Intramolecular Electron Transfer in Tryptophan−Tyrosine Peptide in Photoinduced Reaction in Aqueous Solution

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The formation of chemically induced dynamic nuclear polarization (^{1}H-CIDNP) and its time evolution in course of the photochemical reactions of 2,2′-dipyridyl in its triplet state with the tryptophan−tyrosine dipeptide and with a 1:1 mixture of *N*-acetyl tryptophan and *N*-acetyl tyrosine is studied in acid (pH 3.0), neutral (pH 7.4), and basic (pH 11.4) solution. Time resolution on the microsecond scale allows us to establish the full reaction scheme and to determine all relevant transient intermediates. The stationary value of spin polarization is found to depend strongly on secondary reaction steps and does only in part reflect the primary process of triplet quenching. Degenerate electron exchange, spin relaxation in paramagnetic intermediates, and radical pair reactions in the bulk are identified from the polarization kinetics. In particular, the increase of tyrosine quenching efficiency and the influence of intramolecular electron transfer from the tyrosine moiety to the transient tryptophan radical are revealed when the amino acids are linked in the dipeptide in comparison with the case that they are separated in the 1:1 mixture. The strong effect of linkage on the amplitude and the kinetics of spin polarization is discussed with regard to application of this method to studying structure and folding kinetics of proteins.

Introduction

Recent applications of Chemically Induced Dynamic Nuclear Polarization (CIDNP) to proteins reveal that from the analysis of the nuclear polarization one can get valuable information about structure and dynamic processes in proteins,[1−5] in particular, with respect to the folding processes.[6−10] This nuclear polarization is built up in course of singlet−triplet transitions in the short-lived intermediate radical pairs formed in the photoinduced reversible reactions of electron or hydrogen atom transfer between accessible amino acid residues in proteins and photoexcited dye molecules.[11−13] It was established that tryptophan, tyrosine, histidine and methionine residues can act as active partners in these triplet quenching reactions.[1,3,11] From the analysis of CIDNP spectra, information about accessibility and reactivity of the residues can be obtained. So far, most chemically induced dynamic nuclear polarization (CIDNP) experiments in protein and protein-related compounds have been performed in the so-called continuous wave detection mode (cw-CIDNP) that measures the stationary polarization after completion of the reactions and thus depends on the secondary reaction steps and spin relaxation.[1−5] In contrast, laser flash initiation of chemical reactions followed by synchronized pulsed FT NMR detection of the CIDNP patterns (TR-CIDNP) makes time resolution down to the submicrosecond time scale possible.[6−10] This approach allows us to perform a quantitative analysis of the CIDNP kinetics and to establish in detail the mechanism of radical reactions between the three amino acids and the dye in solution and to determine the structure of the transient radicals.[11−13] Recently, our systematic study of CIDNP in amino acids, complemented by data from optical transient absorption of intermediates detected under closely matched experimental conditions, was extended to the tryptophan−tyrosine dipeptide[24] and to the protein hen egg white lysozyme (HEWL).[25] In particular, it was shown that the main difference between CIDNP in the mixture of individual amino acids and the residues, linked into dipeptide or into protein, is caused by the intramolecular electron transfer (IET) from the tyrosine residues to the tryptophan radical:

$$\mathrm{TrpH^{+\bullet}} - \mathrm{TyrOH} \xrightarrow{k_1} \mathrm{TrpH} - \mathrm{TyrO^{\bullet}} + \mathrm{H^+} \qquad (1)$$

In the time-resolved CIDNP study of protein HEWL in the denatured state and the dipeptide tryptophan−tyrosine, we demonstrated that the efficient IET strongly affects the kinetics of CIDNP.[24,25] The study showed that the tryptophan−tyrosine dipeptide (Trp−Tyr) can serve as a good model system to understand the change of CIDNP for HEWL caused by temperature- and urea-induced denaturizing. For this protein, the reversible unfolding occurs at pH 3.8.[26a] For other proteins that can be denatured under neutral or basic conditions,[26b] the difference between cw-CIDNP in the native and denatured states might also be due to increasing importance of IET upon the denaturizing, because it is known that IET operates under similar conditions in peptides.[28−36] As a consequence, it is necessary to determine the effect of IET on the CIDNP before a reliable analysis of residue accessibility can be performed. On the other hand, a previous cw-CIDNP study of several peptides containing tryptophan and tyrosine at neutral conditions did not reveal any significant deviation from the stationary CIDNP of the binary 1:1 mixtures of the two amino acids.[27] Thus, we aim our present paper at investigating the influence of IET on the CIDNP kinetics in the dipeptide at different values of pH.

The pK_a value for the tryptophan radical $\mathrm{TrpH^{+\bullet}}$ is 4.3 in the free amino acid[37,38] and between 4.6 and 5.4 when in peptides.[39]

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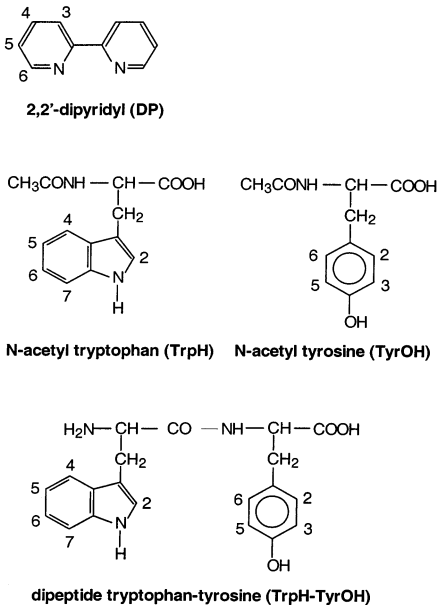
CHART 1



The pK_a value for the phenolic proton of tyrosine is 10.1. It is reasonable to expect that the different structure of reactants in strong acidic, neutral, and strong basic solution results in different efficiency of IET between tyrosine residues and tryptophan radicals.

