Published in final edited form as:

J Phys Chem B. 2009 June 18; 113(24): 8310–8318. doi:10.1021/jp901000z.

¹H Photo-CIDNP Enhancements in Heteronuclear Correlation NMR Spectroscopy

Ashok Sekhar and Silvia Cavagnero*

Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, Wisconsin 53706, USA.

Abstract

Photochemically induced dynamic nuclear polarization (photo-CIDNP) is usually employed as a probe of solvent exposure, in biomolecular NMR. The potential of the photo-CIDNP effect for sensitivity enhancement, however, remains poorly explored. Here, we introduce ¹H-photo-CIDNP in heteronuclear correlation spectroscopy at low laser irradiation power (1 W), and compare the sensitivity of various ¹H-Photo-CIDNP-enhanced- (HPE) ¹H□¹⁵N heteronuclear correlation pulse sequences, including HSQC, HMQC, and SOFAST-HMQC, in terms of their ability to detect the Trp indole H^{ε1} resonance. Both Trp and the Trp-containing protein apoHmpH were analyzed using flavin mononucleotide as photosensitizer in aqueous solutions either containing or lacking urea. We find that ¹H□¹⁵N photo-CIDNP-SOFAST-HMQC, denoted here as HPE-SOFAST-HMQC, yields a two-fold higher signal-to-noise per unit time than the parent SOFAST-HMQC for the solvent-exposed Trp of urea-unfolded apoHmpH. Thus, HPE-SOFAST-HMQC is the most sensitive heteronuclear correlation pulse sequence for the detection of solvent-exposed Trp.

Keywords

Dynamic nuclear polarization; NMR; HSQC; HMQC; SOFAST-HMQC; sensitivity enhancement

Photochemically induced dynamic nuclear polarization, also known as photo-CIDNP, is an NMR-related phenomenon leading to the generation of non-equilibrium longitudinal magnetization for selected nuclei upon laser excitation in the presence of a dye (photosensitizer)¹⁻³. Photo-CIDNP involves the direct interaction of the photo-excited dye with the NMR-active substrate.

According to the most widely accepted mechanism⁴⁻⁷, the dye-substrate interaction results in the formation of a transient radical pair bearing an unpaired electron coupled to the NMR-active nucleus of interest⁸. The nuclear spin states of the NMR-active nucleus modulate the chemical reactivity of the radical pair via electron-nucleus hyperfine interactions, leading to differential fluxes along competing reaction pathways. As a result of the above phenomenon, a net variation in the NMR resonance intensity of the nuclei affected by photo-CIDNP is observed, leading to resonances with absorptive or emissive polarization.

Over the last three decades, photo-CIDNP has been mainly exploited to monitor protein folding and hydrophobic collapse in protein unfolded states⁹⁻¹². This effect serves as a probe of solvent exposure because geminate radical pair formation requires the physical collision of the substrate and the photosensitizer in solution³.

*To whom correspondence should be addressed. Phone: 608-262-5430 FAX: 608-262-9918 Email: cavagnero@chem.wisc.edu.

A poorly explored aspect of photo-CIDNP is its potential to enhance the far-from-ideal sensitivity of NMR spectroscopy. Given that in the presence of an applied magnetic field the difference in the number of ^1H α and β nuclear spin states is very small at equilibrium (of the order of 1 in 10^5), even small variations in the reactivity of molecules carrying α and β spins may in principle lead to large signal enhancements¹⁰. To date, however, there are only few reports testifying on the effectiveness of photo-CIDNP to enhance NMR sensitivity in the context of biomolecular systems¹³.

Among the naturally occurring amino acids in proteins, only Trp, Tyr, His and, to a smaller extent, Met undergo photo-CIDNP enhancement⁸. While this selectivity considerably reduces the generality of any potential sensitivity gains achievable via CIDNP, it may also be viewed as an advantage in that specific resonances can be easily identified even within crowded NMR spectra.

Previous photo-CIDNP studies of biomolecular processes have typically been based on simple 1D ^1H -detected (90°)_{x,y}-acquisition schemes in the presence (*light* conditions) or absence (*dark* conditions) of laser pre-irradiation. Photo-CIDNP enhancements have been recorded as the difference between light and dark spectra, by broadband saturation of the ^1H spectrum before laser irradiation, or by a combination of the two methods^{3,10,14,15}.

Applications of photo-CIDNP to heteronuclear correlation spectroscopy, however, are scarce and limited to the HSQC sequence^{16,17}. Yet, a number of heteronuclear correlation-based sequences have met widespread use in the study of biomolecular conformation and dynamics¹⁸. Heteronuclear correlation approaches selectively detect ^1H s covalently linked to the NMR-active heteroatoms of interest, thus providing invaluable spectral editing in both diluted solutions of pure samples¹⁹ and complex mixtures²⁰.

The combination of photo-CIDNP and ^1H -detected ^1H - ^{15}N heteronuclear correlation pulse trains offers the potential to selectively enhance Trp indole $\text{H}^{\epsilon 1}$ and $^{15}\text{N}^{\epsilon 1}$ resonances, given that backbone amides do not experience appreciable photo-CIDNP⁸. This phenomenon prompted us to explore whether both spectral editing and enhanced sensitivity could be achieved at once, for optimizing the detection of solvent exposure of protein hydrophobic cores (probed via the Trp side chain).

In this work, we explore the photo-CIDNP-based sensitivity enhancement achievable by interfacing a number of ^1H - ^{15}N heteronuclear correlation pulse sequences including SOFAST-HMQC²¹, HMQC²² and HSQC²³ with photo-CIDNP-inducing laser irradiation. The resulting HPE (^1H -photo-CIDNP-enhanced) sequences have been tested on aqueous Trp, Trp in urea, and the Trp-containing protein apoHmpH in its urea-unfolded state, in the presence of 0.2 mM flavin mononucleotide as a photosensitizer. We find HPE-SOFAST-HMQC to be by far the most sensitive photo-CIDNP-based heteronuclear correlation sequence, offering a two-fold enhancement over its counterpart in the absence of photo-CIDNP for the detection of solvent-exposed Trp in apoHmpH. The sequence-dependent trends are rationalized based upon a theoretical framework for photo-CIDNP^{24,25}. The high sensitivity of HPE-SOFAST-HMQC is of interest because SOFAST-HMQC already excels among the known 2D ^1H -detected heteronuclear correlation sequences in terms of its high sensitivity and efficiency for fast data-acquisition^{21,26,27}.

