1 H and 13 C Hyperfine Coupling Constants of the Tryptophanyl Cation Radical in Aqueous Solution from Microsecond Time-Resolved CIDNP

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · OCTOBER 2007

Impact Factor: 3.3 · DOI: 10.1021/jp073385h · Source: PubMed

5 AUTHORS, INCLUDING:

Alexey Kiryutin
Russian Academy of Sciences
23 PUBLICATIONS **181** CITATIONS
SEE PROFILE

Lars Kuhn
European Neuroscience Institute Göttingen
16 PUBLICATIONS **301** CITATIONS
SEE PROFILE

Alexandra V Yurkovskaya
International Tomographic Center
123 PUBLICATIONS **1,389** CITATIONS
SEE PROFILE

^{1}H and ^{13}C Hyperfine Coupling Constants of the Tryptophanyl Cation Radical in Aqueous Solution from Microsecond Time-Resolved CIDNP

Alexey S. Kiryutin,[†,‡,§] Olga B. Morozova,[†] Lars T. Kuhn,[||] Alexandra V. Yurkovskaya,*[,†,‡,⊥] and P. J. Hore*[,||]

International Tomography Center of SB RAS, Institutskaya 3a, 630090 Novosibirsk, Russia, Institute of Experimental Physics, Free University of Berlin, Arnimallee 14, D-14195, Germany, Novosibirsk State University, Pirogova 2, 630090 Novosibirsk, Russia, Department of Chemistry, University of Oxford, Physical and Theoretical Chemistry Laboratory, South Parks Road, Oxford, OX1 3QZ, United Kingdom, and Center of Magnetic Tomography and Spectroscopy, Moscow State University, Leninskie Gory 1-73, 119992 Moscow, Russia

Received: May 3, 2007; In Final Form: July 6, 2007

Relative values of the ^{1}H and ^{13}C isotropic hyperfine couplings in the cationic oxidized tryptophan radical $TrpH^{\bullet+}$ in aqueous solution are determined. The data are obtained from the photo-CIDNP (chemically induced dynamic nuclear polarization) enhancements observed in the microsecond time-resolved NMR spectra of the diamagnetic products of photochemical reactions in which $TrpH^{\bullet+}$ is a transient intermediate. The method is validated using the tyrosyl neutral radical $Tyr^{\bullet}$, whose ^{1}H and ^{13}C hyperfine couplings have previously been determined by electron paramagnetic resonance spectroscopy. Good agreement is found with hyperfine coupling constants for $TrpH^{\bullet+}$ calculated using density functional theory methods but only if water molecules are explicitly included in the calculation.

Introduction

Tryptophanyl radicals are formed by electron-transfer processes in a number of enzymes, including ribonucleotide reductase,[1−3] DNA photolyase,[4−9] peroxidases,[10−19] oxidases,[20−22] and probably also in cryptochromes[23,24] and in some cases have been identified and characterized by electron paramagnetic resonance (EPR), electron−nuclear double resonance (ENDOR) spectroscopy, or both. Supported by ab initio and density functional theory (DFT) calculations of hyperfine interaction tensors and *g* tensors,[25−30] these measurements have yielded valuable information on the protonation state[1,11] and hydrogen bonding of the indole nitrogen, the electrostatic environment of the radical in the protein, and the side chain torsion angles.[29] Although such reactive radicals can evidently be stabilized in a protein environment and at the low temperatures commonly used for EPR and ENDOR, they seem to be very short-lived in solution, to the extent that EPR data for free tryptophanyl radicals appear to be nonexistent.

A source of experimental information on radicals that are too ephemeral for EPR detection is the phenomenon known as CIDNP (chemically induced dynamic nuclear polarization).[31,32] Observed as anomalous spectral intensities in the NMR (nuclear magnetic resonance) spectra of chemically reacting systems, CIDNP arises from the spin-selective reactivity and magnetic interactions of transient spin-correlated radical pairs. Provided these species live long enough for the hyperfine interactions in the radicals to drive the coherent interconversion of the singlet and triplet radical pair states (in practice, ∼1 ns), the diamagnetic products produced when the radicals recombine often exhibit substantial nuclear hyperpolarizations whose magnitudes reflect the hyperfine couplings in the radicals.

Closs et al.[33,34] have shown that relative hyperfine coupling constants may be obtained straightforwardly from CIDNP intensities provided NMR spectra are measured with sufficient time-resolution, for example using a laser flash photolysis technique. The polarizations observed within about a microsecond of the creation of the radicals arise solely from recombination of geminate radical pairs and are free from confounding effects such as nuclear spin−lattice relaxation in the radicals, degenerate electron-transfer reactions, radical recombination reactions, and polarization generated in random encounter pairs (F pairs).[35,36] In many cases, the geminate polarizations of nuclei in the recombination products are simply proportional to the corresponding hyperfine couplings in the radicals.[37]

^{1}H CIDNP, produced in cyclic photochemical reactions, has been extensively used to probe the accessibility of tryptophan, tyrosine, and histidine amino acid residues in proteins.[31,32,38] The magnitudes of the NMR polarizations observed for different residues reveal the ability of a photoexcited dye, commonly either a flavin[31] or 2,2′-dipyridyl,[36] to undergo electron transfer or hydrogen atom abstraction reactions with the (partially) exposed aromatic side chains. Although there have been many applications of photo-CIDNP to the study of protein−ligand and protein−protein interactions and protein folding,[39,40] no attempt has yet been made to extract hyperfine coupling data for the radicals, whether in proteins or the free amino acids.

Here, we report the determination of the relative values of the isotropic ^{1}H and ^{13}C hyperfine coupling constants of the tryptophanyl cation radical ($TrpH^{\bullet+}$) in water using microsecond time-resolved NMR measurements of the CIDNP produced in

* Corresponding authors. E-mail: yurk@tomo.nsc.ru (A.V.Y.) and peter.hore@chem.ox.ac.uk (P.J.H.).
[†] International Tomography Center of SB RAS.
[‡] Free University of Berlin.
[§] Novosibirsk State University.
[||] University of Oxford.
[⊥] Moscow State University.

10.1021/jp073385h CCC: $37.00
Published on Web 09/01/2007

photoinduced flavin-tryptophan and 2,2′-dipyridyl-tryptophan geminate radical pairs. The approach is first validated using the tyrosyl neutral radical $Tyr^{\bullet}$, whose ^1H and ^{13}C hyperfine couplings have previously been determined by EPR. The results for $TrpH^{\bullet+}$ are compared with hyperfine coupling data calculated by DFT methods explicitly including solvent water molecules.

