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Spin-torch experiment on reaction centers of *Rhodobacter sphaeroides*

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Chapter 5

Towards photo-CIDNP spin-torch experiments using protons

Abstract

In chapter 3, spin-torch experiments to transfer the strongly enhanced ^{13}C photo-CIDNP polarization to neighboring ^{13}C labelled atoms by the natural spin diffusion process have been reported. 2D DARR ^{13}C - ^{13}C photo-CIDNP MAS NMR experiments have been performed and a polarization transfer up to a distance of 13.1 Å via relay transfer steps have been observed. Here, an alternative approach for spin-torch experiments is proposed. We aim for transferring the strong polarization of ^{13}C to directly bonded ^1H atoms and observe the ^1H NMR spectra. This experiment is challenging since the influence of homonuclear dipolar interactions from the proton pool is large. Several types of experiments have been performed to overcome this problem. For observing a ^{13}C -polarized proton spectrum, we recognized the importance of efficient phase cycling at the Lee-Goldburg condition. In addition, application of wPMLG techniques allows for improvement of proton resolution. This technique might allow for exploration of the proton contacts in the protein pocket. In particular, neighboring hydrogen bonds could be studied in great detail. We propose to implement the solid-state photo-CIDNP effect to ^{13}C - ^1H heteronuclear correlation experiments to map the entire proton network in a single experiment.

5.1 Introduction

NMR is an insensitive technique. Since it is an invaluable tool to study structure and function of proteins close to their native states, as well as protein-protein interactions, great effort has been spent on increasing the signal-to-noise ratio. To this end, polarization transfer techniques have been developed by different groups both in solid- and liquid- state NMR. Cross-polarization (CP) is probably the most important signal enhancement technique in solid state NMR. It was introduced in 1962 by Hartmann and Hahn for the static condition (Hartmann & Hahn, 1962). Here the polarization is transferred from abundant spins ($I = {}^1\text{H}$) to rare spins ($S = {}^{13}\text{C}$, ${}^{15}\text{N}$, ${}^{29}\text{Si}$) and the signal is observed from the rare spin species. For Hartmann-Hahn matching, both abundant and rare spins are irradiated simultaneously at their Larmor frequencies, with matching according to

$$\gamma_I B_1(I) = \gamma_S B_1(S) \quad (1)$$

In the picture of the doubly rotating frame, this condition is fulfilled if both spins are spin locked. Polarization transfer between I and S spins takes place, when the energy gaps in the rotating frame between the spin states of I and S spins are equal.

This polarization transfer followed by high power decoupling during signal detection resulted in the famous CP experiments introduced by Waugh and coworkers (Pines *et al.*, 1973). The combination of CP with magic angle spinning (MAS), explored by Schaefer's group experimentally and theoretically (Stejskal *et al.*, 1977), allowed for obtaining high resolution spectra of low abundant spins in polycrystalline samples.

The CP transfer can occur in both directions, from $I \rightarrow S$ as well as from $S \rightarrow I$, depending on the relative magnitudes of the initial polarizations of the dipolar coupled spins. Inverse CP experiments from $S \rightarrow I$ are rare and unusual because of broad lines from the proton detection. Haw's was the first group to exploit this counterintuitive technique. Here, CP from the low- γ nuclei to protons allowed to identify the resonance of immobile protons closely associated with ${}^{31}\text{P}$ nuclei in

calcium phosphate samples (Crosby *et al.*, 1988). CP from deuterons to protons provides information on the rapid proton exchange in solids, especially in samples spin diluted by deuteration (Zheng *et al.*, 1993). Kinetics studies using CP in both directions, $^1\text{H} \rightarrow ^{13}\text{C}$ and $^{13}\text{C} \rightarrow ^1\text{H}$, enabled studying molecular motions of fullerene-70 solvated in toluene (Kolodziejski *et al.*, 1996). Collecting high resolution ^1H spectra is always challenging due to strong homonuclear dipolar interactions among protons, and requires both intermediate spinning frequency 12-15 kHz, along with multiple pulse decoupling schemes like LG, FSLG, PMLG etc. The homonuclear decoupling is even more difficult at very high MAS frequencies than at low and intermediate ones. Only in the last two years, it has been shown that homonuclear decoupling at 50-70 kHz is possible but still the resolution is not better than at low frequencies (Leskes *et al.*, 2009). The details of multiple pulse decoupling approach have been discussed in the introduction chapter.

