Tailored flavoproteins acting as light-driven spin machines pump nuclear hyperpolarization

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The solid-state photo-chemically induced dynamic nuclear polarization (photo-CIDNP) effect generates non-Boltzmann nuclear spin magnetization, referred to as hyperpolarization, allowing for high gain of sensitivity in nuclear magnetic resonance (NMR). Well known to occur in photosynthetic reaction centers, the effect was also observed in a light-oxygen-voltage (LOV) domain of the blue-light receptor phototropin, in which the functional cysteine was removed to prevent photo-chemical reactions with the cofactor, a flavin mononucleotide (FMN). Upon illumination, the FMN abstracts an electron from a tryptophan to form a transient spin-correlated radical pair (SCRP) generating the photo-CIDNP effect. Here, we report on designed molecular spin-machines producing nuclear hyperpolarization upon illumination: a LOV domain of aureochrome1a from *Phaeodactylum tricornutum*, and a LOV domain named 4511 from *Methylobacterium radiotolerans* (*Mr*4511) which lacks an otherwise conserved tryptophan in its wild-type form. Insertion of the tryptophan at canonical and novel positions in *Mr*4511 yields photo-CIDNP effects observed by 15N and 1H liquid-state high-resolution NMR with a characteristic magnetic-field dependence indicating an involvement of anisotropic magnetic interactions and a slow-motion regime in the transient paramagnetic state. The heuristic biomimetic design opens new categories of experiments to analyze and apply the photo-CIDNP effect.
Besides photosynthetic RCs, there is another class of proteins acting as molecular spin-machines pumping non-Boltzmann nuclear spin-state upon illumination: biological photoreceptors that contain a flavin cofactor as chromophore with characteristic absorption maxima in the UV-A and blue light regions. Merely one family of flavoproteins, phototropins, was reported to show the photo-CIDNP effect. Generally, phototropins are blue-light receptors that harbor two light-sensing light-oxygen-voltage (LOV) domains, each of which incorporates one flavin mononucleotide (FMN) cofactor non-covalently. Upon excitation, the FMN in a wild-type LOV domain of phototropin undergoes adduct formation with a close-by conserved cysteine. However, so far, no photo-CIDNP effect was observed from a wild-type LOV domain. The first successful solid-state photo-CIDNP effect observation was demonstrated for a frozen cysteine-lacking LOV domain of phototropin from the green alga *Chlamydomonas reinhardtii* (*Cr*-) in the solid state.[41,44] In this case, the photo-excited FMN undergoes inter-system crossing to the triplet state, causes intra-protein electron transfer from a tryptophan to the FMN and thus gives rise to formation of a transient SCRP. Spin evolution of this SCRP allows for the build-up of the solid-state photo-CIDNP selectively on FMN and tryptophan. In Cr-LOV1-C57S, the edge-to-edge distance (*rFW*) between FMN (F) and tryptophan (W) is ~11 Å. Remarkably, Weber et al. first observed photo-CIDNP in a similar LOV protein, the LOV2 domain of phototropin 1 from oat, *Avena sativa* (Asphot1-LOV2-C450A), by 13C liquid-state NMR.[40,45,46] The crystal structure of Asphot1-LOV2-C450A is almost identical to that of Crphot1-LOV1-C57S, given that they share 47% of amino acid sequences. The *rFW* value is very similar for the two proteins. This is a very rare example that a nuclear-spin hyperpolarization effect generated in one system could be observed by both liquid-state and solid-state NMR. To explore the action of anisotropic mechanisms in the liquid sample that are expected to be sensitive to the magnetic field strength,[44,46] a solution-state NMR spectrometer equipped with a field-cycling device[49] was employed to study 1H, 13C and 15N photo-CIDNP generated in Crphot1-LOV1-C57S within a broad field range from 0.01–9.4 T.[49] The LOV domain of wild-type LOV1-LOV2-C450A, is the blue-light mediating module of not only phototropin but also of some other proteins, e.g., aureochromes.[48] Aureochromes represent a class of LOV proteins with an unusual inverted domain arrangement, as they carry, in contrast to the majority of LOV-domain proteins, the functional/signaling domain N-terminally to the LOV domain and not C-terminally; they exhibit great potential in controlling DNA binding as a natural optogenetic module. Despite a different domain organization of the full-length protein, the LOV domain of aureochromes seem to show a high degree of conservation to canonical LOV domains with respect to sequence and secondary structure.[40] Like in other LOV domains, the optical excitation of FMN chromophore leads to formation of an adduct with a conserved cysteine residue. Furthermore, a recently discovered flavoprotein from the radiation-resistant bacterium *Methylobacterium radiotolerans*, *Mr*4511, unusually lacks the single tryptophan conserved in 75% of LOV domains.[41] Due to the absence of tryptophan to quench the triplet state of FMN, FMN, after excitation by light, the cysteine-lacking mutant of *Mr*4511 can serve as an efficient singlet-oxygen generator.[41] The variety of LOV domains raises the question whether LOV domains can be generally constructed in such a way that they show a photo-CIDNP effect.