Experimental Methods

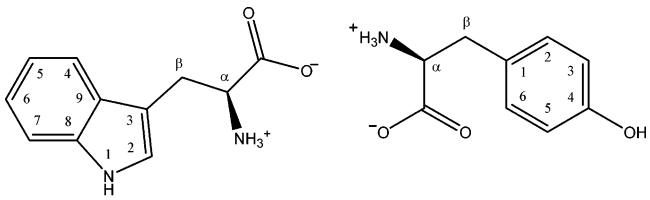
^1H and ^{13}C time-resolved CIDNP experiments were performed on three NMR spectrometers: at the Center of Magnetic Tomography and Spectroscopy, Moscow State University (14.1 T, 600 MHz ^1H frequency); at the International Tomography Center, Novosibirsk (4.7 T, 200 MHz); and at the Free University of Berlin (7.0 T, 300 MHz). The light sources were a frequency-tripled (355 nm, 6 ns, 100−120 mJ) Nd:YAG laser (Brilliant, Quantel) in Moscow and a XeCl excimer laser (308 nm, 15 ns, 100−120 mJ) in Novosibirsk and Berlin. The experimental setup in Novosibirsk and Berlin was as described previously.[41] In Moscow, the light was directed into the NMR sample from above through a cylindrical quartz light guide (outside diameter 4 mm) inserted into the top of the NMR sample tube. To record geminate polarizations, the light flash was immediately followed by a single radiofrequency pulse and acquisition of the ensuing free induction decay. The flip angles and durations of the radiofrequency pulses are quoted in the figure captions. ^1H decoupling was used during ^{13}C signal acquisition only. A long relaxation delay (30 s) between acquisitions ensured that the NMR and CIDNP intensities were proportional to the nuclear polarizations. For the ^1H spectra, the nuclear magnetization prior to the laser flash was destroyed by radiofrequency irradiation so that the resulting spectra had no contribution from the magnetization present at thermal equilibrium. In all cases, a dark spectrum, recorded with a very large number of scans but otherwise under identical conditions, was subtracted from the light spectrum having previously been scaled according to the number of scans. In all other respects, spectra were acquired as previously described.[41]

Uniformly ^{13}C,^{15}N-labeled L-tryptophan (^{13}C 98%, ^{15}N 98%) and L-tyrosine (^{13}C 98%, ^{15}N 98%) were obtained from Cambridge Isotope Laboratories. Samples of L-tryptophan and L-tyrosine at natural isotopic abundance were obtained from Sigma-Aldrich. Flavin mononucleotide (FMN) and 2,2′-dipyridyl (DP; both Sigma-Aldrich) were used as CIDNP dyes. D_2O (^2H 99.9%) was obtained from Deutero GmbH.

The polarizations of the ^1H and ^{13}C nuclei in tryptophan and tyrosine were calculated using the Adrian model for diffusing radical pairs (see Supporting Information for details).[42−44] All ^1H, ^{13}C, ^{14}N, and ^{15}N nuclear spins were included for the radicals $TrpH^{\bullet+}$, $Tyr^{\bullet}$, $FMN^{\bullet-}$, and $DP^{\bullet-}$ using the magnetic parameters listed in Results and in Supporting Information. Apart from two tryptophan carbons that are appreciably strongly coupled (see below), the predicted ^1H and ^{13}C CIDNP intensities were found to be accurately proportional to the corresponding ^1H and ^{13}C hyperfine couplings in the appropriate radicals. This behavior is expected when, as is the case here, the difference in the electron Zeeman interactions of the two radicals is much larger than any of the hyperfine couplings. We have verified that at high magnetic field and for low viscosity solvents, the spectra calculated on the simple Adrian model[45] agree well with simulations made using an exact quantum mechanical treatment with more realistic treatments of the diffusive motion of the radicals.[46,47]

NMR and CIDNP spectral simulations were performed using the program Nuts95 (NMR Utility Transform Software, Acorn NMR). In each of the four cases studied (^1H and ^{13}C spectra of both tyrosine and tryptophan), the best fit to the experimental CIDNP spectrum was obtained by varying the values of the hyperfine couplings of the polarized nuclei (and hence their CIDNP intensities, calculated as outlined in the previous paragraph), keeping all other parameters fixed. This optimization was performed in Matlab 6.1. To ensure the reliability of the relative hyperfine couplings of the polarized nuclei, their absolute values and those of the two g values were varied over a wide range. No change was found in the relative values of the hyperfine couplings that were required to achieve optimum agreement with the experimental spectra. Carbons C4 and C5 in $TrpH^{\bullet+}$ were treated as a strongly coupled AB system; all other nuclear spins were assumed to be weakly coupled. The structures and numbering schemes used for tryptophan and tyrosine are shown in Chart 1.

CHART 1



Tryptophan Tyrosine

DFT methods were used to calculate the geometry and isotropic hyperfine couplings of the zwitterionic forms of the $TrpH^{\bullet+}$, $Trp^{\bullet}$, $TyrH^{\bullet+}$, and $Tyr^{\bullet}$ radicals using Gaussian03.[48] Preliminary geometry optimization was performed in vacuum using the B3LYP/6-31G(d,p) method. The vacuum hyperfine tensor calculation used the resulting geometry directly. After the Hessian positive definiteness check, the hyperfine tensors were computed at the UB3LYP/EPR-II level of theory. The explicit water hyperfine tensor calculation used the vacuum geometry as a starting point for the repeated vacuum reoptimization of the radical with water molecules added one by one to cap the lone pairs and saturate the hydrogen bonds around the polar groups. A PCM (UAKS) B3LYP/6-31G(d,p) reoptimization was performed on the resulting complex. Hessian eigenvalues were checked within the same method, and the hyperfine tensors were then computed on a PCM (UAKS) UB3LYP/EPR-II level of theory. The explicit + PCM approach described above is known to lead to a significant improvement in the quality of hyperfine tensors.[49] Moderate hyperfine couplings are well-predicted, and there are small deviations for the larger ones because of the lack of vibrational averaging in the calculation. Supporting Information contains the isotropic hyperfine coupling constants of all magnetic nuclei in $TrpH^{\bullet+}$, $Trp^{\bullet}$, $TyrOH^{\bullet+}$, and $Tyr^{\bullet}$.