The solid-state photo-CIDNP effect was discovered by Zysmilich and McDermott in 1994 (Zysmilich & McDermott, 1994). They observed light-induced enhancement of NMR signals in frozen and quinone-blocked bacterial reaction

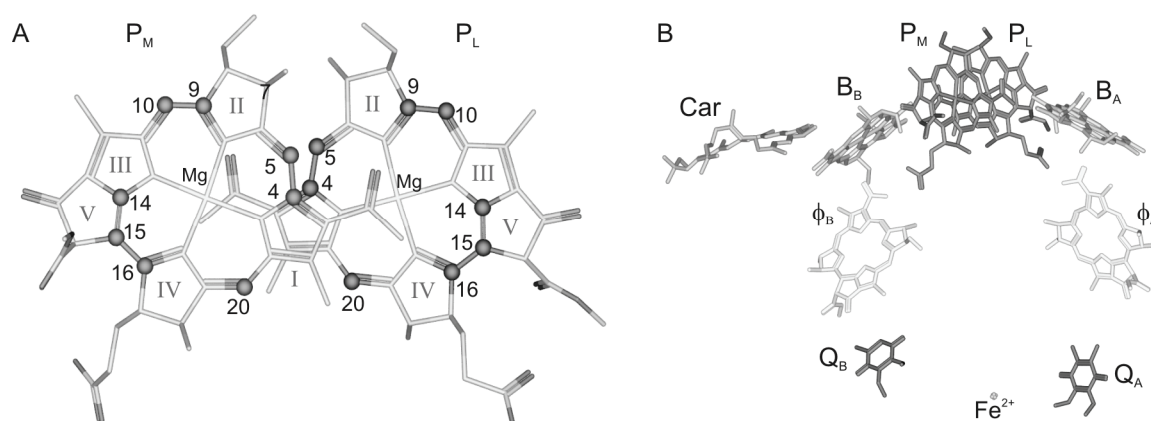


Figure 5.1 (A) Spatial arrangement of the two cofactors P_L (right, isotope labels in blue) and P_M (left, isotope labels in red) forming the Special Pair. The pyrrole rings are numbered with Roman numerals. Pyrrole rings I are overlapping. (B) The arrangement of cofactors in reaction centers (RCs) of *R. sphaeroides* wild type (WT). The primary electron donor, the special pair, is formed by the two bacteriochlorophyll *a* (BChl) molecules P_L and P_M . B_A and B_B are accessory BChl cofactors. Φ_A and Φ_B are bacteriopheophytin (BPhe) cofactors. The acceptor side is formed by two ubiquinone-10 cofactors Q_A and Q_B and a non-heme iron. Side chains are omitted for sake of clarity. The apparent symmetry of the cofactor arrangement is broken by a carotenoid cofactor (Car). The light-induced electron transfer occurs selectively via branch A [PDB entry 1M3X, (Camara-Artigas *et al.*, 2002b) the figure has been made with Accelrys Discovery Studio].

centers (RCs) of *Rhodobacter (R.) sphaeroides* R26 by ^{15}N MAS NMR under continuous illumination with white light, which offered NMR access to the electron-nuclear processes during the charge separation (for reviews, (Jeschke & Matysik, 2003; Daviso *et al.*, 2008b)). By induction of a non-Boltzmann nuclear spin polarization upon photo-reaction in rigid samples, a signal enhancement of a factor of more than 10,000 has been observed by ^{13}C MAS NMR for RCs of the purple bacteria of *R. sphaeroides* wildtype (WT) (Prakash *et al.*, 2005; Prakash *et al.*, 2006). The structural properties of the RCs (Figure 5.1) and mechanisms of photo-CIDNP have been already described in detail in Chapter 2.