Materials and Methods

Sample Preparation

Stock solutions of $^{15}\text{N}_2$ -L-Trp (Isotec Inc., Miamisburg, OH) and riboflavin 5'-monophosphate (flavin, Sigma-Aldrich, St. Louis, MO) were prepared by dissolving the respective powders in

water. The pH of the Trp stock was subsequently adjusted to 7.0. Trp and flavin concentrations were assessed by absorption spectroscopy ($\epsilon_{278, \text{Trp}} = 5,578 \text{ M}^{-1}\text{cm}^{-1}$ 28, $\epsilon_{450, \text{flavin}} = 12,500 \text{ M}^{-1}\text{cm}^{-1}$ 29). Solid urea (up to 6 M, ultrapure, MP Biomedicals, Solon, OH) was added directly to the NMR samples, if needed. The pH of the Trp samples was adjusted to 6.9, unless otherwise stated. The final amino acid concentration of all the Trp NMR samples was 1 mM.

Lyophilized pure ^{15}N -apoHmpH was overexpressed and purified as described ³⁰. NMR samples containing the unfolded protein were prepared by dissolving lyophilized apoHmpH in 25 mM tris- d_{11} (Isotec, Miamisburg, OH) containing 6 M urea followed by pH adjustment to 7.0 with 0.1 M HCl. ApoHmpH concentrations were determined by absorption spectroscopy at 280 nm ($\epsilon_{280, \text{apoHmpH}} = 14,500 \pm 200 \text{ M}^{-1}\text{cm}^{-1}$ 30), and were typically $\sim 300 \mu\text{M}$. In our hands, the presence of tris or tris- d_{11} in NMR samples did not affect the observed photo-CIDNP enhancements (data not shown), consistent with some of the literature reports addressing this topic ³¹.

All NMR samples contained 0.2 mM flavin and 5% D_2O . Urea-containing solutions were used within ca. 24 hrs of sample preparation.

Photo-CIDNP instrumentation

An Omnicrome Ar-ion laser (model 543-MAP, Chino, CA) operating in multi-line mode (main lines: 488 and 514 nm), tuned at 488 nm and operating at 1 W total output power was used as the light source. The laser light was focused into one end of a 10 m-long quartz optical fiber (1 mm diameter, F-MBE, Newport Corporation, Irvine, CA, polished at both ends) using a fiber-coupler (F-91-C1-T, Newport Corporation, Irvine, CA). The other end of the optical fiber was placed into a glass NMR tube coaxial insert (Wilma Labglass, Buena, NJ), introduced into a regular 5 mm NMR tube. The position of the coaxial insert was adjusted so that the tip of the fiber was 4 mm above the top of the receiver coil region. Alternatively, the optical fiber was placed inside a 5 mm Shigemi tube (Shigemi, Inc., Allison Park, PA) insert, with the fiber barely touching the bottom of the insert. A mechanical shutter (modified from HP8452 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA) or the LS055 shutter with a CX2250 controller (NMLaser, San Jose, CA)) controlled by the spectrometer pulse programmer was used for gating the laser irradiation.

NMR spectroscopy: general features

All NMR spectra were acquired on a Varian INOVA 600 MHz spectrometer equipped with a Varian triple resonance $^1\text{H}\{^{13}\text{C}, ^{15}\text{N}\}$ triple axis gradient probe at 24°C. Photo-CIDNP light and dark spectra were acquired with HPE-modified versions of the $^1\text{H}\square^{15}\text{N}$ SE-HSQC ²³, HMQC ²², SOFAST-HMQC ²¹, and g/s-HMQC ³² heteronuclear correlation sequences. The HPE modification consists of a laser irradiation module (500 ms, unless otherwise stated) prior to the first radiofrequency pulse immediately followed by a short (500 μs) delay. PC9 (3 ms) and REBURP (2.03 ms) shaped pulses centered at 10 ppm (4 ppm bandwidth) were used as first and second ^1H pulses, respectively, in the HPE-SOFAST-HMQC sequence. As a comparison, dark and light 1D ^1H data sets were collected with a simple sequence consisting of an initial $(\pi/2)_x$ ^1H pulse followed by a WATERGATE ³³ solvent-suppression scheme.

Relaxation delays of the dark pulse sequences were set to 1.5 s except for the HPE-SOFAST-HMQC sequence, which had a shorter relaxation delay (0.502 s). The photo-CIDNP irradiation time of the light sequences was borrowed from the last portion of the relaxation delay of the dark sequences, leading to an independent relaxation delay decreased by 500 ms.

1D NMR data collection

1D-mode NMR data were collected by setting the ^{15}N chemical shift evolution delay to zero. A spectral window of 9,000 Hz with 1,800 complex data points was used. The data for the free Trp and apoHmpH samples were collected with 4 and 16 transients (preceded by 2 dummy scans), respectively.

Dark and light spectra were processed identically with the VNMR 6.1c software (Varian, Inc., Palo Alto, CA). Spectra were apodized with a 1 Hz exponential linebroadening function and zero-filled to 128k data points to minimize digitization errors. Spectra were baseline-corrected with a spline function using the VNMR6.1c baseline fitting utility.

2D NMR data collection

Representative 2D data sets were collected with a 9,000 Hz ^1H and 250 Hz ^{15}N sweep widths, 2048 complex points and 32 increments with 1 scan per increment. Dark and light spectra were processed identically with VNMR 6.1c software. Time-domain data were apodized with a cosine-square bell in both dimensions and zero-filled to 8,192 points in the direct (^1H) and 128 in the indirect (^{15}N) dimensions before Fourier transformation. S/N measurements were made on the ^1H 2D slice with maximum peak intensity after applying a spline baseline correction.

S/N and $(\text{S/N})_t$ measurements

The S/N of the Trp indole H^{e1} resonance of all samples was calculated from separate measurements of resonance height (S) and peak-to-peak noise amplitudes (N_{PTP}). The N_{PTP} values were measured over a 4 ppm spectral window centered on the Trp resonance. The S/N was estimated from signal intensity (S) and N_{PTP} using the relation ³⁴

$$\frac{S}{N} = \frac{2.5 \cdot S}{N_{\text{PTP}}}, \quad (1)$$

Measurements on two independent samples were averaged and errors were propagated using standard relations ³⁵. For each sample, either 10 (free Trp in water or urea, 4 scans/experiment) or 1 (apoHmpH, 16 scans/experiment, and all 2D spectra) replicate experiments were performed. Data for each set of replicas were averaged and the standard deviations of the mean were employed in the above error propagation analysis. S/N values were normalized to the Trp indole H^{e1} peak intensity in a standard $^1\text{H}/^{15}\text{N}$ HSQC (1D first row or 2D ^1H trace with maximum intensity) to eliminate minor differences in sample preparation.

