shift dimensions. From the build-up of the intensities of the heteronuclear correlations with increasing LG-CP contact time, individual carbon-proton couplings can be characterized. Fig. 5.8 shows several slices that are extracted from the 3-D experiment. The 4’-^{13}C magnetization build-up is associated with heteronuclear dipolar interactions of 4’-^{13}C with 4’-O^{1}H, 1-OO^{1}H, 5’-^{1}H or 3’-^{1}H (Fig. 5.8, dashed lines). The 4’-^{13}C has the strongest interaction with the 4’-O^{1}H proton, which gives rise to a frequency component $\Delta\omega/2\pi$ = 2100 Hz. The initial polarization transfer from the 1-OO^{1}H proton is faster than the transfer from the nearer 5’-^{1}H and 3’-^{1}H and is weakly oscillating with a period of about 2.0 ms, which can account for the observed weak $\Delta\omega/2\pi$ = 1150 Hz component in Fig. 5.5B. The thick solid line in Fig. 5.8 is obtained by adding the separate slices and is very similar to the signal build-up of the 4’-^{13}C extracted from the 2-D experiment (Fig. 5.8, thin solid line). This means that the 4’-^{13}C accepts its polarization predominantly from the 3’-^{1}H, 4’-O^{1}H, 5’-^{1}H and 1-OO^{1}H. The Fourier transforms of the polarization transfer from 5’-^{1}H and 3’-^{1}H to 4’-^{13}C yield LC-CP carbon spectra that are centered around 0 Hz, hence the narrow response in the center in Fig. 5.5B can be assigned to couplings with the 3’-^{1}H and 5’-^{1}H and probably involves relayed transfer of polarization.

5.4 Discussion

The LG-CP carbon spectrum of a CH moiety (Fig. 5.4C) is symmetric around $\omega = 0$ and yields two well resolved maxima. The splitting $\Delta\omega/2\pi$ between the maxima provides a direct measure for the dipolar coupling strength and can be used to resolve internuclear ^{1}H-^{13}C distances. To translate the $\Delta\omega/2\pi$ into a heteronuclear distance r_{CH}, a set of simulations was performed for an isolated CH spin-pair, and a calibration line was produced, in a similar way as discussed for a homonuclear spin-pair on rotational resonance by Verdegem *et al.* [27]. In this set, r_{CH} was varied in steps by shifting the proton away from the carbon along the direction of the internuclear vector connecting the spin-pair. Fig. 5.9 shows a plot of $(\Delta\omega/2\pi)^{-1/3}$ versus r_{CH} for the spin system. $(\Delta\omega/2\pi)^{-1/3}$ varies linearly with

r_{CH} (Fig. 5.9, '○'), which can be used to extract CH distances from the data with good accuracy. The dipolar coupling strength diverges for r_{CH} approaching zero and a linear least-squares fit yields $r_{\mathrm{CH}} = a \cdot (\Delta\omega/2\pi)^{-1/3}$, with $a = 25.86 \pm 0.01$ Å Hz$^{1/3}$ (Fig. 5.9, solid line). When an exponential apodization is applied to the data in the time domain (Fig. 5.9, 'Δ'), $\Delta\omega/2\pi$ decreases slightly. For instance, a 600 Hz exponential apodization yields a shift of about 50 Hz, while an apodization of 1000 Hz produces a shift of approximately 85 Hz. In these cases for large r_{CH} small deviations from linearity can be observed.

Correlations between quaternary carbons and protons that are not directly bonded to a carbon are remarkably easy to detect in the uniformly ^{13}C-enriched system. Apparently, for an ^{1}H directly bonded to a ^{13}C, the magnetization is transferred coherently to its adjacent carbon in a short time of ~ 60-100 μs (cf. Figs. 5.4A and B). After this initial rapid transfer period, the ^{1}H that are not directly bonded to a ^{13}C, for instance O^{1}H or N^{1}H$_3^+$, can transfer magnetization over relatively long distances. For quaternary carbons, CP will be predominantly from protons that are not strongly coupled to a ^{13}C spin, like hydrogen-bonded protons. This offers an attractive route for collecting heteronuclear intermolecular distance constraints in a multispin cluster. In favourable cases such polarization transfer can already be detected without any multi-pulse decoupling scheme, as was demonstrated in Chapter 3 for the 1-COOH and 4'-COH with the CP/WISE technique.

The two high-frequency components observed for the 4'-^{13}C build-up provide information about the heteronuclear dipolar couplings of the 4'-^{13}C with the 4'-O^{1}H and 1-OO^{1}H protons. The relationship between $\Delta\omega/2\pi$ and r_{CH} according to Fig. 5.9 can be exploited to estimate the CH distance from the experimentally observed maxima in the LG-CP carbon spectrum. We have noted above that the effect of remote protons on the LG-CP response of a strongly coupled CH spin-pair is a broadening and a slight increase of $\Delta\omega/2\pi$. Since the relationship between $\Delta\omega/2\pi$ and r_{CH} was obtained for an isolated spin-pair, the frequency shift due to coupling to remote protons in the experiment will introduce an uncertainty in the estimated CH distances. For a strongly coupled spin-pair, this uncertainty will be small. For instance, for the CH moiety in Fig. 5.4E, the differences in $\Delta\omega/2\pi$ for the spin clusters containing one and five protons translate in deviations in r_{CH} that are less than 1 %. On the other hand, the effect of remote protons may be larger on weakly coupled CH pairs. Artificial broadening of the simulated response will not help, since this will shift the maxima to lower frequencies, that is, in the opposite direction. Hence the best results will be obtained if we work with the straight line in Fig. 5.9. A second uncertainty may be introduced by the scaling of the dipolar coupling in the experiment. The coupling strength $\Delta\omega/2\pi$ obtained from the experimental data is slightly lower than the $\Delta\omega/2\pi$ in the simulations. A possible origin for this discrepancy may be an experimental scaling factor that is lower than the theoretical factor $\sin\theta_{\mathrm{m}}$. Comparing the experimental and simulated $\Delta\omega/2\pi$ for the 2-CH moiety in Figs. 5.4C and E, respectively, a correction factor of ~ 1.07 can be estimated for the scaling factor. The effect of this factor is small and it may result in a 2 % deviation in the distance.

Taking the uncertainties in r_{CH} into account in the experimental error, the 2100 ± 25 Hz frequency component yields r_{CH} = 2.02 ± 0.04 Å, while the 1150 ± 100 Hz component gives a CH distance of 2.47 ± 0.07 Å. These distances compare very well with the r_{CH} = 1.982 Å and r_{CH} = 2.521 Å obtained from the neutron diffraction structure (Fig. 5.6) [24]. It demonstrates that the accuracy in the distances r_{CH} obtained with the NMR is good and that the procedure can be applied to extract r_{CH} from the time-oscillating CP build-up curves. This should be particularly useful to characterize hydrogen-bonded protons in a wide range of solids, including biological preparations.

5.5 Conclusions

In this chapter it is shown that the time-oscillatory polarization transfer during LG-CP can be analyzed to determine heteronuclear distances with good accuracy. Fourier transforms of the LG-CP build-up curves provide effective carbon spectra that resemble heteronuclear dipolar powder spectra for non-spinning samples. The LG-CP carbon spectra can be closely reproduced with MAS Floquet simulations. A set of simulations was performed with different r_{CH}, in order to provide an empirical relation between the dipolar coupling strength $\Delta\omega/2\pi$ and the CH distance. We have demonstrated that this relation can be utilized to determine r_{CH} from experimentally observed dipolar coupling strengths by calculating the CH distances to protons that have a hydrogen-bonding interaction or to protons that are not directly bonded to a ^{13}C. The method is also suited to determine r_{CH} of directly coupled CH spin-pairs. This is important, since it is often not possible to obtain good CH distance information from X-ray structures. The measurement of heteronuclear distances sets the stage for a novel route to *de novo* structure determination using uniformly enriched compounds or multispin clusters in ordered systems without long-range translation symmetry, for instance biological systems such as membrane proteins. In addition, a 3-D version of the technique enables an analysis of the polarization exchange within individual CH spin-pairs, offering exciting prospects to study the coherent cross-polarization dynamics and hydrogen-bonding environments in detail on a microscopic scale.

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Chapter 6

The 3-D structure of self-assembled Chl *a* / H_2O from multispin labeling and MAS NMR 2-D dipolar correlation spectroscopy in high magnetic fields

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6.1 Introduction

Magic-angle spinning nuclear magnetic resonance (MAS NMR) dipolar correlation spectroscopy is rapidly forthcoming as a new technique for *de novo* structure determination of microscopically ordered systems without long-range translation symmetry [1]. The development of homonuclear correlation spectroscopy for structural studies has followed the development of MAS NMR dipolar recoupling schemes [2]. Such schemes were initially used in moderately high fields to confirm structural information, that is: to incorporate pairs of labels in structures that were already known to sufficiently high resolution to be verified [3]. It has been shown recently for multiply labeled systems that also *de novo* structure determination is possible with homonuclear MAS NMR dipolar correlation spectroscopy, since the radio-frequency driven dipolar recoupling (RFDR) technique was used to construct an initial model for the parallel stacking in aggregated uniformly ^{13}C-enriched ([U-^{13}C]) chlorophyll *a* / H_2O [4,5]. This first NMR model is based on the measurement of aggregation shifts and the identification of several homonuclear (^{13}C-^{13}C) intermolecular correlations, following the scheme depicted in Fig. 6.1 [1].

Very recently it was discovered that well-resolved MAS NMR heteronuclear (^1H-^{13}C) dipolar correlation spectra of multispin ^{13}C clusters can be acquired at high spinning speeds when frequency-switched Lee-Goldburg (FSLG) [6] irradiation is applied during the ^1H evolution [1,7]. In addition, it was found that ^1H-^{13}C heteronuclear intermolecular correlations can be detected with relatively straightforward 2-dimensional (2-D) techniques in high magnetic fields using fast MAS [8]. It will be shown here how ^1H shift constraints and ^1H-^{13}C heteronuclear intermolecular correlations can be applied in structure refinement studies. This proceeds in a similar way as the concept for structure determination based on ^{13}C homonuclear MAS NMR dipolar correlation spectroscopy presented in Fig. 6.1. First 2-D ^{13}C homonuclear dipolar correlation spectra are recorded and analyzed to arrive at a complete assignment of the carbon chemical shifts. The carbon assignment is subsequently used to assign proton chemical shifts and distance constraints in ^1H-^{13}C heteronuclear dipolar correlation spectra.

The concept of Fig. 6.1 is here applied in a restrained molecular modeling approach to characterize the structure of a solid, self-assembled [U-^{13}C] chlorophyll *a* / H_2O (Fig. 6.2).

Chlorophyll *a* (Chl *a*) is the green pigment involved in photosynthetic harvesting of light and subsequent conversion of light-energy into chemical energy by plants and related species, like algae and cyanobacteria. Chl *a* forms aggregates when it is exposed to H_2O [9]. The molecular arrangement of self-assembled Chl *a* is thought to represent a paradigm for a special type of photosynthetic light-harvesting system, the chlorosome antenna found in some photosynthetic bacteria, which is almost protein-free and is potentially important for artificial photosynthesis research [9].

The condensed Chl *a* / H_2O is microscopically ordered and the molecules constituting the aggregate are rigidly held in place [10]. A systematic determination of solid-state NMR chemical shifts provides detailed information concerning the electronic structure of such a densely packed solid. The analysis of NMR aggregation shifts can already lead to a structural model in an interpretation step (Fig. 6.1, dashed arrow) [5]. Even small shifts can be significant, if the shift effects are correlated in the sense that they follow a pattern or are extended over a region of the molecule [11]. The characterization of non-bonding interactions via their effect on the ^{1}H shift can be quite revealing in the solid state. In particular, ring-current effects for protons in chlorophylls can be very large. It will be shown below that ^{1}H ring-current shifts up to ~ 5 ppm are observed, which is an important effect on the proton chemical shift scale with its range of ~ 15 ppm. In contrast, carbon nuclei have a much larger chemical shift dispersion of ~ 200 ppm, and aggregation effects appear less significant.

In parallel, the identification of heteronuclear (^{1}H-^{13}C) intermolecular correlations from dipolar correlation spectra provides unambiguous structural restraints [12]. Even a limited set of constraints will substantially reduce the number of possibilities to assemble the molecules to form a 3-dimensional (3-D) ordered solid. To arrive at a 2-D sheet of stacks and finally at a 3-D structural model for the aggregated chlorophyll, a homology modeling strategy will be followed. In this approach the NMR shift and distance restraints are used in a comparison with available NMR and structural data for oligomers in solution and high-resolution structural models of crystalline solid chlorophyll derivatives.

6.2 Experimental

Highly enriched Chl *a* (> 95% ^{13}C) was isolated from a cell culture of *Chenopodium rubrum* that was grown on isotope-enriched material [4]. Aggregates of Chl *a* were prepared overnight by hydration in *n*-heptane [4].

Proton data for the monomeric Chl *a* in acetone-d_6 solution, referenced to TMS at 0 ppm were obtained with a DMX-600 spectrometer (Bruker, Karlsruhe, Germany). The chemical shifts were assigned with 2-D proton correlation spectroscopy (^{1}H-^{1}H COSY) and 2-D heteronuclear correlation spectroscopy (^{13}C-^{1}H COSY).

Cross-polarization (CP) MAS dipolar correlation spectra were recorded with a DMX-600 spectrometer, equipped with a 4mm double-resonance CP/MAS probe (Bruker, Karlsruhe, Germany). A home-built spinning-speed controller was used to keep the spinning speed constant to within a few Hz [13]. All correlation spectra were recorded with a ramped CP spin-lock pulse on the carbon nuclei to broaden the CP matching profile at high MAS rates [14]. The protons were decoupled from the carbons by use of the two-pulse phase-modulation (TPPM) decoupling scheme during all ^{13}C acquisition periods and during ^{13}C evolution and mixing in the homonuclear (^{13}C-^{13}C) correlation experiments [15]. A pulse length of 8 μs and a phase-modulation angle of 20 degrees were used for the TPPM decoupling.

2-D homonuclear (^{13}C-^{13}C) dipolar correlation spectra were recorded with the broad-banded RFDR technique with phase-sensitive detection in ω_1 [4,16]. The 2-D heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy employed FSLG irradiation during the proton evolution to suppress the homonuclear ^{1}H interactions (Fig. 6.3) [1,6,7]. Selective heteronuclear polarization transfer during a relatively long mixing time of 2.0 ms was achieved using Lee-Goldburg cross-polarization (LG-CP) [12,17]. The chemical shift scaling factor for the FSLG experiment was determined experimentally by plotting the scaled Chl *a* proton shifts from the FSLG experiment against the corresponding non-scaled proton shifts obtained with low-resolution CP wide-line separation (CP/WISE) correlation spectroscopy [8,18]. The slope in such a plot directly provides the scaling factor. Lee-Goldburg scaling factors are 0.50 and 0.51 for the correlation spectra in Figs. 6.5A and 6.5B below, respectively, less than the theoretical scaling factor of 0.577.

1-dimensional (1-D) solid-state ^1H MAS NMR spectra were recorded in a magnetic field of 9.4 T with an MSL-400 spectrometer, equipped with a 4mm double-resonance CP/MAS probe (Bruker, Karlsruhe, Germany). A home-built spinning-speed controller was used to keep the spinning speed constant [13].

Semi-empirical quantum chemical calculations were performed on a single ethyl-chlorophyllide *a* molecule with various fifth ligands on the Mg, using the MOPAC 97 code incorporated in the Chemoffice package (Cambridgesoft, Cambridge, MA) [19]. The MNDO-d parametrization was used [20,21]. The ethyl-chlorophyllide *a* from the X-ray structure with the Mg coordinated with a single water molecule was used as a starting point and it was verified that MNDO-d optimization reproduces the monomer ring structure found with the X-ray [22]. After a full optimization run, the water was deprotonated or replaced by a THF molecule, followed again by a full optimization of the structure. Estimates of atomic charge densities were evaluated with the Mulliken population analysis on the optimized structures [23].

6.3 Results

6.3.1 ^{13}C-^{13}C homonuclear dipolar correlation spectroscopy

Good progress has been made recently in enhancing the ^{13}C spectral resolution in solid-state NMR by using higher fields, fast MAS rates, a sample geometry that is optimized with respect to RF inhomogeneity and demagnetizing field, and efficient TPPM proton decoupling during ^{13}C acquisition [1]. With these improvements, we have recorded homonuclear (^{13}C-^{13}C) dipolar correlation spectra with MAS rates of 12 kHz and 13 kHz and with different homonuclear mixing times to refine the previously reported ^{13}C assignment [4]. Fig. 6.4A shows a high-field (14.1 T) ^{13}C homonuclear dipolar correlation spectrum recorded with a spinning speed $\omega_r / 2\pi$ = 13 kHz from [U-^{13}C] Chl *a* / H_2O aggregates using the RFDR technique. A ^{13}C homonuclear RFDR mixing time of 2.46 ms was applied to establish the correlations. The resolution in this spectrum is considerably enhanced compared to the earlier work [4]. The ^{13}C lines are narrow and the widths range from 120 Hz to 200 Hz, due to the *J*-couplings between the ^{13}C nuclei in the uniformly labeled Chl *a*.

The improved resolution allows for a refinement of the ^{13}C assignments (Table 6.1). The shifts of the 2^1-C, 7^1-C and the 12^1-C responses were previously reported as approximately 9.6 ppm, 10.5 ppm and 8.9 ppm, respectively. In the high-field data presented here, a doubling of the NMR response from both the 7-C and the 7^1-C can be resolved. This is illustrated with Figs. 6.4B and 6.4C that show in detail the areas indicated with dashed

Table 6.1. ^{13}C *chemical shifts in solution* (σ_{liq}) *of monomeric Chl a in* [U-^{2}H] *tetrahydrofuran [24,25]. The* σ_i *are the isotropic* ^{13}C *chemical shifts for the aggregated Chl a with estimated errors* (ε, *in ppm). The numbering is according to Fig. 6.2.*

Position	σ_{liq} (ppm)	σ_i (ppm)	ε (ppm)
12^1	12.6	10.5	0.3
2^1	12.6	10.2	0.2
7^1	11.2	8.9/11.5	0.2
p17	16.2	17.4	0.1
8^2	18.0	16.5/17.5	0.5
8^1	20.0	19.0/20.5	0.5
p18	20.0	20.0	0.2
p19	20.0	20.0	0.2
p20	23.0	23.3	0.3
p16	23.0	23.3	0.3
18^1	23.9	24.9	0.1
p9	25.2	25.6	0.3
p13	25.6	25.6	0.3
p5	25.8	25.8	0.2
p15	28.7	28.4	0.2
$17^1/17^{2\,a}$	30.1/30.9	32.3	0.1
p7	33.4	33.1	0.1
p11	33.6	33.1	0.1
p6	37.4	37.1	0.5
p12	38.0	38.0	0.2
p8	38.0	38.0	0.2
p10	38.0	38.0	0.2
p14	40.1	40.0	0.2
p4	40.4	40.0	0.2
18	50.0	50.0	0.1
17	51.6	51.7	0.3
13^4	52.0	51.8	0.1
P1	61.3	61.7	0.1
13^2	66.2	64.9	0.1
20	92.8	92.6	0.2
5	100.0	98.3	0.1
15	106.2	103.0	0.1

Table 6.1 (continued)

Position	σ_{liq} (ppm)	σ_{i} (ppm)	ε (ppm)
10	107.1	108.1	0.1
3^2	118.9	113.7	0.1
P2	119.4	121.3	0.1
13	131.5	126.1	0.1
3^1	131.5	126.7	0.1
7	134.0	132.2/133.3	0.1
12	134.2	133.0	0.1
2	135.5	135.6	0.1
3	139.0	136.3	0.1
P3	142.2	138.5	0.1
8	144.1	145.4	0.1
9/11 [a]	146.1/147.7	147.0	0.1
4	148.0	150.7	0.1
6	151.4	154.1	0.2
16	155.8	154.0	0.1
1	154.0	156.1	0.1
14	161.4	162.3	0.1
19	167.4	169.4	0.3
13^3	171.0	169.9	0.1
17^3	172.7	174.3	0.3
13^1	189.3	190.6	0.1

[a] *The differences between 9-C and 11-C, and between 17^1-C and 17^2-C could not be resolved in solution due to ambiguities in the intramolecular correlation pattern.*

rectangles in Fig. 6.4A. The correlations of the 7-C with the 6-C and 8-C in Fig. 6.4B reveal a small but resolved doubling of the 7-C response, with the two resonances separated by 1.1 ppm. A somewhat larger separation corresponding with 2.6 ppm is observed for the 7^1-C response (Fig. 6.4C). The most upfield fraction of the 7^1-C response was previously assigned to the 12^1-C [4]. The correlations of the 7^1-C with the 6-, 7- and 8-C and of the 12^1–C with the 11-, 12- and 13-C (cf. Fig. 6.4C) clearly show that the 7^1-C and 12^1-C responses can be resolved in the high-field datasets. In addition, a doubling of the 8^1-C and/or 8^2-C resonances can be deduced from the data, since three signals are observed, which can be assigned to correlations of the 8^1-C or 8^2-C with the 8-C (cf. Fig. 6.4C). Since two weak correlations appear for the 8^1-C with the 7-C, it may be concluded that at least the signal from the 8^1-C is doubled, while the data suggest an additional doubling of the 8^2-C (Fig. 6.4C). Taking this doubling of signals into account, we arrive at the following new assignment, in ppm: 7-C

(132.2/133.3), 7^1-C (8.9/11.5), 8^1-C (19.0/20.5), 8^2-C (16.5/17.5), 2^1-C (10.2) and 12^1-C (10.5). From the integrated peak volumes of the correlations that are doubled and resolved in the homonuclear correlation spectrum, the relative intensities are estimated as: 7^1(11.5)/8: 47 %, 7^1(11.5)/6: 56 %, 7(132.2)/6: 50 %, 8/7(132.2): 40 %. The full assignment of the ^{13}C resonances of the aggregated Chl *a* is listed in Table 6.1, together with ^{13}C solution chemical shifts in perdeuterated tetrahydrofuran [24,25]. Except for the doubling of a limited set of signals, the high-field assignment is in good agreement with the results that were already obtained with lower fields [4,5].