Results

Tyrosine CIDNP. To validate the use of photo-CIDNP for the determination of relative hyperfine coupling constants of amino acid radicals, we first studied the neutral tyrosyl radical, $Tyr^{\bullet}$ (tyrosine with the phenolic H atom removed), whose magnetic properties are known from EPR.[50,51] [$Tyr^{\bullet}$ $DP^{\bullet-}$] radical pairs were produced in aqueous solution at pH 11.2 by electron transfer from deprotonated tyrosine anion to photoexcited triplet DP (the pK_a of tyrosine is ∼10.3):

$$Tyr^- + {}^3DP \rightarrow {}^3[Tyr^{\bullet}\ DP^{\bullet-}] \leftrightarrow {}^1[Tyr^{\bullet}\ DP^{\bullet-}] \rightarrow Tyr^- + DP$$

Nuclear polarization arises from the back electron transfer of the singlet radical pair which regenerates the ground state reactants, as indicated.[52]

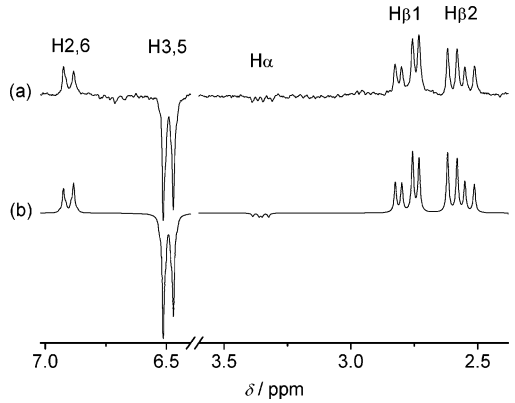


Figure 1. (a) Geminate 200 MHz ^{1}H CIDNP spectrum of 5 mM tyrosine in D_2O with 10 mM DP at pH 11.2. Laser output energy 115 mJ; radiofrequency pulse 1 μs, 18° flip angle; 320 scans. (b) Simulated ^{1}H CIDNP spectrum obtained using the parameter values listed in Table 1 and Supporting Information.

TABLE 1: ^{1}H Hyperfine Coupling Constants for the Tyrosyl Neutral Radical

proton	relative HFC[a] (CIDNP)	HFC[b,d] (EPR)	HFC[b] (DFT, in water)
H2,6	0.20	0.15	0.197
			0.209
H3,5	−0.85	−0.65	−0.580
			−0.654
Hα	−0.16	−0.04	−0.026
Hβ1	1.00[c]	0.77[c]	0.520
Hβ2			0.725

[a] Scaled to 1.00 for Hβ. [b] Millitesla. [c] Two β protons are taken together here. [d] Taken from ref 50. The signs come from the CIDNP sign rule[57] and the DFT calculations presented here.

Figure 1a shows the ^{1}H CIDNP spectrum of tyrosine in which the relative intensities and phases (absorption or emission) of the various resonances reflect the magnitudes and signs of the hyperfine interactions of the corresponding protons in the tyrosyl radical. Similar spectra have been recorded before.[52,53] Figure 1b is a simulation of the experimental spectrum using the NMR parameters (chemical shifts, spin−spin couplings, and linewidths) and EPR parameters (hyperfine couplings and g values) listed in Supporting Information. As anticipated,[37] the values of the hyperfine coupling constants required for an accurate simulation are proportional to the intensities of the corresponding peaks in the CIDNP spectrum once allowance has been made for the various numbers of equivalent protons responsible for each peak. Table 1 and Figure 2a,b compare the relative hyperfine couplings obtained from the simulation with the values of the hyperfine couplings measured previously by EPR[50] and those calculated using DFT methods. Good correlation between the two sets of values is found in both cases (correlation coefficients: $r = 0.997$ and $r = 0.985$, respectively).

Figure 3 shows (a) the ^{13}C CIDNP spectrum of tyrosine using DP as dye together with (b) a simulation. Very similar geminate ^{13}C CIDNP spectra were obtained using 3-carboxybenzophenone, anthraquinone sulfonate, and FMN at 4.7 and 7.0 T (not shown). As in the ^{1}H case, the CIDNP intensities for each carbon are proportional to the corresponding hyperfine interactions in Tyr$^\bullet$ and essentially independent of all other couplings. As shown in Table 2 and Figure 2c,d, the agreement with EPR[51] and DFT data is once again very good (correlation coefficients: $r = 0.916$ and $r = 0.987$, respectively).

In summary, the photo-CIDNP method yields reliable values of the relative ^{1}H and ^{13}C hyperfine couplings in Tyr$^\bullet$, giving confidence that the approach can confidently be used to investigate TrpH$^{\bullet+}$ generated by a similar cyclic photochemical reaction. It should be noted that one cannot determine absolute values of the hyperfine couplings without calibration of the

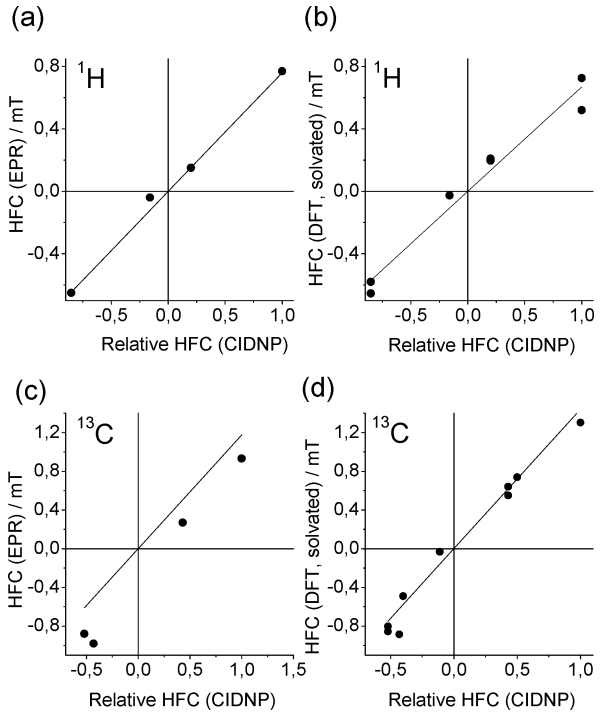


Figure 2. Comparison of the relative hyperfine couplings of Tyr$^\bullet$ obtained from CIDNP data with absolute values of the coupling constants from other sources. (a,b) ^{1}H data. (c,d) ^{13}C data. (a,c) Comparison with EPR data. (b,d) Comparison with DFT values (see Table 1). The sloping lines are linear regression fits to the data, constrained to go through the origin.