In this Chapter, we aim to transfer this strong enhancement of nuclear polarization from labelled carbon atoms to the nearby protons. Such spin-torch experiments might allow for better understanding of the proton environment in the pocket tuning the Special Pair. Since ^1H - ^1H spin diffusion is very efficient, the enhanced ^1H polarization is rapidly transferred to other protons in the sample. In this outlook, I present an exploratory search for the best experimental conditions for detection of enhanced ^1H signals and look for the most suitable pulse scheme, allowing for efficient polarization transfer and fast ^1H detection under MAS conditions. The 5-ALA label pattern (Figure 5.2) is particularly suitable for these studies since the selectively labelled carbon positions C5, C10 and C20 carry directly bound protons. These protons might present the best targets for this initial study. This Chapter shows the present state of the development.

5.2 Materials and Methods

5.2.1 Sample preparation

Selective isotopic labelling in RCs of *R. sphaeroides* is achieved by feeding selectively labelled 5- $^{13}\text{C}_1$ - δ -aminolevulinic acid (5-ALA), which is a precursor for the formation of BChl and BPhe, and leads to a ^{13}C enrichment of $\sim 60\%$. The 5-ALA (Figure 5.2) has been purchased from Buchem B.V. (Apeldoorn, The Netherlands). The RCs were isolated as described earlier (Shochat *et al.*, 1994) and the quinones were removed by incubating the RCs at a concentration of $0.6\ \mu\text{M}$ in

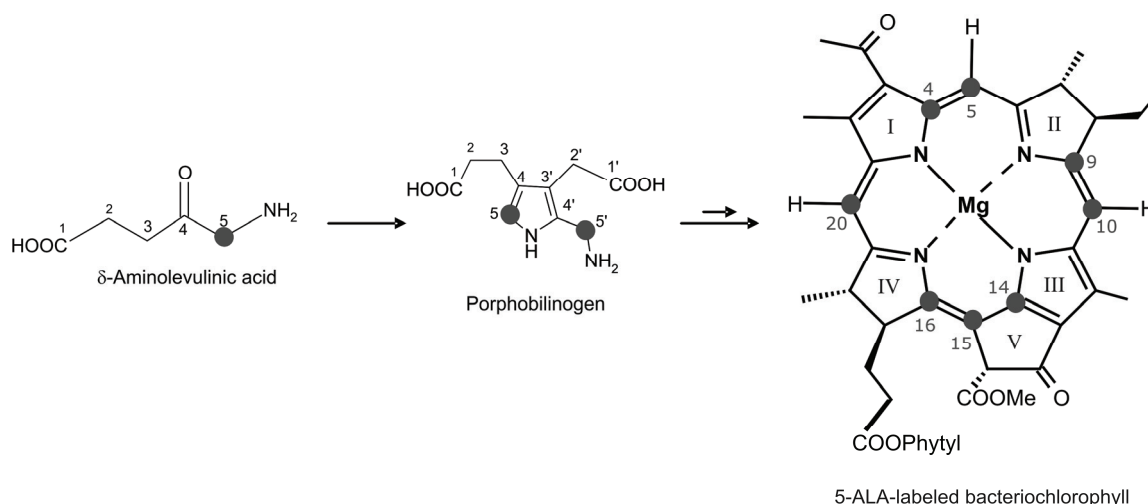


Figure 5.2 Biosynthetic pathway for the formation of selectively ^{13}C isotope labelled bacteriochlorophyll *a* (BChl *a*) by feeding bacteria with 5- ^{13}C - δ -aminolevulinic acid (5-ALA).

4% LDAO, 10 mM o-phenanthroline, 10 mM Tris buffer, pH 8.0, containing 0.025% LDAO and 1 mM EDTA (Okamura *et al.*, 1975). Approximately 15 mg of RC protein complex embedded in LDAO micelles were used in the NMR experiment.

5.2.2 MAS NMR experiments

All NMR experiments were performed with an Avance DMX-400 (9.4 Tesla) NMR spectrometer equipped with a 4 mm MAS probe (Bruker BioSpin GmbH, Karlsruhe, Germany). The sample was loaded into a clear 4 mm sapphire rotor and inserted into the MAS probe. It was frozen slowly at a low spinning frequency of 600 Hz to ensure a homogeneous sample distribution against the rotor wall (Fischer *et al.*, 1992). All experiments were recorded with a MAS frequency of 8 kHz and at a set temperature of 223 K. The probe used for these experiments was a triple resonance probe with a special hole to insert the light fiber inside. The optimum length of the $(\pi/2)$ proton pulse and carbon pulses, determined on uniformly ^{13}C labeled tyrosine, was $\sim 3.1 \mu\text{s}$ and $\sim 5.0 \mu\text{s}$ with a rf-field strengths of 80 kHz and 50 kHz, respectively. A recycle delay of 4 s was used for all experiments.