6.3.2 ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy

Once the ^{13}C assignment is known, individual proton resonances can be assigned from intramolecular correlations and distance constraints can be determined with MAS NMR ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy [12]. There are several advantages in exploring ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy, compared with homonuclear ^{13}C correlation methods. First, the gyromagnetic ratio for protons is four times larger than for carbon nuclei. Since ^{1}H-^{13}C dipolar interactions are strong compared to homonuclear ^{13}C interactions, intermolecular correlations should develop faster than for the homonuclear ^{13}C case. In addition, the heteronuclear spin topology of hydrocarbons generally favours the detection of intermolecular ^{1}H-^{13}C correlations, since protons are always located at the exterior of the molecule. Next, the intramolecular relayed carbon coherence transfer along the carbon backbone can proceed quite efficiently and competes with the intermolecular transfer [5]. A distinction between relayed intramolecular and intermolecular ^{13}C coherence transfer is quite difficult for the relatively small chlorophyll ring system [5]. In heteronuclear correlation spectroscopy this problem can easily be avoided by the application of ^{1}H homonuclear decoupling sequences during the transfer period. In addition, there is another more subtle advantage, which may turn out to be critical in future protein structure determination studies. Heteronuclear correlations between quaternary carbons and protons that are not directly bound to a carbon are easy to detect in uniformly ^{13}C-labeled systems [7,8]. During LG-CP of a proton that is directly bonded to a ^{13}C in a rigid environment the polarization is rapidly transferred in a coherent process on a time scale of ~100 μs from the proton to the adjacent carbon. LG-CP to a quaternary carbon predominantly involves protons in methyl groups or protons that are not strongly coupled to a carbon spin, for instance hydrogen-bonded protons. This provides an attractive route to the detection of intermolecular transfer events, in favourable cases already with the simplest possible pulse schemes like the CP/WISE technique [8].

Intermolecular correlations are generally remote, hence the magnetization has to be transferred over relatively long distances, ≥3 Å, which requires a long CP contact time. On the other hand, in the solid state the interactions between the abundant protons are even stronger than the heteronuclear proton-carbon couplings. To maintain a good selectivity during long CP contact times, homonuclear ^1H decoupling is applied to slow down the proton spin-diffusion processes. We found that the LG-CP [17] can be applied successfully at high MAS rates up to at least 15 kHz while maintaining a good heteronuclear polarization transfer [12]. During the LG-CP, an off-resonance RF field locks the proton spins in the rotating frame along an axis inclined at the magic angle with respect to the static field in the *z*-direction, while the carbon spins are locked on-resonance in the *xy*-plane. In this way, heteronuclear spin-locking with simultaneous suppression of the homonuclear dipolar proton coupling is achieved and polarization can be transferred selectively from the protons to the carbon nuclei.

Fig. 6.5 shows high-field heteronuclear correlation spectra recorded with a MAS rate $\omega_r/2\pi$ = 13 kHz using a pulse sequence with ^{1}H homonuclear FSLG decoupling during proton evolution (Fig. 6.3) [1,6,7]. A relatively short CP contact time of 600 μs was used to build up short-range heteronuclear correlations and to collect the data shown in Fig. 6.5A, while in Fig. 6.5B a long LG-CP mixing period of 2 ms was applied to promote proton polarization transfer over an extended range. The high resolution in the carbon dimension is sufficient to assign nearly all ^{1}H resonances from the intramolecular ^{1}H-^{13}C correlations in the 2-D datasets. The solid-state assignments for 17-H and 13^4-H_3 remain tentative due to spectral overlap in the ^{13}C dimension (cf. Table 6.1). The assignment of the 8^1-CH_2 is difficult because of overlap of the correlations with the strong signals from the P7-Me and P11-Me moieties. The solid-state proton assignment is listed in Table 6.2, together with the proton assignment for monomeric Chl *a* in acetone-d_6 solution. The proton shifts in solution compare well with literature data reported previously for chlorophylls [24,26].

With σ_i the shift for the aggregate and σ_{liq} the shift in solution, aggregation shifts $\Delta\sigma_i = \sigma_i - \sigma_{liq}$ were calculated, reflecting differences in the molecular electronic structure between the monomeric and the aggregated form. The larger proton aggregation shifts $|\Delta\sigma_i| \geq 2$ ppm are upfield and are detected for 2^1-H_3 (-5.4 ppm), 3^1-H (-2.4 ppm), 3^2-H_2 (-3.8 ppm) and 12^1-H_3 (-4.9 ppm). The proton aggregation shifts are visualized in Fig. 6.2, together with the carbon aggregation shifts $|\Delta\sigma_i| \geq 2$ ppm.

6.4 Discussion

6.4.1 Chemical shift and distance constraints on the structural model of the Chl *a* / H_2O stacks

Semi-empirical quantum chemical calculations are often used in the interpretation of chemical shifts [27,28]. For olefinic and aromatic carbons, the effect of variations of the chemical environment on the atomic charge densities can be used to asses the chemical shift variations, since one electronic equivalent of charge difference translates into 150 ppm shift difference [29]. Most likely water molecules that are added in the Chl *a* aggregration process coordinate the Mg in the self-assembled structure. According to the MNDO-d calculations, the direct effect on the atomic charges of the aromatic ring carbons, via the Mg on the macro-

Table 6.2. The σ_{liq} are ^{1}H shifts of monomeric Chl a in acetone-d$_6$, referenced to TMS at 0 ppm. The σ_i are the isotropic ^{1}H shifts for the aggregated Chl a with estimated errors (ε, in ppm). The numbering is according to Fig. 6.2.

Position	σ_{liq} (ppm)	σ_i (ppm)	ε (ppm)
5-H	9.40	8.6	0.3
10-H	9.72	7.8	0.3
20-H	8.56	6.7	0.3
3^1-H	8.12	5.7	0.3
3^2-H_2	6.22/6.01	2.3	0.5
13^2-H	6.20	5.8	0.5
18-H	4.56	2.8	0.5
17-H	4.18	3.9	0.5
13^4-H_3	3.82	3.4	0.5
8^1-H_2	3.79	2	2
12^1-H_3	3.60	-1.3	0.3
2^1-H_3	3.34	-2.1	0.3
7^1-H_3	3.29	1.7^a	0.3
		2.8^b	0.3
17^1-H_2	2.63-2.54/2.49-2.38	3.0	0.5
17^2-H_2	2.05^c	3.0	0.5
18^1-H_3	1.77	1.4	0.5
8^2-H_3	1.70	1	1
P1-H_2	4.36/4.26	4.9	0.3
P2-H	5.01	6.0	0.3
P4-H_2	1.82	2.0	0.5
P5/P9/P13-H_2	1.2	1.2	0.5
P6-H_2	1.2-1.0^d	1.0	0.5
P7/P11-H	1.33	1.2	0.5
P8/P10/P12-H_2	1.2-1.0^d	1.1	0.5
P14-H_2	1.12	2.0	0.5
P15-H	1.51	1.3	0.5
P16/P20-H_3	0.85	1	1
P17-H_3	1.52	1.5	0.5
P18/P19-H_3	0.80	0.9	0.5

[a,b] *Proton signals correlated with the 7^1-C at 8.9 ppm[a] and at 11.5 ppm[b].*

[c] *The 17^2-H_2 resonances are partly overlapping with the acetone signal.*

[d] *broad multiplets.*

aromatic cycle, of replacing the THF in our solution model by a neutral water molecule, should be very small. In the calculations the atomic charge density differences for the macrocycle 1-C - 20-C are well within 0.01 electronic equivalent. This supports conclusions from earlier work on these aggregates that the diamagnetic susceptibility changes observed with the NMR through the aggregation shifts are indeed associated with intermolecular stacking effects in the self-assembled Chl *a* [4].

The proton aggregation shifts from the ^{1}H assignment in Table 6.2 corroborate the stacking model for Chl *a* / H_2O deduced from the ^{13}C homonuclear dipolar correlation spectroscopy [4,5]. The NMR resonances of the 2^1-CH_3, 3^1-CH and 3^2-CH_2 moieties at one side of the molecule and of the 12^1-CH_3, 13-C and 15-C moieties at the opposite side are shifted upfield with respect to the corresponding responses for the monomer in solution (Fig. 6.2). The pattern of proton aggregation shifts in Fig. 6.2 confirms that the regions around the rings I and III/V of the Chl *a* macro-aromatic cycle are predominantly affected upon aggregation. This is well in line with the proposed arrangement of Chl *a* in linear stacks, with the ring I of one molecule placed over rings III/V of a neighbouring molecule. In that case substantial ring-current shifts are expected for protons that reside above the ring of adjacent Chl *a* molecules (Fig. 6.6) [4,5,11].

The positive aggregation shifts observed for the 19-, 1-, 4- and 6-C positions indicate the stabilization of a small amount of positive charge on both sides of the ring I. Although the net charge involved represents only a few percent of an electronic charge equivalent, the pattern of the polarization reflects the charge-alternating characteristics of the molecular orbital contributing to the aromatic ring system [30]. Most likely this charge stabilization is due to mutual polarization effects between the conjugated π system of the ring and an external charge or dipole. The only negative charges that are present in the system are the partial charges that are accumulated on the carbonyl oxygens. One of these, the 13^1 keto carbonyl oxygen of another chlorophyl in the assembly, is located about 3.5 Å above the ring I nitrogen and the 1-C and 20-C in our model of the stack. The mutual polarization effects involving the 13^1 in this position will contribute to the red shift of the Q_y transition and the stabilization of the aggregate.

Chl *a* can also form dimers and we have examined if one out of the several dimer forms that have been observed in solution [31] could serve as building block for the aggregate. The chemical shifts obtained from the ^{1}H and ^{13}C MAS NMR can be used to assess these models as a basic building block for the stack, based on a homology between aggregation shifts of the model and the self-assembled chlorophyll. From the models that have been proposed for the dimeric unit, none could account for the aggregation shifts in Fig. 6.2. The larger proton aggregation shifts for dimeric Chl *a* are consistently observed in the regions around the rings IV and III/V of the molecule, since in the dimer the ring IV of one molecule is located above rings III and V of another molecule [24,31,32]. This contrasts with the pattern of shifts observed for Chl *a* in the solid aggregate that predominantly involves the side chains of ring I (Fig. 6.2).

With the improvement of the ^{1}H resolution that has been established in the last few years, it is now possible to obtain heteronuclear (^{1}H-^{13}C) distance constraints [12]. An example of how the heteronuclear correlations provide distance restraints and structural information is indicated in Fig. 6.5B, which shows the heteronuclear correlations due to magnetization transfer from the 2^1-H_3 and the 12^1-H_3 that resonate around -2.1 ppm (upper trace in Fig. 6.5B) and -1.3 ppm (lower trace), respectively, to ^{13}C in a nearby Chl *a* ring. Due to the pronounced upfield ring-current shifts in the ^{1}H dimension, the correlation signals are well resolved. The 2^1-H_3 have intramolecular correlations to the nearby 2-, 2^1-, 3-, 3^1-, 3^2-, 1- and 20-C. In addition, the 2^1-H_3 correlate with 14-, 15-, 16- and 13^2-C, which are on the opposite side of the Chl *a* molecule. This second set of correlations is due to intermolecular polarization transfer and this shows that the 2^1-H_3 are in a position above the plane of a neighbouring molecule. The 12^1-H_3 have intramolecular correlations with the neighbouring 10-, 11-, 12-, 12^1-, 13- and 14-C, and intermolecular correlations with the 4-, 5- and 6-C.

The heteronuclear intermolecular correlations strongly support a structural homology between the linear stacks of Chl *a* molecules in the aggregate and the stacking in a solid model, crystalline ethyl-chlorophyllide *a* / H_2O [22]. The crystal structure of this analogue predicts intermolecular contacts between methyl protons and aromatic ring carbons, most notably of the 2^1-H_3 with the region centered around the 15-C and of the 12^1-H_3 with the area around the 5-C. They correspond with the overlap regions observed with the MAS NMR, as indicated with the dashed arrows in the model in Fig. 6.6. The average CH distances *d* represent the shortest heteronuclear intermolecular distances in the X-ray structure of the solid model, and are in between 3.7 and 4.1 Å.

6.4.2 Chemical shift and distance constraints on the structural model of the Chl *a* / H_2O layered sheets

Several of the intermolecular correlations were found to arise from intermolecular ring contacts between stacks of Chl *a* (Fig. 6.6, dashed arrow). The 2^1-H_3 are correlated with the 9-C and/or 11-C at 147.0 ppm, and with the 10-C at 108.1 ppm, reflected by a high-field shoulder at -2.1 ppm in the ^1H dimension. The 19-C and 13^3-C resonate around 170 ppm and weakly correlate with protons that have σ_i centered around -1.7 ppm (cf. Fig. 6.7C). The only protons that resonate in this upfield region of the ^1H spectrum are the 2^1-H_3 at -2.1 ppm and the 12^1-H_3 at -1.3 ppm, hence the signal around -1.7 ppm suggests overlapping contributions from the 2^1-H_3 and 12^1-H_3. This provides evidence for magnetization transfer from 2^1-H_3 to 19-C and/or 13^3-C, and from 12^1-H_3 to 19-C [33]. The heteronuclear intermolecular correlations of 2^1-H_3 with 9-C/11-C and 10-C, and of 12^1-H_3 with 19-C provide strong evidence for a layered sheet arrangement according to Fig. 6.6. They confirm the very weak and barely detectable intermolecular polarization transfer that has been reported between 10-C and 1-C, 10-C and 20-C and between 11-C and 19-C in homonuclear (^{13}C-^{13}C) dipolar correlation spectroscopy [5].

The aggregation shifts provide additional support for the layered model. The 10-H and the 20-H responses are shifted upfield by -1.7 ppm and -1.6 ppm, respectively, relative to monomeric Chl *a* in solution (cf. Table 6.2). In the structural model of Fig. 6.6, the 10-H and 20-H are located above the plane of the conjugated ring of a Chl *a* molecule in a neighbouring stack, the 10-H close to the 2-C of an adjacent Chl *a* at a distance d = 3.5 Å and the 20-H near the 11-C and 12-C, with d = 3.9 Å for both. Based on computations of ring-current shifts in macro-aromatic cycles [34], upfield proton aggregation shifts of -1.5 to -2.0 ppm are estimated for the 10-H and 20-H.

In the linear stack the 2^1-H_3 and 12^1-H_3 are located at an average distance of about 3.7 Å above the plane of the ring of an adjacent Chl *a* molecule, with the 2^1-H_3 near the 15-C and the 12^1-H_3 close to the 5-C (Fig. 6.6). According to theoretical estimates of the shifts induced by the ring-current in the conjugated macro-aromatic cycle of a Chl *a* molecule, upfield proton aggregation shifts of -2.0 to -3.0 ppm can be expected for the 2^1-H_3 and 12^1-H_3 in this arrangement [34]. The solid-state proton aggregation shifts of the 2^1-H_3 and 12^1-H_3 of -5.4 ppm and -4.9 ppm, respectively, are therefore too large to be explained by ring-current effects induced by the conjugated ring of a single neighbouring Chl *a* molecule. Additional overlap between stacks forming layered sheets should explain the large ^{1}H aggregation shifts for the 2^1-H_3 and the 12^1-H_3 (Fig. 6.6). Due to the interstack arrangement, the 2^1-H_3 and 12^1-H_3 are also positioned at an average distance d = 3.7 Å above Chl *a* rings from a second stack, which imposes proton aggregation shifts of -2.0 to -3.0 ppm, in addition to the ring-current shifts that result from the stacking in the first dimension. Thus, the aggregation shifts of the 10-H and 20-H, as well as the pronounced upfield shifts of the 2^1-H_3 and 12^1-H_3, can very well be explained in terms of ring-current effects due to additional ring overlap between stacks. They support the model for the sheet depicted in Fig. 6.6.

In the structural model of Fig. 6.6, the overlap between chlorophyll rings is restrained by the aggregation shifts and the intermolecular heteronuclear correlations. This leaves a gap of ~ 4 Å between the Mg and the keto carbonyl of a neighbouring molecule. During the sample preparation, water is continuously added to form the Chl *a* / H_2O aggregates. Two water molecules can fill the open space between the Chl *a* and can stabilize the aggregates by coordination to the Mg and formation of a hydrogen-bonded network to 13^1, 13^3 and 17^3, indicated by the dotted lines in Fig. 6.6. In the X-ray structure of the homologous ethyl-chlorophyllide *a*, the corresponding gap is also filled with two water molecules [22].

Proton slices extracted from a heteronuclear dipolar correlation spectrum recorded with a long LG-CP contact time of 2 ms reveal weak correlations at the 13^1, 13^3 and 17^3 carbonyl ^{13}C frequencies that are not associated with the chlorophyll protons (Fig. 6.7). The 13^1-C=O at 190.6 ppm strongly correlates with the 13^2 proton resonating around 5.8 ppm, and weakly with protons around 0.9 ppm and -1.7 ppm (Fig. 6.7A). The intensity centered around -1.7 ppm reflects overlapping signals associated with transfer from the 12^1-H_3 and with intermolecular transfer from the 2^1-H_3 (Table 6.2). In contrast, the signal at 0.9 ppm was not yet assigned. The 17^3-C=O at 174.3 ppm correlates with protons that have σ_i = 4.7 ppm, most likely the nearby P1-H_2 at a distance of 2.8 Å (Fig. 6.7B). In addition, a broad signal centered around 1.7 ppm is observed. The 13^3-C=O and 19-C around 170 ppm show a correlation centered around 6.3 ppm, suggesting overlapping contributions from 20-H (6.7 ppm) to 19-C and from 13^2-H (5.8 ppm) to 13^3-C (Fig. 6.7C). The 13^4-H_3 can contribute to the intensity around 3.4 ppm, which may be used to refine the assignment of the 13^4-H_3 that was difficult due to the overlap with the 17-H in Fig. 6.5. A correlation with protons having $\sigma_i = 1.0$ ppm is left unexplained. We suggest that the correlations of 13^1-C=O, 17^3-C=O and 19-C/13^3-C=O with protons resonating around 0.9 ppm, 1.7 ppm and 1.0 ppm, respectively, may arise from polarization transferred from protons in the water molecules.

To provide some additional support for this assignment, we collected 1-D ^1H MAS NMR spectra from a preparation of [95%-^2H; 5%-^1H] Chl *a*, aggregated with ^1H_2O (Fig. 6.7D). The main contribution to the ^1H NMR spectrum of the [95%-^2H; 5%-^1H] Chl *a* / ^1H_2O preparation comes from the protons in the water molecules and from the aliphatic protons of the Chl *a*. Since 5% of the ~ 40 aliphatic protons was not labeled with ^2H, the total contribution to the ^{1}H spectrum from the aliphatic protons corresponds with an equivalent of approximately two protons per Chl *a*, while there are four water protons associated with each molecule. The major part of the ^{1}H NMR response that peaks around 1.4 ppm is due to protons from the water molecules, which corroborates our assignment of the signals marked with the arrows in Figs. 6.7A-C.

We have also investigated the possibility of the Mg coordinated by an OH^-, which could occur if two or more water molecules would redistribute their protons and the OH^- would be compensated by an H_3O^+ elsewhere in the network. An OH^- coordination should be considered unlikely. The MNDO-d calculations predict that for an OH^- ligand significant shift effects should arise for the 3^1 and its side chain that alternate with respect to the sign and would be accompanied by shifts, up to ~ 5 ppm, of the 5-, 8-, 12-, and 15-C, relative to the THF form. This is not in line with the experimental data.

Finally, in the model of Chl *a* depicted in Fig. 6.6, a single sliding direction for the stacks is shown. The opposite sliding direction is energetically unfavourable due to steric hindrance of the 13^2 side chain with a neighbouring Chl *a* in the stack [9,35]. In addition, a hydrogen-bonding network supporting the sheet and connecting 13^1-C=O with 13^3-C=O via water molecules can be accomplished most effectively if the 13^1-C=O of a second molecule is at the same side of the Chl *a* ring as the 13^3-C=O of a first molecule.

6.4.3 The bilayer structure of the aggregate.

In the ^{1}H-^{13}C datasets in Fig. 6.5, the intramolecular correlations with carbons of the saturated part of the phytyl chain, P4-P15, systematically appear slightly tilted, while the correlations with the ring carbons are not tilted. The carbon and proton resonances of the tails appear excessively inhomogeneously broadened by ~ 1.5 ppm, and the additional broadening dispersion of the carbon response correlates with the broadening dispersion of the proton response. In addition, in the ^{13}C homonuclear correlation spectrum in Fig. 6.4, most correlations associated with the phytyl chain are similarly broadened. In particular the correlations involving P3 and P4 are broad at the base and extend over 2-3 ppm. The systematic broadening effects indicate that there is moderate conformational heterogeneity of the phytyl chains, which contrasts with the highly ordered structure of the rings in the stacks and the P1-CH_2 and P2-CH moieties. This suggests that the domains comprising the Chl *a* rings and sections with phytyl chains are spatially separate regions in the aggregate.

