Studying hydrogen bonding and dynamics of the acetylate groups of the Special Pair of *Rhodobacter sphaeroides* WT

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Although the cofactors in the bacterial reaction centre of *Rhodobacter sphaeroides* wild type (WT) are arranged almost symmetrically in two branches, the light-induced electron transfer occurs selectively in one branch. As origin of this functional symmetry break, a hydrogen bond between the acetyl group of P₇ in the primary donor and His-L168 has been discussed. In this study, we investigate the existence and rigidity of this hydrogen bond with solid-state photo-CIDNP MAS NMR methods offering information on the local electronic structure due to highly sensitive and selective NMR experiments. On the time scale of the experiment, the hydrogen bond between P₇ and His-L168 appears to be stable and not to be affected by illumination confirming a structural asymmetry within the Special Pair.

The reaction centre (RC) of the purple bacterium *Rhodobacter (R.) sphaeroides* is a membrane protein in which the primary charge separation, the first step of photosynthesis, is taking place. The availability of the x-ray structure of the RCs of this purple bacterium was a major break-through for the understanding of the early processes in photosynthesis¹. The cofactors associated with the M- and L-subunits of bacterial RCs are arranged in two nearly symmetric branches spanning the membrane. Each branch consists of two bacteriochlorophylls a, a bacteriopheophytin a and a quinone. At the end of the branches, a non-heme iron is located (Fig. 1). Close to the B-branch and bound to the M subunit, a carotenoid molecule is present breaking the symmetry of the two branches.

The primary electron-donor P, the so-called Special Pair, is formed by two overlapping bacteriochlorophylls a, P₇ and P₃. Upon illumination, the Special Pair becomes electronically excited and transfers an electron to the ubiquinone Qₐ via an accessory bacteriochlorophyll a (Bₐ) and a bacteriopheophytin a (Φₐ). In the final step, the electron is transferred to Qₐ. Two photocycles coupled with the uptake of two protons reduce Qₐ to QₐH₂ which diffuses out of the protein into the membrane-based quinone pool.

Although the two branches A and B are nearly symmetric, the electron transfer occurs selectively via the A-branch²⁻³. The directional electron transfer is reflected in the asymmetry of the electronic structure of the Special Pair in the various electronic states. In cation radical state P⁺⁺ reflecting the HOMO, techniques as EPR, ENDOR and solid-state photo-CIDNP NMR show more unpaired electron spin density on PL than on PM⁴⁻⁷. The localization of the LUMO mainly on cofactor PM, from which the electron transfer occurs, has been explored by the photo-CIDNP MAS NMR analysis of the donor triplet state ³P₈. This asymmetry is already present in the electronic ground-state of the supermolecule P, as demonstrated by differences in chemical shifts⁹⁻¹³.

Mutagenesis as well as theoretical studies established that the orientation and coordination C-3¹-acetyl groups of P₇ and PM affect the electronic structure and the redox potential of the special pair¹⁴⁻¹⁶. As there is no hydrogen bonding partner available, the x-ray structures and Raman spectroscopy data of the acetyl group of PM also show no involvement in any coordination as the Mg-O distance is about 3.3 ± 0.3 Å, while this distance shrinks to 2.4 Å in QM/MM studies “essentially forming a sixth ligand to the metal”¹⁷⁻²⁰. On the other hand, Raman spectroscopic and QM/MM studies on specifically mutated RC showed that the orientation of the acetyl group of P₇ depends on the protonation state of His-L168 as it is either involved in a hydrogen bond to His-L168 or, if no hydrogen
bond is available, located very close to the magnesium ion of P_M (Fig. 2)\(^{18,21-23}\). It was therefore suggested that a re-orientation of the acetyl group of P_L acts as a valve to block the electron back-transfer upon cleavage of the hydrogen bond to His-L168 and thereby re-tuning of the electronic properties of the Special Pair 16. The acetyl group might therefore be involved in the reorientation of protein polar groups that lead to electric polarization effects during the radical-pair formation\(^{24-26}\). So far, no experimental evidence on the cleavage or the dynamics of the hydrogen bond is known, since no appropriate method with enough sensitivity was available.

