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ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · DECEMBER 2014

Impact Factor: 3.3 · DOI: 10.1021/jp511068n · Source: PubMed

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Modulation of the Rate of Reversible Electron Transfer in Oxidized Tryptophan and Tyrosine Containing Peptides in Acidic Aqueous Solution

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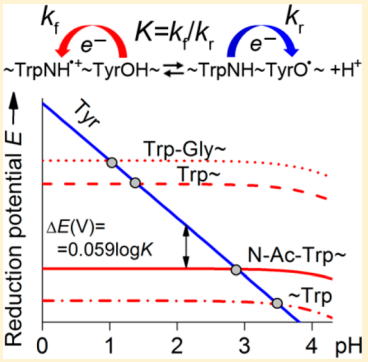
Supporting Information

ABSTRACT: Time-resolved chemically induced dynamic nuclear polarization (CIDNP) was used to investigate reversible intramolecular electron transfer (IET) in short-lived oxidized peptides, which had different structures and contained tryptophan and tyrosine residues, in an acidic aqueous solution with a pH below the pK_a of the tryptophanyl cation radical. The CIDNP kinetic data were obtained at the microsecond scale and were analyzed in detail to calculate the rate constants for electron transfer in both directions: from the tyrosine residue to the tryptophanyl cation radical, k_f, and from the tryptophan residue to the neutral tyrosyl radical, k_r. The charge of the terminal amino group and the presence of glycine and proline spacers were shown to strongly affect the rate constants of the reaction under study. Among these functional groups, the presence and the location of the positive charge on the amino group in close proximity to the cationic indolyl radical had the strongest effect on the rate constant of the forward IET from the tyrosine residue to the tryptophanyl radical cation, k_f. This effect was manifested as an increase of 2 orders of magnitude in k_f for a change in the linkage order between residues in the dipeptide: $k_f = 4 \times 10^3$ s^{-1} for the oxidized Tyr-Trp increased to $k_f = 5.5 \times 10^5$ s^{-1} in oxidized Trp-Tyr. The reverse rate constant for IET was less sensitive to the amino group charge. Moreover, the presence of glycine or proline spacers in the peptides with a tryptophan residue at the N-terminus not only reduced the IET rate constant but also shifted the equilibrium of the IET in the reaction under study toward the formation of tyrosyl radicals with respect to the peptide Trp-Tyr. That is, the glycine or proline spacers affected the difference in the reduction potential of the tryptophanyl and tyrosyl radicals.



INTRODUCTION

Long-range charge transfer in proteins has continuously attracted interest for decades.[1−8] Tryptophan serves as an electron transfer mediator, usually with an additional tyrosine residue as the electron donor or acceptor.[9] It has been established that electron transfer involving tryptophan and tyrosine is required for radical propagation in the functional states of ribonucleotide reductase,[10] photolyases and cryptochromes,[3,11,12] and phototropins.[13] The ability of electrons to find the best pathways from a donor to an acceptor among multiple available paths indicates that the reversibility of fast intramolecular electron transfer (IET) is a fundamental property of the protein network for preserving the functionality of proteins in nature. Thus, it is intriguing and timely to understand how IET reversibility affects the motion of electrons and protons within protein molecules. Such reactions are ubiquitous in biochemistry and difficult to detect by traditional methods; in addition, investigating the reversibility of these reactions can enable us to understand the factors that cause the modulation of the rates of the forward and reverse reactions, the effect of charged groups on the directionality of electron transfer, and the scale of the interaction energies. A schematic of the reversible IET between the most commonly involved tryptophan and tyrosine residues is shown in eq 1.

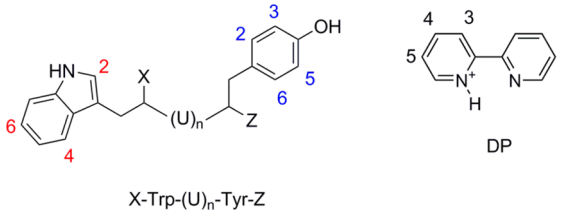
$$\text{X-TrpNH}^{\bullet+}\text{-(U)}_n\text{-TyrOH-Z} \underset{k_r}{\overset{k_f}{\rightleftarrows}} \text{X-TrpNH-(U)}_n\text{-TyrO}^{\bullet}\text{-Z} + \text{H}^+ \quad (1)$$

The structure of the investigated peptides is shown in Chart 1.

Transient radical intermediates of tryptophan and tyrosine, such as the tryptophanyl radical cation and the tyrosyl neutral radical, are characterized by high reactivity under biologically relevant conditions, and therefore, by a notoriously low concentration that precludes their detection by EPR under these conditions. Sensitive optical methods can be used to directly detect the elusive radicals of tryptophan and tyrosine residues, demonstrating that IET results from the appearance of the latter in synchronicity with the disappearance of the former. However, transient optical absorption has not been able to

Received: November 5, 2014
Revised: December 8, 2014
Published: December 9, 2014

Chart 1. General Structure of the Investigated Peptides Containing Tyrosine and Tryptophan[a] and the Structure of the 2,2′-Dipyridyl Dye (DP)



X-Trp-(U)$_n$-Tyr-Z

[a]Trp-Tyr, X = NH_3, n = 0, Z = COOH; Tyr-Trp, X = COOH, n = 0, Z = NH_3; Ac-Trp-Tyr, X = CH_3CONH, n = 0, Z = COOH; Ac-Tyr-Trp, X = COOH, n = 0, Z = CH_3CONH; Trp-Gly-Tyr, X = NH_3, U = Gly, n = 1, Z = COOH; Tyr-Gly-Trp, X = COOH, U = Gly, n = 1, Z = NH_3; Trp-Pro-Tyr, X = NH_3, U = Pro, n = 1, Z = COOH; Trp-$(Gly)_2$-Tyr, X = NH_3, U = Gly, n = 2, Z = COOH).

provide explicit evidence for reversibility even for the simplest system consisting only of primary reactants. Both intermediate radicals are very short-lived; thus, optical studies of transient absorption can only track the attainment of equilibrium in terms of an equivalent rate constant that is equal to the sum of k_f and k_r. Thus, optical studies can only be used to determine the overall directionality of charge transfer and cannot be used to determine the individual k_f and k_r values. In a transient absorption study, the hidden reversibility of the IET does not manifest directly and can be easily misinterpreted as an apparent acceleration of the forward reaction or as a change in the equilibrium concentrations of the transient radicals.