The aim of this work was to extend the range of biological systems amenable to the solid-state photo-CIDNP effect. To reach this goal, we employed *Mr*4511 for an extended protein mutation strategy allowing us to change parameters such as the distance between the partners governing the electron transfer reactions that give rise to SCRP formation and recombination, and to tune magnetic parameters of the SCRP. By doing so, we were able to probe the spin dynamics in the SCRP by magnetic-field dependent photo-CIDNP studies. We could also elucidate the role of different photo-CIDNP mechanisms that are responsible for the formation of nuclear spin-polarization. Last but not least, we discovered a non-tryptophan-induced photo-CIDNP effect generated by the cysteine-devoid *Mr*4511, in which tryptophan is absent.

### Results

To rationalize the key properties of molecular spin-machines that can be used to generate photo-CIDNP, we proposed a design strategy based on mutations, supported by field-dependent CIDNP studies. By using various mutations, as described below, we were able to vary the distance between the electron donor and acceptor. In this way, we affected the rate of SCRP formation and recombination, and also varied the electron–electron spin–spin interaction in the SCRP. To probe the reaction and spin dynamics in the SCRP, we used the field dependence of photo-CIDNP.

**Screening LOV domains for induction of photo-CIDNP.** Aiming for designed molecular spin-machines producing light-induced nuclear hyperpolarization, we have designed a series of protein mutants, which will be presented in parts (i)—(iii) (Table 1).

(i) So far, the occurrence of the solid-state photo-CIDNP effect was limited to cysteine-lacking LOV domains of phototropin[40–41]; for this reason, here we explored other potential LOV-based light-induced hyperpolarization generators. Alignment and comparison of the amino-acid sequences of Asphot1-LOV2,
Crphot-LOV1 and Ptauroe1a-LOV show about 50% of identity (Fig. 1A) and the crystal structures show almost identical tertiary structures (Fig. 1B). In particular, the distance between FMN and tryptophan (Fig. 1C), which determines the strength of the spin–spin coupling in the SCRP and is therefore central to generate photo-CIDNP, is nearly the same, being approximately 11 Å. Therefore, we used the mutant Ptauroe1a-LOV-C287S (rFW ~ 11 Å) for the liquid state photo-CIDNP NMR experiment, in which the conserved cysteine 287 is replaced by serine.

(ii) Formation of an SCRP by electron transfer of excited FMN can occur if a nearby tryptophan is present to act as the electron donor. It has been shown that the amino acid tryptophan is able to provide this function. Therefore, in the LOV protein Mr4511, lacking the conserved tryptophan, we introduced a tryptophan at its canonical position Q112 by mutation resulting in the Mr4511-C71S-Q112W double mutant. Previously, transient absorption experiments have been used to test the function of these mutants. In Mr4511, when the cysteine residue was mutated to serine (C71S) or glycine (C71Q) and no tryptophan was present, the lifetime of \( ^3 \text{FMN}, \tau_T \), was around 240 μs. Introduction of tryptophan to the canonical position, Mr4511-C71S-Q112W, gave rise to faster quenching of \( ^3 \text{FMN} \) reducing \( \tau_T \) to ~24 μs, a value very close to ~27 μs observed in Crphot-LOV1-C57S (rFW ~ 11 Å). Hence, the double mutant Mr4511-C71S-Q112W (rFW ~ 11 Å) is the second candidate for the generation of the solid-state photo-CIDNP effect.