Nuclear magnetic resonance (NMR) spectroscopy can be a major technique to probe local dynamics. The lack of sensitivity usually related to this method, can be overcome by the solid-state photo-CIDNP effect allowing to study the photosynthetic cofactors in their native environment\(^{27,28}\). The solid-state photo-CIDNP effect induces a non-Boltzmann nuclear spin distribution after a photo-cycle in all natural photosynthetic RC as well as in some flavin proteins\(^{29-36}\). The enhancement is sufficiently strong to observe particular carbon positions on the cofactors forming the spin-correlated radical pair (SCRP), which is constituted by the donor and the acceptor cofactors, even in entire plants without any further isolation\(^{37}\). During the lifetime of the SCRP, multiple coherent mixing mechanisms take place leading to observable nuclear hyperpolarisation in the electronic ground state on the donor and the acceptor molecules. These mechanisms can be explained by level anti-crossings and are termed three-spin mixing (TSM) and differential decay (DD)\(^{38-42}\). In case of the quinone-blocked RC of \textit{R. sphaeroides} WT, the Special Pair acts as the donor and the \(\Phi_A\) is the acceptor (Fig. 3).

In this study, we apply photo-CIDNP MAS NMR experiments to investigate the hydrogen-bond interaction between the acetyl group of P_L and His-L168 by measuring the chemical shift anisotropy (CSA) of C-3 of the
13C pulses were applied at radio-frequency (rf) field strength of 40 s and a temperature of 247 K. The sample during the measurement as described in ref. 27. As illumination source, a 488-nm continuous-wave laser (Genesis MX488–1000 STM OPS-Laser-Diode System, Coherent Europe B.V., The Netherlands) operating at 1 W was used. The sample was packed in a clear 4-mm sapphire rotor and frozen in the dark at a slow spinning rate of 8 kHz to ensure a homogenous sample distribution43.

The protein environment was considered by the conductor-like polarisable continuum model (CPCM) for which a dielectric constant of \( \varepsilon = 4 \) was selected57. The two investigated structural models are shown in the Supplementary Figs S1 and S2. Both models differ in the protonation pattern at His-L168 where solely model A forms a hydrogen bond between His-L168 and P\( \delta \). The chemical shifts were calculated with the BLYP functional in combination with a def2-SVP basis set52–54. The empirical dispersion correction of Grimme 3rd version (with Becke/Johnson) was employed to consider dispersion interactions55,56. The protein environment was considered by the conductor-like polarisable continuum model (CPCM) for which a dielectric constant of \( \varepsilon = 4 \) was selected57. The two investigated structural models are shown in the Supplementary Figs S1 and S2. Both models differ in the protonation pattern at His-L168 where solely model A forms a hydrogen bond between His-L168 and P\( \delta \), see Fig. S1. The chemical shifts were calculated with the BLYP functional in ADF 2017 using good numerical quality and no frozen core. The empirical dispersion correction account the scaling factor of 0.15547. The carrier was placed at 192 ppm. A total of 640 scans were averaged per each of the 100 \( t_1 \) increments collected. The \( t_1 \) acquisition time was set to 20 ms. Heteronuclear SWf-TPPM decoupling was used during the \( t_1 \) and \( t_2 \) acquisition44. The carrier was placed at 100 ppm. A total of 32 t1-increments were recorded. The spectral width was set to 32258.1 Hz taking into account the scaling factor of 0.15547. The carrier was placed at 192 ppm. A total of 640 scans were averaged per each of the 100 \( t_1 \) increments collected. The \( t_1 \) acquisition time was set to 20 ms. Heteronuclear SWf-TPPM decoupling was used during the \( t_1 \) and \( t_2 \) acquisition44. The SUPER experiment was performed at a spinning frequency of 8 kHz with a recycle delay of 4 s and a temperature of 247 K. The \( \pi/2 \) 13C pulses were applied at radio-frequency (rf) field strength of 72 kHz, while the rf field strength of the heteronuclear SWf-TPPM decoupling was set to 100 kHz46. For the 1D experiment, 1024 scans were recorded with an acquisition time of 20 ms. The spectral width was set to 30 kHz, with the offset placed in the centre of the spectrum, if not stated otherwise. For the 2D INADEQUATE experiment, the SR26 sequence with an rf field strength of 52 kHz was applied45. One full SR26 cycle was used for DQ excitation and reconversion each, resulting in a total mixing time of 4 ms. To ensure a large spectral width of 46 kHz, STiC phase shifts were used46. The carrier was placed at 100 ppm. A total of 640 scans were averaged per each of the 100 \( t_1 \) increments collected. The \( t_1 \) acquisition time was set to 20 ms. Heteronuclear SWf-TPPM decoupling was used during the \( t_1 \) and \( t_2 \) acquisition44. The SUPER experiment was performed at a spinning frequency of 6 kHz leading to a rf field of 72.72 kHz for CSA reconciliation. 192 scans were averaged during each of the four \( \gamma \)-integral points used, leading to a total number of scans of 768. A total of 32 \( t_1 \)-increments were recorded. The spectral width was set to 32258.1 Hz taking into account the scaling factor of 0.15547. The carrier was placed at 192 ppm. The z-filter for the \( \gamma \)-integral was set to 100 \( \mu \)s. Heteronuclear SWf-TPPM decoupling was used during the \( t_1 \) and \( t_2 \) acquisition45. Frequency discrimination in all 2D experiments was achieved using the States-TPPI method48. The simulation of the CSA line shapes were carried out with SIMPSON49. The script can be found in the supplementary information.