Thus, we develop an alternative means of determining the "ping-pong" transmission of the unpaired electron between tryptophan and tyrosine in oxidized peptides using time-resolved chemically induced dynamic nuclear polarization (CIDNP). Within this approach, a one-electron-deficient peptide (with a radical center at either the tryptophan or the tyrosine residue) is created from a photoinduced reaction of the peptide using a triplet-excited dye.[14] The effect of the magnetic hyperfine interaction in the radical pair of a tryptophan- or tyrosine-centered peptide radical and a photosensitizer radical on the probability of radical recombination produces a nuclear nonthermal polarization that can be detected by NMR of the diamagnetic products. These NMR lines can be strongly enhanced by exploiting the high selectivity of the radical reaction to the total electron spin, which makes CIDNP methods extremely sensitive. A wealth of information about the magnetic resonance parameters of the transient radical and the rate constants of their reactions is contained in the NMR patterns and the polarization kinetics. We used 2,2′-dipyridyl (DP) to efficiently photo-oxidize tryptophan and tyrosine-containing peptides, thereby initiating the IET reaction. DP offered an additional advantage in that its polarization has a different sign in a radical pair with a tryptophanyl radical (emissive, negative) than with a tyrosyl radical (absorptive, positive). Thus, this molecule functions as an "observer", which provides additional information that enables us to accurately determine the rate constants of the reversible IET in short-lived oxidized peptides.

In general, the overall directionality of electron transfer between tyrosine and tryptophan is determined by the difference in the redox potentials of the residues in proteins, which in turn are modulated by the local structure. Figure 1 is a schematic of the pH dependence of the reduction potentials of

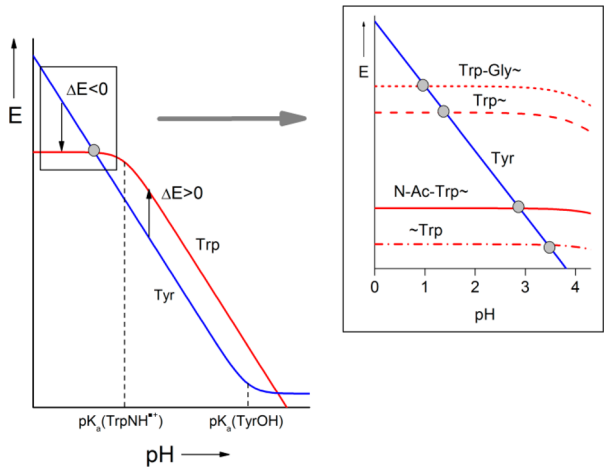


Figure 1. pH dependence of the reduction potentials of the tryptophanyl and tyrosyl radicals and the effect of molecular structure on the difference between these potentials ("~" denotes the peptide backbone).

the tryptophanyl and tyrosyl radicals. The tryptophanyl radical has a constant reduction potential at a pH below its pK_a and is linearly dependent on the pH at a pH above its pK_a, whereas the redox potential of tyrosine is linearly dependent on the pH at a pH below the pK_a of the phenolic proton and remains constant at a pH above this pK_a.[15] The tyrosyl radical is more stable than tryptophanyl radical at a neutral pH, and therefore, forward electron transfer dominates. The directionality of the electron transfer reaction changes in the acidic range below the crossing point, as shown in Figure 1, because the reduction potential of the tyrosyl radical increases, and the neutral tyrosyl radical can be reduced by a tryptophan that results in the formation of the tryptophanyl cation radical, $TrpNH^{\bullet+}$. The pK_a of this radical depends on the surrounding protein structure; thus, slight structural changes that affect the stability of $TrpNH^{\bullet+}$ can be used to tune the reversible electron transfer. In this study, we performed a detailed and sophisticated analysis of the pH dependence of the rates of the reversible IET (reaction 1) for eight peptides to elucidate how fine structural factors, such as the proximity of charged groups to the tryptophan residue, regulate the rate of intramolecular electron transfer. The general structure of these peptides is shown in Chart 1. The study was conducted over the pH range below the pK_a of the tryptophanyl radical cation, as shown in the inset in Figure 1, where the relevant reaction is described by eq 1. The resulting pH dependence of the rate constants enabled us to observe a significant modulation in the equilibrium constant by the positive charges at the nearest terminal groups in the vicinity of the crossing point. We thus determined the effect of the peptide structure on the one-electron reduction potential of the tryptophanyl radical, as shown in the right-hand-side subplot in Figure 1.

We previously performed a detailed study of reversible IET in a Trp-Tyr dipeptide in acidic aqueous solutions.[14] The rate constant of the IET from the tyrosine residue to the tryptophanyl radical cation was determined to be 5.5×10^5 s^{-1}. The rate constant for the reverse IET from the tryptophan residue to the tyrosyl radical was proportional to the proton concentration, in accordance with the reaction scheme for the protonation of the tyrosyl anion that was produced by the electron transfer. Rather intriguing was our finding that different orders of the linkage of the amino acids in the

dx.doi.org/10.1021/jp511068n | J. Phys. Chem. B 2015, 119, 140−149

dipeptide slowed down the forward IET by 2 orders of magnitude, whereas the rate constants for the reverse IET were comparable for both oxidized dipeptides, Trp-Tyr and Tyr-Trp.[16] We used the following strategy to further understand the key factors that control IET in proteins. First, we compared the behavior of nonmodified dipeptides (Trp-Tyr and Tyr-Trp) with dipeptides with an acetylated terminal amino group to determine the effects of the presence and location of the amino group charge. Second, we introduced glycine and proline as spacers to determine whether spacers could be used to scale the rate constants for IET in peptides. Thus, this study was a comparative analysis of IET (according to reaction 1) for a variety of peptides (Chart 1) in acidic aqueous solution.

Kinetic data for CIDNP were obtained from photoreactions of the N-acetylated dipeptides Ac-Trp-Tyr and Ac-Tyr-Trp; the tripeptides Trp-Gly-Tyr, Tyr-Gly-Trp, and Trp-Pro-Tyr; and the tetrapeptide Trp-$(Gly)_2$-Tyr. The results were compared with previously obtained results for Trp-Tyr and Tyr-Trp.[14,16]

The time-dependent CIDNP behavior was highly sensitive to the rate constant of IET in both directions, which enabled us to quantitatively determine the effect of the molecular structure on IET, including minor effects.

EXPERIMENTAL SECTION

A detailed description of our TR-CIDNP setup has been provided elsewhere.[17] The samples were bubbled with argon for 15 min prior to irradiation and then sealed in a standard NMR Pyrex ampule. The samples were irradiated in the probe of a 200 MHz Bruker DPX-200 NMR spectrometer (with a magnetic field of 4.7 T and a proton resonance frequency of 200 MHz) by pulses from a COMPEX Lambda Physik XeCl excimer laser (wavelength 308 nm, output pulse energy up to 150 mJ). Light was guided to the sample through an optical system of a spherical lens, a prism, and a quartz light guide (diameter 5 mm). The TR-CIDNP spectra were obtained by the following procedure: the reactant nuclear spin transitions were saturated using pulses from a broad-band homonuclear decoupler, the laser pulse, followed by a detecting radio frequency (rf) pulse with a 1 μs duration. The laser pulse was synchronized with the front edge of the rf pulse.