The ^{13}C homonuclear correlation spectroscopy (Fig. 6.4) reveals a doubling of NMR responses of the 7-Me and 8-Et region. This shows that two fractions of Chl *a* are present, each of them well-defined. The doubling of the 7-C and 7^1-C resonances is accompanied by a doubling of the 7^1-H_3 signal. From the heteronuclear correlation spectra in Fig. 6.5 it is found that a fraction of ~ 55% of the 7^1-C response has σ_i = 11.5 ppm and correlates with a proton signal around 2.8 ppm, while a part of ~ 45% of the 7^1-C resonance is shifted upfield to 8.9 ppm and correlates with an upfield ^{1}H signal with σ_i = 1.7 ppm. The doubling of the NMR resonances is restricted to the region of the 7-Me and 8-Et. This implies that structural differences between the two pools of Chl *a* molecules are also restricted to the 7-Me and 8-Et side chains and their local chemical environment. The NMR intensities from the Chl *a* conformers in the correlation spectra are roughly equal, leading to the estimate that each fraction constitutes about 50% of the total amount of aggregated Chl *a*.

The doubling of the 7-Me and 8-Et resonances observed with the solid-state NMR represents another chemical shift restraint that can guide the construction of a 3-D model for the structure, following the dashed arrow in Fig. 6.1. At this point, it is important to stress that there *is* a structure. The narrow ^{13}C linewidths of 120-200 Hz for the Chl *a* ring system, the pattern of carbon and proton aggregation shifts relative to Chl *a* in solution and the observation of homonuclear and heteronuclear distance restraints, all provide converging evidence that the Chl *a* is in a highly-ordered, microcrystalline environment. The Chl *a* / H_2O in Fig. 6.6 can form a bilayer by stacking the sheets back to back. This can be achieved by invoking two different conformations of the 8-Et moiety by rotation around the 8-8^1 bond. This is the only conformational degree of freedom on the ring that is left by the shift constraints. The 7-Me moiety is firmly connected to the ring and cannot be manipulated without disturbing the entire Chl *a* ring system. On the other hand, by rotation of all the 8-Et moieties of one layered sheet around the 8-8^1, the 8^2 can be positioned in cavities in the sheet. The 7-Me are the only protuberances of one sheet that fit in the cavities of a complementary layer in which the 8-Et are pointing outwards, and in this way the sheets can form bilayers. This is a different arrangement than for the 3-D crystals of ethyl-chlorophyllide *a*, where the sheets are stacked back to front [22]. There the cavities accommodate the 17^3 ethyl ester of a second layer. For the Chl *a* / H_2O, the different chemical environments of the 7-Me and 8-Et in the bilayers can easily give rise to slightly different shifts, due to a combination of ring-current effects, conformational shifts, steric hindrance associated with the two conformations for the 8-Et, and finally the additional strain resulting from the bilayer formation, while all other signals would be essentially the same. This would explain the local doubling of the 7-Me and 8-Et resonances observed by the NMR. In addition, the relative intensities of the doubled NMR resonances should be approximately 1:1, in agreement with peak-volume estimates for the doubled signals in the correlation spectra in Figs. 6.4 and 6.5. A model summarizing these results is presented in Fig. 6.8A.

From the MNDO-d calculations we estimate an electric dipole moment of 6.2 debye for the chlorophyllide with a water molecule ligated to the Mg. The direction of this electric dipole is from ring III to ring I and ~ 30° out of the plane of the macro-aromatic cycle at the side of the H_2O ligand. In the dimer found in solution [31,32], the dipoles of the two molecules forming the dimer are essentially antiparallel. In the self-assembled stack in the NMR model of Fig. 6.6, however, the dipoles approximately line up, and in the sheet the dipole moments associated with individual stacks are also oriented parallel. In contrast, in the bilayer the resultant electric dipoles associated with the two sheets are partly oriented antiparallel. Probably this arrangement contributes to the stabilization of the bilayer structure and the electrostatic forces between the resultant dipole moments may form an important contribution to driving the self-assembly process on a larger length scale.

6.4.4 The suprastructure of the aggregate

Finally, a tight 3-D suprastructure can be established by arranging the bilayers into a laminar structure (Fig. 6.8B). Random folding of the tails is unlikely, since this would give rise to conformational ^{13}C shifts and substantial inhomogeneous line-broadening, which is not observed, except for the P3 and P4. The broadening of the latter may reflect additional strain associated with a twist of the phytyl chains out of the 2-D sheet in the model. The hydrophobic phytyl chains on the outside can interpenetrate with the phytyl chains of a second bilayer. The Chl *a* rings in the layers are arranged in a 2-D hexagonal lattice, and there is room in between the phytyl chains to accommodate the chains from the next bilayer. In this way a densely packed 3-D laminar structure can be formed, in which layers of highly ordered rings are spatially separated from aligned phytyl regions, that have a higher degree of structural disorder, according to the NMR.

It is fair to state here that this suprastructure model is deduced from shift constraints and not yet confirmed with distance constraints. However, the 3-D model of Fig. 6.8 is corroborated by complementary experimental data. In terms of a crystal system, the bilayer in Fig. 6.8A in a laminar arrangement according to Fig. 6.8B is triclinic with space group $p1$, with two Chl *a* molecules per unit-cell and cell parameters $a = c = \sim 8.9$ Å and $\beta = 120.0°$. The MAS NMR model of Chl *a* / H_2O presented in Fig. 6.8B is well in line with low-resolution X-ray data and electron microscope photographs of hydrated Chl *a* [10,36,37]. First, the electron microscopy revealed thin triangular plates forming layered structures [10]. The thickness of a single plate was ~ 50 Å, corresponding with a single bilayer sheet of hydrated Chl *a* [10]. In line with the NMR data, it was concluded that such sheets are 2-D ‘crystals’, with two molecules of Chl *a* per unit-cell [10,36]. In the electron micrographs it was observed that Chl *a* is capable to form a layered structure of at least ten plates of hydrated Chl *a* in the dimension perpendicular to the sheets, although it was noted that the capacity to grow in three dimensions was much smaller than for microcrystals of ethyl-chlorophyllide [10]. The X-ray diffraction powder patterns of such multilayers of hydrated microcrystalline Chl *a* consist of several sharp lines that can be indexed by a triclinic system with space group $p1$, and unit-cell parameters $a = c = 8.75$ Å, $b = 42.1$ Å, $\beta = 120.0°$, in agreement with the NMR model [36,37]. In contrast, crystals of ethyl-chlorophyllide *a* have $p3_1$ symmetry [22]. It was concluded that the two Chl *a* molecules in the unit-cell are not symmetry related to each other [36]. This corroborates the model in Fig. 6.8, since the relative orientation of the Chl *a* molecules is different in the two sheets.

The 3-D arrangement in Fig. 6.8B is in agreement with lines in the X-ray diffraction pattern corresponding with long periodicities of 42.1 Å, 21.0 Å, 14.0 Å and 10.5 Å [10,36,37]. These lines were associated with reflections in lattice planes that run parallel to the plane of the bilayers, and were indexed as (010) and as the higher-order (020), (030) and (040) reflections, respectively, with Miller indices in parentheses [36]. A different way of stacking the layers than depicted in Fig. 6.8B is not possible without a doubling of the unit-cell or a change of the symmetry. The periodicity and space group exclude a back to front stacking of the sheets, as is the case for crystals of ethyl-chlorophyllide *a*. Finally, such higher-order reflections can *only* be observed if a certain degree of periodicity is present along the third dimension. The observed periodicity of 42 Å is less than the width of ~ 60 Å estimated for the bilayer in Fig. 6.8. The difference of ~ 18 Å roughly equals the length of a phytyl chain, supporting the NMR model of Fig. 6.8B with interpenetrating phytyl chains.

A conformational symmetry-breaking associated with a rotation of the 8-Et moiety can be considered an important fundamental aspect of the 3-D model. In particular, it raises the question if a small and systematic perturbation at the molecular level affects the suprastructure of the aggregate on a much larger length scale. It was noted from the electron microscope photographs that single bilayer plates of microcrystalline Chl *a* / H_2O have a strong tendency to curl upon drying [10]. In contrast with the X-ray and microscopy experiments, it was proposed based on small-angle neutron scattering that hydrated Chl *a* aggregates in non-polar solvents form tubular micelles with well-defined radii of ~ 57 Å [9,38]. From the scattering data it was inferred that the Chl *a* could be arranged in a monolayer, with the phytyl chains on one side of the cylinder and with solvent molecules in the interior [9]. A hollow monolayer cylinder filled with solvent would be hard to reconcile with the NMR data, since a solvent NMR response was not observed. As an interesting coincidence, the cylinder radius of ~ 57 Å inferred from the neutron data is remarkably close to the width of ~ 60 Å that is estimated for the bilayer in Fig. 6.8A.

In order to form a tubular arrangement from the bilayer without solvent, the two sheets in the bilayer should have an opposite curvature, *i.e.*, one layer should be curved in such a way that the phytyl chains point inwards, while for the second sheet the phytyl chains are on the outside. It can be inferred from the NMR that the radius should be very large if an equal number of molecules is presumed to be present along the inner and outer circumference. The ring-current shifts will be different in the outer layer and in the inner layer, because of the difference in overlap. This would inevitably give rise to a doubling of a considerable number of the resonances, which is not observed. The spacing between the Chl *a* rings in Fig. 6.6 is about 3.7 Å, and translational differences between the sheets constituting the structure should be very small in order to have the variations in chemical shift due to different ring-current effects in the two layers within 0.1 ppm, the accuracy of the ^{13}C assignment. Already the small linewidth of 0.8-1.3 ppm of the ^{13}C NMR response imposes a strong restraint on the minimal radius that a curved bilayer can have. At least 60 molecules are required per circumference to have the translational variations between the two layers less than 1 Å, which is necessary for a linewidth of ~ 1 ppm. This leads to a lower limit for the bend radius of ~ 100 Å. The bend radius of the rolls of microcrystalline Chl *a* / H_2O that can be estimated from the electron microscopy is ~ 500 Å, which is indeed much larger than the threshold value of ~ 100 Å [10]. Such a faint curling of the bilayers should have little effect on the NMR response.

6.5 Conclusions

In this chapter it is demonstrated by a structural study of self-assembled Chl *a*, that protons can be used successfully in assignment and distance determination strategies for *de novo* structure refinement using solid-state MAS NMR dipolar correlation spectroscopy and homology modeling. It has been shown that knowledge about the electronic structure deduced from the solid-state proton assignment, as well as the assignment of several intermolecular heteronuclear (^{1}H-^{13}C) correlations, provide information that can be interpreted consistently into a structural model. In this model the Chl *a* molecules form linear stacks that line up into highly ordered 2-D sheets. Part of the proton response can be attributed to polarization transfer from protons in water molecules forming a hydrogen-bonding network to the carbonyl moieties. Finally, a doubling of some of the solid-state NMR resonances reveals that two conformers of Chl *a* are present that are both structurally well-defined. The structural differences are local and it is deduced that pairs of sheets align back to back, mutually rotated over 180° in a bilayer structure. A densely packed suprastructure can be established by a laminar stacking of bilayers with interpenetrated phytyl chains. Alternatively, for a tubular bilayer supra-structure, the narrow ^{13}C lines of the NMR response provide a structural restraint of ~ 100 Å for the minimal bend radius the cylinders can have.

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Chapter 7

Proton shifts from high-field 2-D and 3-D high-speed CP/MAS ^{13}C dipolar correlation spectroscopy of aggregated BChl *c* in uniformly ^{13}C-enriched chlorosomal antennae of *Chlorobium tepidum*

This chapter will be submitted to *Biochemistry*

7.1 Introduction

The first step of the light-energy conversion in the green photosynthetic bacterium *Chlorobium tepidum* is the absorption of light and transfer of the electronic energy in an extramembraneous antenna system, the chlorosome. This antenna system mainly consists of oblong bodies that contain a number of rod-shaped elements, attached to the inner side of the cytoplasmic membrane [1,2]. A variety of experimental data obtained over the last decade has indicated that in the chlorosome a unique organizational principle for an antenna system is realized, based on the self-organization of bacteriochlorophyll (BChl) not directly mediated by proteins [3,4].

The major chromophore of the chlorosomes of the bacterium *Chlorobium tepidum* is BChl *c* (Fig. 7.1A). It is known that BChl *c* forms aggregates, both in non-polar [5,6] and in aqueous media [7]. However, crystallization of chlorosome-type aggregates was not yet accomplished and therefore high-resolution diffraction techniques cannot be used to study the molecular arrangement of BChl *c* at the atomic level. In contrast, information on both the spatial and the electronic structure of the micro-crystalline bacteriochlorophyll in the chlorosomes can be obtained with solid-state NMR spectroscopy. 2-dimensional (2-D) cross-polarization magic-angle spinning (CP/MAS) ^{13}C homonuclear dipolar correlation spectra collected from intact ^{13}C-enriched chlorosomes and isolated uniformly ^{13}C-enriched ([U-^{13}C]) BChl *c* aggregates revealed that in both samples the BChl *c* is ordered on a microscopic scale [8]. In particular, from the NMR it was concluded that the stacking of BChl *c* in the chlorosomes and in the aggregates is highly similar, which provides convincing evidence that self-organization of the chromophore is the main mechanism to support the structure of the chlorosomes [8]. In addition, the structure of the stack deduced from the ^{13}C homonuclear dipolar correlation spectroscopy [9,10] is in agreement with the chromophore arrangement in the parallel chain model proposed by Holzwarth and Schaffner [11].

Proton chemical shifts can also help to refine the structure of the BChl *c* stack. Considerable proton aggregation shifts can be expected due to ring-currents from adjacent chlorin macrocycles or polarization effects in the region of binding moieties. However, due to the combination of the strong homonuclear dipolar interactions between the abundant protons in the solid state and the small proton chemical shift dispersion, ^{1}H NMR in solids is difficult and has not yet found a widespread application as a tool for structure determination. An improved proton resolution can be obtained by ^{13}C detection and by exploiting the large ^{13}C chemical shift dispersion in heteronuclear (^{1}H-^{13}C) correlation spectroscopy. For instance, it was demonstrated that from straightforward 2-D high-speed MAS NMR CP wideline separation (CP/WISE) ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy performed at a high magnetic field and without any ^{1}H homonuclear decoupling scheme during the proton evolution, resolved intramolecular and intermolecular ^{1}H-^{13}C heteronuclear correlations and proton chemical shift assignments can be obtained directly [12]. In addition, it has been shown that a high magnetic field induces a line-narrowing effect on the proton response [12]. However, when CP/WISE correlation spectroscopy is applied to molecules of the size of BChl *c*, this approach is still of limited use, for instance due to strong spectral overlap between the aliphatic protons.

A straightforward way to accomplish additional resolution enhancement is by adding a third dimension to the heteronuclear MAS NMR correlation experiment. When the CP/WISE ^{1}H-^{13}C heteronuclear correlation spectroscopy is combined with ^{13}C homonuclear broadband radio frequency-driven dipolar recoupling (RFDR) correlation spectroscopy at a high magnetic field, 3-dimensional (3-D) spectra can be recorded with a proton resolution that is sufficient to assign all observable BChl *c* proton resonances.

The solid-state NMR ^{1}H response obtained in this way reveals a doubling of the resonances that is most pronounced in the region around ring II of the BChl *c* molecule. This provides strong evidence for the existence of at least two structurally different well-defined arrangements of the BChl *c* in the chlorosome antenna. For the second component (component **II**), large upfield shifts are observed for the NMR resonances of the 5-C and 7-Me compared to monomeric BChl *c* in solution.

7.2 Experimental

The preparation of uniformly ^{13}C-enriched chlorosomes and the purification of BChl *c* are described previously [8]. Chlorosomes of *Chlorobium tepidum* prepared with the same procedure and growth conditions are found to contain five BChl *c* homologs in the following composition: 55% [8-Et, 12-Et], 38% [8-*n*-Pr, 12-Et], 2% [8-Et, 12-Me] and 2% [8-*i*-Bu, 12-Et] BChl *c*. A small fraction of 3% remains undetermined. The [8-Et, 12-Et] BChl *c* homolog was purified with HPLC and proton solution data for the monomeric [8-Et, 12-Et] BChl *c* homolog in $CDCl_3$ containing 5% CD_3OD were obtained. The ^{1}H chemical shifts σ_{liq} were assigned with 2-D proton correlation spectroscopy (^{1}H-^{1}H COSY) and 2-D heteronuclear correlation spectroscopy (^{13}C-^{1}H COSY). The shifts are listed in Table 7.1 and compare well with literature data for bacteriochlorophylls [13-16].

The solid-state CP/MAS dipolar correlation spectra were obtained using a DMX-600 NMR spectrometer equipped with a 4 mm double-resonance MAS probe (Bruker, Karlsruhe, Germany). A home-built spinning-speed controller was used to keep the spinning speed constant [17]. All BChl *c* spectra were collected with a spinning rate $\omega_{\mathrm{r}}/2\pi = 15.0$ kHz. The 2-D heteronuclear (^{1}H-^{13}C) correlation spectrum was recorded using the CP/WISE technique in the form discussed previously [12,18]. The 3-D (^{1}H-^{13}C-^{13}C) dipolar correlation method combines the 2-D ^{13}C homonuclear RFDR technique [19,20] with 2-D ^{1}H-^{13}C heteronuclear CP/WISE spectroscopy. The pulse sequence for the 3-D spectroscopy is depicted in Fig. 7.2. Following a 90° proton preparation pulse, a time increment t_1 before the cross-polarization allows the observation of the proton evolution with ^{13}C detection. Since the efficiency of CP magnetization transfer is very sensitive to RF power instabilities at high MAS frequencies, a ramped-amplitude CP sequence (RAMP-CP) [21] was used to broaden the matching profile. The phase of the ^{13}C RAMP-CP spin-lock pulse was varied according to a time-proportional phase incrementation (TPPI) scheme to simulate phase-sensitive detection in t_2 [22], while a TPPI supercycle was applied to the proton preparation pulse to simulate phase-sensitive detection in t_1. The exchange of polarization through homonuclear ^{13}C dipolar interactions during τ_m was promoted by the use of an integral multiple of XY-8 phase-alternated rotor-synchronized trains of π-pulses as described previously [20]. Short CP contact times of 100 and 250 μs were used to minimize ^{1}H homonuclear coherence exchange during CP, while the RFDR mixing time of $\tau_m = 1$ ms was kept short to avoid exchange of proton modulation via the recoupling of the carbon spins. During the second evolution time t_2, the mixing period τ_m and the acquisition time t_3, the protons were decoupled from the carbons by using the two-pulse phase-modulation (TPPM) decoupling scheme, which improves the high-field ^{13}C resolution considerably [23]. The phase-modulation angle and pulse length for the TPPM decoupling were 20 degrees and 8 μs, respectively. Typical proton and carbon 90° pulse lengths were ~ 4 μs.

The 2-D heteronuclear (^{1}H-^{13}C) correlation spectrum was recorded with 1024 data points in t_2 and zero-filled to 2048 points. A Lorentz-Gauss window with the maximum at 0.1 of the acquisition time and a broadening of 100 Hz was applied prior to Fourier transformation. In the t_1 dimension, 64 points were recorded, which were zero-filled to 256 points, and a sine-square apodization, phase-shifted by π/5, was used. A baseline correction was performed in the t_2 dimension, applying the linear correction mode from the Aurelia 2.1.1 software package (Bruker, Karlsruhe, Germany) with a baseline-profile file generated with XWIN-NMR 1.3 (Bruker, Karlsruhe, Germany). The free induction decays (FIDs) of the 3-D (^{1}H-^{13}C-^{13}C) dataset were recorded with 512 data points in t_3 and zero-filled to 1024 points. In the t_2 dimension 120 points were recorded and zero-filled to 512 points. A Lorentz-Gauss window with the maximum at 0.1 of the acquisition time and a broadening of 100 Hz was applied in both the t_3 and t_2 dimensions prior to Fourier transformation. In the t_1 dimension 56 points were recorded, zero-filled to 128 points and processed with a sine-square apodization, phase-shifted by $\pi/3$.

Semi-empirical quantum chemical calculations were performed using the MOPAC 97 code incorporated in the Chemoffice package (Cambridgesoft, Cambridge, MA) [24]. The MNDO-d parametrization was used [25,26]. The ethyl-chlorophyllide *a* from the X-ray structure with the Mg coordinated with a single water molecule was used to verify that MNDO-d optimization correctly reproduces the chlorophyllide monomer ring structure found with the X-ray [27].

7.3 Results

In Fig. 7.3, a 2-D CP/WISE heteronuclear (^{1}H-^{13}C) correlation spectrum of [U-^{13}C] BChl *c* chlorosomes is shown, recorded at a high magnetic field of 14.1 T. For clarity we divided the spectrum in two parts, which were plotted with a different range of contour levels. To maintain good spectral selectivity, the data were collected with a short CP contact time of 250 μs. This is sufficiently long to enable observation of proton resonances correlated with quaternary carbons [12].

Several proton resonances correlating with the ring carbons are readily resolved in the 2-D ^{1}H-^{13}C spectrum (Fig. 7.3A). The distances between the protons of the unsaturated macro-aromatic cycle are relatively large, and the dipolar interactions are weaker than for protons in the saturated parts in the side chains of the molecule. The joined effect of an increased proton shift dispersion at a high magnetic field and an effective ‘dilution’ of these protons is to attenuate the ^{1}H homonuclear dipolar line-broadening [12]. For instance, one of the 5 ^{1}H-^{13}C correlation signals and the 10 ^{1}H-^{13}C correlation signal are completely resolved. The proton shifts are 8.4 and 10.0 ppm, respectively, from strong heteronuclear correlations with their respective ^{13}C neighbours resonating around 95.1 and 105.6 ppm in the 2-D spectrum of Fig. 7.3A.