The signal-to-noise per unit time $(\text{S/N})_t$ was calculated by dividing the S/N by the square-root of the total experiment time as described ³⁴. Errors in $(\text{S/N})_t$ were obtained by error propagation of the uncertainties in S/N. Standard errors (see Figs. 3 and 5) were obtained by dividing the standard deviations by $(n)^{1/2}$ where n is the number of independent measurements (i.e., 2, see above) ³⁶.

Computational predictions of photo-CIDNP enhancements

The set of three coupled differential equations (5) used to compute sequence-specific photo-CIDNP enhancements was integrated numerically using the Matlab 7.0 software (The MathWorks, Natick, MA). The $(\text{S/N})_t$ values under dark and light conditions were used as measures of H_{z0} and $H_z(t=0.5\text{s})$, respectively. H_{z0} , scaled by $\gamma_{\text{H}}/\gamma_{\text{N}}$, was used as a measure of N_{z0} . HPE-SE-HSQC and NPE-SE-HSQC ¹⁶ were used as reference sequences to determine photo-CIDNP pumping rates p_{H} and p_{N} as follows. Experimental HPE-SE-HSQC $(\text{S/N})_t$ values under dark conditions, and initial guesses for p_{H} and p_{N} , were used to compute initial estimates of HPE-SE-HSQC and NPE-SE-HSQC $(\text{S/N})_t$ values under light conditions. The p_{H} and p_{N}

values were then iteratively optimized until the numerical integration routine reproduced the experimentally observed $(S/N)_t$ of HPE-SE-HSQC and NPE-SE-HSQC under light conditions. The resulting p_H and p_N values were employed in the computational predictions pertaining to all the other pulse sequences.

Results

HPE heteronuclear correlation pulse sequences

We explored the potential of photo-CIDNP for sensitivity enhancement in heteronuclear correlation spectroscopy by integrating a photo-CIDNP laser irradiation module into some of the $^1H-^{15}N$ HSQC- and HMQC-type sequences of most widespread use.

Within the HSQC class, we targeted the sensitivity-enhanced HSQC with gradient coherence selection²³. Sensitivity-enhanced HSQC is the most common sequence to detect $^1H-^{15}N$ correlations. HSQC modules are also extensively used in multidimensional NMR pulse schemes.

The conventional HMQC with jump-return solvent suppression²², the gradient/sensitivity-enhanced HMQC (g/s-HMQC)³² and the SOFAST-HMQC²¹ were chosen within the HMQC class. In biomolecular NMR, HMQC is often employed to detect $^1H-^{13}C$ correlations in large proteins and protein complexes^{37,38}.

A continuous laser irradiation (488 nm) step was inserted before the first rf pulse of each of the above pulse sequences, to render the proton polarization photo-CIDNP-enhanced (Fig. 1). Hence the name of the resulting pulse schemes is preceded by the HPE prefix. A 500 μs delay was inserted between laser irradiation and the first rf pulses to allow for the decay of the transient paramagnetic species generated in solution.

The Fourier-transformed data resulting from the sequences in the presence or absence of laser pre-irradiation are denoted as light and dark spectra, respectively. Trp indole resonances were taken as a measure of the achievable photo-CIDNP in heteronuclear correlation spectroscopy. Resonances of free Trp or a Trp-containing protein were detected by recording data in 1D mode, i.e., collecting only the first row of the 2D sequences thereby turning off ^{15}N chemical shift evolution. Signal-to-noise (S/N) and signal-to-noise per unit time $(S/N)_t$ measurements were made as outlined in the Materials and Methods.

Optimization of laser irradiation time

The intensity (I_{mn}) of a photo-CIDNP-enhanced NMR transition connecting nuclear spin states m and n exhibits an increase with laser irradiation time (t), which is often described by the relation

$$I_{mn}=I_{mn}^0+CrT_1(P_m-P_n)\left(1-\exp\left(-\frac{t}{T_1}\right)\right), \quad (2)$$

where I_{mn}^0 is the NMR intensity of the m -to- n transition in the absence of photo-CIDNP, C is a proportionality constant, r is the rate of formation of radical pairs (assumed to be time-independent), T_1 is the longitudinal relaxation time of the diamagnetic nucleus of interest, and P_m and P_n are the total nuclear spin state-dependent recombination probabilities of the radical pair.³⁹ For optimal sensitivity, laser pulse durations must be of the order of T_1 .³¹

In order to optimize the laser irradiation time of our experiments, we studied the S/N of the HPE-g/s-HMQC light spectrum of Trp in 6 M urea (pH 7.5) as a function of laser irradiation

time (Fig. 2). Relaxation delay and total experiment time were kept constant. Data fitting to a single-exponential of the form

$$y=a+b(1-\exp(-t/\tau)) \quad (3)$$

yields a time constant τ of 350 ms. Hence, laser irradiation times significantly shorter than ~ 350 ms are not sufficient for the buildup of appreciable photo-CIDNP. Between 500 ms and 1 s, the S/N of the light spectrum levels off. At laser irradiation times ≥ 500 ms, a steady-state is reached where the rate of radical pair formation (modulated by the recombination probabilities) balance out the longitudinal relaxation effects. No further intensity variations are expected under these conditions other than potential photodegradative processes unaccounted for by relation (2). Therefore, we did not analyze the behavior of the systems at laser irradiation times > 1 s. Based on the fact that the S/N of the light spectrum does not increase significantly beyond 500 ms laser irradiation and that no significant sample heating effects were observed (assessed from the thermocouple response and spectral quality), we settled on a laser irradiation time of 500 ms for the experiments shown here.

Photo-CIDNP enhancements

Photo-CIDNP enhancements P are defined here as the $(S/N)_t$ ratio for the Trp indole resonance in the light and dark spectra. These enhancements range from 1.2 – 2 for the Trp sample in water at pH 6.9 (Fig. 3a).

The observed photo-CIDNP enhancements are pulse sequence-dependent. Specifically, HPE-g/s-HMQC shows the largest enhancement (ca. 2). However, the parent dark version of g/s-HMQC is less sensitive than the other heteronuclear correlation pulse schemes, in our hands, in apparent contrast with the literature report introducing g/s-HMQC³². The other tested pulse sequences show a moderate enhancement of ca. 1.2.

From the viewpoint of sensitivity, the best sequence for free Trp in water proved to be HPE-SOFAST-HMQC, with a $(S/N)_t$ 70% higher than HPE-SE-HSQC and 20% higher than HPE-HMQC for Trp in water.