All of the ^{1}H CIDNP measurements were performed in D_2O. The acidity of the samples was adjusted by adding DCl. The pH in the D_2O solutions was measured with an H_2O-calibrated pH-meter; thus, the pH readings corresponded to so-called pH* values, which are pD values according to the formula pD = pH* + 0.4.[18] Although all of the exchangeable protons in the CIDNP experiments were substituted by deuterons, the more common term "proton" is used throughout the text.

The concentrations of the reactants were 5.0×10^{-4} M (DP) and 2.5×10^{-3} M (peptides).

Trp-Tyr, Tyr-Trp, Trp-Gly-Tyr, Tyr-Gly-Trp, Trp-$(Gly)_2$-Tyr, and Trp-Pro-Tyr were purchased from Bachem and used without further purification. The N-acetylated peptides Ac-Trp-Tyr and Ac-Tyr-Trp were synthesized by Prof. Dr. Vladimir N. Silnikov and Ms. Lubov A. Yarinich (Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russia) using standard methods with activated ester BOC strategies.

RESULTS AND DISCUSSION

We investigated IET in oxidized peptides using the following procedure.

1. Peptide radicals were photochemically generated in the quenching reaction of a triplet-excited dye (reactions 2a and 2b).
2. The CIDNP kinetics for the radical reactions were obtained at different pH values in acidic solutions.
3. The bidirectional IET rate constants were determined by quantitatively analyzing the CIDNP kinetics of the reactions for all three participating radicals: tryptophanyl, tyrosyl, and the dye radical.

We now consider these steps in detail.

Quenching Reaction of Triplet Excited 2,2′-Dipyridyl by the Peptides. One-electron-deficient peptides, which participate in IET, were generated from a photoreaction with the triplet-excited dye, 2,2′-dipyridyl. In acidic solution, this dye is present in its protonated form, ^{T}DPH$^+$ (Chart 1) with pK_a = 5.8,[19] which is known to be quenched via electron transfer from tyrosine and tryptophan with comparable rate constants.[20] When tryptophan and tyrosine are components of the peptide, two types of peptide radicals are formed: one in which the radical is centered at the tryptophan and another in which the radical is located at the tyrosine residue:

$$^T\mathrm{DPH}^+ + \text{X-TrpNH-(U)}_n\text{-TyrOH-Z} + \mathrm{H}^+ \xrightarrow{k_q^{\mathrm{Trp}}} \mathrm{DPH_2}^{\bullet+} + \text{X-TrpNH}^{\bullet+}\text{-(U)}_n\text{-TyrOH-Z} \quad (2a)$$

$$^T\mathrm{DPH}^+ + \text{X-TrpNH-(U)}_n\text{-TyrOH-Z} \xrightarrow{k_q^{\mathrm{Tyr}}} \mathrm{DPH_2}^{\bullet+} + \text{X-TrpNH-(U)}_n\text{-TyrO}^{\bullet}\text{-Z} \quad (2b)$$

In the investigated pH* range (1.0 < pH* < 3.1), the radical DPH$^\bullet$ that was produced by electron transfer was protonated to form $DPH_2^{\bullet+}$ (pK_a = 8.5[19]), and the tyrosyl cation radical TyrOH$^{\bullet+}$ quickly deprotonated (pK_a < −1[21]), whereas the tryptophanyl cation radical TrpNH$^{\bullet+}$ remained unchanged (pK_a = 4.3 for free tryptophan[22] and 4.6 < pK_a < 5.4 for tryptophan in peptides[23]).

CIDNP Effects in the Photoreaction of 2,2′-Dipyridyl and the Peptides. The CIDNP spectra that were obtained for the photoreaction of DP with various peptides at pH 3.1 are shown in Figure 2 (for clarity, only the aromatic region is shown, and the full-range spectra are provided in the Supporting Information). Four out of eight peptides correspond to four groups of peptides with equivalent thermodynamic properties (vide infra). The spectra were obtained immediately after the laser pulse and corresponded to the so-called geminate spectra. We briefly describe the processes that produce the NMR spectra with nonequilibrium signal intensities, such as those shown in Figure 2. The quenching reaction results in the formation of triplet spin-correlated radical pairs. Intersystem crossing changes the multiplicity of these radical pairs to a singlet, providing an opportunity for the radicals to recombine. In the cyclic photoreactions, radical recombination restores the initial compounds via back electron or hydrogen transfer, i.e., the reactants have the same structures as the products. A hyperfine interaction between the unpaired electron and the nuclei alters the rate of the intersystem crossing (ISC) from state T_0 to S for different nuclear spin configurations ($S-T_0$ mechanism of CIDNP formation in high magnetic fields). Due to high selectivity of the recombination probability of the radical pairs (as a rule recombination is allowed only from the singlet state), the geminate products are enriched in nuclear spin states with higher intersystem crossing

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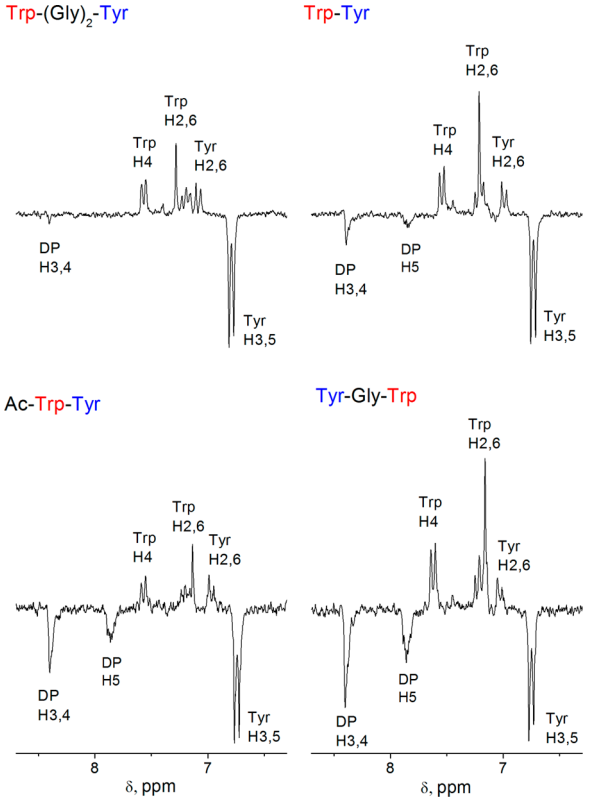

Figure 2. Aromatic region of 200 MHz ^{1}H CIDNP spectra that were obtained for photoreactions of 2,2′-dipyridyl with different peptides containing tryptophan and tyrosine at pH* 3.1 immediately following a laser pulse; the spectra were scaled individually to obtain equal CIDNP intensities of the H3,5 signal of the tyrosine residue.

rate. Thus, the CIDNP contains information about the effect of the nuclear spin configuration on the ISC rate that is encoded in the polarization pattern of the NMR spectra of the reaction products. The sign of the geminate CIDNP signal for each nucleus depends on the sign of the difference in the *g*-factors of the participating radicals and the sign of hyperfine interaction constant of this nucleus.[24] The radicals that escaped geminate recombination have nuclear polarizations of the same magnitude but opposite signs. The geminate stage lasts only a few nanoseconds in nonviscous solutions and is not resolved in our TR-CIDNP experiment. The bulk processes that occur after the geminate stage result in CIDNP formation during free radical encounters (so-called F-pairs).