In several experiments, TPPM proton decoupling (Bennett *et al.*, 1995) with a pulse length of $5.5 \mu\text{s}$ and phase of 15° was used. For the spin lock experiments, the contact time used was 1 ms. The effective fields on proton and carbon used for

the Lee-Goldburg (LG-) CP experiments were 73 kHz and 65 kHz, respectively. The offset used for the LG-CP condition on the protons was 42.42 kHz. A proton pulse length of 1.1 μ s for the wPMLG3 homonuclear decoupling was used. The phases of these pulses are 34.63, 103.90, 173.17, 353.17, 283.90 and 214.63 degrees. The magic flip angle used for the wPMLG experiment was around 1.3 μ s. The acquisition window during wPMLG was 0.5 μ s.

5.3 Results & discussion

5.3.1 Carbon polarization

To study whether the enhanced nuclear polarization of the carbons can be transferred to a nearby proton, a series of five experiments was performed. The first experiment (Figure 5.3A), the starting point for the further development of pulse schemes, was the standard Hahn echo scheme for the direct observation of the strong polarization on the carbon nuclei in a one-dimensional experiment. This pulse sequence is usually employed in one-dimensional photo-CIDNP ^{13}C MAS NMR experiments with continuous illumination. The obtained ^{13}C spectra are displayed in Figure 5.3A'. The spectrum measured in the dark is shown in black,

Table 5.9 : Chemical shifts of ^{13}C labels in the cofactors for 5-ALA-labelled RCs of *R. sphaeroides* WT

^{13}C position	BChl in solution ^a (ppm)	P _L ^b (ppm)	P _M ^b (ppm)	BPheo ^b (ppm)
4	150.0	136.3	144.5	137.2
5	99.9	97.3	105.4	97.6
9	158.5	160.8	158.8	162.8
10	102.4	100.4	98.3	101.9
14	160.8	157.2	-	148.7
15	109.7	110.6	106.9	107.9
16	152.0	145.7	149.5	151.6
20	96.3	108.8	103.1	94.9

while the spectrum observed with continuous illumination is shown in grey. The spectrum in the dark contains only noise. In the spectrum obtained with CI (shown in grey), strong light-induced signals occur in the range between 80 and 180 ppm. due to the strong enhancement of the solid-state photo-CIDNP effect. The total experimental time used for such a spectrum was 10 minutes under CI with a recycle of delay of 4 s. The observed light-induced signals have been assigned previously (Prakash *et al.*, 2007) and are summarized in Table 5.1.

5.3.2 Single-pulse proton experiments

Until now, the solid-state photo-CIDNP effect has been observed for ^{13}C and ^{15}N . Previous attempts to observe this effect directly on protons failed. In such