Interestingly, the majority of the tested sequences shows statistically significant increases in both photo-CIDNP enhancement and $(S/N)_t$ as Trp is switched from pure water to 6 M urea (Fig. 3b). The statistical significance of the difference between pairs of means (for corresponding Fig. 3a and 3b data with unknown and unequal population variances) was tested at 90% confidence level³⁶. The effect of urea is further examined below.

Next, we demonstrated the applicability of our results to a biomolecular system of relevance. We probed the nature and magnitude of Trp indole photo-CIDNP enhancements in the context of apoHmpH, a Trp-containing model protein. ApoHmpH is the apo- form of the heme-binding domain of *E.coli* flavohemoglobin⁴⁰. It is a monomeric globin containing only one Trp residue (W_{120}). Fluorescence emission spectra of apoHmpH indicate that the Trp is solvent-exposed in the urea unfolded state but buried in a hydrophobic core in the native protein³⁰. Urea-unfolded ¹⁵N-apoHmpH is thus an appropriate model system to evaluate the photo-CIDNP enhancements available from various heteronuclear correlation pulse sequences.

1D photo-CIDNP light and dark spectra of ¹⁵N-apoHmpH in 6 M urea (HPE-SOFAST-HMQC sequence) are shown in Figure 4. The S/N of the light spectrum for the Trp indole H^{e1} resonance is 1.8-2.5 fold higher than the S/N of the dark spectrum. Figure 5 compares the S/N and photo-CIDNP enhancements of unfolded apoHmpH across all heteronuclear correlation pulse sequences. The apoHmpH enhancements are significantly larger than the enhancements of free

Trp in water (Fig. 3a). The $(S/N)_t$ trends for the light spectra as a function of pulse sequence are, however, very similar.

HPE-SOFAST-HMQC is by far the most sensitive photo-CIDNP heteronuclear correlation sequence. It is 2.1 fold more sensitive than the parent SOFAST-HMQC for the detection of Trp in apoHmpH (Fig. 4) and 30% more sensitive than HPE-SE-HSQC (Fig. 5). The relative sensitivity increases for the remaining sequences are more moderate. In summary, incorporation of photo-CIDNP laser irradiation into heteronuclear correlation pulse sequences provides up to two-fold sensitivity enhancements relative to the parent sequences lacking photo-CIDNP (at 1 W laser power), for the detection of solvent-exposed Trp in a protein.

The photo-CIDNP data on Trp in 6 M urea (Fig. 3b) serve the additional purpose of testing whether the larger photo-CIDNP enhancements observed for urea-unfolded apoHmpH could be due to the fact that Trp is embedded into the protein scaffold or, more simply, to the presence of urea in solution. As seen above, the photo-CIDNP enhancements of Trp in urea are significantly higher than those of Trp in water for the majority of the sequences analyzed (Fig. 3a and b). This result suggests that the presence of urea is at least partially responsible for the observed increased enhancements. In addition, other effects may be more specifically due to the presence of a protein-type context, especially in the case of HPE-SOFAST-HMQC (see also Discussion).

Understanding photo-CIDNP enhancement trends: Theoretical considerations

The observed pulse sequence-dependent trends in photo-CIDNP enhancements can be rationalized on the basis of a simple theoretical model for photo-CIDNP^{24,25}. In the presence of continuous low power illumination in a strong magnetic field, photo-CIDNP can be modeled as a source of constant variation in the time-dependent longitudinal ^1H and ^{15}N magnetizations. Photo-CIDNP pumping is thus regarded as a zeroth order process occurring at rates p_H and p_N , for ^1H and ^{15}N , respectively.

The equations of motion for the longitudinal operators H_z , N_z and $2H_zN_z$ in the case of a ^1H - ^{15}N spin pair and in the absence of the photochemical reaction (i.e., under dark conditions) were introduced by Goldman⁴¹. In the presence of photo-CIDNP, these equations are modified as^{24,25}:

$$\begin{aligned}\frac{dH_z}{dt} &= p_H - \rho_H (H_z - H_{z0}) - \sigma_{NH} (N_z - N_{z0}) \\ \frac{dN_z}{dt} &= p_N - \rho_N (N_z - N_{z0}) - \sigma_{NH} (H_z - H_{z0}) - \eta_z (2H_zN_z) \\ \frac{d(2H_zN_z)}{dt} &= \rho_{NH} (2H_zN_z) - \eta_z (N_z - N_{z0})\end{aligned}\quad (5)$$

where ρ_H , ρ_N and ρ_{NH} are the autorelaxation rate constants for the H_z , N_z and $2H_zN_z$ operators; σ_{NH} is the ^1H - ^{15}N cross-relaxation rate constant, η_z is the interference rate constant that describes the buildup of the non-equilibrium longitudinal two spin-order operator $2H_zN_z$.

Numerical values for the above rate constants were estimated from spectral density functions ($J(\omega)$), given the experimental N-H bond length ($r_{NH} = 1.02 \text{ \AA}$) and rotational correlation times (τ_c) for global tumbling.

$$\rho_H = \frac{d^2}{4} (3J(\omega_H) + J(\omega_H - \omega_N) + 6J(\omega_H + \omega_N)) \quad (6)$$

$$\rho_N = \frac{d^2}{4} (3J(\omega_H) + J(\omega_H - \omega_N) + 6J(\omega_H + \omega_N)) + c^2 J(\omega_N) \quad (7)$$

$$\rho_{NH} = \frac{3d^2}{4} (J(\omega_H) + J(\omega_N)) + c^2 J(\omega_N) \quad (8)$$

$$\sigma_{NH} = \frac{d^2}{4} (6J(\omega_H + \omega_N) - J(\omega_H + \omega_N)) \quad (9)$$

$$\eta_z = \sqrt{3}cd(J(\omega_N)), \quad (10)$$

where $c = \gamma_N \eta (\sigma_{||} - \sigma_{\perp})$ and $d = -\frac{\mu_0}{4\pi} \left(\frac{\gamma_H \gamma_N \hbar}{r_{NH}^3} \right)$. γ_H and γ_N are the gyromagnetic ratios of 1H and ^{15}N and ω_H and ω_N , the corresponding Larmor frequencies, respectively. The chemical shift anisotropy (CSA) tensor of ^{15}N is assumed to be axially symmetric, with its symmetry axis parallel to the N-H bond vector⁴². The ^{15}N CSA tensor value for the Trp indole NH is assumed to be -89.2 ppm⁴³.