Thus, CIDNP signals were observed for nonexchangeable protons that had nonzero hyperfine couplings in the transient radicals: enhanced absorption for the H2,6 and β protons of Tyr, as well as for the H2, H6, and H4 protons of Trp and emissions of the H3,5 of Tyr and the β protons of Trp (the proton numbering is shown in Chart 1). The DP protons were polarized in two types of radical pairs with either tryptophanyl or tyrosyl radicals. Opposite signs were found for Δg for the DP radical in these pairs: $\Delta g > 0$ for the pair with a tryptophanyl radical, and $\Delta g < 0$ for the pair with a tyrosyl radical.[14] Thus, pairs with a tryptophanyl radical had negatively polarized DP protons, whereas pairs with a tyrosyl radical were positively polarized. Two factors determine the resulting sign of a geminate CIDNP of DP in the various reaction systems: (i) the CIDNP enhancement factors for these two different pairs, which are determined by the magnetic properties of the radicals, and (ii) the fraction of the radical pairs of each type. The second factor depends on the ratio of the quenching rate constants of the triplet-excited DP by tryptophan and tyrosine (reactions 2a and 2b). The DP protons are negatively polarized in the CIDNP spectra detected immediately after the laser pulse. A relatively small negative CIDNP was detected for peptides with glycine spacers, and the largest CIDNP was found for Ac-Tyr-Trp. This CIDNP sign showed the predominance of the CIDNP created in radical pairs with tryptophanyl radical. The difference in the relative intensities of the DP signal in the spectra in Figure 2 depended on the change in the relative reactivity of the residues in the peptides of different structures toward the triplet-excited dye. Thus, CIDNP was a sensitive tool for determining these differences. This topic is discussed below in terms of the difference in the reduction potentials of the tryptophanyl and tyrosyl radicals, which were determined from the equilibrium constants for IET.

CIDNP Kinetics. The reaction under study, IET, was not activated at the geminate stage. IET was initiated on the time scale of the bulk reactions.

In the cyclic photochemical reactions, the CIDNP time dependence after the completion of the geminate stage is determined by the following processes.[25−28] (1) Radical recombination via back electron or hydrogen transfer: when the recombining radicals are correlated in their singlet state, the sign of the polarization of their products is opposite to that of the geminate product, which eventually results in CIDNP cancellation. However, because the radicals could alternatively form a radical pair in the T_0 state, a polarization in F-pairs with the same sign as the geminate polarization is produced, thus enhancing the geminate polarization. (2) Degenerate electron exchange (DEE), which involves one of the radical partners and its parent molecule. There is no net chemical transformation during this reaction. However, in this reaction, the free radical becomes a diamagnetic species and vice versa. Consequently, DEE results in a polarization transfer from a radical to a diamagnetic molecule, which leads to CIDNP cancellation. Because the concentration of radicals is usually much lower than that of their parent molecules, degenerate electron exchange can be considered to be a pseudo-first-order reaction. In the CIDNP kinetics, this reaction manifests as a decay in the polarization at a rate that is proportional to the concentration of the parent molecules. (3) Paramagnetic nuclear relaxation, which is operative during the radical lifetime, reduces the CIDNP in the free radicals and therefore interrupts the CIDNP cancellation. These three basic processes determine the CIDNP kinetics for the simplest cyclic photoreaction in which a triplet-excited dye reacted with a quencher to form radical pairs, and radical recombination restores the initial molecules.

IET is the fourth process that affects the CIDNP time dependence. The electron acceptor radical (which was a tryptophanyl radical for the forward IET and a tyrosyl radical for the reverse IET) is converted by IET into its diamagnetic state. This process results in CIDNP cancellation: the polarization of the radicals that have not participated in geminate recombination is superimposed on the geminate polarization of diamagnetic products, which manifests as the CIDNP decay, similarly to the decay from degenerate electron exchange. The difference between electron exchange and IET is that a radical of the same type is produced during DEE, whereas IET serves as an additional source of electron donor radicals. In the second-order termination reaction of these radicals with dye radicals, an additional polarization is created

dx.doi.org/10.1021/jp511068n | J. Phys. Chem. B 2015, 119, 140−149

that increases the CIDNP intensity of the donor with time. Special attention should be paid to the CIDNP kinetics of the DP. Although the DP radical does not participate in IET, DP polarization is produced in the reactions with the tryptophanyl and tyrosyl radicals, the ratio of the concentrations of which could change during IET. The opposite sign of the DP polarization for the two types of radical pairs makes the CIDNP kinetics of DP highly sensitive to the direction and efficiency of IET.

The CIDNP kinetic data for all three participating particles are shown in Figures 3−5 for two out of the eight peptides,

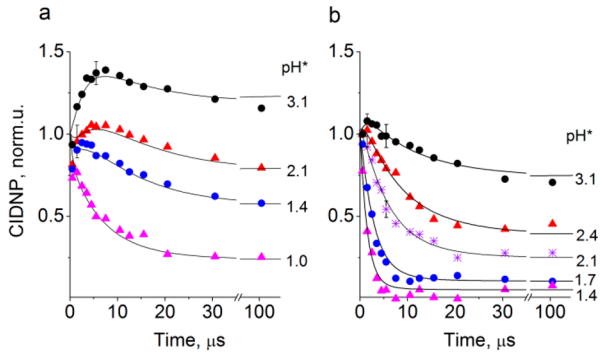

Figure 3. ^{1}H CIDNP kinetics for H3,5 of the tyrosine residue, which were obtained for the photoreactions of 2,2′-dipyridyl with the peptides (a) Trp-$(Gly)_2$-Tyr and (b) Ac-Trp-Tyr at different pH* values; the amplitude of the polarization (emission) is plotted; solid lines represent model simulations; see the Supporting Information for parameters and details of the simulations.