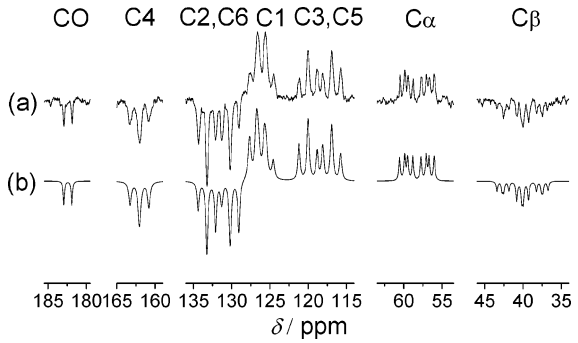


Figure 3. (a) Geminate 50.2 MHz ^{13}C CIDNP spectrum of uniformly ^{13}C, ^{15}N labeled 5 mM tyrosine in D_2O with 10 mM DP at pH 11.2. Laser output energy 115 mJ; radiofrequency pulse 4 μs, 90° flip angle; 1024 scans. (b) Simulated ^{13}C CIDNP spectrum obtained using the parameter values listed in Table 2 and Supporting Information.

TABLE 2: ^{13}C Hyperfine Coupling Constants for the Tyrosyl Neutral Radical

carbon	relative HFC[a] (CIDNP)	HFC[b,c] (EPR)	HFC[b] (DFT, in water)
C1	1.00	0.93	1.301
C2,6	−0.52	−0.88	−0.804
			−0.857
C3,5	0.43	0.27	0.550
			0.639
C4	−0.43	−0.98	−0.885
Cα	0.50	n/a[d]	0.739
Cβ	−0.40	n/a[d]	−0.492
CO	−0.11	n/a[d]	−0.033

[a] Scaled to 1.00 for C1. [b] Millitesla. [c] Taken from ref 51. [d] Not available in ref 51.

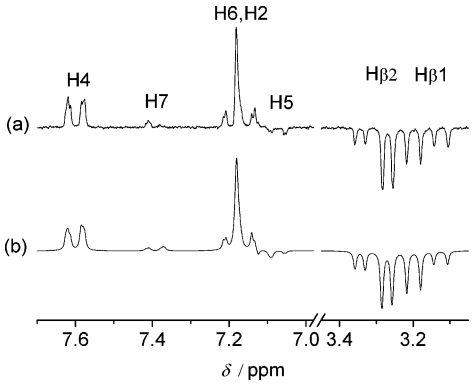


Figure 4. (a) Geminate 200 MHz ^{1}H CIDNP spectrum of 1 mM tryptophan in D_2O with 0.4 mM DP at pH 3.0. Laser output energy 85 mJ; radiofrequency pulse 1 μs, 13° flip angle; 384 scans. (b) Simulated ^{1}H CIDNP spectrum obtained using the parameter values listed in Table 3 and Supporting Information.

CIDNP intensities, a nontrivial task unless the CIDNP spectrum contains signals from hyperpolarized nuclei whose hyperfine couplings are independently known.

Tryptophan ^{1}H CIDNP. The tryptophanyl cation radical $TrpH^{\bullet+}$ was generated in the same way as $Tyr^{\bullet}$ using either FMN or DP as the dye (D):

$$TrpH + {}^3D \rightarrow {}^3[TrpH^{\bullet+}\ D^{\bullet-}] \leftrightarrow {}^1[TrpH^{\bullet+}\ D^{\bullet-}] \rightarrow TrpH + D$$

Back electron transfer in the singlet radical pair, $^1[TrpH^{\bullet+}\ D^{\bullet-}]$, is responsible for the nuclear polarization such that the observed CIDNP intensities reflect the magnetic properties of $TrpH^{\bullet+}$ rather than its deprotonated form, $Trp^{\bullet}$ (minus the N1 indole proton).[36,53] The deprotonation of $TrpH^{\bullet+}$ ($pK_a \approx 4.3$[54]) at pH 7−9 is expected to occur on a much slower time scale than the lifetime of the primary radical pair which is likely to be ~10 ns under the conditions of our measurements. For example, Brydin et al.[55] have found that the cation radical of a solvent-exposed tryptophan residue in *E. coli* DNA photolyase loses its indole proton to bulk water with a time constant of 300 ns that is independent of pH in the range 5.4−8.6. The radicals that escape from the geminate $[TrpH^{\bullet+}\ D^{\bullet-}]$ radical pair will deprotonate, but they cannot affect the CIDNP observed at ~1 μs because the recombination of bulk $Trp^{\bullet}$ and $D^{\bullet-}$ radicals is roughly 2 orders of magnitude too slow (rate constant $\approx 10^9\ M^{-1}\ s^{-1}$, radical concentration $\approx 10^{-5}$ M)[56] to interfere with the measurement. Further evidence for the lack of interference from $Trp^{\bullet}$ comes from the microsecond time-resolved ^{1}H CIDNP spectra (not shown) for tryptophan at a pH (3.0) where $TrpH^{\bullet+}$ does not deprotonate, which are essentially identical to those presented below at pH 9.2. This observation also excludes the possibility that degenerate electron exchange between TrpH and $TrpH^{\bullet+}$ has a significant effect on the spectra at ~1 μs.

Figure 4 shows (a) the geminate 200 MHz ^{1}H photo-CIDNP spectrum of tryptophan using DP as dye together with (b) a simulation calculated using the relative hyperfine coupling constants given in Table 3 and the chemical shifts, spin−spin coupling constants, and linewidths listed in Supporting Information. An essentially identical spectrum was observed using FMN instead of DP (not shown). No other resonances were observed in Figure 4a except those from the dye confirming that the photochemical reactions are indeed cyclic and that the polarization arises solely from tryptophan−DP or tryptophan−FMN radical pairs, with no significant side reactions.

Once again, the relative hyperfine couplings needed to match the simulation to the experimental spectrum were found to be directly proportional to the corresponding CIDNP intensities. Kaptein's rule[57] for the nuclear polarization in the recombination product of a triplet radical pair, with $g(FMN^{\bullet-}) > g(TrpH^{\bullet+})$ or $g(DP^{\bullet-}) > g(TrpH^{\bullet+})$ predicts absorptive polarization for nuclei with negative hyperfine coupling constants and emissive polarization for those with positive couplings.