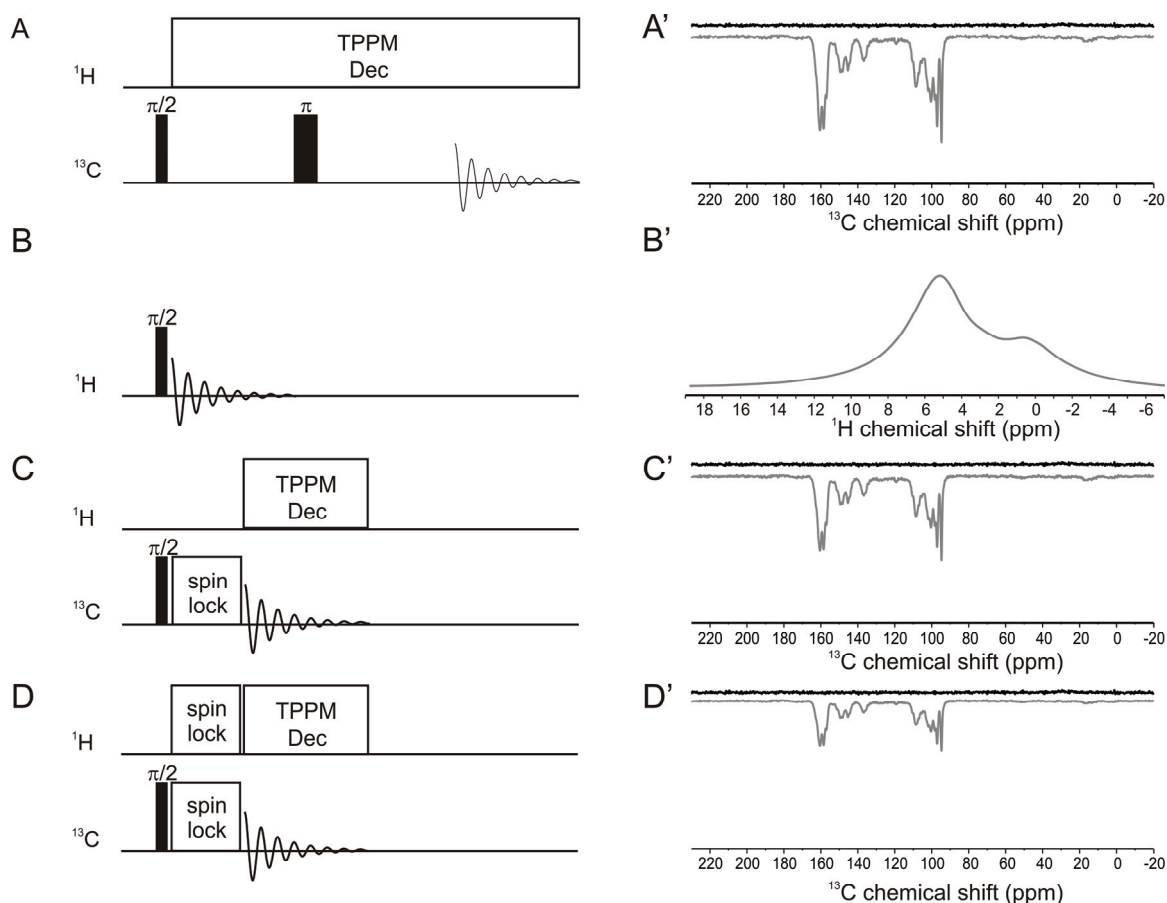


Figure 5.3 Pulse programs used for the experiments and their respective spectra are displayed adjacent to it (black spectra are obtained when the light is off and the grey colored spectra are obtained when the light was on). (A) Hahn echo experiment with carbon acquisition. (B) Single $\pi/2$ degree pulse on proton and acquisition on the same channel. (C) Spin lock pulse on carbon channel only after the $\pi/2$ pulse on carbon. (D) Spin lock pulse on both carbon and proton channel after the $\pi/2$ pulse just on carbon.

experiments a single $\pi/2$ pulse is used to excite the protons as shown in Figure 5.3B. The ^1H data obtained with this procedure are displayed in Figure 5.3B'. The spectra measured in the dark and with continuous illumination are almost identical. In both spectra, two broad peaks at around 0.5 and 5.0 ppm occur, having a FWHH of 2000 Hz. These signals are attributed to protons in the protein backbone and in the frozen water molecules of the buffer, and there is no evidence for direct solid-state photo-CIDNP of the protons.

To quantify the efficiency of polarization transfer from carbons to the protons, we first performed two spin lock experiments (Figure 5.3C & D). The first one consists of a $\pi/2$ pulse on the carbons, followed immediately by a spin lock pulse of 1 ms (Figure 5.3C) on the ^{13}C channel only. In the second experiment, spin lock pulses were applied in parallel on both the carbon and the proton channel (Figure 5.3D). In both experiments, the acquisition was performed on the ^{13}C channel. Comparing the two spectra obtained with continuous illumination (in grey), the ^{13}C photo-CIDNP signal intensity is reduced by $\sim 30\%$ when the spin lock field is applied to the ^1H channel (Figure 5.3C' & D'). This loss of intensity could imply that $\sim 30\%$ of photo-CIDNP polarization of carbons has been transferred to protons. Hence, this observation suggests that polarization transfer to the proton pool occurs and might be experimentally observable.