While the resolution for the correlations with the unsaturated carbons in the 2-D heteronuclear correlation spectrum is good (Fig. 7.3A), the aliphatic signals strongly overlap (Fig. 7.3B). The ^{13}C resolution is considerably better than for the protons, due to the large carbon shift dispersion and weak ^{13}C homonuclear interactions. To improve the resolution, a second ^{13}C dimension can be added. Fig. 7.4 shows a 3-D heteronuclear (^{1}H-^{13}C-^{13}C) dipolar correlation spectrum recorded with the pulse sequence in Fig. 7.2. In the plot of the 3-D spectrum, one contour level is shown, well above the noise. Although the gain of resolution is best for the correlations with the unsaturated ^{13}C, the improvement is sufficient to perform an assignment of proton signals in the aliphatic region.

Figs. 7.4B and 4C show how the 3-D correlation spectra build up. First, transverse magnetization is created with a proton 90° pulse and evolves during the proton evolution time t_1 (cf. Fig. 7.2). Subsequently, the proton coherence is transferred to nearby carbon atoms by the CP. TPPM decoupling is applied to the protons and the carbon coherence is allowed to evolve during the first carbon evolution time t_2. Next, the carbon magnetization is rotated to the *z*-axis and the polarization is exchanged with its ^{13}C neighbours in the RFDR mixing cycle. Finally, the magnetization is brought back into the *xy*-plane and the FID is detected during t_3. The ω_2,ω_3 homonuclear carbon slice shown in Fig. 7.4B is the plane associated with an ω_1 proton chemical shift of ~ 3 ppm, while the ω_1,ω_3 heteronuclear (^{1}H-^{13}C) slice in Fig. 7.4C correlates with an ω_2 carbon shift near 64 ppm. The line in Fig. 7.4B indicates the correlations of the 3^1-^{13}C at 63.7 ppm with the 3-^{13}C (139 ppm) and 3^2-^{13}C (22.2 ppm). Fig. 7.4C shows the same pair of correlations in the proton dimension, from which the chemical shift assignment of the 3^1-^{1}H can be obtained directly.

Fig. 7.4C clearly illustrates for the aliphatic region the gain of resolution obtained in the proton dimension by adding a second ^{13}C dimension. In contrast, the ^{1}H-^{13}C-^{13}C correlation on the diagonal of the carbon-carbon slice (Fig. 7.4B), *i.e.* the signal with an ω_3 carbon shift of 63.7 ppm in Fig. 7.4C, corresponds with the signal obtained with the 2-D CP/WISE (Fig. 7.3) and the resolution is insufficient for an unambiguous assignment of the proton signals. It has substantial wings and is broadened by signals from other components in the sample. For instance, the shoulder with a ^{13}C chemical shift of ~ 60 ppm in Fig. 7.4C can be identified as a signal that originates from a C-OH moiety of monogalactosyl diacylglyceride in the lipid monolayer that surrounds the chlorosomes [8,10]. On the other hand, the correlations of the 3^1-^{1}H with the 3-^{13}C and 3^2-^{13}C in Fig. 7.4C are completely resolved and lead to an unambiguous assignment of the 3^1-^{1}H.

The signal intensity observable at a ^{13}C chemical shift of about 30 ppm in Fig. 7.4C is related to an artifact in the 3-D spectrum. In the 3-D spectrum we observe a noise band, that runs parallel to the ω_2-axis and is connected to the strongest correlations in the spectrum, arising from the 17^1-CH$_2$ and 17^2-CH$_2$ moieties. We have identified this band as t_2-noise, which is the 3-D analog of t_1-noise in 2-D NMR spectroscopy [28]. A vertical trace at 30 ppm due to this noise artifact is also visible in Fig. 7.4B. In the 3-D spectrum the maximum of the t_2-noise intensity corresponds with a proton chemical shift of about 1.5 ppm, which parallels the assignment of the 17^1-^{1}H$_2$ and the 17^2-^{1}H$_2$ response.

It was reported that the overall linewidth in ^{13}C CP/MAS NMR spectra recorded from [U-^{13}C] BChl *c* chlorosomes and from *in vitro* [U-^{13}C] BChl *c* aggregates is about twice the width observed for the ^{13}C resonances of self-assembled hydrated [U-^{13}C] chlorophyll *a* (Chl *a*) [8-10,20], which may reflect conformational heterogeneity in the chlorosomes. Such disorder will also have an inhomogeneous broadening-effect on the proton response, which can not be relieved with NMR multipulse decoupling techniques. For instance, in dipolar-coupled systems the ^{1}H resolution can be further enhanced at high MAS rates and at high magnetic fields by the application of frequency-switched Lee-Goldburg (FSLG) irradiation during the proton evolution [29,30]. We have explored the effect of FSLG line-narrowing in the chlorosomes and found that additional improvement of resolution is only moderate compared to the resolution enhancement already obtained from the high field and the high MAS speed [31]. The ^{1}H line-broadening in the high-field data collected from the chlorosomes is therefore predominantly inhomogeneous, and most likely due to structural heterogeneity and moderate disorder. A comparison between the linewidth of ^{1}H resonances of self-assembled BChl *c* and Chl *a* / H$_2$O will be given below.

The increased resolution in the 3-D spectrum of Fig. 7.4 allows the assignment of 29 observable proton resonances. In Table 7.1, the solid-state proton assignment σ_i of the BChl *c* in the chlorosomes is listed, together with the proton assignment σ_{liq} for the largest fraction, the [8-Et, 12-Et] BChl *c* homolog, as a monomer in solution. From both the 2-D and the 3-D dipolar correlation spectra, it is found that the 5-CH and the 7-Me responses are doubled and give rise to two components in the spectra, designated component **I** and **II**. In Fig. 7.3, a fraction of ~ 57 % of the 5-^{13}C (**I**) response is found at σ_i = 101.2 ppm and correlates with a proton that has σ_i = 7.5 ppm, while a part of ~ 43 % has σ_i = 95.1 ppm for the 5-^{13}C (**II**) with σ_i = 8.4 ppm for the correlated proton. Although the σ_i = 101.2 ppm is

Table 7.1: Proton chemical shifts (ppm) of BChl c in solution and in chlorosomes. The σ_{liq} are the solution shifts of the monomeric [8-Et, 12-Et] BChl c homolog in $CDCl_3$ with 5% CD_3OD and the σ_i are the isotropic shifts of the chlorosomes with estimated errors (ε, in ppm). The numbering is according to Fig. 7.1A.

Position	σ_{liq} (ppm)	σ_{i} (ppm)	ε (ppm)
5-H (**I**)	9.56	7.5[a]	0.3
5-H (**II**)	9.56	8.4[b]	0.3
10-H	9.43	10.0	0.3
3^1-H	6.25	3.0	0.5
13^2-H$_2$	5.09	4.5	0.6
18-H	4.52	4	1
17-H	4.08	4	1
12^1-H$_2$	3.97	2[c]	1
20^1-H$_3$	3.72	3[d]	1
8^1-H$_2$	3.67	2[e]	2
2^1-H$_3$	3.30	1.1	0.3
7^1-H$_3$ (**I**)	3.19	3.1[f]	0.3
7^1-H$_3$ (**II**)	3.19	-0.5[g]	0.3
17^2-H$_2$	2.35/2.42	1.4	0.3
17^1-H$_2$	2.00/2.20	1.4	0.3
3^2-H$_3$	2.01	1.0	0.6
12^2-H$_3$	1.79	2[c]	2
8^2-H$_3$	1.62	2[e]	2
18^1-H$_3$	1.43	2	1
F1-H$_2$	4.31	4.5	0.3
F2-H	5.05	5.4	0.3
F3^1-H$_3$	1.5	1.2	0.6
F4-H$_2$	1.88	1.8	0.3
F5-H$_2$	1.88	2.0	0.3
F6-H	4.95	5.0	0.3
F7^1-H$_3$	1.5	1.2	0.6
F8-H$_2$	1.88	1.8	0.3
F9-H$_2$	1.88	2.0	0.3
F10-H	4.95	5.0	0.3
F11^1-H$_3$	1.50	1.5	1
F12-H$_3$	1.54	1.2	0.6

*[a,b]Proton signals correlated with the 5-C at 101.2 ppm[a] (**I**) and at 95.1 ppm[b] (**II**). [c]proton signals correlated with the 12^1- and 12^2-Et carbons at 18 and 16 ppm, respectively, resolved from 2-D NMR. [d]A possible splitting of the 20^1-H_3 was not resolved from the data. [e]Proton signals correlated with the 8^1- and 8^2-Et carbons at 19 and 17 ppm, respectively, resolved from 2-D NMR. [f,g]Proton signals correlated with the 7^1-C at 10.4 ppm[f] (**I**) and at 6.6 ppm[g] (**II**).*

close to the shift of the 5-^{13}C of the monomeric [8-Et, 12-Et] BChl *c* homolog in solution, it is unlikely that the signal arises from free BChl *c* in the chlorosome preparation. The correlated proton resonance is shifted upfield more than 2 ppm with respect to the 5-^{1}H of BChl *c* in solution, suggesting substantial ring-current effects for this proton associated with a well-defined BChl *c* organization in the chlorosome. From Fig. 7.3 it is found that a fraction of ~ 70 % of the 7^1-^{13}C (**I**) response has $\sigma_i = 10.4$ ppm and correlates with a proton signal with $\sigma_i = 3.1$ ppm. A weaker component **II** of ~ 30 % is observed that has $\sigma_i = 6.6$ ppm for the 7^1-^{13}C and $\sigma_i = -0.5$ ppm for the ^{1}H. This doubling of resonances was confirmed by high-resolution homonuclear (^{13}C-^{13}C) RFDR spectroscopy of the [U-^{13}C] BChl *c* chlorosomes [30]. In Table 7.2, the carbon chemical shifts are listed for the resonances that are doubled for the two components, and are compared with the ^{13}C shifts of the monomeric [8-Et, 12-Et] BChl *c* homolog in $CDCl_3$ with 10% CD_3OD [8].

Although the spectral heterogeneity is most pronounced for the 5-CH and the 7-Me moieties, it is not restricted to this part of the molecule. The ^{13}C homonuclear correlation spectroscopy of the chlorosomes [30] revealed resolved doublings by ~ 2.3 ppm of the 19-^{13}C and ~ 1.5 ppm of the 20-^{13}C responses. The doubling of the 19-^{13}C is confirmed by the 3-D heteronuclear correlation data. The chemical shifts for the 19-^{13}C and the 20-^{13}C are included in Table 7.2. In contrast, a doubling of the 20^1-$^1H_3$ or the 18^1-$^1H_3$ was not resolved from the data.

Table 7.2: Carbon chemical shifts σ_i (ppm) of BChl c in chlorosomes for the resonances that are divided over two components. The numbering is according to Fig. 7.1A. The error of the solid-state assignment σ_i is estimated at about 0.3 ppm. The σ_{liq} (ppm) are the solution chemical shifts of the monomeric [8-Et, 12-Et] BChl c homolog in $CDCl_3$ with 10% CD_3OD [8].

	σ_{liq} (ppm)	σ_i (ppm)	
Position		component **I**	component **II**
4-C	145.37	143.5	144.4
5-C	100.03	101.2	95.1
6-C	150.72	149.8	150.5
7-C	133.45	132.3	131.3
7^1-C	10.37	10.4	6.6
8-C	143.39	142.1	140.1
9-C	146.01	146.1	146.8

Position			
19-C	167.76	169.0	166.7
20-C	104.74	105.0	103.5

Finally, the 13^1-carbon resonance is visible at 195.8 ppm in Fig. 7.3A, due to magnetization transfer during CP over larger distances. It has two weak but well-resolved correlations with protons resonating at 1.2 (0.5) and 4.1 (0.5) ppm. In particular the correlation with proton(s) that have $\sigma_i = 4.1$ ppm is interesting, since apparently there are no protons in the neighbourhood of the 13^1-^{13}C=O that can account for this correlation. For instance, the most obvious assignment would be to the nearby 13^2-$^1H_2$ that resonate around 4.5 ppm. However, the 13^2-$^1H_2$ give rise to a much broader proton response of ~ 4 kHz due to the strong ^{1}H homonuclear dipolar couplings within the CH_2 moiety, which is difficult to reconcile with the relatively small linewidth of ~ 2.4 kHz observed in the proton dimension for the correlation with the 13^1-^{13}C. Other protons near the 13^1-^{13}C fail to account for the correlation at 4.1 ppm. The results suggest that it is associated with intermolecular polarization transfer (see below).

7.4 Discussion

The carbon and proton chemical shifts can be used to extract information at the atomic level about how the electronic structure of the BChl *c* is affected by the self-aggregation. Following the same approach as used previously for the interpretation of the ^{13}C results, aggregation shifts $\Delta\sigma_i$ are calculated, which for each site in BChl *c* are defined as $\Delta\sigma_i = \sigma_i - \sigma_{liq}$. The larger proton aggregation shifts $|\Delta\sigma_i| \geq 1.5$ ppm are upfield and are detected for 2^1-$^1H_3$ (-2.2 ppm), 3^1-^{1}H (-3.3 ppm), 5-^{1}H (**I**) (-2.1 ppm), 7^1-$^1H_3$ (**II**) (-3.7 ppm) and 12^1-$^1H_2$ (-2 ppm). These aggregation shifts are visualized in Fig. 7.1. Smaller aggregation shifts are observed for the 3^2-$^1H_3$ (-1 ppm) and for the 5-^{1}H (**II**) (-1.2 ppm). In the same figure we have included the carbon aggregation shifts with $|\Delta\sigma_i| \geq 1.5$ ppm. For clarity, the carbon aggregation shifts for the 5-, 7-, 7^1- and the 8-^{13}C, and the proton aggregation shifts for the 7^1-$^1H_3$ for component **II** are visualized separately in Fig. 7.1B.

The proton aggregation shifts of the 2^1-$^1H_3$, 3^1-^{1}H and the 12^1-$^1H_2$ observed for component **I** and **II** (Fig. 7.1A) confirm that for both components the region of the 3 side chain, the 2^1-methyl (ring I) and the region around the 12-C and 13-C (rings III/V) are affected similarly by the aggregation processes. This is well in line with the results obtained with ^{13}C homonuclear dipolar correlation spectroscopy, since it complements the fingerprint pattern of aggregation shifts observed for aggregated BChl *c* in intact chlorosomes and *in vitro* aggregates [8,30]. The proton aggregation shifts provide additional support for the parallel chain model, which comprises an arrangement of the BChl *c* in linear stacks by coordination of the oxygen of the 3^1-OH to the Mg of the next molecule in the stack [11]. In this model, ring I of one molecule is placed over rings III/V of a next BChl *c* in the stack

(Fig. 7.5), and substantial ring-current effects are expected for protons that reside above the ring of an adjacent molecule, in line with the pattern of aggregation shifts in Fig. 7.1A.

In addition, the upfield aggregation shifts for the 2^1-$^1H_3$, 3^1-^1H and 12^1-$^1H_2$ for both components are in close agreement with the proton aggregation shifts reported from ^{1}H NMR studies of oligomeric BChl *c* model aggregates in solution. These oligomers were observed to absorb at 740–750 nm, *i.e.*, in the same range of wavelengths as reported for chlorosomes [32,33]. The assignment of the 10-^{1}H at $\sigma_i = 10.0$ ppm (Table 7.1) suggests a small downfield aggregation shift for this proton, in agreement with the work of Mizoguchi *et al.* [32]. The results from the solid-state proton assignment corroborate the conclusion that chlorosome-type BChl *c* aggregates may already be formed in solution [6,33].

The heteronuclear and homonuclear solid-state NMR results can be used to construct a refined model for the self-assembled BChl *c* (Fig. 7.5). First, the structure of a single BChl *c* molecule with the Mg coordinated with a methanol was optimized in a semi-empirical calculation using the MNDO-d parameterization. It should be emphasized that the 20-Me moiety was included in these calculations. This methyl group was not included in the early modeling studies of the BChl *c* stacking by Holzwarth and Schaffner [11]. The methanol can approach the Mg from two sides of the ring, and both situations were taken as a starting point for the calculations. After a full energy minimization, the Mg was located ~ 0.30 Å above or below the plane formed by the four nitrogen atoms, depending on the side from which the methanol provided the fifth ligand to the Mg. We denote the configuration in which the Mg is out of plane at the same side of the macro-aromatic ring system as the 17^1-C by *syn*, while the situation in which the Mg and 17^1-C are on opposite sides of the ring plane is labeled *anti*. Interestingly, we found that the steric hindrance between the 2-Me and 20-Me can induce a pronounced nonplanarity of the macro-aromatic cycle in the region of ring I. The lowest energy in the calculations is obtained when the distortion due to the steric hindrance between the two methyl groups and the metal out of plane distortion are cooperative, leading to a strong out of plane deformation in the region of ring I. This confirms the importance of including the 20-Me in the calculation of the structure of the monomer.

Next, two bent *syn* or two bent *anti* configurations can be placed together in a docking operation to build an optimal dimer structure restrained by the NMR shift and distance information [8-10,30]. For both *syn* and *anti* BChl *c*, a dimer could be formed without any difficulty by coordination of the oxygen of the 3^1-OH to the Mg of the second molecule, with the BChl *c* molecules nearly parallel. In a third step a trimer was built either from three *syn* or from three *anti* molecules, with the relative position of the rings approximately the same as in the docked dimer, and with the relative orientation of the rings taken parallel.

The semi-empirical modeling also suggests the possibility of a flat ring structure, albeit at a higher energy, if the distortion due to steric hindrance between the two methyl groups is globally balanced by the puckering of the ring. However, a parallel dimer model with the OH of one molecule coordinating the Mg leads to significantly different distances between *e.g.* the 13^1 and the plane of the ring of the other molecule in the dimer than for the structure shown in Fig. 7.5. Since this would lead to a doubling of the NMR signals, already based on differences in ring-current shifts between the two structures, we have not considered the two flat higher-energy configurations as additional possibilities in the construction of a structural model for the chlorosome antenna.

With *syn* or *anti* stacks it is possible to form a 2-D layered sheet, stabilized by the formation of interstack hydrogen bonds between the 3^1-OH moieties in one stack to the 13^1=O in a neighbouring stack [11]. This interaction between the stacks of BChl *c* is different than the interstack interaction found in 2-D sheets of ethyl-chlorophyllide *a*. For ethyl-chlorophyllide *a*, the interaction between the stacks is provided by the 17^3=O via hydrogen bonding to one of the two water molecules in a hydrogen-bridging network [27].

For BChl *c*, however, there is no experimental evidence that the 17^3=O is also involved in an interaction. First, FTIR measurements provided evidence for a hydrogen-bonding interaction of the 13^1=O to the 3^1-OH of a second BChl *c*, while no indication was found for an interaction at the 17^3=O (see *e.g.* Ref. [11], and Refs. therein). Second, complementary evidence is provided by the fact that no water is required for the aggregation of BChl *c*, in contrast with ethyl-chlorophyllide *a*. In absence of water, it is not possible to have a second hydrogen-bonding interaction that involves the 17^3-C=O.

In the 2-D layered sheets, there is no relevant ring overlap between BChl *c* molecules in different stacks that can lead to pronounced additional ring-current shifts. This is in line with the pattern of aggregation shifts in Fig. 7.1A that can be accommodated assuming ring-current effects due to overlap within the stack. This contrasts with 2-D layered sheets of self-assembled Chl *a* / H_2O studied in Chapter 6, where the 2-Me and 12-Me are located both above the ring of an adjacent Chl *a* in the stack and above the ring of a Chl *a* in a neighbouring stack. As a result of this extended overlap, large ring-current shifts of –5 ppm are observed for the NMR resonances of the 2^1-H_3 and 12^1-H_3 of the Chl *a* molecule.

The *anti* BChl *c* stack shown in Figs. 7.5A and B has the same sliding direction as in the model proposed by Holzwarth and Schaffner [11]. The *syn* trimer in Figs. 7.5C and D has an opposite sliding direction. This is in line with the solution work of Mizoguchi *et al.* [34], in which two different types of stacked structures with opposite sliding directions were proposed, that both can be arranged within the concept of the parallel chain model. A set of ^{1}H-^{1}H intermolecular NOE correlations was obtained that could be interpreted in terms of two different sliding directions for the aggregated BChl *c* in solution [34]. Interestingly, we found that the reported set of NOE correlations is in line with the two types of stacks in Fig. 7.5. In the model in Fig. 7.5, the overlap between the BChl *c* rings is virtually the same for the two sliding directions, and the ring-current shifts in the regions of overlap should be the same for both. A doubling of resonances was not reported for the solution NMR spectra, which indicates that the *syn* BChl *c* stack is essentially the mirror image of the *anti* configuration. On the other hand, there is also a difference between the two configurations, since in the *syn* stack in Figs. 7.5C and D, the 3^2-C is very close to the 5-H at a distance $d \approx$ 2.0 Å. The large upfield aggregation shift of -4.9 ppm for the 5-^{13}C of component **II** may possibly be related to this contact [35,36]. In solution the 5-^{13}C shows first a slight downfield shift, followed by a large upfield shift, as function of the size of the oligomer [34].