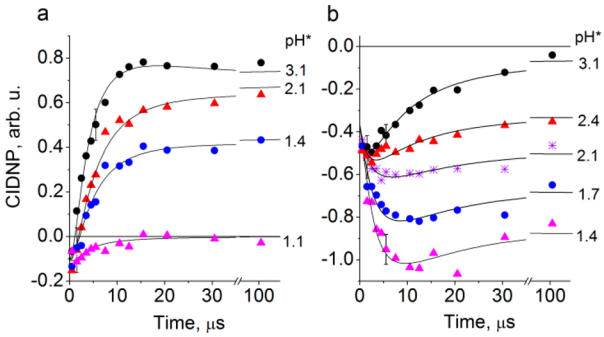

Figure 4. ^{1}H CIDNP kinetics for H3,4 of 2,2′-dipyridyl, which were obtained for photoreactions of 2,2′-dipyridyl with the peptides (a) Trp-$(Gly)_2$-Tyr and (b) Ac-Trp-Tyr at different pH* values; solid lines represent model simulations; see the Supporting Information for parameters and details of the simulations.

Trp-$(Gly)_2$-Tyr and Ac-Trp-Tyr. All of the kinetic data sets can be found in the Supporting Information. The rate constant k_r of the reverse reaction 1 was proportional to the proton concentration.[14,16] The kinetics were obtained at pH* 3.1, at which the contribution of the reverse reaction was negligibly small, and at lower pH* values, at which the rate constants of reaction 1 fell within the "kinetic window" of our TR CIDNP experiment (from $\cong 2 \times 10^4$ s^{-1} to $\cong 10^6$ s^{-1}). The quantitative analysis was performed using the signals of H3,5 of Tyr, of β protons of Trp, and of H3,4 protons of DP.

The initial growth of the CIDNP for Tyr clearly observed for the peptide Trp-$(Gly)_2$-Tyr at pH* 3.1 and the sign change of the CIDNP for DP were indications of IET from the tyrosine residue to the tryptophanyl cation radical. The tyrosyl radicals

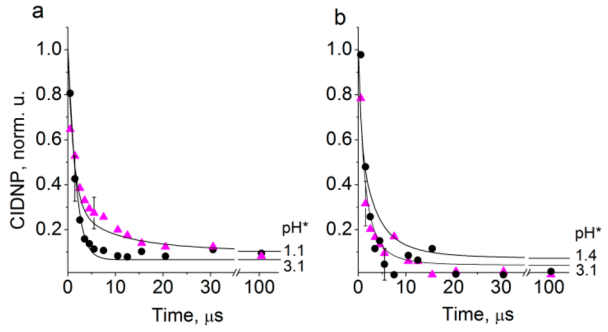

Figure 5. ^{1}H CIDNP kinetics of the β protons of the tryptophan residue for photoreactions of 2,2′-dipyridyl with the peptides (a) Trp-$(Gly)_2$-Tyr and (b) Ac-Trp-Tyr at different pH* values; the amplitude of the polarization (emission) is plotted; solid lines represent model simulations; see the Supporting Information for parameters and details of the simulations.

that had formed up to that point contributed in the pairs with a DP radical to the polarization of both partners, tyrosine and dipyridyl. An additional polarization (emission) was created for Tyr, whereas the change in the sign of polarization for the DP resulted from the increase in the relative concentration of radical pairs with a tyrosyl radical compared to the initial radical pair distribution. The model simulation results showed that the rate constant for the forward IET was 5.1×10^5 s^{-1} for this tetrapeptide, which was almost as high as for the previously studied Trp-Tyr peptide.[14] The aforementioned characteristic features of IET were not pronounced for the peptide Ac-Trp-Tyr. However, the model simulation results helped to explicate the IET reaction, although the rate constant was an order of magnitude lower than that of the tetrapeptide: $k_f = 5.1 \times 10^4$ s^{-1}. Without this reaction, the CIDNP of Tyr would have decayed to a lower value, and the negative CIDNP of the DP would be higher at later time.

In the pH range studied, the tryptophanyl radical participates in a degenerate electron exchange with its parent molecule for which the pseudo-first-order rate constant is a product of the second-order rate constant k_{ex} and the peptide concentration ("P" denotes nuclear polarization):

$$^{P}\mathrm{TrpNH}^{\bullet+} + \mathrm{TrpNH} \xrightarrow{k_{ex}} {}^{P}\mathrm{TrpNH} + \mathrm{TrpNH}^{\bullet+} \qquad (3)$$

This reaction is the main process that determines the form of the CIDNP kinetics for Trp, which is a fast decay to a stationary value (Figure 5). This rapid decay makes the CIDNP kinetics of Trp almost insensitive to the IET rate. However, when the reversible IET is considered, the degenerate exchange also begins to affect the CIDNP kinetics of Tyr as follows. Let us first consider a peptide radical that has its radical center at the tyrosyl residue, the protons of which carry "escape" nuclear polarization that is undetectable by NMR because of the paramagnetic frequency shift. This polarization decreases via relaxation but can increase by a reaction with DP radicals. The reverse IET removes the radical center from the tyrosyl radical, which makes the instantaneous polarization of the radical detectable by NMR. The forward IET makes this polarization "invisible" again but susceptible to the processes of relaxation and the creation of F-pair polarization. Degenerate electron exchange converts the peptide that has its radical center at the tryptophanyl residue and the nuclear polarization at the tyrosyl residue into the diamagnetic state. The newly formed peptide radical has no nuclear polarization at both residues. The reverse

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IET generates a peptide radical with its radical center at the tyrosyl residue, which can gain nuclear polarization in a second-order reaction with DP radicals.

Thus, a global fitting procedure for all three participating particles with a common set of parameters was required to obtain the values of k_f and the pH-dependent k_r for the oxidized peptides of different structures. The simulations were based on a model that was developed by Fischer et al. for the CIDNP kinetics in cyclic reactions[29] and modified by taking the reversible IET into account. The simulation procedure was tested for IET in oxidized Trp-Tyr[14] and successfully applied to oxidized Tyr-Trp.[16] Details of the simulation procedure can be found in the Supporting Information.

