Towards photo-CIDNP MAS NMR as a generally applicable enhancement method

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Chapter 4

Solid-state photo-CIDNP effect observed in phototropin LOV1-C57S by ^{13}C magic-angle spinning NMR spectroscopy

4.1 *Abstract*

Until now, the solid-state photo-CIDNP effect, discovered in 1994 by Zysmilich and McDermott, has been observed exclusively in natural photosynthetic centers. The present work demonstrates the first observation of this effect in another system, the blue-light photoreceptor phototropin LOV1-C57S using ^{13}C magic-angle spinning (MAS) NMR.

This chapter is published in *J. Am. Chem. Soc*., 2010, 132 (44), pp 15542–15543.

4.2 *Introduction*

4.2.1 *The solid state photo-CIDNP effect*

The solid-state photochemically induced dynamic nuclear polarization (photo-CIDNP) effect was observed for the first time in 1994 by McDermott's group in frozen and quinone-blocked bacterial RCs of ^{15}N-labeled *Rb. sphaeroides* R26 by MAS NMR.[1] The effect allows for enhancement of NMR signals by a factor of more than 10,000 due to non-Boltzmann nuclear polarization.[2,3] Since then, the effect has also been shown in various other RCs including those of algae[4] and plants,[5,6] and even at a cellular level for RC-antenna complexes when in the

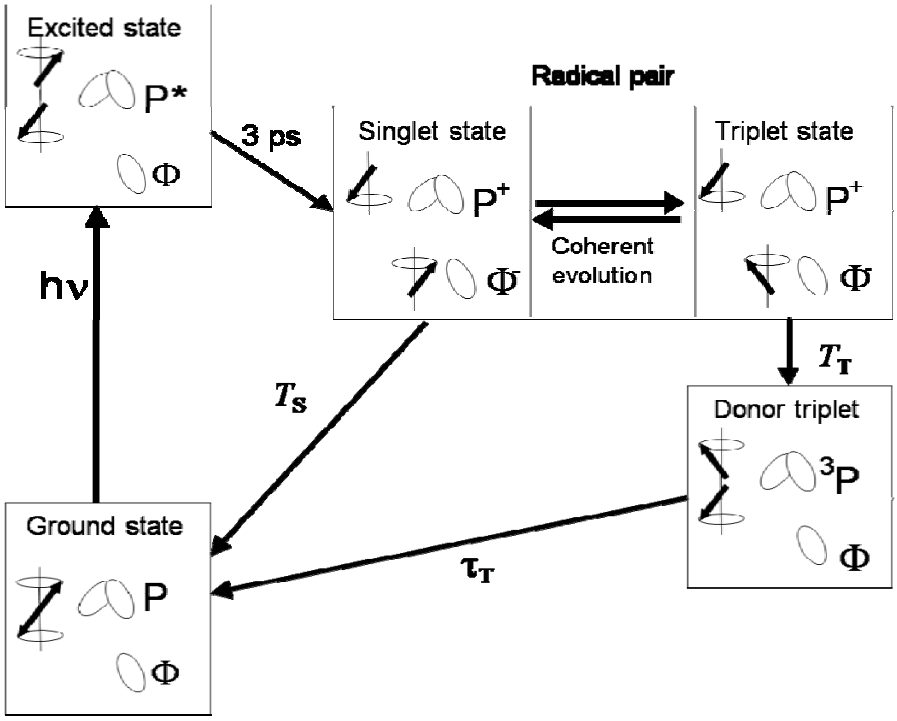

Figure 4.1.Photocycle in quinone-depleted RCs of *Rb. sphaeroides* WT and R26. Upon illumination and fast electron transfer from an excited singlet state, a radical pair is formed in a pure singlet state having high electron spin order. The radical pair is formed by a radical cation at the two donor BChls (Special pair, P) and a radical anion on the BPhe acceptor cofactor (Φ) of the active branch. The chemical fate of the radical pair depends on its electronic spin state: the singlet state is allowed to recombine with the lifetime of T_S= 20 ns and this recombination pathway is termed singlet channel. For the triplet state a direct recombination is spin-forbidden and a donor triplet (^{3}P) is formed with a lifetime of T_T= 1 ns. The lifetime of the donor triplet state (τ_T) is 100 ns for WT RCs and 100 μs for R26 RCs. The recombination pathway of the donor triplet to the ground state is termed triplet channel. Mechanisms building up photo-CIDNP under steady-state conditions are TSM, DD and DR.