It was noted before that the correlation of the 13^1-^{13}C=O with proton(s) resonating around 4.1 ppm may be associated with intermolecular magnetization transfer, and it could involve polarization transfer from the hydrogen-bonded 3^1-OH. From studies on hydrogen bonding in a [U-^{13}C] tyrosine·HCl model compound, it was observed that heteronuclear correlations between quaternary carbons and protons that are not directly bonded to a carbon are easy to detect in uniformly ^{13}C-labeled systems [12,29]. The polarization of a proton that is directly bonded to a ^{13}C in a rigid environment is rapidly transferred during CP in a coherent process within the short time of ~ 100 μs to its adjacent carbon. Polarization transfer to a quaternary carbon predominantly involves protons in methyl groups or protons that are not strongly coupled to a carbon spin. For instance, hydrogen-bonded protons can transfer polarization in ~ 250 μs over extended distances, > 2.5 Å. In particular, it turned out that the CP magnetization transfer along a hydrogen bond from an -OH proton can be quite efficient [12].

Protons in a hydroxyl group typically resonate in the range 6-10 ppm [37]. A 3^1-O^{1}H signal at 4.1 ppm would imply a significant upfield shift of ~ 2-5 ppm. In the stacks in Fig. 7.5, the 3^1-OH is located close to the Mg above the macro-aromatic cycle of an adjacent BChl *c* molecule. For such an arrangement, pronounced upfield shifts up to 5 ppm can be expected due to the BChl *c* ring-currents [38]. Finally, the correlation of the 13^1-^{13}C=O with protons resonating around 1.2 ppm may involve transfer from the 3^2-$^1H_3$. These protons resonate around 1.0 ppm, and are close to the 13^1-^{13}C. It is shown below that the proximity of the 3^2-CH_3 and 13^1-C=O can provide a mechanism that induces a curvature in the sheets.

The upfield ^{1}H and ^{13}C aggregation shifts of about -4 ppm for the 7-Me in component **II** (Fig. 7.1B) are intriguing, since the resonances of the 7-Me moiety are not much affected by *e.g.* ring-current shifts for oligomeric BChl *c* model stacks in solution [32-34]. The formation of 2-D sheets of aggregated stacks of BChl *c* can not easily explain these large aggregation effects. In the 2-D models, there is no strong overlap of the 7-Me region with a nearby BChl *c* ring. In this configuration ring-current shifts induced by overlap within the sheet can not account for the large upfield 7-Me aggregation shifts observed with the NMR.

The doubling of the resonances in the region around rings II and IV provides strong evidence for the existence of at least two structurally different well-defined arrangements of the aggregated BChl *c*. The 7-Me region can be identified as the part of the molecule for which the difference between the components **I** and **II** is most pronounced in the solid-state NMR. No doubling of NMR responses is observed for the region of the 3 side chain and the 2^1-CH_3 (ring I) and the region around 12-C and 13-C (rings III/V), which means that the aggregation shifts in these regions are essentially the same for all BChl *c* molecules in the NMR. Since the pattern of aggregation shifts for the regions around rings I and III/V (Fig. 7.1A) supports the structural model for the linear aggregates in Fig. 7.5, this strongly suggests that for both components the BChl *c* forms stacks according to the parallel chain model.

Recently, we have investigated the possibility that the observed spectral heterogeneity is induced by high MAS frequencies $\omega_r/2\pi = 13$-15 kHz that are required to obtain well-resolved MAS NMR correlation spectra [31]. At these high rotation frequencies, the sample experiences a considerable centripetal force, which could lead to a deformation of the structure. 1-dimensional (1-D) ^{13}C CP/MAS NMR spectra were recorded from a freshly prepared [U-^{13}C] BChl *c* chlorosome preparation at a low MAS speed of 5300 Hz, corresponding with a radial acceleration of about 150,000 × *g*. This is a characteristic value commonly utilized in chlorosome preparation procedures [39]. By comparing spectra recorded with a MAS rate of 5300 Hz before and after spinning the sample for ~ 20 hours at 13 kHz, we found that high MAS rates, at least up to 13 kHz, are not perturbing the sample on the scale of the NMR [31]. In particular, it was confirmed that a doubling of the 7^1-^{13}C resonances was present in the first spectrum recorded at a low MAS rate, from a freshly prepared sample [31]. In addition, we demonstrated that the spectral heterogeneity is essentially independent of the degree of hydration of the sample, since the doubling of resonances is observed both for a wet chlorosome preparation and for a dehydrated sample [8,31].

A doubling of the 5-CH and the 7-Me NMR responses is also observed for *in vitro* BChl *c* aggregates prepared from the natural mixture of BChl *c* homologs and diastereomers isolated from *Chlorobium tepidum* cells [8] and for *in vitro* aggregates prepared from the [8-Et, 12-Et] BChl *c* homolog. The doubling of the resonances for the pure homolog indicates that the formation of two structurally different BChl *c* arrangements in the chlorosomes is not related to the presence of different BChl *c* epimers and homologs in the chlorosome preparation. In addition, we observed a doubling of the NMR signals in the 7-Me region for *in vitro* [U-^{13}C] Chl *a* / H_2O aggregates (Chapter 6). These results suggest that the formation of more than one well-defined chromophore arrangement appears to be a more general feature, and may represent an intrinsic property of the self-organization mechanism driving the chlorophyll aggregation process.

The evidence provided by the solid-state NMR data for the existence of two types of chlorosomal BChl *c* in different well-defined arrangements is corroborated by other experimental data. For instance, the observation of spectrally distinct types of aggregated BChl within the chlorosomes from *Chlorobium tepidum* and other green bacteria has been reported by several authors. First, a strong spectral inhomogeneity was observed in chlorosomes of *Chlorobium vibrioforme* and *Chlorobium phaeovibrioides* that was ascribed to unlike spectral properties of different oligomers [40-42]. Next, by deconvolution of linear and circular dichroism spectra, Matsuura *et al.* [43] demonstrated the presence of two major spectral forms of aggregated BChl *c* in chlorosomes of *Chloroflexus aurantiacus* that were attributed to differences in the local chromophore arrangement. Subsequently a schematic model was proposed in which the two forms of BChl *c* coexist in the rods with the direction of the transition dipole moment of both forms nearly parallel to the long axis of the cylinders [44,45]. Somsen *et al.* confirmed that the variability of the circular dichroism (CD) of chlorosomes reflects the presence of at least two structurally different BChl *c* species [46]. These were explained in terms of small variations in the cylindrical structure of the rod-shaped BChl *c* subunits. Finally, Steensgaard *et al.* provided evidence for two spectrally different types of BChl *c* within chlorosomes from *Chlorobium tepidum* by monitoring the spectral changes during acid treatment [39]. The existence of two spectral forms of BChl *c* absorbing at ~ 740 and ~ 760 nm was demonstrated, with the short wavelength form being more susceptible to reaction with protons [39]. It is thus tempting to conclude that the observation with solid-state NMR of two types of chlorosomal BChl *c* is related to the spectral inhomogeneity in chlorosomes reported in the literature.

At this point the question arises: what is the molecular arrangement of BChl *c* in the chlorosomes? From the present NMR data alone, it is difficult to arrive at a full high-resolution 3-D structure, and the detection of more intermolecular distance restraints, between the stacks and sheets, will be necessary to model a highly resolved structure. On the other hand, the number of possible arrangements of the BChl *c* can be reduced substantially already in a homology approach, by comparing the present results with the NMR and structural data for a related system, self-assembled Chl *a*. In Chapter 6, we have studied the arrangement of [U-^{13}C] Chl *a* / H_2O with ^{13}C homonuclear and ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy. It was found that Chl *a* forms linear arrays that line up in 2-D layered sheets of aggregated stacks. Second, the resonances in the 7-Me region of the Chl *a* were doubled in the NMR, which provided evidence for two structurally different well-defined arrangements of the Chl *a* in the sheets that can be accommodated by the formation of a bilayer. Finally, the Chl *a* bilayers can form a 3-D laminar structure by interpenetration of the Chl *a* phytyl chains or roll up into a tubular arrangement.

Electron microscopy revealed rod-shaped structures with a diameter of ~ 10 nm in the chlorosomes, with a central hole of about 3 nm in diameter [1,2,47]. There are several ways to form tubular arrangements with sheets of BChl *c*. In the model for the tube proposed previously [11], the cylinder is formed by a monolayer sheet of aggregated *anti* BChl *c* that is curved with the farnesyl chains pointing outward. However, this arrangement leaves a large amount of space empty inside the tubes, which contrasts with the relatively small hole observed by Cruden and Stanier [47]. Alternatively, in analogy with Chl *a*, the cylinder wall can be a back to back bilayer of sheets of BChl *c*. In this case the farnesyl tails extending from the inner sheet can fill up the space inside the tube almost completely, leaving a small hole, in agreement with the electron microscopy results.

In the NMR experiments, the signal intensity of the 7-Me resonances of component **I** is systematically higher than that of component **II**. For instance, from 1-D ^{13}C CP/MAS NMR spectra of chlorosome preparations, an integrated signal intensity of 57 % is found for the 7^{1}-^{13}C (**I**) response relative to the signal from 7^{1}-^{13}C (**II**). In addition, integrated peak-volume estimates of the 7-Me (**I**) correlation in the heteronuclear correlation experiment in Fig. 7.3 provides a relative fraction of 70 %, while an FSLG-decoupled ^{1}H-^{13}C correlation experiment collected from a freshly prepared control sample showed that the 7-Me (**I**) contributes a fraction of 61 % [31]. Likewise, in RFDR experiments from both preparations, correlations with 7^1-^{13}C (**I**) are stronger than with 7^1-^{13}C (**II**) [30]. This provides converging evidence that the upfield shifted 7-Me (**II**) resonances are associated with a minor fraction of BChl *c* in the chlorosomes.

On the other hand, for the 5-CH moiety the situation appears less well-defined. In the ^{1}H-^{13}C CP/WISE spectrum in Fig. 7.3, the 5-CH (**I**) correlation comprises the largest fraction of 57 %, while in a ^{13}C homonuclear RFDR spectrum collected from the same sample, correlations with 5-^{13}C (**I**) are weaker than correlations with 5-^{13}C (**II**) [30]. This strongly suggests that factors other than the absolute amounts of spectrally different types of BChl *c* in the chlorosomes may contribute to the intensity of the doubled signals in the correlation spectra, for instance the efficiency of coherence transfer during a short CP contact or during the RFDR mixing. In a ^{13}C homonuclear dipolar correlation experiment, correlation networks were resolved that couple the 5-^{13}C (**I**) with the 7^1-^{13}C (**I**) and the 5-^{13}C (**II**) with the 7^1-^{13}C (**II**) [30]. This strongly suggests that the upfield shifted 5-^{13}C (**II**) and 7-Me (**II**) resonances are from the same type of BChl *c*. Since the 7-Me (**II**) is associated with the minor fraction of BChl *c* molecules, we propose that the components **I** and **II** in the spectra represent major and minor fractions, respectively, of structurally different BChl *c* in the chlorosomes.

It was suggested above that the upfield shift of -4.9 ppm of the 5-^{13}C of component **II** is related to the close contact of the 3^2-CH_3 with the 5-CH in the *syn* stacked BChl *c* [35,36]. From this it is inferred that the minor fraction of BChl *c* is associated with *syn* stacks, and the major fraction with *anti* stacks. In addition, in a bilayer tube the number of molecules in the inner layer should be smaller than in the outer layer, which strongly suggests that the minor component **II** in the NMR spectra corresponds with the BChl *c* in the inner tube. The alternative would be the presence of the flat species, however, in that case doubling at the side of the ring III and ring V is expected, which was not observed.

For the *anti* stacks, hydrogen bonding of the 13^1-C=O to the 3^1-OH is difficult if the stacks have the same orientation, due to steric hindrance of the 3^2-CH_3 with the 13^1-C=O. However, if the stacks are slightly rotated relative to each other, the 13^1-C=O can approach the 3^1-OH without difficulty from the side that is opposite to the direction of the 3^2-CH_3. As a result, the sheet of *anti* stacks will have a tendency to curl in such a way that the farnesyl tails are directed towards the outside of the tube (Fig. 7.6A). For the *syn* stacks, the situation is reversed. Here the 3^2-CH_3 is oriented towards the 5-CH, in a direction that is opposite compared to the orientation of the 3^2-CH_3 in the *anti* stack, where it is directed away from the 5-CH. Again a sheet can be formed most easily by a slight relative rotation of the *syn* stacks. The *syn* sheet will also have an inclination to curl, but in a direction that is opposite to the one of the *anti* sheet, *i.e.*, with the farnesyl chains directed towards the inside (Fig. 7.6B). Hence, the steric hindrance with the 3^2-CH_3 moiety that has different conformations for the two types of stacks may provide a simple mechanism that forces the sheets to curve in opposite directions.

A curved *anti* and *syn* sheet can be combined to a bilayer tube, with the *anti* sheet at the outside and the *syn* sheet at the inside. This is in line with the considerations made above that the minor component is associated with an inner sheet of *syn* stacks. Finally, the reorientation of the *anti* stacks rotates the 7-Me away from the *syn* sheet. In contrast, the *syn* stacks rotate in such a way that the 7-Me region of the molecule is directed towards the *anti* sheet, which makes the 7-Me region of the *syn* BChl *c* prone to perturbations in the contact region between the two layers (cf. Fig. 7.6). This can easily give rise to different shifts of the two 7-Me moieties, due to the combination of ring-current effects, conformational shifts and the additional strain resulting from the bilayer formation.

Additional support for a bilayer arrangement is obtained by comparing ^{1}H linewidths of BChl *c* with those of self-assembled Chl *a* / H_2O. To this end, several vertical slices representing BChl *c* proton responses correlated with separate carbons were extracted from the 2-D heteronuclear correlation experiment in Fig. 7.3 and are shown with dashed lines in Fig. 7.7. In the same figure, the solid lines represent proton slices from a CP/WISE experiment collected from aggregated [U-^{13}C] Chl *a* / H_2O, recorded with a MAS rate $\omega_r/2\pi$ = 13.0 kHz at a field of 14.1 T. For an objective comparison of the linewidths of the resonances of the two different species, the data have been processed without apodization in t_1.

Figs. 7.7A and B show the BChl *c* proton responses correlated with a 7^1-^{13}C (**I**) and 5-^{13}C (**II**), respectively, which mainly represent the 7^1-$^1H_3$ (**I**) and 5-^{1}H (**II**) signals, and a 7^1-$^1H_3$ and 5-^{1}H signal from Chl *a* / H_2O. From the figure it transpires that the linewidths of the 7^1-$^1H_3$ (**I**) and 5-^{1}H (**II**) resonances of BChl *c* are virtually the same as the widths of the corresponding signals form self-assembled Chl *a* / H_2O. For the Chl *a* / H_2O, these ^{1}H lines are from protons that are located in a unique chemical environment. Hence, the similarity between the linewidths suggests that also for the BChl *c* the 7^1-$^1H_3$ (**I**) and 5-^{1}H (**II**) signals are associated with protons that are in a well-defined environment. Similar results are obtained for the 5-^{1}H (**I**) response of BChl *c*. On the other hand, for the 7-Me (**II**) the situation is different. In 1-D ^{13}C CP/MAS NMR spectra it was observed that the linewidth of the 7^1-^{13}C (**II**) signal is about 350 Hz, while the width of the response from 7^1-^{13}C (**I**) is ~ 220 Hz, which suggests additional inhomogeneous broadening of the 7^1-^{13}C (**II**) signal. In the ^{1}H-^{13}C dataset in Fig. 7.3, the correlation with 7^1-^{13}C (**II**) is tilted. Also here the 7-Me carbon and proton resonances appear inhomogeneously broadened, and the additional broadening dispersion of the carbon response correlates with the broadening dispersion of the proton response. This is in line with the assignment of the 7-Me (**II**) resonances to the *syn* BChl *c*, since in our model the 7-Me protrude from the *syn* sheet and are more exposed and subject to aggregation shifts, in a similar way as was found previously for the Chl *a*, albeit less pronounced.

The 10-^{1}H resonance correlated with 10-^{13}C of BChl *c* in Fig. 7.7C is broadened compared to the 10-^{1}H response of Chl *a*. For BChl *c*, the 10-^{1}H is not doubled in the NMR, hence the total 10-^{1}H response reflects the contributions from BChl *c* molecules in at least two different arrangements. This suggests that the structural heterogeneity indeed contributes to the increased linewidth of the proton resonances that are not doubled in the NMR, and that the different micro-crystalline arrangements resulting from the opposite sliding directions in the bilayer and different radii for the inner and outer layer may partly explain the conformational heterogeneity and associated inhomogeneous linewidth in the spectra of [U-^{13}C] chlorosomes and *in vitro* [U-^{13}C] BChl *c* aggregates.

Finally, comparison of the proton responses of the farnesyl tail of BChl *c* with the resonances of the phytyl chain of Chl *a* (Figs. 7.7D and E) reveals a significant line broadening of the BChl *c* farnesyl proton signals. For Chl *a*, the phytyl chains of neighbouring bilayers form an interpenetrating network and have a relatively high degree of order. The broadened signals of the BChl *c* tail strongly suggest that the farnesyl chains are more disordered and may exhibit a random folding. In addition, we have demonstrated recently that the NMR relaxation parameters of the rigid BChl *c* ring system and the farnesyl chain are highly similar, from which it was concluded that at least a part of the farnesyl chains should be relatively immobile [31]. This is in line with the model of a bilayer cylinder, where the farnesyl chains on the inside will be rigidly held in place.

The formation of a bilayer arrangement is a feature that is observed for self-assembled BChl *c* and Chl *a*, and may be present in other aggregated chromophores as well. In powder X-ray diffraction patterns of self-assembled Chl *a* / H_2O, lines were observed that reflected a periodicity of 42 Å [48-50]. This periodicity can be explained in terms of bilayer sheets of Chl *a* in a laminar arrangement. A comparable periodicity of 47 Å was reported for aggregated BChl *a*, which may also provide evidence for a bilayer structure [48]. Complementary support for a structural analogy between aggregated BChl *c* and self-assembled Chl *a* / H_2O is provided by the high degree of similarity between the solid-state NMR data of both species. In particular the doubling of resonances in the region of the 7-Me that is observed for both types of assemblies may provide a ‘fingerprint’ related to a bilayer formation.

A bilayer tube composed of an *anti* outer layer and a *syn* inner layer of aggregated BChl *c* can thus be reconciled with the solid-state NMR results and other available structural data. The model may explain the variety of spectroscopic data that demonstrated a spectral heterogeneity in the chlorosomes. For example, the presence of two spectrally different types of BChl *c* absorbing at ~ 740 and ~ 760 nm according to Steensgaard *et al.* may be related to the formation of two different types of sheets [39]. The observation that the short wavelength form was more susceptible to reaction with protons can be easily explained if the sheet absorbing at ~ 740 nm is the one at the outside, which in our model is the sheet of *anti* stacks. Finally, the presence of more than one well-defined structural organization with different cylindrical structure is in agreement with the findings of Somsen *et al.*, who showed that a small rotation of the chromophores around the axis parallel to the long axis of a tube can explain the variability of the CD of chlorosomes [46].

7.5 Conclusions

The arrangement of BChl *c* was studied in uniformly ^{13}C-labeled intact chlorosomes of the green photosynthetic bacterium *Chlorobium tepidum* using high-field 2-D (^1H-^{13}C) and 3-D (^1H-^{13}C-^{13}C) heteronuclear MAS NMR dipolar correlation spectroscopy. From the correlation spectra it was possible to assign all observable proton resonances. The solid-state

^1H assignment was used to calculate proton aggregation shifts relative to monomeric BChl *c* in solution. The pattern of aggregation shifts corroborates the parallel chain model. A doubling of the 5-CH and the 7-Me NMR resonances was observed, which provides strong evidence for the presence of at least two structurally different well-defined arrangements of the BChl *c* in the chlorosomes. The NMR data for [U-^{13}C] BChl *c* chlorosomes and *in vitro* [U-^{13}C] BChl *c* aggregates are remarkably similar to those obtained from aggregates of [U-^{13}C] Chl *a* / H_2O. By comparing the NMR results with other available NMR and structural data in a homology study, a bilayer tube is proposed for the arrangement of BChl *c* that is composed of sheets of stacks with opposite sliding direction.

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Chapter 8

Binding of Q_A and Q_B in *Rhodobacter sphaeroides* R26 reaction centers

8.1 Introduction

The primary processes of photosynthesis in the photosynthetic bacterium *Rhodobacter* (*Rb.*) *sphaeroides* R26 comprise a light-induced charge separation by which excitation-energy is converted into chemical energy. The charge-separation process occurs in the photosynthetic reaction center (RC), a large transmembrane protein complex consisting of three polypeptide chains, the L, M, and H sub-units that support nine cofactors (Fig. 8.1) [1]. Upon excitation with light, an electron is transferred from the special pair P via the accessory chlorophyll B_A

and bacteriopheophytin Φ_A to the primary quinone Q_A. Within about 200 μs the electron is transferred to the secondary quinone Q_B. After a second excitation, Q_B is doubly reduced and doubly protonated and is released from the RC as a Q_BH_2 diquinol. Although Q_A and Q_B are both identical ubiquinone-10 molecules (UQ_{10}, Fig. 8.2), their electrochemical function and binding properties are different. While Q_A is tightly and permanently bound to the protein, it temporarily accepts single electrons and serves as a one-electron gate. Q_B, on the other hand, is weakly bound. During the photosynthesis process it accepts two electrons and is chemically converted in the RC. To resolve the mechanisms behind the molecular electronics of the photosynthetic charge-separation process, the characterization of differences in quinone-protein interactions between the Q_A and Q_B, and differences in binding between the neutral and the reduced forms of the quinones is important.

