Communication

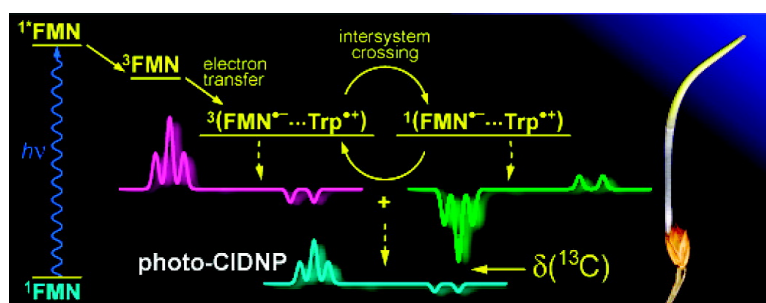# Natural Abundance Solution C NMR Studies of a Phototropin with Photoinduced Polarization

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Natural Abundance Solution ^{13}C NMR Studies of a Phototropin with Photoinduced Polarization

Wolfgang Eisenreich,*,† Monika Joshi,† Stefan Weber,*,‡ Adelbert Bacher,† and Markus Fischer*,§

Technische Universität München, Lehrstuhl für Biochemie, Lichtenbergstr. 4, 85747 Garching, Germany, Albert-Ludwigs-Universität Freiburg, Institut für Physikalische Chemie, Albertstr. 21, 79104 Freiburg, Germany, and Universität Hamburg, Institut für Lebensmittelchemie, Grindelallee 117, 20146 Hamburg, Germany

Received August 8, 2008; E-mail: wolfgang.eisenreich@ch.tum.de; Stefan.Weber@physchem.uni-freiburg.de; markus.fischer@uni-hamburg.de

We previously reported exceptionally strong blue-light induced nuclear spin polarization in solution ^{13}C NMR spectra of a flavin-based photoreceptor.[1] The resulting anomalous spectral intensities commonly known as photochemically induced dynamic nuclear polarization (photo-CIDNP) can be interpreted in terms of a radical-pair (RP) mechanism.[2−5] Provided that RP species that have been generated by light-induced electron transfer (ET) live long enough for the electron−nuclear hyperfine interactions in the radicals to drive the coherent interconversion of singlet and triplet RP states, then the diamagnetic products produced by radical recombination or backward ET often exhibit substantial nuclear hyperpolarizations whose magnitudes reflect the hyperfine couplings in the radicals.[4,6,7] Our previous NMR studies were done using blue-light irradiated mutant phototropin LOV2 domains with specifically ^{13}C-labeled flavin mononucleotide isotopologues.[1] From the relative signs of the hyperfine couplings derived from the enhanced absorptive or emissive ^{13}C NMR lines we concluded that a flavin anion radical (Fl$^{\bullet-}$) is transiently generated as a result of light-induced intraprotein ET from an as yet to be identified redox-active amino acid to the initially fully oxidized flavin (Fl^{ox}) situated in the core of the protein. In addition to the flavin resonances, a very strong emissively polarized NMR line has been observed around 110 ppm. This signal is not due to the resonance of any of the carbons in the flavin as the protein is unlabeled and the signal was not observed in the NMR spectra recorded in the dark. Hence, it must originate from spin-polarized ^{13}C nuclei occurring at natural abundance in the apoprotein. Based on its chemical shift, the resonance has been proposed to arise from a tryptophan residue involved in light-induced ET,[1] where C(3) (IUPAC numbering scheme for the indole group of tryptophan) of the aromatic side chain is typically observed at 109 to 112 ppm downfield from TMS.[8]