membrane, in the natural environment.[3,4] Several attempts were made to observe polarization in other proteins, however, other systems with photoreaction kinetics that allows for the observation of the photo-CIDNP effect were not yet identified.[7] Here we demonstrate that the solid-state photo-CIDNP effect can also be observed in a rather different, non-photosynthetic protein, a mutant of the light-, oxygen-, or voltage-sensitive (LOV) domain of the blue-light photoreceptor phototropin. Signals were recorded from the flavin cofactor without isotopic enrichment.

Photo-CIDNP is well known in solution NMR and was explained by the radical pair mechanism (RPM) soon after its discovery in 1969. [8,9] The classical RPM in solution is based on molecular diffusion. The solid-state photo-CIDNP effect is interpreted by up to three mechanisms running in parallel,[10] three-spin mixing (TSM),[11] differential decay (DD),[12] and differential relaxation (DR)[13] (Figure 4.1), which allow for the conversion of the initial electron-spin zero quantum coherence of the spin-correlated radical pair[14] into nuclear polarization by symmetry breaking. In illuminated RCs, an electron is transferred from the excited electron-donor P^* to a primary acceptor Φ forming an spin correlated radical pair, initially in singlet state $^1(P^{+\bullet}\Phi^{-\bullet})$ and it converts to triplet state $^3(P^{+\bullet}\Phi^{-\bullet})$ and back by coherent evolution. The high electron spin order of initial singlet state is transferred to net nuclear polarization by two parallel and competing mechanism termed TSM[11] and DD[12]. The TSM mechanism is explained by the combined action of electron-electron dipolar coupling or exchange coupling, the nuclear Zeeman interaction and the pseudosecular hyperfine coupling (hfc) $B = (A_{zx}^2 + A_{zy}^2)^{1/2}$. This breaks the antisymmetry of $<I_z>$nuclear spin population in coherent spin evolution of the spin correlated radical pair. The different lifetimes of singlet (T_S) and triplet states (T_T) breaks the antisymmetry of the nuclear spin population in the spin correlated radical pair by a buildup of net nuclear polarization via the B in a DD mechanism. In cyclic reactions, net nuclear polarization can occur if the products have different nuclear longitudinal relaxation rates. Such a type of RPM can also occur in solids, as in frozen R26 RCs having a long-lived donor triplet state, and is called the DR mechanism.