There is converging general evidence that the binding of the quinones in the RC proceeds by the formation of hydrogen bonds to the UQ_{10} carbonyls. However, while there is a vast amount of spectroscopic data on the quinone binding and the hydrogen bonding for the reduced forms, the information about the ground-state quinone-protein interactions from X-ray diffraction, NMR and Fourier-transform infrared (FTIR) spectroscopy is not yet conclusive, in particular with respect to the H-bonding interactions for the 1-C=O of the Q_A. 1-Dimensional (1-D) ^{13}C cross-polarization magic-angle spinning (CP/MAS) NMR spectroscopy of *Rb. sphaeroides* R26 RCs reconstituted with [1-^{13}C] UQ_{10} or [4-^{13}C] UQ_{10} for Q_A, revealed highly asymmetric interactions of the carbonyls with the protein in the ground state [2]. While the [1-^{13}C] Q_A R26 RC MAS NMR provided narrow lines with a short CP time, both at low temperatures $210 \leq T \leq 250$ K from frozen detergent-solubilized and precipitated preparations, and at ambient temperatures from precipitated samples, a [4-^{13}C] Q_A signal could be observed only in partially hydrated samples at temperatures $T \leq 255$ K and for longer CP times of ~ 3 ms [2,3]. The temperature dependence of the NMR responses of the [4-^{13}C] carbonyl and the adjacent [5-^{13}C] in Q_A (Fig. 8.2) were attributed to destructive interference of dynamic Q_A-protein interactions with the CP efficiency [2-4]. In addition, a shortening of T_1^ρ for hydrated-precipitated [4-^{13}C] Q_A RCs was observed for temperatures approaching $T \approx 255$ K from below, indicating characteristic frequencies around 50-60 kHz for the restricted dynamics at the 4-C=O side [3]. It was observed that the linewidth of the label response was highly sensitive to the degree of hydration of the precipitated RCs. A narrow [4-^{13}C] Q_A response was only observed for a 'wet paste', while for precipitated RCs that were either dry or completely soaked with water the NMR label response showed a substantial line-broadening. These findings corroborate a subtle interplay between dynamic Q_A-protein interactions at the 4-C=O side of the Q_A and the degree of hydration of the RCs that has an important effect on the CP efficiency and linewidth of the NMR response [3].

In contrast, a good CP efficiency and a narrow NMR signal provide convincing evidence for a tight fit at the 1-^{13}C=O Q_A. The tight fit suggests the possibility of strong hydrogen bonding at this carbonyl functionality. However, the isotropic shift of the 1-^{13}C=O of the Q_A in the RC is very similar to the shift observed for unbound UQ_{10}, in the solid state or in solution [2]. Model studies and semi-empirical calculations were performed, to address the effect of hydrogen bonding on the charge density and on the NMR shift and its anisotropy [5]. The calculations indicate that the C=O carbon charge is not particularly sensitive to the strength of the hydrogen bond [5]. Downfield shifts of only a few ppm are predicted upon hydrogen bonding, which can easily be compensated by other shift effects from the protein environment [5].

The binding at the 1-C=O side of the Q_A in the ground-state RC was also investigated with FTIR [6-8]. The absence of a shift of the mode dominated by the 1-C=O Q_A vibration was initially interpreted as an essentially free or weakly bound carbonyl group. However, like for the shifts in the NMR, C=O bond orders and vibrational modes of neutral quinones may be not very sensitive to the polarity and hydrogen-bonding capability of solvents [9]. It is thus difficult to draw conclusions about specific Q_A-protein interactions from both NMR shifts and FTIR, and more elaborate spectroscopic methods are needed to establish the presence or absence of a hydrogen bond to the 1-C=O of the Q_A.

In this chapter, a novel strategy is followed to exploit CP/MAS NMR in a characterization of the binding at the 1-C=O side of the Q_A and a comparison with the 1-^{13}C of the Q_B in the ground state of the RC. First, 2-dimensional (2-D) heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy is used to investigate R26 RCs reconstituted with [1-^{13}C] UQ_{10} for Q_A or Q_B. From the heteronuclear correlation spectra, the isotropic chemical shifts of proton(s) that participate in polarization transfer to 1-^{13}C Q_A or Q_B are assigned by indirect detection via the carbon label. In Chapter 5, it was demonstrated that in particular hydrogen-bonded protons can be involved in a rapid magnetization build-up of a quaternary carbon. Protons in a hydrogen bond typically resonate in the range 8-15 ppm, hence characterization of the correlated protons in terms of their chemical shift will already be instrumental in assessing possible hydrogen-bonding interactions. In a next step, Lee-Goldburg CP (LG-CP) build-up curves are recorded from the [1-^{13}C] UQ$_{10}$ reconstituted RCs. From these build-up curves, the distances r_{CH} between the 1-^{13}C carbon and the proton(s) involved in the polarization transfer will be estimated, using the novel methodology discussed in Chapter 5.

8.2 Experimental

UQ$_{10}$ specifically labeled at position 1 (Fig. 8.2) was obtained by total-synthesis [10]. The NMR measurements of hydrated-precipitated RCs reconstituted with [1-^{13}C] UQ$_{10}$ for Q$_A$ were recorded from a sample prepared and described previously by van Liemt *et al.* [2]. The NMR data of detergent-solubilized RCs and from hydrated precipitated RCs reconstituted with [1-^{13}C] UQ$_{10}$ for Q$_B$ were recorded from freshly prepared samples.

8.2.1 Preparation of [1-^{13}C] Q$_A$ *Rb. sphaeroides* R26 RCs

A batch of 150 ODV at a wavelength of 800 nm (ODV^{800}) [11] of isolated Q$_A$ and Q$_B$-depleted (dQ) *Rb. sphaeroides* R26 RCs was prepared following the procedures described in [12]. The quinones were removed by washing the RCs anaerobically for about 24 hours with a solution of 4% LDAO, 10 mM TRIS (pH 8) and 10 mM *o*-phenanthroline [13]. Fig. 8.3A shows an electronic absorption spectrum recorded from the dQ RCs. The bacteriopheophytin band at 755 nm is relatively high compared to the band at 865 nm from the bacteriochlorophylls forming the special pair P. This suggests a partial conversion of bacteriochlorophyll into bacteriopheophytin, which could imply that a fraction of the RCs is denatured. The RCs were rapidly reconstituted with UQ_{10}, since dQ RCs are unstable and highly sensitive to light. The denatured RC fraction does not bind the quinone and was removed at a later stage.

The RCs were diluted to an OD of ~ 18 at a wavelength of 800 nm (OD^{800}) in 8 ml of a standard 'TL' buffer solution of 0.025% LDAO, 10 mM Tris (pH 8) and 1 mM EDTA. To this RC solution, 460 µl [1-^{13}C] UQ_{10} (1 mg/ml) in 1% Triton X-100 was added in three steps. The final Triton concentration was 0.05%. After each step the RC-quinone mixture was allowed to incubate for 20 minutes at room temperature. After the incubation, the relative amounts of reconstituted Q_A and Q_B were determined by monitoring the recombination kinetics of the absorption changes at 865 nm following a flash of actinic light. The incorporation of Q_A was close to 100%, while for about 30% of the RCs, [1-^{13}C] UQ_{10} was also incorporated at the Q_B-position.

To remove both the labeled Q_B and the denatured RCs, the mixture was diluted to an OD^{800} of 1 in 150 ml of a 0.5% LDAO and 50 mM Tris (pH 8) solution. To reduce the RCs, solid $NaBH_4$ was added to a $NaBH_4$ concentration of 5 mM. After incubating this solution for 10 minutes at room temperature, the RCs were loaded on a DEAE Sephacel column and washed several times with a 0.5% LDAO and 50 mM Tris (pH 8) solution in order to remove the released Q_BH_2 and traces of reduced UQ_{10}, the denatured RCs, the Triton X-100 and the excess of $NaBH_4$. After washing, the effluent was coloured light-blue. An absorption spectrum showed that the effluent did not contain intact RCs. A salt gradient (0-500 mM NaCl) was applied to elute the RCs from the column. The RCs eluted from the column at about 250 mM NaCl. Fig. 8.3B shows the electronic absorption spectrum recorded from the RCs. The bands of the bacteriopheophytin (755 nm) and the special pair (865 nm) are of equal intensity and a typical A_{280} / A_{800} ratio of 1.28 was measured, indicating that all the denatured RCs have been successfully removed.

Fig. 8.4 shows the biological activity following a flash of actinic light. The activity of the RCs (Fig. 8.4A) can be fitted with a single exponential decay $A \cdot \exp(-t/\tau)$ with a decay constant $\tau = 0.108$ s, which is the typical rate for the $P^+Q_A^{\bullet-} \rightarrow PQ_A$ back-reaction. The absence of a slowly-decaying component with $\tau \approx 1$ s for the $P^+Q_B^{\bullet-}$ back-reaction means that the excess of Q_B has been successfully removed by the reduction with $NaBH_4$. Fig. 8.4B shows kinetics of the same sample after adding a 5-fold excess of UQ_{10}. Since both Q_A and Q_B are fully occupied in this preparation, the decay is bi-exponential, $A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2)$. From this measurement, the incorporation of Q_A can be calculated as $A/(A_1+A_2)$*100% and is found to be about 95%.

The NaCl was removed by dialyzing the RCs for 24 hours at 4°C against a 0.1% LDAO TL-buffer, and two times 24 hours against a 0.025% LDAO TL buffer. The RC solution was pre-concentrated by using a 100 kDa filter (Amicon, Ca.). Subsequently, the

RC mixture was super-concentrated by drying the preparation in a sealed container above a saturated salt solution at 4°C, to a final volume of 80 µl with an OD^{800} of ~ 1000. About 60 µl of this highly concentrated RC solution was used for the solid-state NMR experiments. During the concentration steps, and before and after the NMR experiment, absorption spectra were monitored that confirmed the maintenance of a good sample quality.

8.2.2 Preparation of [1-^{13}C] Q_B *Rb. sphaeroides* R26 RCs

100 ODV^{800} of isolated *Rb. sphaeroides* R26 RCs were diluted in 100 ml of a 0.5% LDAO and 50 mM Tris (pH 8) solution. Solid KBH_4 was added to a concentration of 5 mM to reduce the RCs. After incubating this solution for 10 minutes at room temperature, the RCs were loaded on a DEAE Sephacel column and were washed with a 0.5% LDAO, 50 mM Tris (pH 8) solution to remove the released Q_BH_2 and the excess of KBH_4. Subsequently, the Q_B-depleted (dQ_B) RCs were eluted from the column with a 1 M NaCl (0.1% LDAO) solution. The RCs were dialyzed overnight at 4°C against a 0.025% LDAO TL buffer. The recombination kinetics following a flash of actinic light were recorded from the dQ_B RCs before and after adding a 5-fold excess of UQ_{10}. By comparing the recombination kinetics, a Q_A depletion of 10% and a Q_B depletion of 90% was calculated.

The reconstitution of the RCs with [1-^{13}C] UQ_{10} for Q_B was accomplished by adding a 5-fold excess of [1-^{13}C] UQ_{10} to the dQ_B RCs and allowing the RC-quinone mixture to incubate for one hour at room temperature and overnight at 4°C. The reconstituted RCs were loaded on a DEAE Sephacel column and washed with 0.025% LDAO TL buffer to remove the excess of [1-^{13}C] UQ_{10}. The RCs were eluted with a 0.025% LDAO TL buffer containing

1 M NaCl, and dialyzed overnight at 4°C against a 0.025% LDAO TL buffer. About 85 ODV^{800} of reconstituted RCs were obtained. The RC solution was concentrated using a 100 kDa filter (Amicon, Ca.) to a final OD^{800} of about 500. After concentration, 85 % of the RCs contained [1-^{13}C] UQ$_{10}$ at the Q$_B$ side. Due to the 10% Q$_A$ depletion before adding the [1-^{13}C] UQ$_{10}$, up to 10% of the RCs will also be reconstituted with [1-^{13}C] UQ$_{10}$ for Q$_A$. From the [1-^{13}C] Q$_B$ RCs, 50 ODV^{800} was used for the solid-state NMR experiments.

8.2.3 NMR spectroscopy

The NMR spectra were recorded using MSL-400 and DMX-600 spectrometers, equipped with 4mm CP/MAS probes (Bruker, Karlsruhe, Germany). A home-built spinning-speed controller was used to keep the spinning speed constant to within a few Hz [14]. All data were recorded with the sample confined to the center of the NMR coil by using spacers in the rotor. During the data acquisition, the protons were decoupled from the carbons by use of the two-pulse phase-modulation (TPPM) decoupling scheme [15]. Low-temperature NMR measurements were performed using liquid nitrogen-cooled bearing gas [16]. The temperature of the bearing gas T_B was measured just before the gas entered the spinning assembly, and the temperature of the sample can be approximated by $T \approx 0.86 \cdot T_B + 50$ K (± 5 K). The detergent-solubilized samples were cooled down to 267 K at a relatively low spinning rate $\omega_r / 2\pi \approx 1.5$ kHz. The freezing of the sample while spinning ensures a homogeneous sample distribution against the rotor wall.

The 2-D ^{1}H-^{13}C heteronuclear dipolar correlation spectra were recorded at a magnetic field of 14.1 T at ambient temperature. The correlation spectrum of the [1-^{13}C] Q$_A$ R26 RCs was obtained from hydrated-precipitated RCs [2], using the CP/WISE technique described in Chapter 3 with a short CP time of 200 μs. In the t_1 dimension 32 points were collected. The correlation spectrum of hydrated-precipitated [1-^{13}C] Q$_B$ R26 RCs was recorded with the technique described in Chapter 4, with frequency-switched Lee-Goldburg (FSLG) ^{1}H homonuclear decoupling during the proton evolution. A LG-CP contact time of 4.5 ms was applied and 32 slices were recorded in t_1.

The LG-CP build-up curves were obtained with the technique discussed in Chapter 5, with the spectrometer adjusted for the $n = -1$ Hartmann-Hahn matching condition. The ^{13}C signal intensities for the build-up curves were determined with the speedy-fit programming [17] using the MINUIT (CERN, Geneva, Switzerland) fitting package [18], following the same procedure as described in Ref. [2]. The same phase correction was applied to all spectra in the polarization build-up series. The carbonyl region (190-160 ppm) of the spectrum with the best signal to noise ratio for the 1-^{13}C UQ$_{10}$ response was first deconvoluted with a superposition of two Gaussian lines, one for the label signal around 183 ppm and one for the natural-abundance peptide carbonyl background signal around 173 ppm. The isotropic chemical shifts and linewidths were fixed at the values determined from this deconvolution. In this way, the integrated intensities of the label and carbonyl background signal are left as the only free parameters in the fit of the other spectra in the series, and they can be determined accurately.

8.3 Results

From 1-D ^{13}C CP/MAS NMR spectra of hydrated-precipitated RCs, reconstituted with [1-^{13}C] UQ_{10} for Q_A, the [1-^{13}C] Q_A R26 RC resonance was assigned with a chemical shift of 183.8 ppm [2]. Fig. 8.5 shows a contour plot of a 2-D ^{1}H-^{13}C heteronuclear dipolar correlation spectrum of hydrated-precipitated [1-^{13}C] Q_A RCs, recorded at ambient temperature, at a high magnetic field of 14.1 T and using a MAS rate $\omega_r/2\pi$ = 15.0 kHz. The [1-^{13}C] Q_A resonance around 183.8 ppm has a correlation signal with a proton with σ_i =

9.5 ± 0.5 ppm, indicated with an arrow in the 2-D correlation spectrum. This correlation signal predominantly involves intermolecular polarization transfer. The nearest protons in the UQ_{10} molecule are a proton of the nearby CH_2 moiety of the quinone tail (r_{CH} = 2.7 Å, [21]) and possibly one of the 6'-methoxy protons, depending on the orientation of the methoxy substituent (Fig. 8.2). These protons all resonate more upfield and can not account for the observed ^1H shift of ~ 9.5 ppm.

Fig. 8.6A shows a LG-CP build-up curve of [1-^{13}C] Q_A, recorded from frozen detergent-solubilized [1-^{13}C] Q_A R26 RCs at a temperature of 235 K and at a magnetic field of 9.4 T. A MAS rate $\omega_r/2\pi$ = 11.0 kHz was used. The LG-CP build-up curve is slowly oscillating, and its Fourier transform (Fig. 8.6B) provides the LG-CP carbon spectrum with two resolved maxima. The build-up of the [1-^{13}C] Q_A signal was compared with simulated build-up curves for CH spin-pairs with different r_{CH}, which were calculated in Chapter 5.

The best correspondence between the experimental and simulated data is found for a spin-pair that has r_{CH} = 2.03 Å (Fig. 8.6A). Using the procedures described in Chapter 5 to correlate the splitting $\Delta\omega/2\pi$ = 1950 Hz between the maxima in the Fourier transform in Fig. 8.6B to a distance r_{CH}, the 1950 Hz component translates into a short r_{CH} = 2.1 ± 0.1 Å.

The NMR spectral characteristics of the [1-^{13}C] UQ$_{10}$ in the Q$_{A}$ site can be compared with the signals from the same label in the Q$_{B}$ site. Fig. 8.7A shows a 1-D ^{13}C CP/MAS NMR spectrum of frozen detergent-solubilized RCs, reconstituted with [1-^{13}C] UQ$_{10}$ for Q$_{B}$. The spectrum is recorded at a field of 9.4 T, at a temperature T = 244 K and using a MAS rate $\omega_{\mathrm{r}}/2\pi$ = 7.0 kHz. The asterisk indicates the response of the ^{13}C label. The [1-^{13}C] Q$_{B}$ R26 RC signal resonates with a chemical shift of 182.4 ppm relative to TMS, which compares well with the shifts reported for [1-^{13}C] Q$_{A}$ (183.8 ppm) and [4-^{13}C] Q$_{A}$ (183.1 ppm, at T = 190 K) [2]. The Q$_{B}$ signals are consistently broader than the corresponding resonances for the Q$_{A}$. A Gaussian linewidth of 300 Hz is measured for the [1-^{13}C] Q$_{B}$ R26

RC response. The linewidth of the [1-^{13}C] Q_A signal is 150 Hz at room temperature and 250 Hz at $T = 230$ K, while for the [4-^{13}C] Q_A response a linewidth of 220 Hz is reported at $T = 190$ K [2]. The relatively large width of the [1-^{13}C] Q_B R26 RC signal suggests substantial heterogeneity in the chemical environment of the Q_B, more than for the Q_A. Fig. 8.7B displays a ^{13}C CP/MAS NMR spectrum of hydrated-precipitated [1-^{13}C] Q_B R26 RCs, collected at ambient temperature at a magnetic field of 14.1 T, using a MAS rate $\omega_r/2\pi$ = 12.0 kHz. The precipitated RCs have essentially the same characteristics as the detergent-solubilized RCs, like for the Q_A side. In particular, the chemical shift and linewidth are comparable to those observed for the detergent-solubilized preparation, indicating that a good sample quality is maintained during the precipitation of the RCs.

A contour plot of a 2-D FSLG decoupled heteronuclear (^{1}H-^{13}C) dipolar correlation spectrum of the precipitated [1-^{13}C] Q_B R26 RCs is shown in Fig. 8.8. The data were collected at room temperature with a MAS speed $\omega_r/2\pi$ = 12.0 kHz at a magnetic field of 14.1 T. The [1-^{13}C] Q_B at 182.4 ppm correlates with a proton signal around 9.5 ± 0.5 ppm, indicated with an arrow in the spectrum. The downfield ^{1}H response provides strong evidence for intermolecular heteronuclear polarization transfer, similar to the [1-^{13}C] Q_A.

Finally, Fig. 8.9A shows the LG-CP build-up curve recorded for the [1-^{13}C] Q_B in a preparation of hydrated-precipitated [1-^{13}C] Q_B R26 RCs at a field of 14.1 T, at ambient temperature. A spinning frequency $\omega_r/2\pi$ = 12.0 kHz was applied. Using the same procedure as for the [1-^{13}C] Q_A, the build-up curve of the [1-^{13}C] Q_B signal can be compared with simulations of the LG-CP process for isolated CH spin-pairs. The best correspondence is found for a heteronuclear spin-pair with a separation r_{CH} = 1.96 Å (Fig. 8.9A). From the analysis of the Fourier transform of the [1-^{13}C] Q_B signal build-up, a weak splitting $\Delta\omega/2\pi$ of ~ 2000 Hz is observed, which translates into a distance r_{CH} of approximately 2.1 Å.

8.4 Discussion

A number of 3-dimensional (3-D) structures of the *Rb. sphaeroides* RC, refined from X-ray diffraction data from crystallized RCs, have been reported in the literature [1,19-24]. There are ambiguities regarding the position of the quinones in the binding pockets, in particular the positioning of Q_B varies significantly among the various structures [23,24]. According to all structures, the 1-C=O of Q_A is proposed to hydrogen bond to the backbone amide nitrogen of Ala M260. The 4-C=O of Q_A is at hydrogen-bonding distance from the side-chain oxygen of Thr M222 [1,19,22] or the side-chain nitrogen of Trp M252 [1] in some structures, while other structures place it within hydrogen-bonding distance to $N\delta_1$ of the imidazole ring of His M219 [20,21]. For ground-state Q_B, it is suggested that the 1-carbonyl hydrogen bonds to the backbone amide nitrogen of Ile L224 [1,20,22] or to the side-chain oxygen of Ser L223 [1,19,20], while in another structure the 1-C=O should not be specifically involved in the formation of a hydrogen bond to the protein [21]. The 4-carbonyl of Q_B is proposed to have a hydrogen-bonding interaction with the imidazole $N\delta_1$ of His L190 [1,19,20,22] or the backbone amide nitrogen of Ile L224 [21]. Recently, it was demonstrated that Q_B can be found in two different positions, depending on the extent of dark adaptation [24]. It was argued that this may form one of the sources for the widely different positioning of Q_B reported by the various groups [24]. In conclusion, it is clear that the characteristics of the hydrogen bonding is beyond the resolving power of the 3-D structures, since they are not conclusive with respect to the hydrogen-bonding interactions of both the Q_A and the Q_B.