5.3.3 *Inverse CP from carbons to protons*

After observation of the loss of ^{13}C polarization, presumably to the proton pool in the CP experiment, the next aim is to observe the proton spectrum after the spin lock pulse on both channels. For optimal selectivity of the carbon to proton transfer during CP, the spin lock pulse on the protons was modified (Figure 5.4A) to satisfy the Lee-Goldburg (LG-) CP condition. At this condition the proton-proton interactions are largely suppressed by applying an off-resonance rf field resulting in an effective field in the rotating frame pointing along an axis tilted by the magic angle with respect to the direction of the external field (Lee & Goldburg, 1965). At the same time, transfer by the heteronuclear carbon-proton dipolar

interaction is allowed via a Hartmann-Hahn condition imposed on the effective rf fields experienced by the nuclei (Hartmann & Hahn, 1962). Additionally the phase cycling has been optimized to suppress the huge water peak coming from the buffer solution. It has been shown that under this condition the polarization can be transferred within a particular ^1H - ^{13}C spin pair (van Rossum *et al.*, 2000).

The spectrum obtained with inverse LG-CP is displayed in Figure 5.4A'. The spectrum obtained with continuous illumination is shown in grey as a broad peak, while in the dark experiment, there were no signals observed in the spectrum displayed in black color. This broad peak occurs at 8.5 ppm, which is where the response from the protons at C5, C10 and C20 is expected, but with FWHH of 2800 Hz. For such a spectrum, 10 k scans and approximately 11 hrs were required. When the pattern of this spectra were compared to that of the spectra in Figure 5.3B, these data clearly prove that the photo-CIDNP polarization from ^{13}C has been transferred to ^1H and can be observed in a one-dimensional ^1H spectrum.

5.3.4 Inverse CP from carbon to proton with wPMLG

Caused by strong dipolar coupling, ^1H NMR in the solid-state is still a challenging task. To improve the proton resolution, various methods have been developed as, for example, MREV8, BR24, BLEW12, FSLG, PMLG, DUMBO, R-symmetry etc.

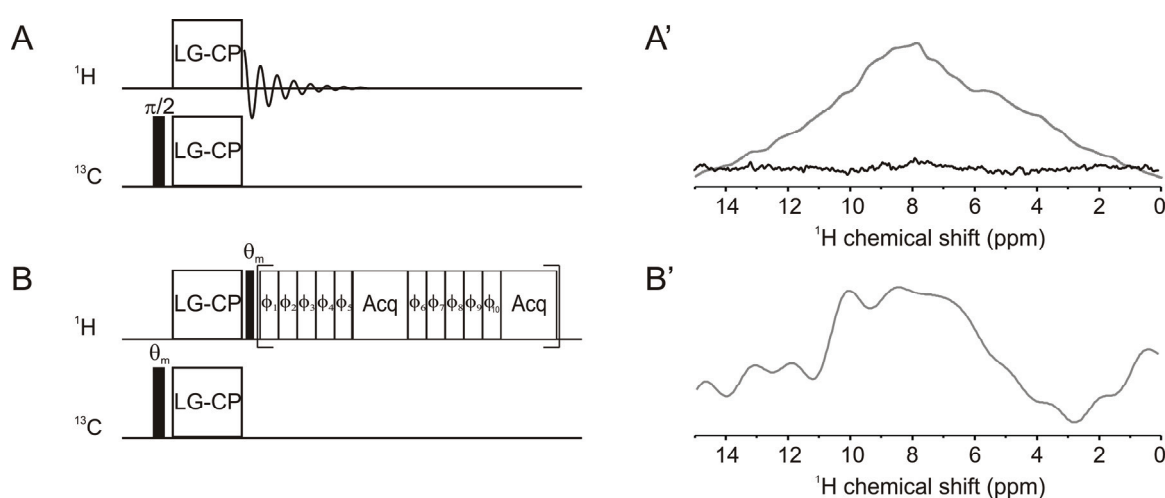


Figure 5.4 Pulse programs used for the experiments and their respective spectra are displayed adjacent to it (only the spectra with CI is displayed). (A) Polarization transfer from ^{13}C → ^1H with LG-CP condition. (B) Polarization transfer from ^{13}C → ^1H with LG-CP with wPMLG method. The corresponding spectra are displayed adjacent to the respective pulse program.