DFT calculations. Geometry optimization calculations were based on the crystal structure of Camara-Artigas et al. (PDB: 1M3X) and were carried out with the program ORCA 4.0.1.250,51. The resolution of identity approximation in combination with the corresponding auxiliary basis set was employed to speed up the calculation based on the BLYP functional in combination with a def2-SVP basis set52–54. The empirical dispersion correction of Grimme 3rd version (with Becke/Johnson) was employed to consider dispersion interactions55,56. The protein environment was considered by the conductor-like polarisable continuum model (CPCM) for which a dielectric constant of \( \varepsilon = 4 \) was selected57. The two investigated structural models are shown in the Supplementary Figs S1 and S2. Both models differ in the protonation pattern at His-L168 where solely model A forms a hydrogen bond between His-L168 and P\( \delta \), see Fig. S1. The chemical shifts were calculated with the BLYP functional in ADF 2017 using good numerical quality and no frozen core. The empirical dispersion correction...
of Grimme 3rd version (with Becke/Johnson) was employed to consider dispersion interactions. For protons a single-zeta basis set without polarization was applied. For the carbon atoms a double-zeta singly polarized Slater-type basis set (DZP) was used. Application of a triple-zeta singly polarized Slater-type basis set (TZP) lowered the obtained agreement in isotropic chemical shifts.

Results and Discussion
So far, the assignment of the resonances of the cofactor signals has been performed by comparison of the resonances with the relevant chlorophyll in solution state or by homonuclear DARR or RFDR experiments. Comparison to model molecules can lead to wrong assignments due to the drastic effect of the protein matrix on the electronic structure of the Special Pair. Two-dimensional homonuclear experiments on samples with several tetrapyrole macrocycles might struggle from signal overlap. We therefore apply INADEQUATE experiments to unambiguously assign the resonances as it has already been performed on the 5-ALA labelling pattern. Since the distances are significantly larger in the 3-ALA labelling pattern (Fig. 4A), we used the SR26 sequences which recouples weak dipolar interactions efficiently.45

Figure 4B shows the 1D spectrum as well as a detailed view on the 2D INADEQUATE spectrum (Fig. 4C,D). As can be seen, a clear correlation between neighbouring labeled carbons up to two bonds apart can be established. This connectivity, the fact that P₇ carries more electron density leading to more shielding, as well as the already known assignments from the DARR spectra allow to assign all resonances unambiguously as shown in Table 1. In course of this, due to the observed correlation signal with C-7₄ of P₇ at 64 ppm, we assign the resonance at 19 ppm to C-8₄ of P₇ which has been erroneously denoted as C-7 in ref.32. We do not observe the resonances of C-8₄ of P₇ at 32.1 ppm, C-12 of P₈ at 128.8 ppm and C-18 of P₉ at 50.9 ppm as it was reported earlier. This might be due to the strong field dependence of the solid-state photo CIDNP effect and the different magnetic fields used for both experiments. Nevertheless, the SR26 sequence shows a good performance and allows for recoupling over about 2.6 Å (i.e., two bonds) making it suitable for even sparsely labeled samples as they are used in photo-CIDNP MAS NMR.