From the ^{1}H-^{13}C heteronuclear correlation spectroscopy, it is found that both [1-^{13}C] Q_A and [1-^{13}C] Q_B have a correlation signal with a proton that resonates around 9.5 ppm (cf. Figs. 8.5 and 8.8). These shifts imply that the correlations arise from intermolecular polarization transfer, and the shifts are representative for hydrogen-bonded protons, for instance, a hydroxyl or amide proton in the protein environment. In addition, the CH distances between the ^{13}C label and the correlated protons are short, $r_{\mathrm{CH}} = 2.1 \pm 0.1$ Å for both the [1-^{13}C] Q_A and the [1-^{13}C] Q_B.

These distances are very short for intermolecular transfer. Generally, the shortest intermolecular distances between protons and carbons are ~ 3 Å. The molecular vibrations prevent in practice closer contacts, with the exception of hydrogen-bonding interactions, which involve the stabilization of orbital overlap between the atoms O and X involved in the hydrogen bond. In this way a short intermolecular distance between hydrogen-bonding partners is realized, in between the covalent bond $r_{\mathrm{OX}} \leq 1.5$ Å, and the ionic bond $r_{\mathrm{OX}} \geq 3$ Å. Since both quinones are not protonated in the ground state, the proton that stabilizes the hydrogen bridge is bonded to the X-atom, which implies that $r_{\mathrm{XH}} \sim 1$ Å [25]. Since we measure r_{CH} = 2.1 Å for both the Q_A and the Q_B, and the r_{CO} = 1.2 Å, our measurements indicate that in addition r_{OH} ~ 1 Å. This would imply a strong hydrogen bond for both quinones at the 1-C=O side, with r_{OX} ~ 2 Å. In such a strong hydrogen bond there is a single minimum in the proton potential energy, which favours a localization of the proton around the center of the bond [25]. The observation of a short r_{CH} = 2.1 Å for both the Q_A and the Q_B may indicate that the strong hydrogen bonding is a generic property of the quinone in the RC. It may be a prerequisite for the tight binding of the Q_A. The narrow line for the [1-^{13}C] response is in line with the well-defined binding environment that transpires from the X-ray data, while the broader [1-^{13}C] response for the Q_B side indicates that a structural heterogeneity is not necessarily accompanied by weaker binding interactions.

An alternative explanation for a strong heteronuclear dipolar coupling and a short r_{CH} = 2.1 Å can in principle be a hydrogen bond in which the proton jumps between two local minima. Since the CP proceeds through the dipolar interactions, only the shortest distance will effectively contribute to the CP process. However, a proton at the oxygen side of the H-bond for a large fraction of the time is difficult to reconcile with the [1-^{13}C] chemical shift, since it could involve charge transfer and a pronounced shift of the ^{13}C, which is not observed in the NMR experiments. In addition the dipolar interaction will be scaled according to the fraction involved in the LG-CP, and there is no evidence for such a scaling phenomenon from the data or the simulations. In contrast, a strong hydrogen bond is well in line with the rapid CP and tightly held cofactor transpiring from the MAS NMR studies. The observation of strong H-bonding interactions at the 1-C=O side for both quinones not accompanied by large isotropic shifts of the carbonyls in the RC relative to the shifts of free UQ_{10}, also corroborates the semi-empirical calculations, according to which there is no conclusive correlation between the quinone C=O carbon charge and the strength of the hydrogen bond [5].

To address the question which protons can contribute to the heteronuclear polarization transfer for the [1-^{13}C] Q_A, the X-ray structures can be still of some help. For instance, Fig. 8.10 shows the residues close to the 1-C=O of Q_A, according to the 3-D structure of Ermler *et al.* [21]. According to this and other X-ray structures, the protons that in principle could participate in the intermolecular polarization transfer to the [1-^{13}C] Q_A, based on characteristic ranges of chemical shifts, are the amide proton of alanine M260, and the $H\varepsilon_1$ of tryptophan M252. The amide proton of alanine and the $H\varepsilon_1$ of tryptophan are expected to resonate in the ranges 4.0-11.4 ppm and 6.8-11.9 ppm, respectively [26]. However, the CH distance between the 1-C Q_A and $H\varepsilon_1$ Trp M252 is about 4.4 Å. This is far too long to allow for the rapid polarization build-up of the 1-^{13}C Q_A response observed in the CP/MAS NMR experiments. The shortest ^{1}H-^{13}C distance in the X-ray structural models is 2.6-2.9 Å, between the amide H of Ala M260 and the 1-C Q_A.

Hence, the r_{CH} = 2.1 Å measured with the LG-CP NMR appears less than the shortest heteronuclear distance in the X-ray structural models that are based on datasets with a resolution in the range of 2.6-3.1 Å. On the NMR side, interactions with more distant protons lead to a minor apparent shortening of the r_{CH} calculated from the analysis of the LG-CP response to first order with an isolated heteronuclear spin-pair. In Chapter 5 it was demonstrated that the heteronuclear dipolar couplings to distant protons produce a minor increase of 100-200 Hz of the splitting $\Delta\omega/2\pi$ between the maxima in the LG-CP carbon response of a strongly coupled CH pair. As a result, the r_{CH} is probably underestimated by ~ 0.1 Å. On the other hand, we know from the 2-D heteronuclear correlation data that the contribution from the remote protons is small. For instance, there is not a strong correlation with the CH_2 protons at a distance of ~ 2.7 Å in the Q_A tail itself, resonating with a chemical shift in the range of ~ 1-4 ppm [21].

Although the couplings to remote protons may introduce a minor uncertainty in the distance, we have the 'hard' experimental observation that the 1-^{13}C Q_A signal builds up fast during the LG-CP, which is only possible in the presence of at least one nearby proton (cf. Fig. 8.6A). In the X-ray models, the Ala M260 N is positioned about 0.5-1.0 Å above the plane of the Q_A quinone ring, with the NH vector inclined at 45-60° with respect to the normal of the plane and directed towards the 1-carbonyl of Q_A [21,22]. In contrast in the quinhydrone, which is a complex formed by benzoquinone and hydroquinone and stabilized by hydrogen bonding between the OH of the hydroquinones and the keto functionalities of the benzoquinones, the OH of the hydroquinone is located within the plane of the adjacent benzoquinone, with the OH inclined at an angle of ~ 135º with respect to the C=O bond [27,28]. In addition, *ab initio* calculations performed with a small basis set to model the configuration of a small (HCl) molecular dipole oriented towards a benzoquinone demonstrated that an energetically stable configuration is found for the O···HCl vector in the plane of the benzoquinone, inclined at ~ 135° with respect to the C=O bond [29]. Hence it transpires that a planar configuration of the O···HX vector and the quinone ring may be favourable for hydrogen bonding.

In the X-ray structures the quinone ring is placed in a position to match the experimentally determined electronic density profile. Since our earlier NMR results have provided evidence that the 4-C=O side is subject to dynamics, the positioning of the quinone ring may have to be reconsidered. In reality the ring may be closer to one of the H-bond partners suggested by the X-ray, in particular the Ala M260 NH for the 1-C=O of the Q_A. If this is combined with a 15-20° rotation of the quinone, the NH vector can be brought within the plane of the Q_A ring. Hence, a small repositioning of the Q_A can bring the 1-C=O in a tight and probably favourable hydrogen-bonding configuration with Ala M260 NH [30].

The r_{CH} ~ 2 Å for the 1-C=O Q_B is similar to the Q_A, which suggests that the quinone binding generally involves a strong interaction with a hydrogen-bonding partner. However, for the Q_B, it is difficult to identify a unique proton that has a H-bonding interaction with the 1-^{13}C=O due to the ambiguities between the various X-ray models. For instance, in the structure of Arnoux *et al.*, the peptide N of Ile L224 is at a distance of 3.5 Å from the 1-C=O of Q_B, with the amide H about 3.1 Å from the 1-C of Q_B [22]. The same hydrogen-bonding partner was found in other structures, that in addition propose the hydroxyl moiety of Ser L223 as a possible hydrogen-bond donor [1,20]. Both the amide H of Ile L224 and the hydroxyl H of Ser L223 can in principle account for the downfield ^{1}H shift of 9.5 ppm observed from the correlation with the [1-^{13}C] Q_B [26], and it is not possible to assign the interacting proton unambiguously. In contrast, according to the structure of Ermler *et al.* [21] and the dark-adapted structure of Stowell *et al.* [24], the 1-C=O of Q_B is not involved in the formation of a hydrogen bond to the protein. In this case a proton relay system involving one or more water molecules may have to be invoked in order to explain the rapid polarization transfer during the CP.

The observations of a strong hydrogen-bonding interaction at the 1-C=O side and of a more dynamic interaction at the 4-C=O side of Q_A in ground-state RCs contrast with the binding of the primary quinone probed in the charge-separated or semi-quinone state $Q_A^{\bullet-}$. In the state $Q_A^{\bullet-}$ there is converging experimental evidence for hydrogen-bonding interactions of different strength to both the Q_A carbonyls of RCs. Already at an early stage, electron nuclear double-resonance spectroscopy has indicated the presence of a hydrogen bond to the 4-C=O of $Q_A^{\bullet-}$ [31]. From electron-spin echo envelope modulation (ESEEM) measurements on Zn-substituted RCs, it was inferred that the $N\delta_1$ of the imidazole ring of His M219 is involved in a hydrogen-bonding interaction to the 4-C=O of $Q_A^{\bullet-}$ [32]. It was concluded from electron paramagnetic resonance (EPR) studies from RCs reconstituted with selectively labeled quinones in the Q_A side that the hydrogen bonding to $Q_A^{\bullet-}$ is asymmetric, the 4-C=O forming the strongest hydrogen bond with the protein [33,34]. Recently, ESEEM and EPR spectroscopy on Zn-substituted RCs, in combination with site-directed mutagenesis, provided evidence for additional weaker hydrogen bonding between the 1-^{13}C=O of the $Q_A^{\bullet-}$ and the peptide nitrogen of Ala M260 [35]. Finally, in the reduced $P^+Q_A^{\bullet-}$ state, librational motion of the ring has been detected with an axis over the carbonyl carbons [36].

The difference of the Q_A binding in the ground-state RC and in the charge-separated state $Q_A^{\bullet-}$ suggests that the hydrogen bonding and the dynamics depend on the oxidation state of the primary quinone. This is of functional importance, since it would imply a rearrangement of the H-bonding of the Q_A upon electron transfer. In ground-state RCs, the Q_A ring appears to be tightly networked to Ala M260, with the 1-C=O of Q_A in a tight hydrogen-bonding configuration involving Ala M260 NH. When an electron arrives at Q_A, a rearrangement of $Q_A^{\bullet-}$ and the formation of a strong hydrogen bond between $N\delta_1$ of His M219 and 4-C=O of $Q_A^{\bullet-}$ creates an efficient screening of the electron charge, via hydrogen bonding to His M219 and possibly prohibiting the $P^+Q_A^{\bullet-} \rightarrow PQ_A$ back-reaction. After transferring the electron to Q_B, Q_A can move back to its original position, thereby weakening the coupling with His M219. Hence, a H-bond switching of Q_A and $Q_A^{\bullet-}$ can act as a subtle mechanism that contributes to the establishment of an efficient forward electron transfer and the prevention of a wasteful back-reaction.

8.5 Conclusions

Solid-state heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy has been used to study the binding of the quinones in photosynthetic RCs in the ground state. It was found that the 1-^{13}C of both Q_A and Q_B have intermolecular correlations with protons that resonate downfield, in the region of hydrogen-bonding protons. From the LG-CP build-up curves of the [1-^{13}C] Q_A and Q_B signal, it is calculated that the distances between the carbon labels and the correlated protons are short, 2.1 ± 0.1 Å. The NMR provides evidence for strong hydrogen-bonding interactions at the 1-C=O of both Q_A and Q_B for RCs in the ground state. An unambiguous assignment of the protons that correlate with the [1-^{13}C] is not possible from the X-ray structures. However, it is proposed that the 1-C=O of Q_A in the ground state has a strong H-bonding interaction with Ala M260 NH and is disconnected from the direct pathway for electron transfer. A rearrangement of $Q_A^{\bullet -}$ in the semi-quinone state may contribute to the efficiency of the forward electron transfer reaction, while it may prohibit the wasteful back-reaction.

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^{13}C MAS NMR evidence for structural similarity of L162YL mutant and *Rhodobacter sphaeroides* R26 RC, despite widely different cytochrome c_2-mediated re-reduction kinetics of the oxidized primary donor

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Chapter 10

General discussion and outlook

The main target of the PhD research described in this thesis is to acquire access to protons and hydrogen bonds in moderately sized organic solids and proteins with MAS NMR. In addition, it is demonstrated that protons can be used for assignment and structure-determination strategies of biological systems in the solid state. Concerning the accomplishment of the first part of this goal, several NMR techniques have been developed that can be applied successfully with rapid MAS (≥ 10 kHz) to suppress the strong homonuclear dipolar interactions among the abundant protons. It is found that Lee-Goldburg irradiation provides a versatile tool to suppress the ^{1}H dipolar couplings under various experimental conditions. It is demonstrated that frequency-switched Lee-Goldburg can be implemented during the proton evolution to acquire highly resolved heteronuclear correlation spectra at high MAS rates. In addition, with Lee-Goldburg cross-polarization (LG-CP) heteronuclear spin-locking under simultaneous suppression of the homonuclear dipolar proton interactions is easily achieved and the technique can be applied to build up correlations in heteronuclear dipolar correlation spectroscopy in a selective manner. The selectivity is preserved for long LG-CP contact times and the method can be exploited to detect long-range intermolecular heteronuclear correlations, including transfer via hydrogen-bonded protons, to provide ‘hard’ structural restraints. In a different approach, the use of the LG-CP can be extended to measure ^{1}H-^{13}C heteronuclear dipolar couplings at high MAS speeds, which can be translated into CH distances. In principle the technique should not be limited to measure CH distances but may include other nuclei as well, for instance to detect ^{1}H-^{15}N distances.

Concerning the second main target, concepts for structure determination using dipolar correlation spectroscopy are presented and applied successfully in a 3-D structural study of the arrangement of pigments in artificial aggregates and in intact chlorosomes, and to study the binding by hydrogen bonding of quinones in photosynthetic reaction centers in the ground state. It is demonstrated that a comprehensive 3-D structural model for self-assembled chlorophyll can be obtained from knowledge about the electronic structure deduced from the solid-state proton assignment, and from the assignment of several intermolecular heteronuclear correlations. It is expected that this approach can be followed in general to study self-assembled systems and the interaction or binding of cofactors to a protein, by use of complexes in which the native cofactor has been reconstituted with a partly or uniformly labeled counterpart. In this respect, a solid-state NMR correlation spectroscopy study of the protein-pheophytin interactions in the photosynthetic reaction center has also been performed outside the context of the thesis [1].

It is demonstrated that high magnetic field gives a non-linear line-narrowing effect on the NMR response. This offers promising prospects for structure research of compounds in the solid state, as increasingly higher magnetic fields will become available in the future. It is anticipated that assignment strategies exploiting correlation spectroscopy will benefit from the resolution enhancement that can be reached at ultra-high magnetic field strengths (> 17 T) with fast MAS, and that combinations of 2-D correlation methods into novel 3-D dipolar correlation techniques may lead to the assignment of many, if not all, of the resonances of moderately large proteins. Currently, work is in progress to obtain a full assignment of the NMR resonances of a moderately large uniformly ^{13}C,^{15}N-enriched solid protein, the α-spectrin SH3 domain (7.2 kDa, 62 amino acids) [2]. Already in 2-D ^{1}H-^{13}C heteronuclear dipolar correlation spectra numerous correlations can be resolved which can be attributed to individual CH moieties in the protein [3].

Another interesting application of the solid-state MAS NMR dipolar correlation techniques for structure refinement and structure-function research is the study of the binding of agonists and antagonists to receptors. For example, knowledge about the precise mechanism of the antagonistic binding is invaluable for rational drug-design and can be exploited to control pathologic symptoms. A better understanding of the structure-function relationship and interaction between ligands and receptors can be provided by detailed structural information of the receptor-bound state, using partly or uniformly labeled complexes. Already at the level of the chemical shift assignment structural information can be obtained. First, it is expected that changes of chemical shifts induced by the binding of a ligand to a receptor will be predominantly observed for residues that reside in the contact area. Hence, a comparison between chemical shifts of the bound and the unbound complex can be used to map out the part of the surface where the interaction takes place. Second, the chemical shift assignment can be assessed to identify intermolecular correlations which provide distance constraints. Even a limited set of such constraints will substantially reduce the number of possibilities to assemble the receptor-hormone complex in a modelling step.

As a concluding remark, the methodology described in the thesis sets the stage for a wide range of applications exploring the relatively uncharted field of protons in resolving ordered structures without translation symmetry with solid-state NMR.

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Summary

In the study described in this thesis it is demonstrated that protons can be utilized in assignment and structure refinement studies of biological systems in the solid state using cross-polarization (CP) magic-angle spinning nuclear magnetic resonance (MAS NMR) dipolar correlation spectroscopy. A set of novel solid-state NMR spectroscopic techniques is developed that enables the use of protons in multidimensional heteronuclear dipolar correlation spectroscopy of multiply enriched systems. In addition, concepts for structure refinement using ^{13}C homonuclear and ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy are presented. Protons are important. For instance, they form the hydrogen bonds that establish and stabilize the structure of many compounds. In particular, hydrogen-bonding interactions determine the secondary structure of proteins, they stabilize the binding of cofactors to the protein environment and the self-assembly of pigments. Solution NMR techniques can not always be exploited for structural investigations, for instance when the system of interest can not be dissolved or when it has to be studied in an intact or native environment, such as a membrane. In addition, crystals are often not available and high-resolution diffraction techniques can not be used for structural studies. In these cases, solid-state NMR in general, and the study of protons with NMR in particular, can be utilized to provide structural information.

The new solid-state NMR approach for structure determination is applied in studies of the electronic and spatial structure of organelles involved in bacterial photosynthesis. In particular the self-aggregation of pigments in the native chlorosome antenna system and in artificial aggregates, and details of the structure of the bacterial reaction center are investigated. However, it should be mentioned here that the applicability of the solid-state NMR techniques described in this thesis is much broader and the methods are suited to study protons in a wide range of multiply ^{13}C-enriched microscopically ordered systems without long-range translation symmetry.

In **Chapter 2** a brief theoretical background is provided to describe the effect of off-resonance radio-frequency irradiation during the proton evolution period in heteronuclear correlation spectroscopy and during polarization transfer with CP. It is demonstrated in **Chapter 3** that high magnetic fields have a line-narrowing effect on the proton response. It has been shown for a preparation of uniformly ^{13}C-enriched L-tyrosine·HCl salt that the resolution enhancement in the dimension of the protons in 2-D heteronuclear dipolar correlation spectra obtained with strong magnetic fields and fast MAS is sufficient to

determine the proton chemical shifts directly from the 2-D experiment, without any ^{1}H homonuclear decoupling scheme during the proton evolution. In addition, intermolecular heteronuclear correlations and hydrogen-bonding characteristics in the solid state can already be determined with very simple pulse schemes at high field. It is anticipated that further improvement of the proton resolution can be obtained with increasingly higher magnetic fields. This will be of advantage for heteronuclear dipolar correlation spectroscopy exploiting multipulse proton decoupling techniques to achieve additional attenuation of the proton linewidths, and will be particularly useful for the development of future proton assignment and structure determination strategies.

It is demonstrated in **Chapter 4** that well-resolved MAS NMR heteronuclear (^{1}H-^{13}C) dipolar correlation spectra of multispin ^{13}C clusters can be acquired at high spinning speeds when frequency-switched Lee-Goldburg irradiation is applied during the ^{1}H evolution. The resolution enhancement obtained in this way makes it possible to incorporate protons in concepts for assignment strategies and structure determination of uniformly ^{13}C-enriched spin clusters in biological systems, using 2-D or 3-D dipolar correlation spectroscopy. In addition, it is shown that Lee-Goldburg CP (LG-CP) in combination with heteronuclear dipolar correlation spectroscopy can be exploited to detect ^{1}H-^{13}C heteronuclear intermolecular correlations and to provide unambiguous structural restraints

In **Chapter 5** it is shown that the time-oscillatory polarization transfer during LG-CP recorded at high MAS rates with simultaneous suppression of the ^{1}H homonuclear dipolar interactions can be analyzed to determine heteronuclear distances with good accuracy in uniformly ^{13}C-enriched compounds. Fourier transforms of the LG-CP build-up curves provide effective carbon spectra that resemble heteronuclear dipolar powder spectra for non-spinning samples. The LG-CP carbon spectra can be closely reproduced with MAS Floquet simulations. A set of simulations was performed for CH spin-pairs with different separations, in order to provide an empirical relation between the heteronuclear dipolar coupling strength and the CH distance r_{CH}. It is demonstrated that this relation can be utilized to determine r_{CH} from experimentally observed dipolar coupling strengths, by calculating the CH distances to protons that have a hydrogen-bonding interaction. The method is also suited to determine r_{CH} of directly coupled CH spin-pairs. This is important, since it is often not possible to obtain good CH distance information from X-ray structures. The measurement of heteronuclear distances sets the stage for a novel route to *de novo* structure determination using uniformly enriched compounds or multispin clusters in ordered systems without long-range translation symmetry, for instance biological systems like membrane proteins. In addition, a 3-D version of the technique enables an analysis of the polarization exchange within individual CH spin-pairs, offering exciting prospects to study the coherent cross-polarization dynamics and hydrogen-bonding environments in detail on a microscopic scale.