(iii) Finally, we introduced the electron-donating tryptophan at non-canonical positions. The introduction of tryptophan to a new position of the protein allows to change the distance between FMN and tryptophan, their relative orientation and chemical environments and, therefore, to affect the kinetic and magnetic parameters, critical for the formation of the solid-state photo-CIDNP effect. It is difficult to fine-tune all relevant reaction and magnetic resonance parameters simultaneously, therefore, we focused on creating mutants with different rFW. Lacking a crystal structure of Mr4511, the design relied on a structural model created using SWISS-MODEL and the crystal structure of aureochrome1a-LOV (PDB: 3UE6) from a eukaryotic photosynthetic stramenopile as template. The report of the modeling parameters is provided in Supplementary Information Table S1. Additionally, comparison of the amino-acid sequence (Fig. 1A) allowed us to predict the occurrence of α-helix and β-sheet secondary structures and to reconstruct the tertiary structure of Mr4511. As targets for mutation, we considered amino acids that do not interact with the FMN and also have a bulky side chain similar as tryptophan. Using these ideas, we have designed the following mutants with different positions of tryptophan with various rFW values: Mr4511-C71S-F130W (~6 Å), Mr4511-C71S-Y116W (~9 Å), Mr4511-C71S-Y129W (~11 Å), and Mr4511-C71S-K57W (~17 Å) (Table 1).

Another aspect relevant for rational design of a biomimetic light-driven spin-machine for production of photo-CIDNP is the possibility to introduce isotope labels. In particular, for the measurement of the \( ^15 \text{N} \) photo-CIDNP we employed \(^{15}\text{NH}_3\text{Cl}\) as the sole nitrogen source in the bacterial growth medium during protein expression and produced uniformly \( ^{15}\text{N} \)-labelled protein and cofactor (see “Methods”). For the \( ^{13}\text{C} \) photo-CIDNP experiment, different labelling strategies were previously applied, either by incorporating the \( ^{13}\text{C} \)-labelled FMN into a natural abundant protein moiety of the phototropin-LOV domain or by selective \( ^{13}\text{C} \)-labelling of the single tryptophan of the phototropin-LOV domain. This enables unambiguous assignment of hyperpolarized \( ^{13}\text{C} \) signals and analysis of the photo-CIDNP effect generated by electron donor and acceptor separately. A complete picture of the effect, however, involving both electron donor and acceptor is still missing. Therefore, in the present work we produced a uniformly \( ^{13}\text{C} \)-labelled Crphot-LOV1-C57S (rFW ~ 11 Å), aiming to compare the photo-CIDNP effect of FMN and tryptophan under the same conditions. The hyperpolarization effect in combination with isotope labelling paves the way to field-dependent NMR measurements, providing knowledge

| Mutants | rFW (Å)* | Species | Amino acid numbers | Weight (kDa) | Structure | Percentage identityb |
|---------|----------|---------|-------------------|--------------|-----------|----------------------|
| Crphot-LOV1-C57S | ~11 | C. reinhardtii | 16–133 | ~15 | PDB 1N9L | – |
| Ptauroe1a-LOV-C287S | ~11 | P. tricornutum | 238–378 | ~18 | PDB 5A8B | 53% |
| Mr4511-C71S-F130W | ~6 | | | | | |
| Mr4511-C71S-Y116W | ~9 | | | | | |
| Mr4511-C71S-Q112W | ~11 | M. radiotolerans | 1–164 | ~18 | | 43% |
| Mr4511-C71S-Y129W | ~11 | | | | | |
| Mr4511-C71S-K57W | ~17 | | | | | |
| Mr4511-C71S | – | | | | | |

Table 1. LOV proteins employed for photo-CIDNP NMR in this work. *The FMN-Trp distance (rFW) is given as edge-to-edge distance and estimated based on a structural model obtained by SWISS-MODEL. The percentage identity is compared with the amino-acid sequence of Crphot-LOV1-C57S.
of the relationship between enhancement factor and magnetic field, which might provide the key data for future theoretical analysis of the exact photo-CIDNP mechanism.

Comparison of the photo-CIDNP effect between phototropin and aureochrome. Figure 2A shows the \( ^1\)H photo-CIDNP effect and its field dependence observed in Crpho1-LOV1-C57S (\( r_{FW} \sim 11 \, \text{Å} \)). The protein produced for the experiment initially contained all the nuclei in their natural abundance. Then the protonated buffer of the sample was exchanged to a deuterated buffer (see "Methods" section). The final protein solution may contain \( \sim 0.4\% \) residual \( ^1\)H. From this sample, the effect has not been observed directly on the protons of FMN and tryptophan, however, the light-minus-dark spectra show a negative enhancement (emissive signal, i.e., opposite to the thermal polarization) of the HDO signal at 4.7 ppm, which agrees with the previous publication\(^{39}\). A closer look at the light-minus-dark spectra shows that the entire range (–2 to 10 ppm) in the protons of FMN and tryptophan, however, the light-minus-dark spectra show a negative enhancement (emissive polarization at all fields and maximal polarization at around 3 T in agreement with previous results\(^{39}\). The selected \( ^1\)C atoms of the isoalloxazine ring of FMN and the side chain of tryptophan.