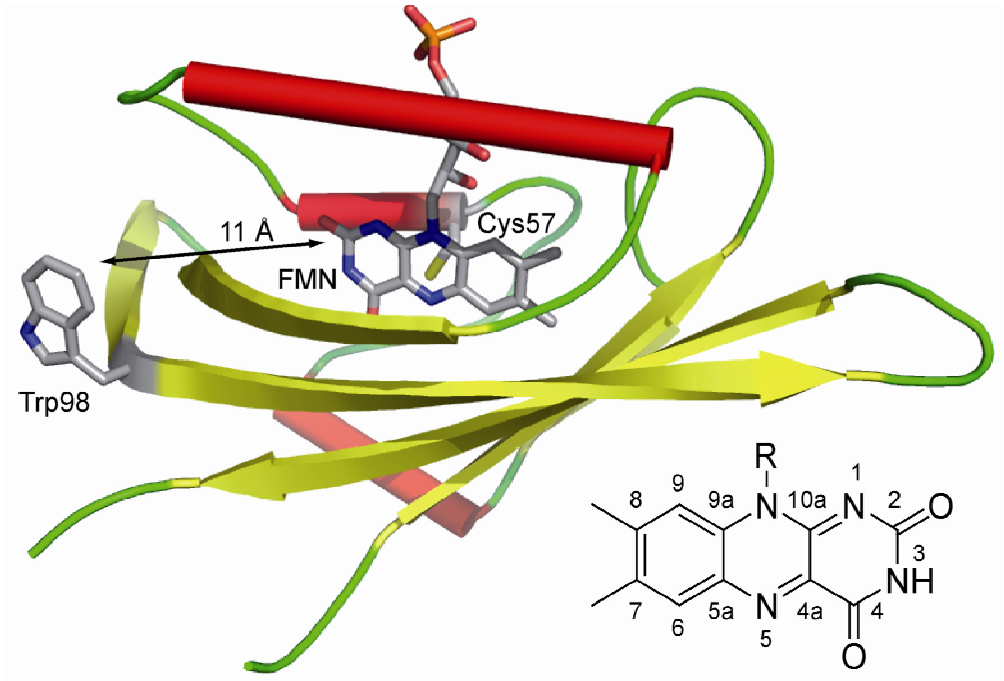

Figure 4.2: Structure of the phototropin LOV1 domain in the dark (PDB 1N9L).[15] Cysteine 57 was replaced by serine in this study. The numbering of the flavin chromophore is included.

4.1.2 *Phototropin LOV1-C57S*

Phototropin is a member of the family of flavin-containing blue-light photoreceptors and regulates key responses of plants to light, such as phototropic movement and chloroplast relocation.[16]Phototropin comprises two LOV domains, each binding non-covalently a flavin mononucleotide (FMN), and a kinase domain. Upon illumination, the triplet excited state of the flavin reacts with a nearby cysteine residue to form a covalent adduct as the signaling state.[17] Mutation of the reactive cysteine to serine or alanine abolishes this adduct formation. Instead, a less efficient competing pathway of electron transfer from a tryptophan leads to transient accumulation of a flavin anion radical on illumination[18,19] and finally to formation of a flavin neutral radical.[20] The radical is re-oxidized by oxygen. We investigated the mutant, C57S, of the phototropin-LOV1 domain from the green alga *Chlamydomonas reinhardtii* (Figure 4.2). [21]

4.3 *Materials and Methods*

4.3.1 *LOV1 expression and purification*

The C57S mutant of the LOV1 domain (amino acids 16-133) of *Chlamydomonas reinhardtii* was expressed in *E. coli* strain BL21(DE3) carrying an N-terminal 10x His-tag as described before.[21] The cells were disintegrated using a French Press and the protein was purified via affinity chromatography using His-bind resin (Novagen) loaded with copper sulphate. Protein was transferred into 50 mM potassium phosphate, pH 8, containing 300 mM NaCl by repeated ultrafiltration using an Amicon Ultra-15 filter device with a 10 kDa cutoff (Millipore). The final solution was concentrated to an optical density of ~20 at 450 nm (optical path length, 1 cm).

4.3.2 *MAS NMR experiment*

An Avance 100 MHz spectrometer equipped with 4-mm MAS probe (Bruker, Karlsruhe, Germany) was used for the ^{13}C MAS NMR experiments both in the dark and using continuous illumination with white light. The sample was packed into a 4-mm sapphire rotor and inserted into the MAS probe. For a homogeneous sample distribution against the rotor wall, the sample was frozen at a very low spinning frequency of 500 Hz.[22] The spinning frequency was increased to 8 kHz after the sample is completely frozen at 235 K. This frequency and set temperature were used for all ^{13}C MAS NMR measurements. A simple Hahn echo pulse sequence[23] with TPPM proton decoupling[24] was used for all NMR measurements. A cycle delay of 2 s was used for all NMR experiments and signals were accumulated for 12 h. An artificial line-broadening of 10 Hz was applied before Fourier transformation. All ^{13}C MAS NMR spectra were calibrated with respect to the ^{13}COOH chemical shift of solid tyrosine.HCl at 172.1 ppm. The same phase-correction parameters required to properly phase a tyrosine spectrum were used for the dark and photo-CIDNP spectra.