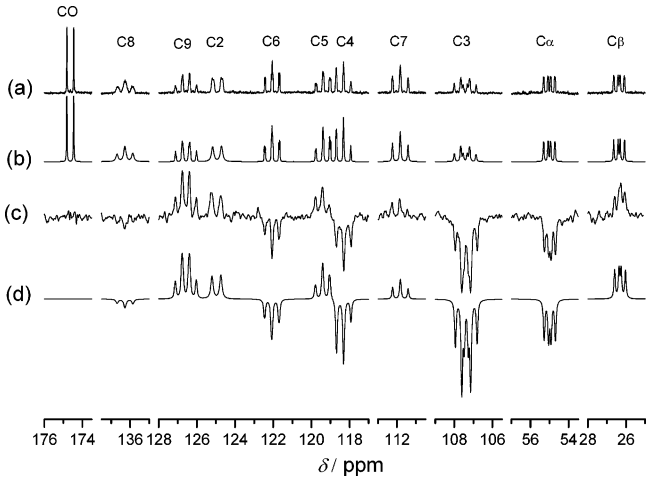


Figure 5. (a) Experimental 150.9 MHz ^{13}C NMR spectrum of 3 mM uniformly ^{13}C,^{15}N labeled L-tryptophan in D_2O with 0.3 mM FMN at pH 9.0 recorded with ^{1}H decoupling during signal acquisition. 4096 scans. (b) Simulated ^{13}C NMR spectrum. (c) Geminate ^{13}C CIDNP spectrum, laser output energy 100 mJ; radiofrequency pulse 4 μs, 90° flip angle; 512 scans. (d) Simulated ^{13}C CIDNP spectrum obtained using the parameter values listed in Supporting Information. The intensities of spectra a and c have been scaled independently.

TABLE 3: ^{1}H Hyperfine Coupling Constants for the Tryptophanyl Cation Radical

proton	relative HFC[a] (CIDNP)	HFC[b] (DFT, in vacuo)	HFC[b] (DFT, in water)
H1	n/a[c]	−0.598	−0.413
H2	−0.525	−0.278	−0.421
H4	−0.495	−0.488	−0.504
H5	0.145	−0.040	0.124
H6	−0.49	−0.208	−0.412
H7	−0.07	−0.364	−0.050
Hα	0	−0.093	0.001
Hβ1	1.00	1.605	2.544
Hβ2	0.69	0.046	1.189

[a] Scaled to 1.00 for Hβ1. [b] Millitesla. [c] Not available in D_2O.

The observed pattern of signal enhancements matches that observed previously using continuous wave irradiation: significant polarization for H2, H4, H6, and Hβ and little for H5, H7, and Hα.[32] Strong multiplet CIDNP effects are not expected or observed because the difference in EPR frequencies of the two radicals greatly exceeds all of the hyperfine coupling frequencies. Further discussion of the hyperfine couplings is given below.

Tryptophan ^{13}C CIDNP. Figure 5 shows (a) the ^{1}H-decoupled ^{13}C NMR spectrum and (c) the geminate photo-CIDNP spectrum of uniformly ^{13}C, ^{15}N labeled L-tryptophan in D_2O. The assignments were confirmed by recording a two-dimensional ^{1}H−^{13}C HMQC spectrum (see Supporting Information). As expected, ^{13}C polarization is not observed for the FMN molecule at natural ^{13}C isotopic abundance. The NMR data obtained by simulation of the NMR spectrum, Figure 5b, are tabulated in Supporting Information. To within 10%, all 11 multiplets in the NMR spectrum (Figure 5a) have identical integrals.

Once again, absorptive (emissive) ^{13}C polarization is expected for nuclei with negative (positive) hyperfine coupling con-

TABLE 4: ^{13}C Hyperfine Coupling Constants for the Tryptophanyl Cation Radical

carbon	CIDNP intensity[a]	relative HFC[a] (CIDNP)	HFC[b] (DFT, in vacuo)	HFC[b] (DFT, in water)
C2	0.19	−0.19	−0.280	−0.227
C3	−1.00	1.00	0.629	1.254
C4	−0.41	0.46	0.475	0.619
C5	0.24	−0.29	−0.233	−0.499
C6	−0.27	0.27	0.032	0.443
C7	0.11	−0.11	0.290	−0.182
C8	−0.07	0.07	−0.359	0.092
C9	0.43	−0.43	−0.545	−0.891
Cα	−0.45	0.45	0.008	0.025
Cβ	0.28	−0.28	−0.357	−0.512
CO	0.00	0.00	0.027	−0.045

[a] Scaled to ± 1.00 for C3. [b] Millitesla.

stants.[57] The use of 90° flip angle pulses suppresses any small multiplet polarizations except for strongly coupled pairs of nuclei (see below). The signal enhancement arising from the CIDNP effect can be estimated as approximately 300.

Figure 5d shows the best-fit simulation of the experimental CIDNP spectrum using the chemical shifts, spin−spin couplings, and linewidths obtained by simulating the NMR spectrum and varying only the ^{13}C hyperfine coupling constants of TrpH$^{\bullet+}$ (which control the intensities of the individual carbon multiplets, given in Table 4). The following g values were used in the simulation: TrpH$^{\bullet+}$, $g = 2.0027$[58] and FMN$^{\bullet-}$, $g = 2.0034$.[59,60] The relative values of the ^{13}C hyperfine couplings so obtained are also shown in Table 4. Apart from the strongly coupled pair of carbons (C4 and C5), the relative hyperfine couplings were found to be identical to the corresponding relative CIDNP intensities. The ^{1}H-coupled NMR and CIDNP spectra could be very satisfactorily simulated using identical parameters to Figure 5d, with the addition of the ^{1}H−^{13}C spin−spin coupling constants (not shown). Using the same parameter values, we were also able to simulate very satisfactorily the ^{13}C CIDNP spectra of tryptophan recorded at 4.7 T with DP and at 7.0 T with FMN (not shown).

Tryptophan DFT Calculations. Tables 3 and 4 show the results of DFT calculations of respectively the ^{1}H and ^{13}C hyperfine coupling constants for TrpH$^{\bullet+}$ both in vacuo and with explicit water molecules. Although the relative couplings derived from CIDNP show a rather poor correlation with the in vacuo data (Figure 6a,c, $r = 0.629$ and 0.817), the agreement is much more impressive when water molecules are explicitly included (Figure 6b,d, $r = 0.990$ and 0.981). The correlation coefficients for b, c, and d were calculated with the constraint that the best-fit line went through the origin. The data for the Cα and Hβ nuclei have been omitted from both Figure 6 and the calculation of the correlation coefficients. The lack of agreement here is not surprising given that the hyperfine couplings have been calculated for the lowest energy conformation of TrpH$^{\bullet+}$ with no attempt to perform a thermal average over the Cα−Cβ or Cβ−C1 torsion angles. Supporting Information contains the full set of DFT isotropic hyperfine coupling constants.