\[ \text{Figure 1. (A) Alignments of the amino-acid sequence of wild-type phototropin 1-LOV2 from } \textit{Avena sativa} \text{ (Asphot1-LOV2), phototropin-LOV1 from } \textit{Chlamydomonas reinhardtii} \text{ (Crpho1-LOV1), aureochrome1a-LOV from } \textit{Phaeodactylum tricornutum} \text{ (PtAureo1a-LOV), and 4511 from } \textit{Methylobacterium radiotolerans} \text{ (Mr4511): segments highlighted in green and blue refer to } \alpha\text{-helices, and } \beta\text{-sheets, respectively, indicating the secondary structure of the proteins. The conserved positions of cysteine located in } E_\text{loops}, \text{ and tryptophan in } \text{H}_\text{loops} \text{ in the LOV domains are typeset in orange. The five positions to introduce tryptophan in Mr4511 via mutagenesis are highlighted in red. (B) Alignment of the crystal structures of Crpho1-LOV1 (PDB: 1N9L, green)\(^{39}\), PtAureo1a-LOV (PDB: 3A8B, red)\(^{37}\), and the simulated structure of Mr4511 (yellow) without FMN. The simulation is performed with SWISS-MODEL based on the crystal structure of aureochrome1a-LOV from \textit{Vaucheria frigida} \text{ (PDB entry: 3UE6)}\(^{38}\). The information about structural modeling is listed in Supplementary Information Table S1. Five mutants of cysteine-lacking Mr4511 were generated, one with tryptophan placed at the canonical position, Q112W, \( r_{FW} \sim 11 \, \text{Å} \), the other four at non-canonical positions F130W, Y116W, Y129W and K57W with increasing \( r_{FW} \). The figure was created by the PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. (C) IUPAC numbering of atomic positions in the isoalloxazine ring of FMN and the side chain of tryptophan.} \]
Figure 2. (A) $^1$H, (B) $^{13}$C and (C) $^{15}$N photo-CIDNP spectra of Crphot-LOV1-C57S (~11 Å) detected at 289 K at different magnetic field strengths by using a 9.4 T liquid-state NMR spectrometer equipped with a field-cycling device. Hereafter, all photo-CIDNP spectra are light-minus-dark NMR spectra. The upper trace includes signal assignments (blue) with $^{13}$C chemical shifts of hyperpolarized signals mentioned in Supplementary Information Table S2 and $^{15}$N chemical shifts in Table 2. The lower trace shows stacked spectra of the CIDNP effects of the corresponding nuclei measured at five different magnetic fields.

Figure 3. Intensity of selected hyperpolarized signals of Crphot-LOV1-C57S ($r_{FW}$ ~ 11 Å), integrated and plotted against the magnetic field strength for (A) $^1$H, (B) $^{13}$C and (C) $^{15}$N. For convenient comparison, the magnitude of the maximum polarization of each nucleus is arbitrarily set to −1 and the signal enhancement is normalized to this value. The plus and minus signs at the y-axis represent the enhanced absorptive (positive) and emissive polarization (negative) relative to the thermal polarization. Uniformly $^{13}$C- and uniformly $^{15}$N-enriched Crphot-LOV1-C57S (~11 Å) samples were employed for the $^{13}$C and $^{15}$N photo-CIDNP NMR experiments, respectively. Regarding $^1$H photo-CIDNP, the signal of HDO, the aliphatic region (0 to 3 ppm), and the broader region (~2 to 10 ppm) of the $^1$H photo-CIDNP spectrum were integrated for comparison.
and the 1H spectrum intensity integrated from −2 to 10 ppm of Crphot-LOV1-C57S (rFW ~ 11 Å) (Fig. 4A). The field at which the 1H, 13C and 15N photo-CIDNP production reached its maximum follows the relationship: 0.6 T (1H) < 3 T (13C) < 5 T (15N). Under similar conditions, the field maxima for photo-CIDNP enhancement of the corresponding nuclei in Pt aureo1a-LOV-C287S (rFW ~ 11 Å) (tryptophan is selectively isotope-labelled) were obtained. The positions of the maxima are in the same order (Fig. 4B); the corresponding 1D spectra are shown to guide the eye. The maximum emissive polarization of all nuclei is scaled to −1. The minus sign of the y-axis implies that the hyperpolarization is emissive. The 1D spectra of the photo-CIDNP effect observed in Pt aureo1a-LOV-C287S are shown in Supporting Information Figure S1.