The well-known expression for the spectral density function of an isotropically tumbling molecule in the absence of local motions is

$$J(\omega) = \frac{2}{5} \left(\frac{\tau_c}{1 + \omega^2 \tau_c^2} \right). \quad (11)$$

Relation (11) was used to estimate spectral densities at the different relevant frequencies (ω_H , ω_N , $\omega_H - \omega_N$ and $\omega_H + \omega_N$) from known Larmor frequencies and rotational correlation times. The τ_c for Trp in water is 50 ps²⁴. The τ_c for Trp in 6 M urea (68 ps) was computed from the τ_c in water estimated from the Stokes-Einstein equation and the 1.36-fold larger viscosity of aqueous 6 M urea over pure water. The resulting numerical values of ρ , σ and η for Trp in water and in 6 M urea are listed in Table 1. In addition to affecting rotational correlation times and spectral densities, increases in viscosity also result in larger p_H and p_N (see Discussion).

HPE-SE-HSQC and NPE-SE-HSQC (^{15}N -photo-CIDNP-enhanced HSQC¹⁶) were used as reference sequences to compute the 1H and ^{15}N photo-CIDNP pumping rates p_H and p_N (see Materials and Methods), respectively. Experimental values of $(S/N)_t$ under dark conditions and p_H and p_N , determined as mentioned above, were entered in the numerical integration routine to yield predicted $(S/N)_t$ values under light conditions at $t=0.5s$ for each of the different pulse sequences.

Comparison between experimental and computed photo-CIDNP enhancements

Table 2 compares the experimental and computed 1H photo-CIDNP enhancements P for all the HPE sequences discussed in this study. The fully experimentally determined values of P are overall in very good agreement with the values computed using the theoretical photo-CIDNP model outlined above.

The data in Table 2 illustrate the fact that, while the extent of photo-CIDNP is pulse sequence-independent, the observed sensitivities of the HPE pulse sequences can indeed be different. This is a consequence of the pulse sequence dependence of $(S/N)_t$ under dark conditions. The above explains why the application of ^1H photo-CIDNP to the highly sensitive SOFAST-HMQC results in the large $S/N)_t$ observed for HPE-SOFAST-HMQC under light conditions. The agreement between computed and experimental P_s is excellent for all pulse sequences except for HPE-H. The reasons behind the lack of agreement in the latter case are not entirely clear at present.

The application of computational predictions of photo-CIDNP enhancements to the case of the urea-unfolded apoHmpH protein is more complex, and would involve several approximations and assumptions. For instance, the motions of unfolded proteins are typically highly anisotropic and cannot generally be described by a unique effective rotational correlation time describing global correlation time and rapid site-specific independent internal motions. Therefore, model-free formulas⁴⁴ for spectral density functions cannot generally be applied, rendering any estimation of ρ , σ and η unreliable. In addition, the nature of the Trp side chain local motions is poorly studied and it would be hard to postulate any model for the interpretation of these motions. We have therefore chosen not to report predicted photo-CIDNP enhancements of unfolded apoHmpH.

Trp Photodegradation in photo-CIDNP experiments

Hoch and coworkers reported that the photochemical modification of free Trp and Trp residues in peptides upon laser irradiation in the presence of flavin can be a major problem in photo-CIDNP NMR⁴⁵. Therefore, we tested for evidence of Trp photodegradation in the low power laser irradiation experiments described here. The integrity of Trp and apoHmpH samples was probed before and after the photo-CIDNP experiments by both NMR and analytical HPLC. No evidence of photodegradation was observed within the detection sensitivity of either of the above techniques in free Trp or in apoHmpH, for the duration of laser illumination used in this work (~ 120 1 W laser pulses of 500 ms duration each; HPLC data not shown). The total exposure of the Trp sample to laser irradiation is 25% longer than the one used by Connolly *et al.*⁴⁵, who observed evidence of photodegradation with a 2 W laser.

To further characterize photodegradation at even longer irradiation times, we acquired a series of 1D ^1H spectra of Trp in water, irradiating the sample with 6 pulses of 500 ms 1 W laser light between each 1D spectrum. The $\text{H}^{\epsilon 3}$, $\text{H}^{\zeta 2}$, $\text{H}^{\zeta 3}$ and $\text{H}^{\eta 2}$ Trp resonance intensities are plotted against number of laser pulses in Figure 6a. A significant ($\sim 10\%$) decrease in resonance intensity is observed only after prolonged laser irradiation (600 pulses) at the low 1 W laser power. Less than 5% decrease in resonance intensity is observed up to 200 laser pulses, indicating that photodegradation in this irradiation regime is negligible.

Both photobleaching of flavin and photodegradation of Trp may result in the reduction of photo-CIDNP enhancements and overall sensitivity. The effect of the above parameters was probed by collecting a series of alternating light and dark HPE-SOFAST-HMQC data on Trp in water. The indole $\text{H}^{\epsilon 1}$ intensities of light and dark HPE-SOFAST-HMQC spectra as a function of number of laser irradiation pulses are plotted in Figure 6b. The plot is linear and less than 5% reduction in sensitivity is detected, over 200 laser pulses (i.e., the maximum number of pulses used for the experiments reported in Figures 2-5). The average reduction in signal intensity is moderate even for experiments involving more laser pulses, with an overall 0.025 % reduction per laser pulse. The fact that the dark and light spectra have similar slopes also suggests that the observed small reduction in intensity is due primarily to photodegradation of Trp. Any concurrent flavin photobleaching, if present, is not responsible for the slightly reduced photo-CIDNP enhancements. In summary, photodegradation and photobleaching do

not significantly influence photo-CIDNP enhancements arising from solvent-exposed Trp side chains for samples exposed to low power laser irradiation up to a few hundred laser pulses.

2D ^1H photo-CIDNP-enhanced heteronuclear correlation spectroscopy

In order to demonstrate the feasibility of HPE heteronuclear correlation spectroscopy in 2D data collection mode, we acquired representative 2D photo-CIDNP dark and light data. The most sensitive HPE-SOFAST-HMQC sequence was used and 2D dark and light data were collected on ^{15}N -apoHmpH (Fig. 7). As evidenced from the stacked plot of the light spectrum (Fig. 7d), no significant t1 noise is observed.

Photo-CIDNP enhancements were computed from ^1H slices of the 2D spectra as detailed in the Materials and Methods. The resulting photo-CIDNP enhancement of 1.79 ± 0.01 (averaged over two independent samples) in 2D HPE-SOFAST-HMQC spectra of Trp in 6 M urea are identical, within experimental error, to the enhancements (1.7 ± 0.1) obtained from the corresponding 1D spectra.

In summary, we have shown that the HPE-SOFAST-HMQC pulse sequence is amenable to 2D data collection.