Discussion

Relative values of the isotropic ^{1}H and ^{13}C hyperfine coupling constants of the cationic tryptophan radical in water have been determined. This appears to be the first characterization of the magnetic properties of a tryptophan radical outside of the stabilizing environment of a protein. The lack of reported EPR observations of free TrpH$^{\bullet+}$ (or of its neutral form, Trp$^{\bullet}$) may be due to a number of factors that render the spectra broad and weak and therefore difficult to detect. (a) Both TrpH$^{\bullet+}$ and Trp$^{\bullet}$ are likely to be rather short-lived in solution, perhaps <100 ns. Protein-bound TrpH$^{\bullet+}$ deprotonates in 200−300 ns[55,61] and presumably more rapidly for free TrpH$^{\bullet+}$. (b) Both radicals have a multitude of hyperfine splittings which are unlikely to be well-resolved, causing the available EPR intensity to be spread over a broad spectral range. (c) Rapid degenerate electron exchange with the parent tryptophan molecule can lead to further line broadening in the case of the cation radical. The rate constant of this reaction is essentially diffusion controlled[36] (9×10^8 $M^{-1}s^{-1}$).

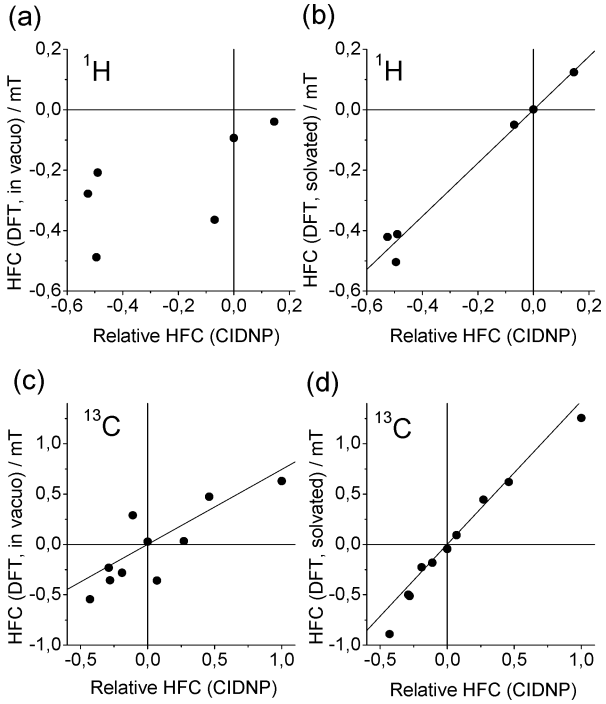


Figure 6. Comparison of the relative hyperfine couplings of Trp$^{\bullet+}$ obtained from CIDNP data compared with DFT values. (a,b) ^{1}H data. (c,d) ^{13}C data. (a,c) DFT calculations in vacuo. (b,d) DFT calculations in water. The sloping lines are linear regression fits to the data, constrained to go through the origin.

The method used here to obtain hyperfine coupling data for TrpH$^{\bullet+}$ entails quantitative detection of nuclear polarization in the diamagnetic products formed by recombination of spin-correlated [TrpH$^{\bullet+}$ D$^{\bullet-}$] radical pairs. Even though the radicals are very short-lived, their legacy is straightforwardly detectable "downstream" in the time-resolved NMR spectra of their chemically stable reaction products, provided the experiment has sufficient time resolution (in practice ∼1 μs) to avoid confounding effects from F-pair polarization, degenerate electron-transfer reactions, radical recombination, and nuclear spin relaxation in the radicals.[32] This approach should be generally applicable, provided one can be confident that the observed polarizations derive exclusively from the radical of interest. Determination of the relative hyperfine coupling constants is particularly simple at high field, where the difference in electron Larmor frequencies of the two radicals greatly exceeds their hyperfine couplings, because the relative values of the couplings are simply identical to the relative nuclear polarizations in the absence of significant strong coupling among the nuclei. Absolute values of the ^{13}C hyperfine couplings could in principle be determined if the experiment could be repeated using a ^{13}C-labeled flavin (whose hyperfine couplings are known[59,60]) to provide a calibration.

Unfortunately, it is not possible to compare the relative hyperfine couplings measured here for free $TrpH^{\bullet+}$ with those for radicals derived from Trp residues in proteins. To the best of our knowledge, the only protein-bound tryptophan radicals that have been characterized by EPR/ENDOR are neutral Trp radicals, in particular, in mutants of ribonucleotide reductase.[3] Although cation Trp radicals in proteins have been reported, there is almost no experimental information on their hyperfine interactions.[8,61]

As far as we are able to tell, all previously published ab initio and DFT calculations for indole-based radicals have been performed either in vacuo or in PCM (polarizable continuum model) water.[3,25−30] The hyperfine coupling data for $TrpH^{\bullet+}$ obtained from CIDNP correlate very well with DFT calculations but only if water molecules are included explicitly. The most significant differences between the calculated results in vacuo and with explicit water molecules are as follows (using $a(i)$ to denote the coupling constant of nucleus i): a(C6) is approximately 10 times larger in water; a(C7) has opposite sign in the two media; a(C8) has opposite sign and is roughly 4 times smaller in water; a(H7) is about 7 times smaller in water. All other $a(i)$ values are similar to within a factor of ∼2. The differences between the two calculations, which are most pronounced in the region of the molecule close to the indole NH group, reflect the solvating interactions of the water molecules with that polar group. The ^{14}N1 isotropic hyperfine interaction in $TrpH^{\bullet+}$ also differs in the two media: 0.321 mT in vacuo and 0.195 in water (Supporting Information). The observed differences between the calculated hyperfine coupling constant of $TrpH^{\bullet+}$ in vacuo and that in water suggest that their sensitivity to the electrostatic environment and details of solvation could be used more extensively as a probe of $TrpH^{\bullet+}$ radicals in proteins. Explicit solvation was found to have a much less striking effect on the ^{1}H, ^{13}C, and ^{14}N couplings of the neutral radical $Trp^{\bullet}$ (Supporting Information).

The largest differences in hyperfine couplings calculated for $TrpH^{\bullet+}$ and $Trp^{\bullet}$ in water are for C2 (∼4 times smaller in $TrpH^{\bullet+}$) and H2 (∼8 times larger in $TrpH^{\bullet+}$). Together with the absence of a H1 coupling in $Trp^{\bullet}$, these two interactions are probably the most reliable way of distinguishing the two radicals on the basis of their hyperfine couplings.