**Table 2.** Photo-CIDNP experimental results and obtained 15N chemical shifts from the eight LOV proteins studied in this work. Trp tryptophan, + tryptophan-derived photo-CIDNP effect, + (new) non-tryptophan-derived photo-CIDNP effect, − no photo-CIDNP effect, n.a. not available.

| Mutants                  | rFW. (Å) | 15N photo-CIDNP | 1H photo-CIDNP | 15N chemical shift (ppm) | FMN N-5 | FMN N-10 | Trp N-1 |
|--------------------------|----------|-----------------|----------------|-------------------------|--------|----------|---------|
| Crphot-LOV1-C57S         | ~ 11     | +               | +              | 344.1                   | 155.0  | 127.6    |
| Pt aureo1a-LOV-C287S     | ~ 11     | +               | +              | n.a.                    | n.a.   | 127.8    |
| Mr4511-C71S-F130W        | ~ 6      | =               | =              | n.a.                    | n.a.   | n.a.     |
| Mr4511-C71S-Y116W        | ~ 9      | +               | +              | 345.1                   | 156.2  | 127.7    |
| Mr4511-C71S-Q112W        | ~ 11     | +               | +              | 345.3                   | 156.4  | 127.2    |
| Mr4511-C71S-Y129W        | ~ 11     | +               | +              | 345.5                   | 126.2  | 127.7    |
| Mr4511-C71S-K57W         | ~ 17     | + (new)         | + (new)        | 345.2                   | n.a.   | n.a.     |
| Mr4511-C71S              | No Trp   | + (new)         | + (new)        | 345.3                   | n.a.   | n.a.     |

**Figure 4.** Comparison of the field dependencies of the 1H and 13C and 15N photo-CIDNP effects generated by (A) Crphot-LOV1-C57S (rFW ~ 11 Å) and (B) Pt aureo1a-LOV-C287S (rFW ~ 11 Å). Detailed experimental parameters are provided in the “Methods” section. The hyperpolarized 1H signal has been obtained by integrating the spectral region of −2 to 10 ppm (black square); the 13C signal from C-3 of tryptophan (red triangle) and the 15N signal from indole of tryptophan (blue circle) are also shown. The dashed lines are added to guide the eye. The maximum emissive polarization of all nuclei is scaled to −1. The minus sign of the y-axis implies that the hyperpolarization is emissive. The 1D spectra of the photo-CIDNP effect observed in Pt aureo1a-LOV-C287S are shown in Supporting Information Figure S1.

**Tryptophan and non-tryptophan derived photo-CIDNP effect generated in Mr4511 mutants.** To generate a photo-CIDNP effect in Mr4511, the conserved cysteine was first replaced with the inactive serine resulting in the mutant Mr4511-C71S. Furthermore, tryptophan was introduced to different locations of the Mr4511-C71S protein, generating five additional mutants (Table 1). The magnetic field dependence of the photo-CIDNP effect under liquid-state conditions obtained by 15N and 1H NMR is summarized in Fig. 5, plotted in the same way as in Fig. 3. The corresponding 15N NMR spectra are shown in Figure S2 and the 15N chemical shifts are provided in Table 2.
The mutant $M_r4511-C71S-F130W$ (rFW $\sim 6$ Å), which has the shortest distance between the redox partners of the SCRP, shows neither a $^1$H nor a $^{15}$N photo-CIDNP in the magnetic field range from 0.1 to 9.4 T (Fig. 5A1,A2; Supplementary Information Figure S3 and S4). Time-resolved optical absorption analysis on F130W suggests that the triplet state $^3$FMN is not formed to an observable extent in this protein, most likely, because of the ultrafast electron transfer between FMN and tryptophan (Aba Losi, personal communication). Accordingly, for the construction of light-induced artificial spin-machines pumping nuclear hyperpolarization, the information of a minimum distance of the redox partners of the SCRP is highly relevant.