Figure 4. (A) Labelling pattern in bacteriochlorophyll a achieved by feeding 3-δ-aminolevulinic acid (3-ALA). The atom numeration is according to IUPAC. (B) 1D ¹³C spectra of 3-ALA labeled RC of R. sphaeroides WT. (C,D) Detailed views on the low (C) and high (D) field regions of the INADEQUATE spectrum of 3-ALA labeled RC of R. sphaeroides WT. The double-quantum peak of C-12 P₇ correlated to C-1₃ of P₇ (marked with an asterisk) was in the range of noise.
Hence, the two C-31-acetyl carbons of PM and PL have different isotropic chemical shifts, occurring at 194.5 ppm (PL) and 196.3 ppm (PM), pointing towards a different chemical environment as, for example, that the acetyl group of PL has hydrogen bond interaction with His-L168, while the acetyl group of PM coordinates the magnesium of PL. To obtain further insight into the chemical environment, we investigated two different DFT models in which His-L168 is protonated at either the $\tau$ or the $\pi$ position (Fig. 5). Depending on the protonation state of His-L168, the acetyl group of PL is either involved in a hydrogen bond to His-L168 (model A) or it coordinates to the magnesium ion of PM. The acetyl group of PM is always coordinated to the magnesium ion of PL.

To verify the existence of the hydrogen bond and to explore possible dynamics, we measured the chemical shift anisotropy (CSA) pattern of both groups via the SUPER technique \(^{47}\). In highly enriched samples, SUPER reintroduces homonuclear dipolar interactions which in conjunction with J-coupling leads to broadening of the CSA patterns at slow spinning speeds \(^{47,65}\). In our case, moderately fast spinning, only very few labels, weak dipolar interactions (~390 Hz) and the absence of J-couplings should not lead to significant broadening of the CSA pattern as it was verified by SIMPSON simulations (Supplementary Figure S4).

Figure 6 shows the experimental powder patterns of C-31 in the acetyl groups of PL and PM as well as their simulations which matched the experimental data best. Table 2 shows the experimentally obtained isotropic and anisotropic chemical shifts of the Special Pair in the 3-ALA $^{13}$C labeled bacterial RCs of \(R.\ sphaeroides\) WT.

### Table 2. Experimentally determined isotropic $^{13}$C chemical shifts of the Special Pair in the 3-ALA $^{13}$C labeled bacterial RCs of \(R.\ sphaeroides\) WT.

| Position | PL   | PM   |
|----------|------|------|
| 1        | 134.1 ppm | 136.9 ppm |
| 2        | 194.1 ppm | 196.3 ppm |
| 7        | 46.0 ppm  | —     |
| 8        | 19.7 ppm  | 28.9 ppm |
| 12       | 120.0 ppm | —     |
| 13       | 188.0 ppm | 190 ppm |
| 17       | 30.1 ppm  | 29.2 ppm |
| 18       | 49.3 ppm  | —     |

Hence, the two C-3-acetyl carbons of PM and PL have different isotropic chemical shifts, occurring at 194.5 ppm (PL) and 196.3 ppm (PM), pointing towards a different chemical environment as, for example, that the acetyl group of PL has hydrogen bond interaction with His-L168, while the acetyl group of PM coordinates the magnesium of PL.

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Figure 5 shows the detailed view on the acetyl group of PM (blue) and PL (pink) in the two DFT models. On the left-hand side (model A), His-L168 is protonated in the $\tau$-position forming a hydrogen bond of 1.6 Å to the acetyl group of PL suggesting a moderate hydrogen bonding interaction. The acetyl group of PM does not have a hydrogen bonding partner and is therefore coordinated to the magnesium ion of PL. On the right-hand side (model B), His-L168 is protonated in the $\pi$-position. Lacking a partner for hydrogen bonding, the acetyl group of PL coordinates to the magnesium ion of PM. The acetyl group of PM is always coordinated to the magnesium ion of PL. The extended presentations of the two models are shown in the Supplementary Information (Supplementary Figs S1 and S2).