Discussion

Sensitive detection of Trp in proteins

The sensitivity of all the heteronuclear correlation pulse sequences tested here is enhanced by photo-CIDNP. This effect supports the applicability of photo-CIDNP as a sensitivity enhancement technique in heteronuclear correlation spectroscopy for the detection of solvent-exposed Trp in polypeptides and proteins. This tool can prove particularly useful in ligand-binding and protein folding investigations involving modifications in the degree of Trp solvent exposure, especially when high sensitivity is desirable.

Enhancement factors range from 1.8 to 2.5, depending on the sequence. HPE-SOFAST-HMQC is the most sensitive sequence for the detection of solvent-exposed Trp, offering two-fold greater $(\text{S/N})_t$ than the parent SOFAST-HMQC, and a 30% larger $(\text{S/N})_t$ than HPE-SE-HSQC.

While the enhancement factors obtained in this study are significant but not dramatically large, it is important to notice that they are expected to become significantly larger at higher laser powers. Recently introduced modifications of the fiber optic tip may further help improving NMR sensitivity by allowing the fiber's tip to reach further into the NMR sample ⁴⁶.

Two-fold sensitivity enhancement translates into a four-fold shorter data collection time. Such reduction in experiment duration is generally beneficial for data collection on samples that need to be dilute either due to lack of material or to scarce solubility. Shorter data collection times are also critical in the case of unstable samples. Urea-unfolded proteins, for instance, need to be handled within 24-28 hrs due to slow urea-induced chemical modifications of Lys and Cys ⁴⁷.

Importantly, our experiments show that HPE data can be collected with up to 200 1 W laser irradiation pulses without incurring significant photodegradation or photobleaching.

The 500 ms relaxation delay (embedding the optimal 500 ms laser irradiation time), employed here in conjunction with a 120° PC9 shaped pulse, is ideally suited for excellent SOFAST-HMQC sensitivity. This value is indeed very close to the 350 ms relaxation delay that provides the maximal achievable SOFAST-HMQC sensitivity in the absence of photo-CIDNP ²⁶.

Hence, the HPE- variant of SOFAST-HMQC enhances the already high sensitivity of this sequence, for the detection of solvent-exposed Trp.

However, if optimal performance towards fast data acquisition is desired (e.g., kinetic or H/D exchange studies²⁷), higher power lasers may be employed to shorten the laser irradiation (and relaxation delay) time necessary to achieve optimal photo-CIDNP enhancements in a shorter time. Clearly, this type of application is most suitable for samples where low concentration is not an issue.

1D versus 2D photo-CIDNP heteronuclear correlation spectroscopy

The data shown here were acquired on 2D pulse sequences with the t_1 evolution delay set to zero, resulting in 1D sequences appropriate to establish proof-of-concept ideas. The number of Trp residues is small for most proteins. In such cases, the efficient 1D HPE-type pulse sequences suffice to provide the necessary resolution to observe independent Trp resonances. However, it is anticipated that extension of the 1D HPE heteronuclear correlation sequences to a 2D mode may be necessary for proteins with multiple overlapping Trp resonances or when chemical shift or linewidth information is desired in the ^{15}N dimension.

This work demonstrates the feasibility of 2D ^1H - ^{15}N HPE heteronuclear correlation data collection (Fig. 7). The absence of t_1 noise illustrates the fact that laser stability, shutter timing response and sample photodegradation are not an issue, under the data collection conditions adopted here. These results are entirely consistent with the feasibility of 2D photo-CIDNP shown by others for a variety of homonuclear^{13,15,48,49} and heteronuclear^{16,17} pulse sequences. While photodegradation and photobleaching set an upper limit to the number of increments that may be obtained in multidimensional photo-CIDNP, this limit can be stretched by a number of approaches, including sample spinning between increments to optimize dye mixing^{15,48} and addition of hydrogen peroxide to reoxidize reduced flavin¹⁶

In addition, protein ^1H -Photo-CIDNP heteronuclear correlation spectra are expected to have only a few resonances localized to a specific region of the NMR spectrum. Such spectral features lend themselves elegantly to fast data acquisition methods⁵⁰, which enable a reduction in the amount of laser irradiation necessary to acquire a 2D photo-CIDNP spectrum and speedier data collection.

Advantages of low laser power

The power of the light source employed in this work (1 W) is lower than the typical power used in the photo-CIDNP literature (2 W or more). For instance, low laser power is at least partially responsible for the lower enhancements seen here for aqueous Trp relative to Lyon *et al.*¹⁶. However, low laser power is not without advantages. The 1 W light source employed in this report appears to significantly reduce the rate of Trp photodegradation relative to published literature reports⁴⁵. In addition, low power lasers are inexpensive and easy to set up. Finally, sample heating effects are negligible at 1 W laser power.

Perturbation of photo-CIDNP enhancements by urea

The magnitude of the photo-CIDNP enhancement of free Trp in the presence of urea is 1.3 ± 0.2 -fold larger than in its absence (Fig. 3). This effect is statistically significant (90% confidence) for the majority of the sequences studied here.

The viscosity of 6 M urea is 1.36-fold higher than the one of water, at 25° C⁵¹. Higher viscosities result in increases in the rotational diffusion coefficient D_r and consequently, increases in rotational correlation time τ_c . Therefore, increases in viscosity affect ρ , σ and η rate constants in equation 5, as shown in Table 1. Increases in viscosity also lead to a larger

translation diffusion coefficient D_t , thereby perturbing the diffusive dynamics and re-encounter probabilities of Trp-flavin radical ion-pair intermediates⁵². Consequently, photo-CIDNP pumping rates p_H and p_N are also increased in the presence of urea.

The computed photo-CIDNP enhancements in the presence of urea reported in Table 2 take into account the effect of increased viscosity both relaxation rate constants and photo-CIDNP pumping rates. The very good agreement between experimental and computed photo-CIDNP enhancements for Trp in water and urea supports the applicability of the theoretical photo-CIDNP framework used in this work.

Except for HPE-SOFAST-HMQC, none of the sequences shows a statistically significant difference (90% confidence level) between the observed photo-CIDNP enhancement of free Trp in urea and unfolded apoHmpH in urea. Therefore, with the exclusion of HPE-SOFAST-HMQC, the above general solution viscosity effect is sufficient to explain the observed trends in water and 6 M urea for both free Trp and apoHmpH. In the case of HPE-SOFAST-HMQC (compare Fig. 3b and 5), however, some additional protein-specific effects, possibly including urea hydrogen-bonding to the protein chain, may also play a role. Further experiments are necessary to settle this issue.