The data presented here may be useful when interpreting the ^{13}C CIDNP spectra of photoactive proteins. For example, Richter et al. have recently reported measurements on a cysteine-to-alanine mutant of the LOV2 domain of the blue-light receptor phototropin from *Avena sativa*.[62] In addition to strong ^{13}C polarization from the ^{13}C enriched FMN cofactor, blue-light irradiation of the protein produced a natural abundance polarized ^{13}C NMR signal attributed to the C3 carbon of a tryptophan residue, strongly suggesting the involvement of a flavin−tryptophan radical pair. It is noteworthy that C3 has the largest hyperfine coupling of all the carbons in $TrpH^{\bullet+}$, both experimentally and theoretically (Table 4).

Acknowledgment. Financial support from INTAS (Project No. 05-100000-8070), the Royal Society (International Joint Project Grant Program), the Russian Fund for Basic Research (RFBR Project No. 05-03-32370), and FASI (theme RI-112/001/207) is gratefully acknowledged. O.B.M. is indebted to the Young Scientists Grant Program of the President of the Russian Federation (Project No. MK-2547.2005.3) for financial support. L.T.K. thanks the Studienstiftung des deutschen Volkes and the BBSRC for financial support. A.V.Y. acknowledges support from the EU FP6 Marie Curie Actions program (Project No. IIF 2208) for a fellowship held at FU Berlin. P.J.H. and L.T.K. thank the Oxford Supercomputing Centre for generous allocation of CPU time.

Supporting Information Available: Two-dimensional ^{1}H−^{13}C HMQC spectrum of tryptophan; magnetic resonance parameters used for spectral simulations; isotropic hyperfine coupling constants of $TrpH^{\bullet+}$, $Trp^{\bullet}$, $TyrOH^{\bullet+}$, and $Tyr^{\bullet}$ both in vacuo and explicitly solvated; CIDNP spectral simulation methods. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Bleifuss, G.; Kolberg, M.; Potsch, S.; Hofbauer, W.; Bittl, R.; Lubitz, W.; Graslund, A.; Lassmann, G.; Lendzian, F. *Biochemistry* **2001**, *40*, 15362−15368.
(2) Lendzian, F. *Biochim. Biophys. Acta* **2005**, *1707*, 67−90.
(3) Lendzian, F.; Sahlin, M.; MacMillan, F.; Bittl, R.; Fiege, R.; Potsch, S.; Sjoberg, B. M.; Graslund, A.; Lubitz, W.; Lassmann, G. *J. Am. Chem. Soc.* **1996**, *118*, 8111−8120.
(4) Aubert, C.; Mathis, P.; Eker, A. P. M.; Brettel, K. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5423−5427.
(5) Aubert, C.; Vos, M. H.; Mathis, P.; Eker, A. P. M.; Brettel, K. *Nature* **2000**, *405*, 586−590.
(6) Byrdin, M.; Eker, A. P. M.; Vos, M. H.; Brettel, K. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 8676−8681.
(7) Gindt, Y. M.; Vollenbroek, E.; Westphal, K.; Sackett, H.; Sancar, A.; Babcock, G. T. *Biochemistry* **1999**, *38*, 3857−3866.
(8) Kim, S. T.; Sancar, A.; Essenmacher, C.; Babcock, G. T. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8023−8027.
(9) Essenmacher, C.; Kim, S. T.; Atamian, M.; Babcock, G. T.; Sancar, A. *J. Am. Chem. Soc.* **1993**, *115*, 1602−1603.
(10) Ivancich, A.; Dorlet, P.; Goodin, D. B.; Un, S. *J. Am. Chem. Soc.* **2001**, *123*, 5050−5058.
(11) Pogni, R.; Baratto, M. C.; Giansanti, S.; Teutloff, C.; Verdin, J.; Valderrama, B.; Lendzian, F.; Lubitz, W.; Vazquez-Duhalt, R.; Basosi, R. *Biochemistry* **2005**, *44*, 4267−4274.
(12) Blodig, W.; Smith, A. T.; Winterhalter, K.; Piontek, K. *Arch. Biochem. Biophys.* **1999**, *370*, 86−92.
(13) Ivancich, A.; Jakopitsch, C.; Auer, M.; Un, S.; Obinger, C. *J. Am. Chem. Soc.* **2003**, *125*, 14093−14102.
(14) Pogni, R.; Baratto, M. C.; Teutloff, C.; Giansanti, S.; Ruiz-Duenas, F. J.; Choinowski, T.; Piontek, K.; Martinez, A. T.; Lendzian, F.; Basosi, R. *J. Biol. Chem.* **2006**, *281*, 9517−9526.
(15) Sivaraja, M.; Goodin, D. B.; Smith, M.; Hoffman, B. M. *Science* **1989**, *245*, 738−740.
(16) Jakopitsch, C.; Obinger, C.; Un, S.; Ivancich, A. *J. Inorg. Biochem.* **2006**, *100*, 1091−1099.
(17) Hiner, A. N. P.; Martinez, J. I.; Arnao, M. B.; Acosta, M.; Turner, D. D.; Raven, E. L.; Rodriguez-Lopez, J. N. *Eur. J. Biochem.* **2001**, *268*, 3091−3098.
(18) Barrows, T. P.; Bhaskar, B.; Poulos, T. L. *Biochemistry* **2004**, *43*, 8826−8834.
(19) Perez-Boada, M.; Ruiz-Duenas, F. J.; Pogni, R.; Basosi, R.; Choinowski, T.; Martinez, M. J.; Piontek, K.; Martinez, A. T. *J. Mol. Biol.* **2005**, *354*, 385−402.
(20) Wiertz, F. G. M.; Richter, O. M. H.; Cherepanov, A. V.; MacMillan, F.; Ludwig, B.; de Vries, S. *FEBS Lett.* **2004**, *575*, 127−130.
(21) MacMillan, F.; Budiman, K.; Angerer, H.; Michel, H. *FEBS Lett.* **2006**, *580*, 1345−1349.
(22) Rich, P. R.; Rigby, S. E. J.; Heathcote, P. *Biochim. Biophys. Acta* **2002**, *1554*, 137−146.
(23) Giovani, B.; Byrdin, M.; Ahmad, M.; Brettel, K. *Nat. Struct. Biol.* **2003**, *10*, 489−490.
(24) Zeugner, A.; Byrdin, M.; Bouly, J. P.; Bakrim, N.; Giovani, B.; Brettel, K.; Ahmad, M. *J. Biol. Chem.* **2005**, *280*, 19437−19440.
(25) O'Malley, P. J.; Ellson, D. A. *Chem. Phys. Lett.* **1996**, *260*, 492−498.
(26) Walden, S. E.; Wheeler, R. A. *J. Phys. Chem.* **1996**, *100*, 1530−1535.
(27) Walden, S. E.; Wheeler, R. A. *J. Am. Chem. Soc.* **1997**, *119*, 3175−3176.
(28) Himo, F.; Eriksson, L. A. *J. Phys. Chem. B* **1997**, *101*, 9811−9819.
(29) Un, S. *Magn. Reson. Chem.* **2005**, *43*, S229−S236.
(30) Siegbahn, P. E. M.; Eriksson, L.; Himo, F.; Pavlov, M. *J. Phys. Chem. B* **1998**, *102*, 10622−10629.
(31) Kaptein, R.; Dijkstra, K.; Nicolay, K. *Nature* **1978**, *274*, 293−294.