Recently, windowed phase-modulated Lee-Goldburg (wPMLG) homonuclear decoupling has been proposed by Vega's group (Leskes *et al.*, 2006). Here, acquisition is sandwiched by trains of pulses with specific phases reducing dipolar interactions (Figure 5.4B). Thus, by applying the LG-CP experiment followed by wPMLG detection, photo-CIDNP on the carbon atoms has been successfully transferred to attached protons (Figure 5.4B'). As the chemical shift suggests, the small broad light-induced peak at about 8.5 ppm with a FWHH of 2000 Hz originates from the protons directly bound to the carbons C5, C10 and C20.

For this experiment 3k scans were used, which corresponds to a measurement time of three hours. The reduction of time and line width, compared to the previous spectrum, is due to wPMLG detection. Since the signal is still rather broad (4-5 ppm), it is currently difficult to distinguish different hydrogen bonding networks around the Special Pair. Further improvement of the decoupling strategy will presumably yield better resolution.

5.4 Future experiments

The preliminary results presented here could be starting blocks for many new experiments in the future. For instance, two-dimensional ^{13}C - ^1H experiments could be envisaged resolving the hydrogen bonding in the Special Pair of photosynthetic reaction centers. In addition, the solid-state photo-CIDNP effect can be used as a spin-torch to explore for example the protein vicinity of the Special Pair in detail. In particular, the conformational, electronic as well as protonic state of the amino acids surrounding the Special Pair can be studied.

5.4.1 2D photo-CIDNP ^{13}C - ^1H correlation experiment

In the pilot experiments presented in Figure 5.4, the possibility for polarization transfer between the highly polarized ^{13}C and the thermally polarized ^1H has been demonstrated. Careful optimization of more advanced transfer experiments might provide more details on the proton network close to the carbonyl carbons of the cofactors. To obtain two-dimensional data, allowing for assignments, a pulse scheme is proposed in Figure 5.5. In this sequence, carbons are measured in the

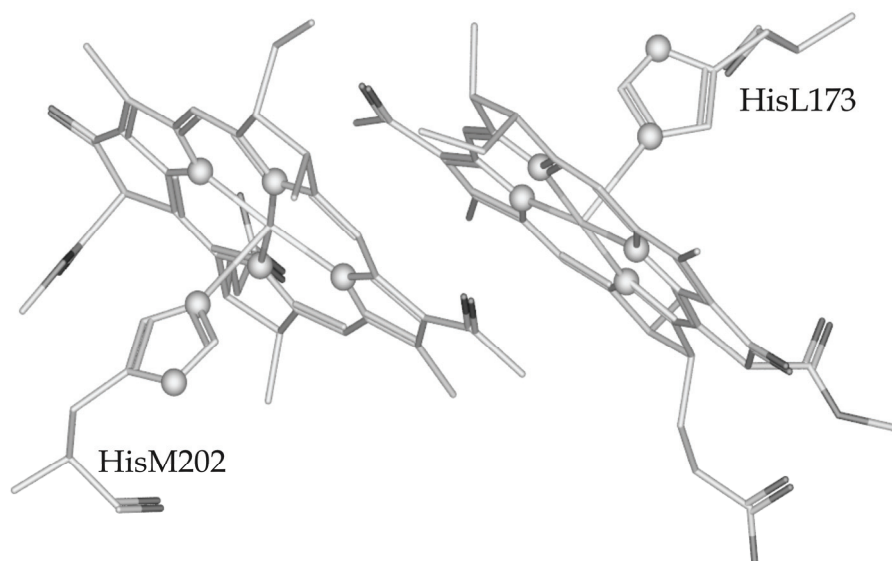


Figure 5.7 Presence of histidines HisL173 and HisM202 near the Special Pair.

5.4.3 ^{15}N labeled RCs with selectively Histidines

The presence of selectively ^{13}C labeled cofactors increases signal and selectivity of the photo-CINDP MAS NMR experiment. RCs also have been selectively isotope-labelled at histidines (Alia *et al.*, 2001). Polarization transfer experiments might be applied to study the axial histidines (HisL173 and HisM202) of the Special Pair, displayed in Figure 5.7. In particular, it would be of interest to determine their protonation characteristics and whether there is a change upon charge separation as indicated in chapters 2 and 3.