Concluding Remarks

This study investigates the sensitivity enhancements resulting from applying photo-CIDNP to some of the most widely used heteronuclear correlation NMR pulse sequences. Photo-CIDNP-enhanced HPE-SOFAST-HMQC emerges as the most sensitive sequence for detecting solvent-exposed Trp indole side chains. This sequence offers a two-fold sensitivity enhancement (assessed as $(S/N)_t$) of a factor of two for the urea-unfolded apoHmpH protein. Extensions to 2D data acquisition mode and combination of photo-CIDNP heteronuclear correlation sequences with rapid data acquisition approaches show potential for further increasing the sensitivity and rapid detection capabilities of solvent-exposed Trp in biomolecules.

Acknowledgments

We are grateful to Ed Turner for lending us equipment. We thank Charles Fry, Robert Shanks, Andrew Crowther, Stacy Carrier, Gerry Stamm, Tracy Drier and Keith Rein for technical assistance and Fleming Crim, Lucio Frydman, Kevin Gardner and Rolf Boelens for helpful discussions. We thank Jennifer Choi for providing a sample of ^{15}N -labeled apoHmpH. We are also grateful to Nese Kurt and Jamie Ellis for a critical reading of the manuscript. This research was funded by the National Institutes of Health grant GM068538.

References

- (1). Bargon J, Fischer H, Johnsen U. Z. Naturforsch., A: Phys. Sci 1967;22:1551–1555.
- (2). Ward HR, Lawler RG. J. Am. Chem. Soc 1967;89:5518.
- (3). Kaptein R, Dijkstra K, Nicolay K. Nature 1978;274:293. [PubMed: 683312]
- (4). Kaptein R, Oosterhoff JL. Chem. Phys. Lett 1969;4:195.
- (5). Kaptein R, Oosterhoff LJ. Chem. Phys. Lett 1969;4:214.
- (6). Closs GL. J. Am. Chem. Soc 1969;91:4552.
- (7). Closs GL, Trifunac AD. J. Am. Chem. Soc 1970;92:2183.
- (8). Hore PJ, Broadhurst RW. Prog. Nucl. Magn. Reson. Spectrosc 1993;25:345.
- (9). Canet D, Lyon CE, Scheek RM, Robillard GT, Dobson CM, Hore PJ, van Nuland NAJ. J. Mol. Biol 2003;330:397. [PubMed: 12823977]
- (10). Mok KH, Hore PJ. Methods 2004;34:75. [PubMed: 15283917]
- (11). Mok KH, Kuhn LT, Goetz M, Day IJ, Lin JC, Andersen NH, Hore PJ. Nature 447:106. [PubMed: 17429353]
- (12). Hore PJ, Winder SL, Roberts CH, Dobson CM. J. Am. Chem. Soc 1997;119:5049–5050.

- (13). Shapira B, Morris E, Muszkat KA, Frydman L. *J. Am. Chem. Soc* 2004;126:11756. [PubMed: 15382886]
- (14). Goetz M, Mok KH, Hore PJ. *J. Magn. Reson* 2005;177:236. [PubMed: 16153864]
- (15). Scheek RM, Stob S, Boelens R, Dijkstra K, Kaptein R. *Faraday Discuss. Chem. Soc* 1984;78:245.
- (16). Lyon CE, Jones JA, Redfield C, Dobson CM, Hore PJ. *J. Am. Chem. Soc* 1999;121:6505–6506.
- (17). Schlorb C, Mensch S, Richter C, Schwalbe H. *J. Am. Chem. Soc* 2006;128:1802. [PubMed: 16464074]
- (18). Cavanagh, J.; Fairbrother, WJ.; Palmer, AG.; Rance, M.; Skelton, NJ. *Protein NMR spectroscopy*. Vol. Second ed. Elsevier Academic Press; San Diego: 2007.
- (19). Sattler M, Schleucher J, Griesinger C. *Prog. Nucl. Magn. Reson. Spectrosc* 1999;34:93.
- (20). Rajagopalan S, Chow C, Raghunathan V, Fry CG, Cavagnero S. *J. Biomol. NMR* 2004;29:505. [PubMed: 15243181]
- (21). Schanda P, Brutscher B. *J. Am. Chem. Soc* 2005;127:8014. [PubMed: 15926816]
- (22). Szewczak AA, Kellogg GW, Moore PB. *FEBS Lett* 1993;327:261. [PubMed: 7688695]
- (23). Palmer AG, Cavanagh J, Wright PE, Rance M. *J. Magn. Reson* 1991;93:151.
- (24). Hore PJ, Egmond MR, Edzes HT, Kaptein R. *J. Magn. Reson* 1982;49:122.
- (25). Kuprov I, Hore PJ. *J. Magn. Reson* 2004;168:1. [PubMed: 15082243]
- (26). Schanda P, Kupce E, Brutscher B. *J. Biomol. NMR* 2005;33:199. [PubMed: 16341750]
- (27). Schanda P, Forge V, Brutscher B. *Proc. Natl. Acad. Sci. U. S. A* 2007;104:11257. [PubMed: 17592113]
- (28). Mihalyi E. *J. Chem. Eng. Data* 1968;13:179.
- (29). Whitby LG. *Biochem. J* 1953;54:437. [PubMed: 13058921]
- (30). Eun YJ, Kurt N, Sekhar A, Cavagnero S. *J. Mol. Biol* 2008;376:879. [PubMed: 18187151]
- (31). Kaptein, R. Photo-CIDNP studies of proteins. In: Berliner, LJ.; Reuben, J., editors. *Biological magnetic resonance*. Vol. Vol. 4. Plenum Press; New York: 1982. p. 145
- (32). Zhu G, Kong X, Sze K. *J. Magn. Reson* 1998;135:232. [PubMed: 9799699]
- (33). Piotto M, Saudek V, Sklenár V. *J. Biomol. NMR* 1992;2:661. [PubMed: 1490109]
- (34). Ernst, RR.; Bodenhausen, G.; Wokaun, A. *Principles of nuclear magnetic resonance in one and two dimensions*. Oxford University Press; New York: 1989.
- (35). Bevington, PR.; Robinson, DK. *Data reduction and error analysis for the physical sciences*. Vol. Second ed. McGraw-Hill, Inc.; New York: 1992.
- (36). Walpole, RE.; Myers, RH.; Myers, SL.; Ye, K. *Probability and statistics for engineers and scientists*. Vol. Eighth ed. Pearson Prentice Hall; New Jersey: 2007.
- (37). Tugarinov V, Hwang PM, Ollerenshaw JE, Kay LE. *J. Am. Chem. Soc* 2003;125:10420. [PubMed: 12926967]
- (38). Sprangers R, Kay LE. *Nature* 2007;445:618. [PubMed: 17237764]
- (39). Kaptein, R. Chemically induced dynamic nuclear polarization. Theory and applications in mechanistic chemistry. In: Williams, GH., editor. *Advances in free-radical chemistry*. Vol. Vol. V. Academic Press; New York: 1975. p. 319
- (40). Ilari A, Bonamore A, Farina A, Johnson KA, Boffi A. *J. Biol. Chem* 2002;277:23725. [PubMed: 11964402]
- (41). Goldman M. *J. Magn. Reson* 1984;60:437.
- (42). Tjandra N, Szabo A, Bax A. *J. Am. Chem. Soc* 1996;118:6986.
- (43). Cross TA, Opella SJ. *J. Am. Chem. Soc* 1983;105:306.
- (44). Lipari G, Szabo A. *Biophys. J* 1980;30:489. [PubMed: 7260284]
- (45). Connolly PJ, Hoch JC. *J. Magn. Reson* 1991;95:165.
- (46). Kuprov I, Hore PJ. *J. Magn. Reson* 2004;171:171. [PubMed: 15504697]
- (47). Stark GR, Stein WH, Moore S. *J. Biol. Chem* 1960;235:3177.
- (48). Scheek RM, Stob S, Boelens R, Dijkstra K, Kaptein R. *J. Am. Chem. Soc* 1985;107:705.
- (49). Consonni R, Limiroli R, Molinari H, Fusi P, Grisa M, Vanoni M, Tortora P. *FEBS Lett* 1995;372:135. [PubMed: 7556654]