(32) Hore, P. J.; Broadhurst, R. W. *Prog. Nucl. Magn. Reson. Spectrosc.* **1993**, *25*, 345−402.
(33) Closs, G. L.; Miller, R. J. *J. Am. Chem. Soc.* **1979**, *101*, 1639−1641.
(34) Closs, G. L.; Sitzmann, E. V. *J. Am. Chem. Soc.* **1981**, *103*, 3217−3219.
(35) Closs, G. L.; Miller, R. J.; Redwine, O. D. *Acc. Chem. Res.* **1985**, *18*, 196−202.
(36) Tsentalovich, Y. P.; Morozova, O. B.; Yurkovskaya, A. V.; Hore, P. J. *J. Phys. Chem. A* **1999**, *103*, 5362−5368.
(37) Hore, P. J.; Egmond, M. R.; Edzes, H. T.; Kaptein, R. *J. Magn. Reson.* **1982**, *49*, 122−150.
(38) Mok, K. H.; Hore, P. J. *Methods* **2004**, *34*, 75−87.
(39) Mok, K. H.; Nagashima, T.; Day, I. J.; Jones, J. A.; Jones, C. J. V.; Dobson, C. M.; Hore, P. J. *J. Am. Chem. Soc.* **2003**, *125*, 12484−12492.
(40) Mok, K. H.; Nagashima, T.; Day, L. J.; Hore, P. J.; Dobson, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 8899−8904.
(41) Morozova, O. B.; Yurkovskaya, A. V.; Sagdeev, R. Z.; Mok, K. H.; Hore, P. J. *J. Phys. Chem. B* **2004**, *108*, 15355−15363.
(42) Adrian, F. J. *J. Chem. Phys.* **1971**, *54*, 3912−3917.
(43) Closs, G. L. In *Advances in Magnetic Resonance*; Waugh, J. S., Ed.; Academic Press: San Diego, CA, 1974; Vol. 7, p 157.
(44) Lyon, C. E.; Lopez, J. J.; Cho, B. M.; Hore, P. J. *Mol. Phys.* **2002**, *100*, 1261−1269.
(45) Grosse, S.; Yurkovskaya, A. V.; Lopez, J.; Vieth, H.-M. *J. Phys. Chem. A* **2001**, *105*, 6311−6319.
(46) Ivanov, K. L.; Lukzen, N. N.; Vieth, H. M.; Grosse, S.; Yurkovskaya, A. V.; Sagdeev, R. Z. *Mol. Phys.* **2002**, *100*, 1197−1208.
(47) Ivanov, K. L.; Vieth, H.-M.; Miesel, K.; Yurkovskaya, A. V.; Sagdeev, R. Z. *Phys. Chem. Chem. Phys.* **2003**, *16*, 3470−3480.
(48) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03*, revision C.02; Gaussian, Inc.: Wallingford, CT, 2004.
(49) Adhikary, A.; Kumar, A.; Becker, D.; Sevilla, M. D. *J. Phys. Chem. B* **2006**, *110*, 24171−24180.
(50) Tomkiewicz, M.; McAlpine, R. D.; Cocivera, M. *Can. J. Chem.* **1972**, *50*, 3849−3856.
(51) Hulsebosch, R. J.; van den Brink, J. S.; Nieuwenhuis, S. A. M.; Gast, P.; Raap, J.; Lugtenburg, J.; Hoff, A. J. *J. Am. Chem. Soc.* **1997**, *119*, 8685−8694.
(52) Tsentalovich, Y. P.; Morozova, O. B. *J. Photochem. Photobiol. A* **2000**, *131*, 33−40.
(53) Tsentalovich, Y. P.; Lopez, J. J.; Hore, P. J.; Sagdeev, R. Z. *Spectrochim. Acta A* **2002**, *58*, 2043−2050.
(54) Baugher, J. F.; Grossweiner, L. I. *J. Phys. Chem.* **1977**, *81*, 1349−1354.
(55) Byrdin, M.; Sartor, V.; Eker, A. P. M.; Vos, M. H.; Aubert, C.; Brettel, K.; Mathis, P. *Biochim. Biophys. Acta* **2004**, *1655*, 64−70.
(56) Morozova, O. B.; Yurkovskaya, A. V.; Tsentalovich, Y. P.; Forbes, M. D. E.; Hore, P. J.; Sagdeev, R. Z. *Mol. Phys.* **2002**, *100*, 1187−1195.
(57) Kaptein, R. *Chem. Commun.* **1971**, 732−733.
(58) Grosse, S. CIDNP-Untersuchungen an photoinduzierten Radikalpaar-Reaktionen mit Feldzyklisierung im Magnetfeldbereich von 0 bis 7 Tesla. Ph.D. Thesis, Free University of Berlin, 2000.
(59) Ehrenberg, A.; Eriksson, L. E. G.; Müller, F. *Flavins and Flavoproteins;* Slater, E. C., Ed.; Elsevier: Amsterdam, The Netherlands, 1966; p 37.
(60) Ehrenberg, A.; Müller, F.; Hemmerich, P. *Eur. J. Biochem.* **1967**, *2*, 286−293.
(61) Kobori, Y.; Norris, J. R. *J. Am. Chem. Soc.* **2006**, *128*, 4−5.
(62) Richter, G.; Weber, S.; Romisch, W.; Bacher, A.; Fischer, M.; Eisenreich, W. *J. Am. Chem. Soc.* **2005**, *127*, 17245−17252.