To assign the natural abundance NMR line we have in the present study measured strongly spin-polarized ^{13}C NMR spectra from blue-light irradiated recombinant LOV2 domains carrying a C450A mutation and comprising amino acid residues 409−525 (short-LOV2-C450A) or 404−559 (long-LOV2-C450A) of *Avena sativa* phototropin, respectively. The short LOV2 mutant domain does not contain a C-terminal linker helix, Jα, extending over residues 522−560. The Jα region was proposed to be involved in photomechanical switching in wild-type LOV domain signaling.[9−11] The C450A mutant is lacking the reactive cysteine that is required for native flavin-C(4a)-cysteinyl adduct formation.[12−14] Instead, the mutant undergoes light-induced redox reactions that have been studied quite extensively by optical spectroscopy and electron paramagnetic resonance (EPR).[15,16]
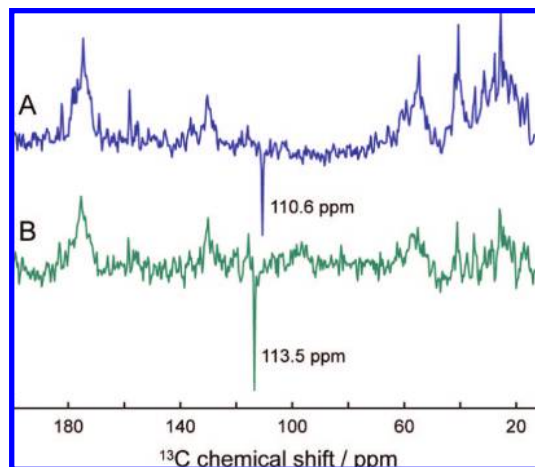
† Technische Universität München.
‡ Albert-Ludwigs-Universität Freiburg.
§ Universität Hamburg.

Figure 1. 125.8-MHz ^{13}C photo-CIDNP spectra of unlabeled LOV2 C450A domains from *A. sativa* phototropin. (A) short-LOV2-C450A comprising amino-acid residues 409−525. (B) long-LOV2-C450A comprising residues 404−559. The spectra were measured at 27 °C under blue-light irradiation (455 nm) from a photodiode in a setup described previously.[1] A four-channel Bruker DRX-500 spectrometer equipped with a 5-mm ^{1}H/^{13}C dual probe head and a pulsed-field gradient accessory was used for all experiments. ^{13}C chemical shift assignments of the hyperpolarized resonances are referenced to internal dioxane (67.84 ppm relative to TMS).

^{13}C NMR spectra of unlabeled protein samples recorded under blue-light irradiation are shown in Figure 1. The short LOV2-C450A domain shows a strong emissive signal at 110.6 ppm (Figure 1A).[1] A similar resonance that is offset to 113.5 ppm (Figure 1B) is observed in the long LOV2-C450A domain. Thus, the presence of the additional Jα helix affects the chemical shift values of the putative tryptophan under consideration most probably via modulation of the π-orbital overlap between its indole ring and the CO group in the amino acid backbone.[8] It must be located within the amino acid sequence of the short LOV2 domain, because the emissive natural abundance ^{13}C NMR line is observed in the spectra of both samples.

Two tryptophan residues, W491 and W557, are found in the long LOV2 C450A domain. The short domain has only W491. To identify the specific tryptophan that gives rise to the emissive 113.5-ppm signal under natural abundance conditions, two double mutant LOV2 domains were constructed. The long-LOV2-C450A/W557A double mutant and the long-LOV2-C450A single mutant yielded virtually identical NMR patterns; see Figure 2A, B. However, the replacement of W491 by alanine, also in the long-protein version, long-LOV2-C450A/W491A, completely quenched the emissive signal (Figure 2C). These observations clearly suggest that W491 is the redox-active amino acid that, upon photoexcitation, donates
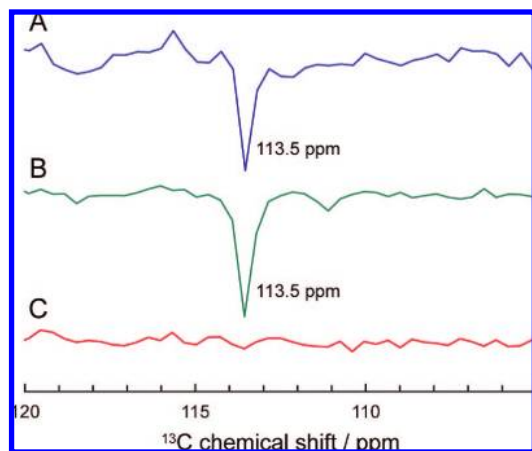
Figure 2. 125.8-MHz ^{13}C photo-CIDNP spectra of unlabeled single and double mutant LOV2 domains comprising amino acid residues 405−559 of *A. sativa* phototropin: (A) long-LOV2-C450A, (B) long-LOV2-C450A/W557A, (C) long-LOV2-C450A/W491A. The experimental conditions are the same as those specified in Figure 1.