Except for $M_r4511-F130W$ (rFW $\sim 6$ Å), all other $M_r4511-C71S$ mutants generated both, the $^{15}$N and $^1$H photo-CIDNP effect under liquid-state conditions. As shown in Fig. 5B2, $M_r4511-C71S-Y116W$ (rFW $\sim 9$ Å) shows hyperpolarization for the nitrogen on FMN and tryptophan, therefore it is referred to as "tryptophan-derived photo-CIDNP". This protein shows a maximal $^1$H hyperpolarization at 2.4 T (Fig. 5B1), which is a higher field than observed for any other mutant (see below).

The rFW value of $\sim 11$ Å in the phototropin and aureochrome LOV domains was known to generate a photo-CIDNP effect. Here we compare the two cases when the tryptophan is at the canonical position, $M_r4511-C71S$ (Fig. 5C1,C2) and the non-canonical position but with nearly the same rFW distance, $M_r4511-C71S-K57W$ (rFW $\sim 11$ Å) (Fig. 5D1, D2). Although $M_r4511-C71S$ and $M_r4511-C71S-Y116W$ exhibit a similar field maximum for the $^1$H photo-CIDNP effect, their field-dependent $^{15}$N photo-CIDNP effects are significantly different. This means that the efficiency of photo-CIDNP formation does not only depend on the spatial distance. Different orientations and different local mobility of the residues might be considered as the origin of this difference. However, the phase of hyperpolarized Trp N-1 signal is always negative for mutants Y116W, Y129W and Q112W.

The tryptophan residue in $M_r4511-C71S-K57W$ (rFW $\sim 17$ Å) is the most remote electron donor from FMN in the studied set of mutants and, thus, the reaction rate constant of electron transfer in this protein mutant is expected to be the smallest in this series. The protein shows a $^{15}$N hyperpolarization solely on the FMN N-5, while the FMN N-10 and Trp N-1 signals do not exhibit any enhancement in a wide range of magnetic fields (Fig. 5E). The same photo-CIDNP experiments were also performed on $M_r4511-C71S$ in which no tryptophan was present leading to very similar results (Fig. 5F) with somewhat weaker single emissive FMN N5 signal (Figure S2 graphs D and E). This surprising result clearly indicates that the $^{15}$N photo-CIDNP effect reported here is not derived solely from the involvement of the added tryptophan residue. It is well-known that tyrosine can also act as an electron donor in biological systems. According to the amino-acid sequence and our structural model of $M_r4511$, there are four tyrosine residues located in proximity to FMN in the range of 9 to 12 Å. In line with this speculation is the fact that the $^{15}$N photo-CIDNP effect is not observable for tyrosine, since tyrosine does not contain $^{15}$N in the side chain. Therefore, the light-driven molecular spin-machines can probably also rely on SCRPs containing a tyrosine radical.

**Discussion**

Here, we show that a photo-CIDNP effect originating from the SCRP of FMN and tryptophan can be produced in artificially designed flavoproteins. We employ a systematic mutation strategy to vary reaction and magnetic parameters of the paramagnetic centers generated by light. It appears that the rFW distance of $\sim 6$ Å between the FMN and tryptophan is too short to provide conditions suitable for photo-CIDNP formation, whereas in the range of $9$ to $11$ Å, the effect has been observed (Fig. 5; Table 2). The data on the field dependence of the photo-CIDNP effect generated by the designed LOV domains show complex dependencies, which are not expected for the liquid-state photo-CIDNP effect. In particular, the differences in field dependence obtained for LOV domains having the same distance of donor and acceptor suggest that anisotropic spin interactions come into play as they are expected for solids. In addition to the field dependence, a distance dependence has been documented. Apparently, further parameters are involved, presumably the relative orientation of donor and acceptor as well as their local dynamics. Both, anisotropy and relaxation effects require further studies.

Furthermore, the effect of different label patterns requires a future study.

The presence of solid-state mechanisms in LOV domains in liquids implies that the transient SCRP occurs in a slow-motion regime, during which the anisotropic electron–nuclear interactions are conserved for the build-up of hyperpolarization. In contrast, on NMR time scale, all the anisotropic nuclear interactions, i.e., nuclear dipolar coupling and chemical shift anisotropy as present in solids are averaged out and thus the hyperpolarized signals in the NMR spectra exhibit no obvious anisotropic features. Such phenomenon was previously observed for a...
photosynthetic RC protein-membrane complex corresponding to ~ 1 MDa, measured by $^{13}$C liquid-state NMR. For LOV proteins, having the molecular weight of less than 20 kDa, the occurrence of anisotropic mechanisms in liquids likely relies on the formation of dimers or higher multimers in solution. The slow tumbling rate may lead to the presence of residual proton-proton couplings which allow for the $^1$H hyperpolarization transfer from the center of the photo-reaction into the environment (Fig. 2A).