A concept for structure determination using ^{13}C homonuclear and ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy is presented in **Chapter 6**. The concept has been applied in a 3-D structural study of self-assembled [U-^{13}C] chlorophyll *a* / H_2O. It is demonstrated that protons can be used successfully in assignment and distance determination strategies for *de novo* structure refinement using solid-state MAS NMR dipolar correlation spectroscopy and homology modeling. It is shown that knowledge about the electronic structure deduced from the solid-state proton assignment, as well as the assignment of several intermolecular heteronuclear (^{1}H-^{13}C) correlations, provide information that can be interpreted consistently into a structural model. In this model the chlorophyll *a* (Chl *a*) molecules are arranged in linear stacks that line up to form highly ordered 2-D sheets. Part of the proton response is attributed to polarization transfer from protons in water molecules forming a hydrogen-bonding network to the carbonyl moieties. Finally, a doubling of some of the solid-state NMR resonances revealed that two conformers of Chl *a* are present that are both structurally well-defined. The structural differences are local and it was deduced that pairs of sheets align back to back, mutually rotated over 180° in a bilayer structure. A densely packed suprastructure can be established by a laminar stacking of bilayers with interpenetrated phytyl chains. Alternatively, for a tubular bilayer supra-structure, the narrow ^{13}C lines of the NMR response provides a structural restraint of ~ 100 Å for the minimal bend radius such cylinders can have.

In **Chapter 7**, the arrangement of bacteriochlorophyll *c* (BChl *c*) in uniformly ^{13}C-enriched intact chlorosomes of the green photosynthetic bacterium *Chlorobium tepidum* is studied with high-field 2-D (^{1}H-^{13}C) and 3-D (^{1}H-^{13}C-^{13}C) heteronuclear MAS NMR dipolar correlation spectroscopy. From the correlation spectra it is possible to assign all observable proton resonances. The solid-state ^{1}H assignment is used to calculate proton aggregation shifts relative to monomeric BChl *c* in solution. The pattern of aggregation shifts corroborates the parallel chain model for the stacking of the BChl *c*. A doubling of the 5-CH and the 7-Me NMR resonances is observed, which provides strong evidence for the presence of at least two structurally different well-defined arrangements of the BChl *c* in the chlorosomes. The NMR data for [U-^{13}C] BChl *c* chlorosomes and *in vitro* [U-^{13}C] BChl *c* aggregates are remarkably similar to those obtained from aggregates of [U-^{13}C] Chl *a* / H_2O. By comparing the NMR results with other available NMR and structural data in a homology study, a bilayer tube is proposed for the arrangement of BChl *c* that is composed of sheets of stacks with opposite sliding direction.

In **Chapter 8**, solid-state heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy is used to study the binding of the quinones in *Rhodobacter sphaeroides* R26 reaction centers (RCs) in the ground state. The RCs were reconstituted with ubiquinone-10, specifically ^{13}C-enriched at the 1-carbonyl position, for Q_A or for Q_B. It is found that the 1-^{13}C of both Q_A and Q_B have intermolecular correlations with protons that resonate downfield, in the region of hydrogen-bonding protons. From the LG-CP build-up curves of the [1-^{13}C] Q_A and Q_B

signal, it is calculated that the distances between the carbon labels and the correlated protons are short, ~ 2.1 Å. The NMR study thus provides evidence for strong hydrogen-bonding interactions at the 1-C=O of both Q_A and Q_B for RCs in the ground state. This contrasts with the reduced state $Q_A^{\bullet -}$, for which a hydrogen bond has been found at the 4 carbonyl position, suggesting that H-bonding of Q_A depends on the oxidation state. This can be of functional importance, since it would imply a rearrangement of the Q_A during the electron transfer, which may be essential to establish an efficient forward electron transfer or to slow down the back-reaction.

Finally, in **Chapter 9**, ^{13}C CP/MAS NMR data collected from [4'-^{13}C] Tyr enriched L162YL mutant *Rhodobacter sphaeroides* RCs revealed that Tyr L162 is in a slightly heterogeneous and probably rigid section of the protein complex. The structural changes in the mutant with respect to *Rhodobacter sphaeroides* R26, as probed by the labels, are minimal on the scale of the NMR. This suggests that the reported dramatic decrease of re-reduction rate of the oxidized primary donor P upon mutation of Tyr L162 cannot be attributed to significant structural changes in the protein. The NMR study supports previous inferences that the function of tyrosine L162 is not to intervene directly and to facilitate the electron-transfer between the cytochrome and the photo-oxidized special pair P^+, but rather to fine-tune a fragile cytochrome-RC docking/reorientation processes.

Samenvatting

In dit proefschrift wordt aangetoond dat protonen benut kunnen worden in toekennings- en structuurverfijningsstudies van biologische systemen in de vaste stof met behulp van *cross-polarization* (CP) *magic-angle spinning* ('magische hoek'-rotatie, MAS) nucleaire magnetische resonantie (NMR) dipolaire correlatiespectroscopie. Een aantal nieuwe vaste-stof-NMR-spectroscopische technieken is ontwikkeld die het mogelijk maken om protonen op te nemen in meerdimensionale heteronucleaire dipolaire correlatiespectroscopie van meervoudig met ^{13}C verrijkte systemen. Daarnaast zijn concepten voor structuurverfijning met behulp van ^{13}C-homonucleaire en ^{1}H-^{13}C-heteronucleaire dipolaire correlatiespectroscopie gepresenteerd. Protonen zijn belangrijk. Ze vormen bijvoorbeeld de waterstofbruggen die de structuur van veel verbindingen bepalen en stabiliseren. In het bijzonder leggen waterstofbindende interacties de secondaire structuur van eiwitten vast en stabiliseren ze de binding van cofactoren aan het omringende eiwit en de zelf-assemblatie van pigmenten. Vloeistof-NMR kan niet altijd benut worden voor structuuronderzoek, bijvoorbeeld wanneer het te onderzoeken systeem niet kan worden opgelost of wanneer het bestudeerd dient te worden in zijn intacte of oorspronkelijke omgeving, zoals in een membraan. Daarnaast zijn kristallen vaak niet beschikbaar waardoor hoge-resolutie-diffractietechnieken niet gebruikt kunnen worden voor onderzoek aan de structuur. In zulke gevallen bieden vaste-stof-NMR in het algemeen, en onderzoek aan protonen met NMR in het bijzonder, uitkomst bij het verkrijgen van informatie over de structuur.

De nieuwe vaste-stof-NMR-benadering voor structuurbepaling is geïllustreerd aan de hand van onderzoek aan de electronische en ruimtelijke structuur van organellen die betrokken zijn bij de bacteriële fotosynthese. In het bijzonder worden de zelf-aggregatie van pigmenten in natieve chlorosoomantennesystemen en kunstmatige aggregaten, en details van de structuur van het bacteriële reactiecentrum onderzocht. Het dient hier echter opgemerkt te worden dat de toepasbaarheid van de NMR-technieken die in dit proefschrift besproken worden veel algemener is en dat de methoden geschikt zijn om protonen te bestuderen in een groot scala aan meervoudig met ^{13}C verrijkte microscopisch-geordende systemen zonder lange-afstandstranslatiesymmetrie.

In **Hoofdstuk 2** wordt een beknopte theoretische achtergrond gegeven om het effect van *off-resonance* radio-frequentie-instraling te beschrijven tijdens de proton evolutieperiode in heteronucleaire correlatiespectroscopie en tijdens de polarisatieoverdracht gedurende CP.

In **Hoofdstuk 3** wordt aangetoond dat hoge magneetvelden een lijn-versmallend effect hebben op de protonrespons. Voor een zout van uniform met ^{13}C verrijkt L-tyrosine·HCl wordt gedemonstreerd dat de resolutieverhoging in de dimensie van de protonen in 2D-heteronucleaire dipolaire correlatiespectra, opgenomen in een sterk magneetveld en met snelle MAS, volstaat om chemische verschuivingen van protonen direct van de 2D-experimenten te bepalen, zonder dat hierbij ^{1}H-homonucleaire ontkoppelingssequenties nodig zijn tijdens de protonevolutie. Daarnaast blijkt dat bij hoog veld met de meest eenvoudige puls-programma's al intermoleculaire heteronucleaire correlaties en karakteristieken van waterstofbruggen in de vaste stof bestudeerd kunnen worden. Er wordt dan ook voorzien dat een verdere verhoging van de protonresolutie verkregen zal worden met de beschikbaarheid van steeds sterkere magneetvelden. Dit zal zeker in het voordeel werken van heteronucleaire dipolaire correlatiespectroscopische methoden die gebruik maken van multi-puls-^{1}H-ontkoppelingstechnieken om extra versmalling van de proton-lijnbreedte te bewerkstelligen, en zal in het bijzonder van pas komen bij de ontwikkeling van toekomstige strategieën om protonen toe te kennen en bij de bepaling van structuren.

In **Hoofdstuk 4** wordt gedemonstreerd dat MAS-NMR-heteronucleaire (^{1}H-^{13}C) dipolaire correlatiespectra van multi-spin-^{13}C-clusters met een goede resolutie opgenomen kunnen worden bij hoge MAS-snelheden, indien frequentie-geschakelde Lee-Goldburg-instraling gebruikt wordt tijdens de ^{1}H-evolutie. De verhoging van de resolutie die op deze manier wordt verkregen maakt het mogelijk om protonen op te nemen in concepten voor toekenningsstrategieën en structuurbepaling van uniform met ^{13}C verrijkte spinclusters in biologische systemen, gebruikmakend van 2D- of 3D-dipolaire correlatiespectroscopie. Daarnaast wordt aangetoond dat Lee-Goldburg-CP (LG-CP) in combinatie met heteronucleaire dipolaire correlatiespectroscopie gebruikt kan worden voor de detectie van ^{1}H-^{13}C-heteronucleaire intermoleculaire correlaties en voor de verkrijging van eenduidige structurele restricties.

Het wordt in **Hoofdstuk 5** aangetoond dat de tijd-oscillerende polarisatieoverdracht tijdens LG-CP bij hoge MAS-snelheid en met gelijktijdige onderdrukking van de ^{1}H-homonucleaire dipolaire interacties, geanalyseerd kan worden teneinde heteronucleaire afstanden met een goede nauwkeurigheid te bepalen in uniform ^{13}C-verrijkte materialen. De Fourier-getransformeerde van de LG-CP-opbouwcurve levert een effectief koolstofspectrum op, welke gelijkenis vertoont met een heteronucleair dipolair poederspectrum voor een niet-spinnend sample. Deze LG-CP-koolstofspectra kunnen nauwkeurig gereproduceerd worden met MAS-Floquet-simulaties. Een reeks simulaties is uitgevoerd voor CH-spinparen met verschillende CH-afstanden r_{CH}, om zo een empirische relatie te verkrijgen tussen de heteronucleaire dipolaire koppelingssterkte en de afstand r_{CH}. Er wordt gedemonstreerd dat deze relatie gebruikt kan worden om r_{CH} te bepalen uit een experimenteel waargenomen dipolaire koppelingssterkte, door middel van het berekenen van CH-afstanden tot protonen in een waterstofbruggende interactie. De methode is ook geschikt om r_{CH} te bepalen van direct gekoppelde CH-spinparen. Dit is belangrijk, omdat het vaak niet mogelijk is om goede

CH-afstandinformatie te verkrijgen uit röntgendiffractiestructuren. Het meten van heteronucleaire afstanden biedt nieuwe mogelijkheden voor *de novo*-structuurbepaling met behulp van uniform-verrijkte verbindingen of multi-spinclusters in geordende systemen zonder lange-afstandstranslatiesymmetrie, bijvoorbeeld biologische systemen zoals membraaneiwitten. Daarnaast maakt een 3D-versie van de techniek een analyse mogelijk van de polarisatie-uitwisseling binnen individuele CH-spinparen, wat interessante mogelijkheden biedt om de coherente CP-dynamica en de vorming van waterstofbruggen gedetailleerd te bestuderen op microscopische schaal.

Een concept voor structuurbepaling met ^{13}C-homonucleaire en ^{1}H-^{13}C-heteronucleaire dipolaire correlatiespectroscopie wordt gepresenteerd in **Hoofdstuk 6**. Het concept wordt toegepast in een 3D-structuurstudie van zelf-geassembleerd [U-^{13}C] chlorofyl *a* / H_2O. Het wordt gedemonstreerd dat protonen succesvol benut kunnen worden in strategieën voor toekennings- en afstandsbepaling ten behoeve van *de novo*-structuurverfijning met behulp van vaste-stof-MAS-NMR-dipolaire correlatiespectroscopie en homologie-*modeling*. Het wordt aangetoond dat zowel de kennis over de electronische structuur afgeleid van de vaste-stof-NMR-protontoekenning, alsmede de detectie van verschillende intermoleculaire heteronucleaire (^{1}H-^{13}C) correlaties, informatie verschaffen die sluitend geïnterpreteerd kan worden tot een structureel model. Volgens dit model vormen de chlorofyl *a* (Chl *a*) moleculen lineaire stapelingen die uitlijnen om zo een hoog-geordend 2D-vlak te vormen. Een gedeelte van de protonrespons kan worden toegeschreven aan polarisatie-overdracht van protonen in watermoleculen die betrokken zijn bij de formatie van een waterstof-bruggend netwerk tussen de carbonylgroepen. Tenslotte, een verdubbeling van een aantal van de vaste-stof-NMR-resonanties laat zien dat er twee conformaties van Chl *a* aanwezig zijn, beide structureel wel gedefinieerd. De structurele verschillen zijn lokaal en het is afgeleid dat paren van vlakken “rug-aan-rug” uitlijnen, 180° gedraaid ten opzichte van elkaar, tot een bilaag-structuur. Een dicht gestapelde super-structuur kan worden verwezenlijkt door een laminaire stapeling van de dubbellagen met in elkaar grijpende fytylstaarten. Als alternatief, in geval van een cilindrische bilaag super-structuur leveren de smalle ^{13}C-lijnen in de NMR-respons een structurele restrictie van ~ 100 Å voor de minimale straal die de cilinders kunnen hebben.

In **Hoofdstuk 7** wordt de rangschikking van bacteriochlorofyl *c* (BChl *c*) in uniform met ^{13}C verrijkte intacte chlorosomen van de groene fotosynthetische bacterie *Chlorobium tepidum* bestudeerd met behulp van hoogveld 2D (^{1}H-^{13}C) en 3D (^{1}H-^{13}C-^{13}C) heteronucleaire MAS-NMR-dipolaire correlatiespectroscopie. Het blijkt mogelijk om alle waarneembare protonresonanties toe te kennen op grond van de correlatiespectra. De vaste-stof-^{1}H-toekenning kan vervolgens gebruikt worden om aggregatieverschuivingen van de protonen te berekenen ten opzichte van BChl *c* als monomeer in de vloeistof. Het patroon van de aggregatieverschuivingen bevestigt het *parallel chain*-model voor de stapeling van BChl *c*. Een verdubbeling van de 5-CH en de 7-Me NMR-resonanties is waargenomen, wat overtuigend bewijs levert voor de aanwezigheid van op zijn minst twee structureel-verschillende wel gedefinieerde ordeningen van het BChl *c* in de chlorosomen. De NMR-data van [U-^{13}C] BChl *c*-chlorosomen en *in vitro* [U-^{13}C] BChl *c*-aggregaten vertonen opvallend veel overeenkomsten met die van aggregaten van [U-^{13}C] Chl *a* / H_2O. Door de resultaten van de vaste-stof-NMR te vergelijken met andere beschikbare NMR- en structuurgegevens in een homologiestudie, is een dubbellaag-cilinder voorgesteld voor de organisatie van het BChl *c*, waarbij de dubbellaag is samengesteld uit twee vlakken opgebouwd uit stapelingen met tegenovergestelde glij-richting.

In **Hoofdstuk 8** wordt vaste-stof-heteronucleaire (^{1}H-^{13}C) dipolaire correlatie-spectroscopie gebruikt om de binding van de chinonen te bestuderen in *Rhodobacter sphaeroides* R26-reactiecentra (RCs) in de grondtoestand. De RCs zijn gereconstitueerd met ubichinon-10, specifiek met ^{13}C verrijkt op de 1-carbonylpositie, voor Q_A of voor Q_B. Er is ontdekt dat de 1-^{13}C van zowel Q_A als Q_B een intermoleculaire correlatie heeft met proton(en) die resoneren in een spectraal gebied dat specifiek is voor waterstofbruggende protonen. Uit de LG-CP-opbouwcurves van het [1-^{13}C] Q_A- en Q_B-signaal is berekend dat de afstanden tussen de koolstoflabels en de gecorreleerde protonen kort zijn, ~ 2.1 Å. De NMR-studie levert dus bewijs voor sterke waterstofbindende interacties naar de 1-C=O van zowel de Q_A als de Q_B voor RCs in de grondtoestand. Dit contrasteert met de gereduceerde toestand $Q_A^{\bullet -}$, waar een waterstofbrug is gevonden naar de 4-carbonylpositie, wat suggereert dat de waterstofbindende interacties naar Q_A afhankelijk zijn van de oxidatietoestand. Dit kan van functioneel belang zijn, omdat dit een herschikking van Q_A tijdens het electrontransport zou betekenen, wat essentieel zou kunnen zijn om een efficiënte voorwaartse electronoverdracht te bewerkstelligen of om de terugval naar de grondtoestand te vertragen.

Tenslotte, in **Hoofdstuk 9** laten ^{13}C-CP/MAS-NMR-data opgenomen van met [4'-^{13}C] Tyr verrijkte L162YL-mutant *Rhodobacter sphaeroides* RCs zien dat Tyr L162 zich in een enigszins heterogeen en waarschijnlijk rigide gedeelte van het eiwitcomplex bevindt. De structurele veranderingen in de mutant ten opzichte van *Rhodobacter sphaeroides* R26, waargenomen via de labels, zijn minimaal op de schaal van de NMR. Dit suggereert dat de waargenomen substantiële afname van de re-reductiesnelheid van de geoxideerde primaire donor P na de mutatie van Tyr L162 niet toegeschreven kan worden aan significante structurele veranderingen in het eiwit. De NMR-studie ondersteunt de eerdere conclusies dat de functie van tyrosine L162 niet zozeer ligt bij het direct ingrijpen en het vergemakkelijken van de electronoverdracht tussen het cytochroom en de foto-geoxideerde donor P^+, maar meer bij het fijn-afstellen van het kwetsbare cytochroom-RC-*docking*/reoriëntatieproces.

Curriculum vitae

Na het behalen van het VWO diploma in 1987 aan de Rijksscholengemeenschap Noord-Kennemerland te Alkmaar, ben ik in september van hetzelfde jaar begonnen aan de studie natuurkunde aan de Universiteit Leiden. De laatste anderhalf jaar van mijn studie heb ik een experimentele stage gedaan bij de vakgroep ‘Metalen’ onder leiding van prof. J.A. Mydosh. Tijdens deze stage werd de magnetische ordening van verschillende overgangs-metalen in uraan-germanium verbindingen onderzocht. In 1993 behaalde ik het doctoraal diploma in de experimentele natuurkunde.

In september 1994 werd ik aangesteld als promovendus in de vakgroep Organische Chemie, sectie vaste stof NMR, onder begeleiding van prof. dr. H.J.M. de Groot en prof. dr. J. Lugtenburg. Tijdens het promotieonderzoek ontwikkelde ik vaste-stof-NMR-technieken ten behoeve van structuur-functie onderzoek aan organische verbindingen zoals membraan-eiwitten en onderzocht ik de structuur van diverse fotosynthetische systemen. Een gedeelte van de metingen heb ik verricht in het buitenland (Bruker Analytik GmbH in Karlsruhe; Institut für Organische Chemie und Biochemie aan de Technische Universität München; Forschungsinstitut für Molekulare Pharmakologie (FMP) in Berlijn; Weizmann Institute of Science, Rehovot, Israel).

De resultaten van mijn onderzoek heb ik kunnen presenteren middels een posterbijdrage op diverse nationale en internationale bijeenkomsten en congressen: *IXth* en *Xth International Congress on Photosynthesis* in Montpellier (1995) en Boedapest (1998), *3rd European NMR Conference*, Oxford (1998); SON werkgemeenschappen Moleculaire Spectroscopie in Lunteren (1995-1997); BIOMAC bijeenkomst in Oegstgeest (1996) en door het geven van een aantal lezingen: BIOMAC bijeenkomst in Oegstgeest (1997); congres *Solid-state NMR towards Structural Biology* in Berlijn (1997); SON werkgemeenschap Moleculaire Spectroscopie in Lunteren (1998); SLW bijeenkomst in Leiden (1998).

Tijdens de promotie heb ik drie keer deelgenomen aan een summerschool (BIOMAC Graduate Schools in Leiden; ESF summerschool in Londen).

Vanaf mei 1999 ben ik werkzaam bij het FMP in Berlijn, waar ik met behulp van vaste-stof-NMR de binding van liganden aan eiwitten bestudeer.

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Patents:

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