When the pH is lowered, the reverse IET begins to affect the CIDNP kinetics. Evidence for this behavior was provided by the CIDNP decay for tyrosine to a stationary value and the increase in the contribution of the negative polarization to the DP (Figures 3−5). For Trp, the CIDNP kinetics should have accelerated when the rate of the reverse reaction 1 increased. A highly efficient degenerate electron exchange smooths out the effect of the reverse IET. However, a pronounced reverse reaction was observed for peptides with relatively low k_{ex} values, such as Trp-$(Gly)_2$-Tyr (Figure 5), Trp-Gly-Tyr, or Trp-Pro-Tyr. In the simulation procedure, which is described in the Supporting Information, the fitting parameters were the product of the initial radical concentration and the second-order radical termination rate constant, k_tR_0 (the individual value at each pH*), k_{ex} (which was common for all pH* values), the vertical scaling factor, and the rate constants k_f (which were common for all pH values) and k_r (the individual value at each pH*). The k_f values were used to classify the peptides into two groups, depending on whether this rate constant fell within the "kinetic window" of the TR CIDNP experiment. The first group of peptides included Trp-Tyr, Trp-$(Gly)_n$-Tyr, Trp-Pro-Tyr, and Ac-Trp-Tyr. The second group consisted of the peptides Tyr-Trp, Tyr-Gly-Trp, and Ac-Tyr-Trp, with the latter representing a boundary case. For the peptides in the first group, the CIDNP kinetics that were obtained at pH* 3.1 were largely determined by the forward IET, which resulted in highly accurate k_f values. The kinetic data at lower pH* values were simulated using the aforementioned k_f values and a variable k_r. In all cases, the pH* dependences of log k_r and, therefore, log K = log(k_f/k_r) demonstrated good linearity (Figure 6). For the peptides in the second group, k_f was estimated using this linearity property. Each case of CIDNP kinetics was simulated for different pairs of k_f and k_r: the changes in the CIDNP kinetics resulting from varying one of these values could be compensated to a certain extent by varying the other value, i.e., a high k_f required a high k_r. The sets of pH*-dependent k_r values were obtained using different values of k_f that produced good agreement between experiment and simulation. We estimated k_f by the value that produced the best linearity for the pH* dependence of log K (Figure 6).

The k_f and k_r values (at pH* 2.6, which corresponded to pD 3) that resulted from the best-fit simulations are given in Table 1. All of the simulation parameters, including the k_r value at each pH*, can be found in the Supporting Information. The influence of the charge of terminal amino group and the presence of spacers of the rate constants of reversible IET is discussed below in the corresponding subsection.

The rate constant of $k_f = 5.1 \times 10^4$ s^{-1} for IET in the peptide Ac-Trp-Tyr was in agreement with the value of 1.2×10^4 s^{-1} that was obtained using pulse radiolysis[30] and by considering the expected 2-fold difference in the rate constants from the kinetic isotope effect.[31] In ref 30, an acceleration of the forward IET was observed upon lowering the pH from ≅3 to ≅2. This result contradicts the acidity independence of the forward IET, whereby a decrease in the pH should have shifted the equilibrium to the reverse reaction. The most likely explanation of the discrepancy between our results and those reported in ref 30 is that, in the latter study, the equilibration process with the rate constant $k_f + k_r$ was detected, and the reversibility of the IET was not considered.

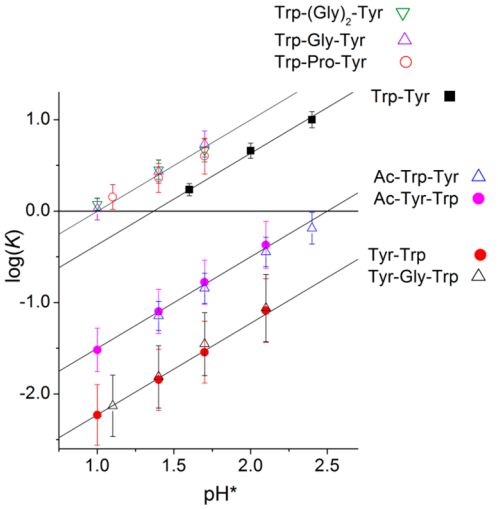

Figure 6. pH* dependence of the decimal logarithm of the equilibrium constant K for intramolecular electron transfer in different oxidized peptides containing tryptophan and tyrosine.

The Difference of the Reduction Potentials between the Tryptophanyl and the Tyrosyl Radicals. The equilibrium constant, $K = k_f/k_r$, was used to calculate the difference in the reduction potential between the tryptophanyl and the tyrosyl radicals, $\Delta E = E(\mathrm{TrpNH^{\bullet+}/TrpNH}) - E(\mathrm{TyrO^{\bullet}},\mathrm{H^+/TyrOH})$ as follows:[16]

$$\Delta E = 0.059 \times \log K \quad (4)$$

The difference in the reduction potential increases by 0.059 V per pH unit. Table 1 shows the ΔE values at pH* 2.6 (pD 3). It is reasonable that the peptides with neutral terminal groups exhibited log K values with identical pH dependences despite the different rate constants. For the oxidized peptides that had a positive charge at the terminal amino group, the IET was was shifted more strongly toward this charge, that is, toward the tryptophanyl radical for the peptides Trp-Tyr, Trp-$(Gly)_n$-Tyr, and Trp-Pro-Tyr and toward the tyrosyl radical in the peptides Tyr-Trp and Tyr-Gly-Trp, relative to the equilibrium state of neutral oxidized peptides. The difference in the K values at a given pH between charged and neutral peptides was more pronounced when tryptophan was at the N-terminus. Note that the same acidity dependences of log K were observed for peptides that began with tryptophan and contained glycine or proline spacers unlike for the Trp-Tyr peptide. No such effect was observed for peptides with the reverse order of the amino acid residues, i.e., no difference in the pH dependence of log K was observed between Tyr-Trp and Tyr-Gly-Trp. That is, the reduction potential of the tryptophanyl cation radical was more sensitive to its chemical surroundings in the presence of a nearby positive charge, whereas the reduction potential of the

dx.doi.org/10.1021/jp511068n | J. Phys. Chem. B 2015, 119, 140−149

Table 1. Rate Constants k_f and k_r for Intramolecular Electron Transfer[a] in Oxidized Peptides Containing Tryptophan and Tyrosine, the Values of pH* at Which the Equilibrium Constant $K = 1$, and the Difference in the Reduction Potential of Tryptophanyl and Tyrosyl Radicals at pH* 2.6[b]

peptide	k_f, s^{-1}	k_r (pH* 2.6),[c] s^{-1}	pH* at which $K = 1$	ΔE (pH* 2.6), mV
Trp-Tyr[d]	$(5.5 \pm 0.5) \times 10^5$	$(3.3 \pm 1.0) \times 10^4$	1.4	72
Tyr-Trp[e]	$(4 \pm 3) \times 10^3$	$(1.7 \pm 0.5) \times 10^4$	3.2	−37
Ac-Trp-Tyr	$(5.1 \pm 1.5) \times 10^4$	$(4.5 \pm 1.4) \times 10^4$	2.5	6
Ac-Tyr-Trp	$(2 \pm 1) \times 10^4$	$(1.7 \pm 0.5) \times 10^4$	2.5	6
Trp-Gly-Tyr	$(3.8 \pm 0.5) \times 10^5$	$(9 \pm 3) \times 10^3$	1.0	94
Trp-$(Gly)_2$-Tyr	$(5.1 \pm 0.5) \times 10^5$	$(1.2 \pm 0.4) \times 10^4$	1.0	94
Trp-Pro-Tyr	$(3.0 \pm 0.6) \times 10^5$	$(9 \pm 3) \times 10^3$	1.0	94
Tyr-Gly-Trp	$(2.0 \pm 1.5) \times 10^3$	$(7.8 \pm 2.5) \times 10^4$	3.2	−37

[a] k_f and k_r are defined in eq 1. [b] pH* 2.6 corresponds to pD 3. [c] Obtained from linear extrapolation of the values determined at lower pH*. [d] Data taken from ref 14. [e] Modified data from ref 16.

neutral tyrosyl radical was not affected by the neighboring positive charge. This result fully confirmed the assumption that the positive charge of potassium ions destabilized the tryptophanyl radical that formed during the catalytic cycle of cytochrome *c* peroxidase (CcP).[32] Trp191 was found to be less stable in an engineered K^+ site of CcP than in wild-type CcP, and in a mutant CcP signal of Tyr was observed in the EPR spectrum instead of Trp, which was consistent with a shift of the equilibrium of reaction 1 toward the formation of a tyrosyl radical.