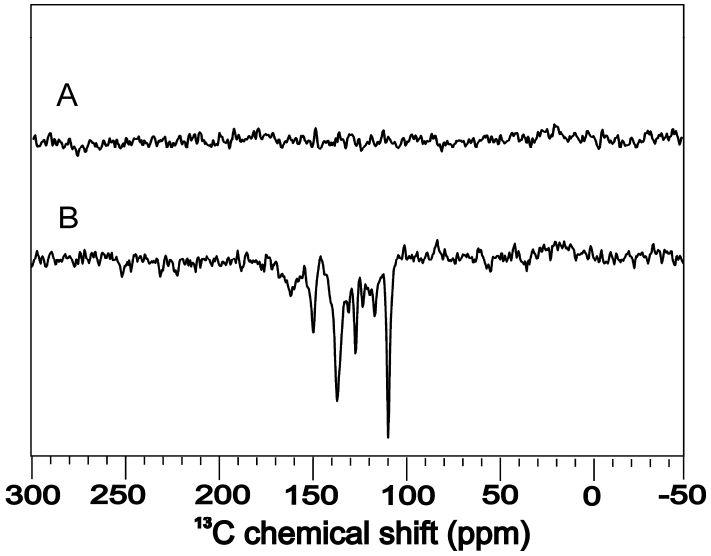

Figure 4.3. ^{13}C MAS NMR spectra of phototropin LOV1-C57S obtained with 8 kHz MAS in a magnetic field of 2.3 T in the dark (A) and using continuous illumination with white light (B).

4.4 *Results and discussion*

4.4.1 *Comparison of Photo-CIDNP and dark MAS NMR spectra*

In Figure 4.3, the ^{13}C MAS NMR spectra of phototropin LOV1-C57S obtained in the dark (A) and with illumination (B) are shown. Both datasets were measured at 2.3 T (*i.e.*, 100 MHz ^{1}H frequency) using a spinning frequency of 8 kHz. At a set temperature of 235 K, the sample was entirely frozen as monitored by the NMR tuning frequency. A simple Hahn-echo pulse sequence with two-pulse phase-modulation protondecoupling was used. Continuous illumination was supplied by a 1 kW xenon lamp.[23] The cycle delay was 2 s and the measurement time was about 12 h. In the dark no resonance signal was detected but under illumination several strong signals appear in the aromatic region.

An enlarged view of the aromatic region of the solid-state photo-CIDNP spectrum is presented in Figure 4.4. All light-induced ^{13}C NMR peaks are emissive (negative). This pattern is reminiscent of the photo-CIDNP MAS NMR spectra obtained from RCs of *Rb. sphaeroides* WT[2] and of photosystem I[5] and contrasts

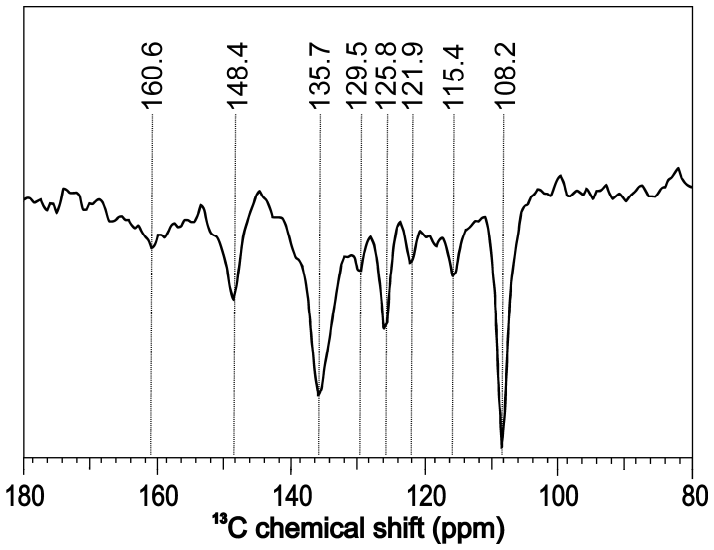

Figure 4.4. Expanded view on the aromatic region of the ^{13}C MAS NMR spectrum of phototropin LOV1-C57S showing the solid-state photo-CIDNP effect (Spectrum 4.3B).