The observed experimental anisotropy values $\delta_{\text{aniso}}$ of both powder patterns (12 kHz $\approx$ 120 ppm (PL) and 11.3 kHz $\approx$ 112 ppm (PM)) are larger than the anisotropy values of the carboxylate group in glycine ($\delta_{\text{aniso}} \approx$ 7.5 kHz) providing strong evidence for the high rigidity of the system \(^{47}\). Motions with a correlation time $\tau_c \ll 85$ µs would lead to an averaging of the CSA, which is in the timescale of multiple photocycles in RC of \(R.\ sphaeroides\) WT \(^{46}\). Since the sample is under continuous illumination and therefore passes multiple photocycles during each scan, the size of the anisotropy implies that the acetyl group, if there is any structural change related to this group, does not remain changed on the timescale of nanoseconds or longer. If the acetyl group of PL would be changing its orientation, the movement in both directions need to be on a ps time scale that is not observable with this experiment.
The calculated isotropic chemical shifts of C-31 of PL show a reasonable agreement for model A. If His-L168 is protonated in the $\pi$-position (model B), the calculated isotropic chemical shift is off by about 10 ppm as C-31 of PL is coordinated to the magnesium ion of PM. The isotropic chemical shift as well as the principal values of the chemical shift anisotropy of C-31 of PM are independent of the protonation state of His-L168. We note, however, that the calculated values are off by about 5 ppm which is within the expected error of the employed approach. Unfortunately, we are limited to GGA calculations due to the size of the system. Since the principal CSA values are caused by the electronic environment of the observed nucleus, the differences in the principal CSA values might therefore also be caused by differences in geometry of the model compared to the experimental case which is assumed to be close to the crystal structure. In this case, the high accuracy of the NMR data might be used to recalculate the orientation of the acetyl group and therefore for refinement of the arrangement of the cofactor in the protein pocket.

The findings are also in agreement with the observations of Li and Hong stating that the $\pi$-tautomer of histidine is only formed as an anionic tautomer at high pH and is metastable in the presence of water suggesting a short lifetime. Hence, a stabilization of the $\pi$-tautomer of His-L168 can only be achieved by further metal-ion coordination or H-bonding, which is implausible in this case. Therefore, we assume that His-L168 is protonated in the $\tau$-position.

**Table 2.** Experimental (best fit from Fig. 2) and calculated isotropic chemical shift $\delta_{\text{iso}}$, reduced anisotropy $\delta_{\text{aniso}}$ and principal values $\delta_{11} - \delta_{33}$ of the CSA tensor according to IUPAC of the acetyl groups of the two special pair molecules P_L and P_M.

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| Model | Experiment P_L | DFT P_L | DFT P_M | Experiment P_M | DFT P_M | DFT P_M |
|-------|----------------|---------|---------|----------------|---------|---------|
|       | B              | A       | B       | B              | A       | B       |
| $\delta_{\text{iso}}$ [ppm] | 194.1 | 195.3 | 204.6 | 196.3 | 201.9 | 200.8 |
| $\delta_{\text{aniso}}$ [ppm] | $-120 \pm 2$ | $-107$ | $-120$ | $-112 \pm 2$ | $-114$ | $-114$ |
| $\eta$ [-] | 0.60 $\pm$ 0.04 | 0.24 | 0.09 | 0.58 $\pm$ 0.04 | 0.09 | 0.16 |
| $\delta_{11}$ [ppm] | 290 | 262 | 270 | 285 | 269 | 267 |
| $\delta_{22}$ [ppm] | 218 | 236 | 259 | 220 | 249 | 249 |
| $\delta_{33}$ [ppm] | 74 | 88 | 85 | 85 | 88 | 87 |
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Conclusion

We used the selectivity and the strong enhancement of solid-state photo-CIDNP MAS NMR to identify and probe the dynamics of the two acetyl groups in the Special Pair of R. sphaeroides WT by measuring the isotropic chemical shifts and principal values of their CSA tensors. In conjunction with DFT calculations, we showed that a rigid hydrogen bond between His- L168 and the acetyl group of P$_2$ is present. The high values of the reduced anisotropy of the CSA of both acetyl groups imply that they are not changing their orientation on the time scale of ns to ms. This suggests that if the acetyl group of P$_2$ is flipping to act as a valve preventing fast charge-recombination, the flip has to happen on the ps time scale after the light-induced electron transfer.

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Daniel Gräsing: performed experiments, wrote the paper. Katarzyna M. Dziubińska-Kühn: performed the DFT calculations. Stefan Zahn: designed the research, performed the DFT calculations, wrote the paper. A. Alia: prepared the sample, wrote the paper. Jörg Matysik: designed the research, wrote the paper.

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