So far, flavoproteins and photosynthetic RCs are the only reported electron transfer protein systems that show solid-state photo-CIDNP effect. Even despite the different cofactor arrangements and spin-dynamics, they might share the same mechanisms. Consequently, similar features of CIDNP are expected regarding the sign change of nuclear spin hyperpolarization and the similar field at which the maximum polarization occurs. LCs and LACs analysis suggested that a solid-state photo-CIDNP effect is not only field-dependent, but also strongly orientation-dependent because of the anisotropic interactions governing in spin dynamics of the SCRPG in solids. To the present experiments conducted under liquid-state conditions, the same theory will be applied to understand the sign change that occurred in the LOV proteins as shown in Fig. 3B.C as well as Fig. 5B2-D2.

Summarizing these considerations, we can propose the following interpretation of the experimental findings.

By increasing $r_{FW}$, we decrease two parameters: The SCRPG recombination rate and the electron–electron spin–spin coupling, $J_{SCRPG}$, within the SCRPG. When the $r_{FW}$ distance is too short, photo-CIDNP formation is suppressed, most probably, due to two reasons: The first reason is that $J_{SCRPG}$ is too large, introducing an energy gap between the singlet and triplet SCRPG spin states. This gap cannot be overcome by the relatively small hyperfine couplings, and singlet–triplet interconversion in the SCRPG is thus suppressed. The second reason is that the spin-evolution of the SCRPG requires sufficient time for photo-CIDNP formation: fast SCRPG recombination interrupts this process and thus no photo-CIDNP is formed.

As $r_{FW}$ increases further, we enter the regime in which the SCRPG lifetime is sufficiently long and $J_{SCRPG}$ is sizeable, but not too large to suppress singlet–triplet interconversion giving rise to photo-CIDNP formation. In this situation, the TSM scenario comes into play and the maximum position, $b_{max}$, in the photo-CIDNP field dependence is given by the matching condition $|\gamma N|b_{max} \approx J_{SCRPG}$, with $\gamma N$ being the nuclear gyromagnetic ratio; the sign of polarization of the three different kinds of nuclei ($^1$H, $^{13}$C and $^{15}$N) is also consistent with previous theoretical considerations. Hence, the $b_{max}$ field is different for different nuclei, which is consistent with the experimental data.

When $r_{FW}$ increases further, $J_{SCRPG}$ is decreased and other photo-CIDNP mechanisms come into play. In this situation, polarization formation is no longer sensitive to the $\gamma N$ value, i.e., to the nuclear spin isotopes, so that different kinds of nuclei exhibit a similar behavior. The sign changes of photo-CIDNP can be rationalized in terms of changing dominance of enhancement mechanisms, as it happens in RCs.

The design strategy also leads to the discovery of a new-type of photo-CIDNP effect generated by Mr4511-C71S in which no triphyrin is present. The same effect (Fig. 5E,F) also occurs in Mr4511-C71S-K57W ($r_{FW} \sim 17$ Å). Based on the present results, we are unable to unravel the origin of the new-type photo-CIDNP effect. Recent research on a designed cysteine-lacking Asphot1-LOV2 domain indicated that, without presence of the triphyrin, the FMN was reduced, however to less extend compared to the case when the triphyrin was present. Kinetic data suggested that one of the tyrosines in the LOV domain acts as counter radical. Therefore, we proposed that tyrosine might act as electron donor in the absence of triphyrin also in our case.

With this, the present work significantly extends the class of light-driven molecular spin machines, which pump nuclear spin-hyperpolarization. The LOV systems are particularly suitable for such biomimetic design, while photosynthetic RCs due to their structural complexity allow for limited manipulations only. The biomimetic design that affects the parameters of the photo-CIDNP effect provides new categories of experiments to analyze the conditions for its occurrence.