an electron to the flavin, thus forming an RP ^{3}[Fl$^{\bullet-}$ ⋯ W491$^{\bullet+}$] presumably in a triplet electron-spin configuration.[1,16] No photo-CIDNP signal is observed from the more distant W557 (35 Å) at the protein surface of long-LOV2-C450A/W491A. Hence, in contrast to other solution NMR studies, where exogenous flavins have been added as photosensitizers to probe the surface accessibility of tryptophans, tyrosines, and histidines,[3−5] free chromophore diffusion is not a necessary ingredient for the observation of photo-CIDNP.

Since the flavin and W491 in *A. sativa* LOV2 are separated by ∼14 Å,[9,17] the spin−spin interaction between the two radicals in the RP is expected to be sufficiently small so that electron−nuclear hyperfine couplings of magnetic nuclei can influence triplet-to-singlet interconversion in the RP state. The initially generated triplet RP has then two possible fates: (i) ^{3}[Fl$^{\bullet-}$ ⋯ W491$^{\bullet+}$] may either evolve into an electronic singlet state, ^{1}[Fl$^{\bullet-}$ ⋯ W491$^{\bullet+}$], which subsequently undergoes spin-allowed backward ET to regenerate the ground-state reactants (Fl and W491), or (ii) so-called "escape products" are formed directly from ^{3}[Fl$^{\bullet-}$ ⋯ W491$^{\bullet+}$] because radical recombination is spin-forbidden from the triplet configuration. These products will ultimately also react and regenerate the ground-state reactants, however, on a longer time scale. If we assume that the frequency of the coherent interconversion between singlet and triplet RP states depends on the spin configuration (characterized by the quantum number m_I) of the ^{13}C nuclei in the flavin and W491 via the hyperfine interaction, and furthermore, if efficient nuclear spin relaxation takes place on one of the two competing pathways,[4] then nuclear spin polarization can be accumulated during one or several reaction cycles, thus giving rise to strongly enhanced absorptive or emissive resonance NMR lines. That only the resonance of ^{13}C(3) from W491 is observed is most likely due to slow nuclear relaxation in the electronic ground state of W491, and fast relaxation in the radical form (W491$^{\bullet}$ or W491$^{\bullet+}$)

of this quaternary carbon, in combination with its large hyperfine coupling as compared to the other carbons in the indole ring.[18] Together with the continuous sample irradiation, the enormous net polarization of ^{13}C(3) in W491 could be further increased through repetitive sample excitation leading to a balance of CIDNP pumping and decay.[1]

With W557 being too distant from the flavin for direct ET, and W491 sufficiently remote, it appears as if the wild-type LOV2 is engineered such that alternative ET reactions to the flavin cannot compete with functional ET from the reactive C450 that leads to the flavin-C(4a)-cysteinyl adduct.

In summary, we emphasize that, despite strong efforts, the proposed RP state [Fl$^{\bullet-}$ ⋯ W491$^{\bullet+}$] has so far escaped direct detection by optical spectroscopy and time-resolved EPR. The latter technique can probe radical intermediates on a time scale as short as 10 ns. Thus, [Fl$^{\bullet-}$ ⋯ W491$^{\bullet+}$] may be either too short-lived (<10 ns) or its electron-spin polarization relaxes too fast for direct detection. This confirms the potential of photo-CIDNP as a tool to yield information on transient paramagnetic states by measuring nuclear-spin polarizations of diamagnetic reaction products. We anticipate that this technique can be applied generally to other ET proteins of moderate size with not too close redox partners to unravel ET pathways and thus to gain information on protein reaction mechanisms.

Supporting Information Available: Details of the protein preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

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