with the mixed absorptive/emissive enhancement pattern observed by photo-CIDNP of a LOV2 sample in solution.[19]

4.3.2 *Preliminary assignments of light induced ^{13}C signals*

A preliminary assignment of these peaks can be obtained by comparison with the ^{13}C chemical shifts of FMN in solution and in the LOV2 domain of *Avenasativa* phototropin obtained by ^{13}C liquid-state NMR (Table 4.1). Six of the eight light-induced signals can be assigned to the ten aromatic carbons in the FMN cofactor, with four pairs of overlapping resonances (C2 and C4, C5a and C9a, C6 and C9, C7 and C8). The two additional signals at 108.2 and 115.4 ppm appear upfield of the others and probably do not arise from FMN. As known from solid-state photo-CIDNP studies on RCs, polarized signals are observed from both the electron-donor and the electron-acceptor. Hence, we assume that at least these two signals arise from the electron-donor in the spin-correlated radical pair. In fact, the

	^{13}C chemical shift in ppm			
	Free FMN in D_2O solution[a]	FMN bound to LOV2 in the dark[b]	FMN bound to LOV2 under illumination[b]	FMN bound to LOV1-C57S under illumination[c]
2	159.8	159.2	159.3	160.6
4	163.7	161.0	165.9	160.6
4a	136.2	134.5	65.0	129.5
5a	136.4	136.3	130.3	125.8
6	131.8	132.8	120.7	121.9
7	140.4	139.0	130.3	135.7
8	151.7	150.7	136.2	135.7
9	118.3	119.3	118.7	121.9
9a	133.5	134.2	127.7	125.8
10a	152.1	150.8	156.9	148.4

[a] ref. 25.
[b] LOV2 domain of *Avena sativa* phototropin reconstituted with [u-^{13}C^{15}N]-FMN, ref.26.
[c] This work.

Table 4.1. Preliminary assignment of ^{13}C chemical shifts of FMN in LOV1-C57S.

two peaks can be attributed to C^{γ} and $C^{\zeta 2}$ of a tryptophan residue (Trp) (Table 4.2), [27] while no match for the resonance at 108.2 ppm would be expected from the two other possible electron donors, histidine and tyrosine. Hence, the data can be reconciled with observed nuclear polarization originating from a light-induced [$FMN^{-\bullet}Trp^{+\bullet}$] radical pair. Trp at position 98 (Figure 4.1), the only Trp in the protein, is about 11 Å edge-to-edge distance from FMN and, thus, at a suitable distance for efficient electron transfer.

Carbon number	**^{13}C chemical shift in ppm**	
	Trp[a]	Trp[b]
C	176.18	-
C^{α}	57.71	-
C^{β}	29.97	-
C^{γ}	110.6	108.2
$C^{\delta 1}$	126.5	125.8
$C^{\delta 2}$	127.49	129.5
$C^{\varepsilon 2}$	138.62	135.7
$C^{\varepsilon 3}$	120.46	121.9
$C^{\zeta 2}$	114.23	115.4
$C^{\zeta 3}$	121.40	121.9
$C^{\eta 2}$	123.83	121.9

[a]ref.27
[b]This work

Table4.2. ^{13}C chemical shifts of Tryptophan (Trp)

4.4 *Conclusion*

The data presented in this Chapter provide a first experimental demonstration that the solid-state photo-CIDNP effect is not only a peculiarity of photosynthetic systems but can arise in at least one other photo-active protein. In the same way that photo-CIDNP MAS NMR has provided detailed insights into photosynthetic electron transport in RCs, [1-7] a variety of applications in studies of the functionality of blue-light photoreceptors can be anticipated. For example, it may be possible to characterize in detail the photo-induced flavin and tryptophan radicals in cryptochrome, the flavoprotein that has been proposed as the radical pair magnetoreceptor for the avian magnetic compass.[29]

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