As previously mentioned, we measured the kinetic isotope effect of ≅2 for the forward IET for the oxidized Trp-Tyr peptide.[31] It is reasonable to assume that the same result is valid for the reverse IET. Thus, log *K* should be identical on the pH and pD scales, which enabled us to compare our thermodynamic data with those obtained for *N*-acetyl tryptophan and tyrosine amides using pulsed voltammetry.[33] From the data on pH dependences of the reduction potentials given in ref 33, it could be found that the condition $K = 1$ was met at pH 2.9. Our measurements showed that, for the oxidized *N*-acetyl derivatives of the dipeptides, $K = 1$ at pH* 2.5, which corresponded to pD 2.9. Thus, there was excellent agreement for the thermodynamic properties of the species with uncharged terminal groups. The pH dependences of the reduction potentials of the tryptophanyl and tyrosyl radicals that are presented in Figure 1 enabled us to confirm that the reduction potential of the tryptophanyl cation radical varied significantly with its chemical surroundings, which was not the case of the reduction potential of neutral tyrosyl radical. Moreover, our detailed and sophisticated analysis of the nuclear polarization kinetic data produced sufficiently precise forward and reverse rate constants, enabling us to calculate the exact values of the pH* at which the equilibrium conditions were met (the equilibrium constant $K = 1$). These data are shown in Table 1 and denoted by circles in the inset in Figure 1. Thus, remarkable advances were made in this study. In previous publications, different values were reported for tryptophan with different substituents in the backbone;[21,34] however, only the results of the present study have enabled us to conclusively identify the differences that shift the equilibrium point ($K = 1$) over a wide pH* range from 1 to 3.2.

The relative quenching rate constants and the difference in reduction potential are associated with electron removal from the amino acid residues. Therefore, we used the signal intensities in the geminate CIDNP spectra, which reflected the ratio of the reactivities of the residues toward the triplet-excited dye, to correlate the relative quenching rate constants with the difference in the reduction potential. The degenerate electron exchange made the tryptophan signal unsuitable for this analysis. The CIDNP signal for tryptophan that was detected in the first microsecond after the laser pulse was reduced proportionally to the value of k_{ex}, which varied noticeably among the different peptides. The CIDNP intensities of DP and Tyr were free from the effect of degenerate electron exchange and were better reflections of the true geminate CIDNP values. The CIDNP pattern of the Trp-Tyr peptide was used as a reference. A 1.5-fold predominance of k_q^{Trp} over k_q^{Tyr} was previously calculated for this peptide by comparing the CIDNP pattern with that obtained for a mixture of *N*-acetyl derivatives of amino acids, for which the quenching rate constants were known from laser flash photolysis measurements.[20] We calculated a range of k_q^{Trp}/k_q^{Tyr} values from 1.08 for Trp-$(Gly)_n$-Tyr peptides to 2.70 for the Ac-Tyr-Trp peptide. The dependence of $\log(k_q^{Trp}/k_q^{Tyr})$ on ΔE (pH* 2.6) in shown in Figure 7. A good linear correlation can be observed except for Ac-Tyr-Trp. For this peptide, the CIDNP signal of DP was the highest with respect to the signal of Tyr,

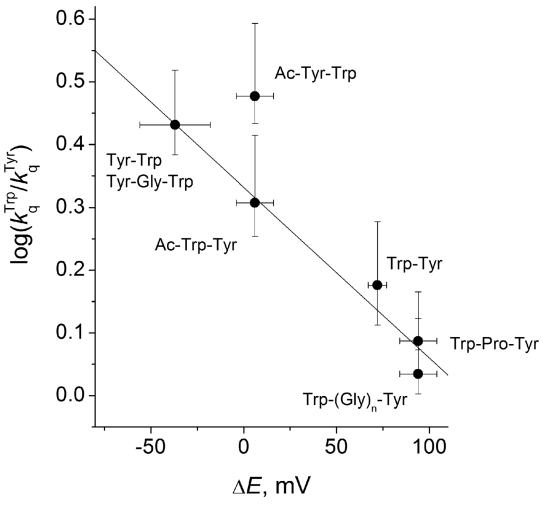

Figure 7. Correlation between the ratio of the quenching rate constants for triplet 2,2′-dipyridyl via electron transfer from tryptophan and tyrosine residues in different peptides, as estimated from the analysis of geminate CIDNP intensities, with the difference in the reduction potential between tryptophanyl and tyrosyl radicals for the corresponding peptides at pH* 2.6, which was calculated from the pH* dependences of the equilibrium constant for intramolecular electron transfer in the oxidized peptides.

dx.doi.org/10.1021/jp511068n | J. Phys. Chem. B 2015, 119, 140−149

whereas the signal intensity distribution should be similar to that for Ac-Trp-Tyr to lie within the range of applicability of the correlation. A possible explanation for this behavior is the *N*-acetyl group, which reduced the intermolecular quenching rate constant (sterically hindered the reaction of the triplet-excited DP with the tyrosine residue), whereas this restriction did not affect the intramolecular reaction steps. However, we have no direct proof of this mechanism.

The Influence of Charges and Spacers on IET Rate Constants. We now analyze the effects of the charges in the dipeptides and the presence of spacers in the tri- and tetrapeptides on the IET rate constants. Figure 8a shows the logarithms of the rate constants at conditions where $K = 1$ for the N-acetylated dipeptides (pH* 2.5). The vertical position of the star corresponds to k_f and k_r for Ac-Trp-Tyr, and the position of the cross corresponds to k_f and k_r for Ac-Tyr-Trp (the values of k_f and k_r coincide at this pH*). The vertical gap between the star and the cross reflects the difference between the IET rate constants, which most likely resulted from the differences in the molecular structure of the peptides. The acetylated peptides were used as a reference for the nonacetylated peptides with the same order of residues. The forward reaction (solid symbols) was noticeably attenuated in the presence of a positive charge at the terminal amino group; when electrons moved toward the positive charge, the rate constant increased by an order of magnitude (solid circle). For electron transport in the direction opposite to the positive charge, a ≅4-fold deceleration in the IET rate (solid triangle) was observed. The reverse IET rate constant (open symbols) for electron migration in the direction opposite to the positive charge was only slightly lowered (open circle) or remained unchanged (open triangle). These observations prove that the amino group charge had a larger effect on the tryptophanyl cation radical than on the neutral tyrosyl radical.