- (50). Freeman R, Kupce E. J. Biomol. NMR 2003;27:101. [PubMed: 12962120]
- (51). Kawahara K, Tanford C. J. Biol. Chem 1966;241:3228. [PubMed: 5912116]
- (52). Burri J, Fischer H. Chem. Phys 1989;139:497.

Fig. 1. ^1H photo-CIDNP-enhanced (HPE) pulse sequences introduced in this study: a) HPE-SE-HSQC, b) HPE-HMQC, c) HPE-SOFAST-HMQC, d) HPE-g/s-HMQC, and e) reference HPE-H. Pulse phases for the first scan are shown. Phase cycling is identical to that of the parent sequences.

Fig. 2.

Variation in S/N of the Trp indole $H^{\epsilon 1}$ resonance as a function of laser irradiation time. Data were acquired on a sample of Trp in 6 M urea (pH 7.5) with the $^1H/^{15}N$ HPE-g/s-HMQC pulse sequence. The relaxation delay, which includes the 500 ms laser irradiation time, was kept constant at 1.5 s. Error bars represent ± 1 standard error of the mean. Measurements were performed on two independent samples.

Fig. 3. Sensitivity $(S/N)_t$ of various $^1\text{H}/^{15}\text{N}$ heteronuclear correlation pulse sequences, defined as the S/N divided by the square root of the total experiment time, for a) ^{15}N -Trp in water and b) ^{15}N -Trp in 6 M urea at pH 6.9. Measurements were averaged over two independent samples, and 10 replicate experiments were carried out for each sample. Uncertainties are expressed as ± 1 standard error of the mean.

Fig. 4.

First row 1D photo-CIDNP a) dark and b) light spectra of urea-unfolded ^{15}N -apoHmpH acquired using the $^1\text{H}/^{15}\text{N}$ HPE-SOFAST-HMQC pulse sequence. 16 transients were collected for each spectrum (preceded by 2 dummy scans). The relaxation delay was set to 502 ms, which includes 500 ms of laser irradiation at 1 W power for the light spectrum. The light spectrum displays a two-fold higher sensitivity than the dark spectrum.

Fig. 5. Sensitivity $((S/N)_t)$, defined as in the Figure 3 legend, of $^1\text{H}\text{--}^{15}\text{N}$ HPE heteronuclear correlation pulse sequences for the indole $\text{H}^{\epsilon 1}$ resonance of Trp_{120} in urea-unfolded apoHmpH. Error bars represent ± 1 standard error of the mean of measurements performed on two independent samples. The vertical scale is not directly comparable to that of Figure 3 due to the different number of transients and sample concentrations.

Fig. 6.

a) Intensity variation of selected $H^{\epsilon 3}$, $H^{\zeta 2}$, $H^{\zeta 3}$ and $H^{\eta 2}$ Trp resonances (95% water, 5% D_2O) upon exposure to an increasing number of laser pulses. Data were collected with a 1D 1H -pulse-acquire sequence with solvent presaturation (1.5 s, 198 Hz rf field strength). Data points were collected consecutively. Each sample was exposed to six 500 ms 1 W laser pulses followed by a 1 s delay before each data acquisition step. b) Intensity variations of Trp indole $H^{\epsilon 1}$ resonances for 1H and ^{15}N HPE-SOFAST-HMQC dark (■) and light (□) data collected in 1D mode (no ^{15}N chemical shift evolution) as a function of increasing exposure to laser irradiation. Dark and light spectra were acquired in an interleaved fashion on the same sample. A 1 min time delay was applied between each light and dark experiment. The solid lines represent linear least-squares fits.

Fig. 7. 2D ^1H - ^{15}N HPE-SOFAST-HMQC spectra of urea-unfolded ^{15}N -apoHmpH (300 μM in 25 mM tris- d_{11} , pH 7.0 containing 6 M urea and 0.2 mM flavin). Contour plots of dark and light spectra are shown in panels a and c, respectively. Corresponding stacked plots are shown in panels b and d.

Table 1

Calculated NMR relaxation rate constants used in the computational prediction of photo-CIDNP enhancements according to (3)._—

Samples	NMR relaxation rate constants			
	ρ_H (s ⁻¹)	ρ_N (s ⁻¹)	ρ_{NH} (s ⁻¹)	σ_{NH} (s ⁻¹)
Trp in water	0.26	0.26	0.16	0.13
Trp in 6 M urea	0.36	0.36	0.22	0.18
				δ (s ⁻¹)
				0.09
				0.12

Table 2

Experimental and computationally predicted photo-CIDNP enhancements.

NMR Pulse Sequence	Samples			
	Trp in water		Trp in 6 M urea	
	Observed	Computed	Observed	Computed
HPE-SE-HSQC ^a	1.3 ± 0.1	1.3	1.8 ± 0.1	1.8
HPE-HMQC	1.3 ± 0.1	1.2	1.5 ± 0.1	1.6
HPE-SOFAST-HMQC	1.2 ± 0.1	1.2	1.7 ± 0.1	1.8
HPE-g/s-HMQC	2.1 ± 0.1	1.8	2.6 ± 0.2	3.2
HPE-H	1.3 ± 0.1	1.4	1.7 ± 0.1	2.4

^aUsed as a reference sequence for computing pH.