Methods

Protein preparation. The plasmid (i) encoding the LOV-C287S mutant of aureochrome1a from P. tricornutum comprising the flanking Jα and A ‘α helices (238–378) was received from Peter Kroth (University of Konstanz). The plasmid (ii) that encodes Mr4511 from M. radiotolerans (1–164) was generated in our own group by genome cloning. On that basis, we first constructed the cysteine-lacking Mr4511-C71S mutant. Subsequently, additional five mutants encoding triphyrin situated at different positions were created, $Mr4511-C71S$ (6 Å), $Mr4511-C71S$ (9 Å), $Mr4511-C71S$ (11 Å), $Mr4511-C71S$ (12 Å) and $Mr4511-C71S$ (17 Å) with primers shown in the Supporting Information Table S3. All genetic manipulations were according to standard protocols. Plasmid (iii) encodes the LOV1-C57S mutant of phototropin from C. reinhardtii (16–133) carrying a 15×His tag at the N-terminus. Further information about all the mutants employed in this work is summarized in Table 1. The protocol of heterologous overexpression and isoype-labelling of all the mutants in Chonrichia variabilis has been reported elsewhere. Isotopically enriched material, $^{13}$NH$_4$Cl, $^{15}$N indole, [u-$^{13}$C$_6$] glucose, and [u-$^{13}$C$_8$] indole employed in this research were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Use of $^{13}$NH$_4$Cl or [u-$^{13}$C$_6$] glucose as the sole source in the growth medium yields a uniformly $^{13}$N or $^{13}$C labelled protein, while supplementation of $^{13}$C or $^{15}$N isotope-enriched indole as a precursor to the normal medium results in a selective labelling of the triphyrin side chain. The $^{13}$N and $^{13}$C labelled proteins were used for corresponding $^{13}$N and $^{13}$C NMR measurements. For $^{1}$H NMR measurement, the employed proteins are in their natural abundance, and they were washed with deuterated phosphate buffer (300 mM NaCl, 50 mM K$_2$PO$_4$ in D$_2$O, pH 8.0) to a final solution containing approximately 0.4% residual protons. The final concentration of the flavoproteins were controlled at about 16 μM ($^{450nm}=12,500$ M$^{-1}$ cm$^{-1}$) before photo-CIDNP measurement.
Photo-CIDNP solution-state NMR. The field-dependent \(^{15}N\), \(^{13}C\), and \(^1H\) photo-CIDNP experiments of the LOV proteins were carried out on an NMR spectrometer operating at a magnetic field of 9.4 T (\(^1H\) frequency of 400 MHz) (Bruker Avance III HD) equipped with a home-built field-cycling device\(^7\). It transfers the sample to variable magnetic fields within the range 10 mT < B0 < 9.4 T at which the sample is illuminated and returns it for the NMR detection at 9.4 T. For the \(^{13}C\) and \(^{15}N\) photo-CIDNP experiments, pulse-acquire with WALTZ-16 proton decoupling was employed. The pulse sequence of the \(^1H\) photo-CIDNP experiments starts with a pre-saturation composite pulse sequence\(^8\) at 9.4 T, followed by the sample shuttle cycle that includes the sample transfer to the chosen magnetic field for illumination by LED (called “light”) or the same cycle without illumination (called “dark”) during the fixed time and the return to 9.4 T, and it ends with the detection sequence. For all photo-CIDNP experiments, the samples were measured in dark and light, respectively, with the same number of scans. The illumination source was a 400-nm 2-W LED (Chanzon, China) and the illumination time was optimized to 0.5 s. A fresh aliquot of the sample stock was taken for a measurement at each magnetic field to compensate the effect of photo-bleaching. The temperature was 289 K for all samples with the exception that P. aureo1a-LOV-C287S was measured at 277 K. The line-broadening for \(^{15}N\) and \(^{13}C\) NMR spectra were set to 30 Hz and for \(^1H\) spectra was set to 1 Hz. The \(^{15}N\) and \(^{13}C\) NMR spectra were phased to the external standard, a mixture of 0.1 M \(^{15}N\) labelled urea and 0.1 M \(^{13}C\) labelled methanol in DMSO. The chemical shifts of \(^{15}N\) NMR spectra were relative to liquid ammonia and referenced externally to urea \(^{15}N\) at 76.3 ppm\(^9\). To present the uncertainty of the \(^{13}C\) and \(^{15}N\) photo-CIDNP data represents the noise level relative to the corresponding hyperpolarized signal area. The spectra shown in Fig. 2 and Supplementary Information Figure S1, S2, and S4 were created with OriginPro Version 2017.

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Author contributions
R.Z.S., K.L.I., A.V.Y. and J.M. designed the research. The sample preparation was mainly performed by Y.D., Z.Z., Q.X., P.K., S.B. and T.K. Most of the field-dependent photo-CIDNP experiments are done by Y.D. and A.S.K. Y.D., A.S.K., K.L.I., A.V.Y. and J.M. interpreted field-dependent CIDNP data. The figures are created by Y.D. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Competing interests
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