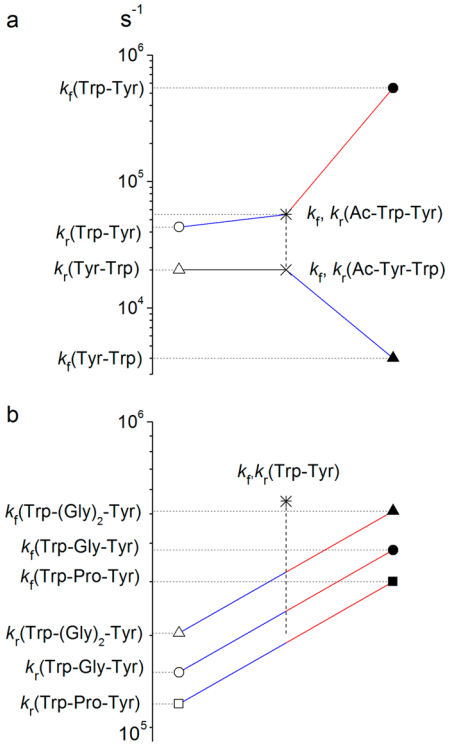

Figure 8. Diagram illustrating the dependence of k_f and k_r on (a) the terminal amino group charge in dipeptides containing tryptophan and tyrosine; (b) the presence of glycine or proline spacers in peptides containing tryptophan and tyrosine; data correspond to conditions at which (a) $K = 1$ for the oxidized peptides Ac-Trp-Tyr and Ac-Tyr-Trp (pH* 2.5) and (b) $K = 1$ for oxidized Trp-Tyr (pH* 1.4). Note the different scales of the subplots.

Figure 8b illustrates the effect of spacers on IET in oxidized peptides with tryptophan at the N-terminus. The logarithms of the rate constants that are presented here correspond to pH* 1.4, where $K = 1$ for Trp-Tyr. The IET rate constants for these peptides were reduced to different extents for the forward and back reactions relative to the corresponding values for the Trp-Tyr peptide: for Trp-Gly-Tyr, k_f and k_r were 1.4 and 3.5 times lower, respectively; for Trp-$(Gly)_2$-Tyr, these factors were 1.1 and 2.7, respectively; for Trp-Pro-Tyr, these factors were 1.8 and 4.1, respectively. The difference in these pairs of factors shifted the acidity dependence of log K for peptides with spacers relative to the reference peptide Trp-Tyr. We identified two separate contributions to the observed IET rate constants: geometry and energetics. The vertical decrease shows the geometric contribution by indicating the degree of separation of the residues. Using this hypothetical rate constant as a reference, the forward IET was accelerated (solid symbols) and the reverse IET was decelerated (open symbols), which was in accordance with the direction of electron transport to or from the positive charge. Both these rate constants were two times lower for the peptide Tyr-Gly-Trp (not shown) than those of Tyr-Trp, showing that only the geometry affected the IET rate constants and that these two peptides were thermodynamically equivalent in terms of the IET. Note that the geometric factor was 2.3 for Trp-Gly-Tyr, which was close to the factor of 2 for Tyr-Gly-Trp. The effect of spacers on the peptides of reverse order provided additional evidence that the chemical surroundings had a larger effect on the tryptophanyl cation radical than on the neutral tyrosyl radical. Thus, we were able to detect the fine-tuning effect of the structure of neighboring groups on IET for the one-electron-deficient systems of tryptophan and tyrosine.

CONCLUSION

The results of the present study demonstrate that the CIDNP method, which is an indirect means of detecting short-lived radical species, is a powerful tool for determining the rate constants of radical reactions and is especially useful when other methods are inapplicable. We used a refined simulation procedure to determine the effects of the charge of the terminal amino group and the presence of glycine or proline spacers on both the kinetic and thermodynamic parameters of reversible intramolecular electron transfer rate constants for oxidized peptides containing tryptophan and tyrosine. This analysis involved modeling the CIDNP kinetic data for all of the three radicals that produced CIDNP in the system under study, namely, the tryptophanyl and the tyrosyl radicals, as well as the dye radical, which was not directly involved in the IET. However, the sign of the dye polarization, which was negative when paired with a tryptophanyl radical and positive when paired with a tyrosyl radical, reflected the ratio of the two types of peptide radicals, where different locations of the radical center made the dye radical act as a type of "wind vane" for the direction and efficiency of IET.

Several pieces of evidence were used to verify the IET rate constants that were determined from simulations of the kinetic CIDNP data: (i) the rate constants that were obtained by the

dx.doi.org/10.1021/jp511068n | J. Phys. Chem. B 2015, 119, 140−149

CIDNP method corresponded with available constants that were obtained by transient optical spectroscopy (similar rate constants were obtained when both experiments were carried out in D_2O, demonstrating a 2-fold difference when different water isotopes were used); (ii) the pH dependence of the logarithm of the equilibrium constant of the IET was linear; (iii) similar pH dependences were obtained for the IET equilibrium constant for oxidized peptides with uncharged terminal groups, and these thermodynamic data agreed with those for *N*-acetyl tryptophan and tyrosine amides with uncharged terminal groups; and (iv) the estimated ratio of the triplet dye quenching rate constants via electron transfer from tryptophan or tyrosine residues correlated satisfactorily with the difference in the reduction potential between the tryptophanyl and tyrosyl radicals.

The results of the present study convincingly demonstrate that the chemical environment can be used to modulate the rate of reversible electron migration via different pathways in peptides. It should be challenging to incorporate these effects into theoretical treatments of charge transfer processes in proteins.

■ ASSOCIATED CONTENT

Supporting Information

Modeling of the CIDNP kinetics in photoreactions of 2,2′-dipyridyl with peptides containing tryptophan and tyrosine; CIDNP spectra, CIDNP kinetics, and parameters of simulations of the CIDNP kinetics that were obtained during the photoreaction of 2,2′-dipyridyl and peptides containing tryptophan and tyrosine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by RFBR (Project No. 13-03-00437), Program of the Division of Chemistry and Material Science RAS (Project 5.1.1), and the Alexander von Humboldt Foundation.

■ ABBREVIATIONS

CIDNP, chemically induced dynamic nuclear polarization; IET, intramolecular electron transfer; DEE, degenerate electron exchange

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