In this paper, we present a time-resolved study of CIDNP formation in the photoreaction of the dipeptide tryptophan−tyrosine with the aza-aromatic dye 2,2′-dipyridyl (DP) at three values of pH: 3.0, 7.4, and 11.4. The time resolution allows us to separate the polarization of products of geminate termination from the polarization formed in the reactions between radical partners in the bulk and to perform a full kinetic analysis of such radical reactions in solution. From the comparison of the results obtained for the dipeptide and for a 1:1 mixture of individual amino acids, *N*-acetyl-L-tyrosine, and *N*-acetyl-L-tryptophan under the same experimental conditions, additional information about the reactivity of radicals and the importance of IET reactions between the residues in dipeptide at different pH is obtained. The structure of the compounds under study is shown in Chart 1.

Experimental Section

A detailed description of our TR-CIDNP apparatus was given previously.[16,17] The samples, purged with argon and sealed in a standard NMR Pyrex ampule, are irradiated by a COMPEX Lambda Physik excimer laser (wavelength 308 nm, pulse energy up to 150 mJ) in the probe of an DRX-200 Bruker NMR spectrometer. TR-CIDNP experiments are carried out using the usual pulse sequence: rf saturation pulses, laser pulse, evolution time τ, rf detection pulse, and free induction decay. As the background signals in the spectrum originating from Boltzmann polarization are suppressed by the saturation pulses, only signals of the polarized products formed during the variable delay τ between the laser and the NMR radio frequency pulse appear in the CIDNP spectra. In all kinetic measurements, an rf detection pulse with a duration of 1 μs was used.

D_2O (Aldrich), DP (Aldrich), L-tryptophan-L-tyrosine, and the amino acids *N*-acetyl-L-tryptophan and *N*-acetyl-L-tyrosine (Sigma) were used as received. The concentration of the peptide used was 3 mM. In the experiments on the mixture of the amino acids, the concentration of *N*-acetyl tryptophan and *N*-acetyl tyrosine was 3 mM each. The molar extinction coefficient of DP at 308 nm is 12 900 M^{-1} cm^{-1} for the protonated form, DPH^+, and 1200 for the nonprotonated form of DP.[21] The concentration of DP was 5×10^{-4} M at pH 3.0 and 1.4×10^{-2} M at pH 7.4 and 11.4.

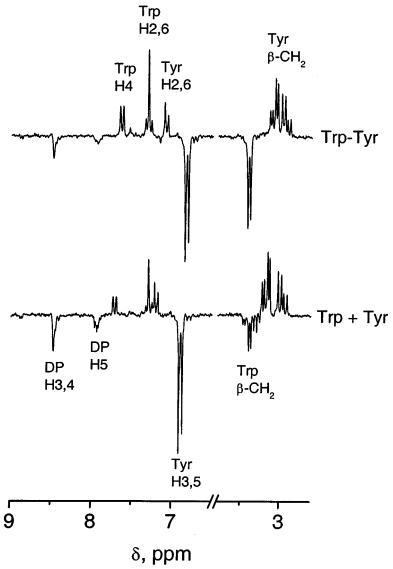


Figure 1. ^{1}H CIDNP spectra obtained immediately after the laser pulse for the solution containing DP with the tryptophan−tyrosine dipeptide (top) and with an equimolar mixture of *N*-acetyl derivatives of tryptophan and tyrosine (bottom) at pH 3.0.

Results

CIDNP effects in the reaction between the photoexcited triplet dye and the dipeptide tryptophan−tyrosine are detected only for protons of the initial compounds; therefore, one can conclude that the reaction is reversible and leads to restoration of the reactants. The CIDNP spectra for the 1:1 mixture of the two amino acids reacting with DP and for the dipeptide reacting with DP, all taken at zero delay τ after the laser pulse, at pH 3.0, 7.4, and 11.4, are shown in Figures 1, 3, and 5, respectively.

In aqueous solution, the ground state and the excited triplet state of dipyridyl can exist in the protonated and in the neutral form (DPH^+ and $^TDPH^+$, DP and ^TDP, respectively). The pK_a for the triplet state is 5.8,[23] while for the ground state the pK_a value is 4.3.[40,41] At the chosen values of pH (3.0, 7.4, and 11.4), one can neglect the protonation reactions in the triplet state. For each pH, only the prevailing form of the triplet dye is taken into consideration in the description given below.

Irrespective of the pH, the following CIDNP effects are observed for the amino acids in the mixture and in the dipeptide. For tryptophan, the protons at the positions H4, H2,6 show enhanced absorption, and the β-CH_2 protons show emission; for tyrosine, H2,6 exhibit enhanced absorption, H3,5 exhibit emission, and β-CH_2 exhibits enhanced absorption. H2 and H6 of tryptophan are not equivalent in the intermediate radical; however, in most cases, they could not be separated in the NMR spectrum. Thus, only the sum of their polarization is considered. In the aromatic region, which is important for CIDNP analysis of proteins, the most intense polarization is detected for H2,6 of tryptophan and H3,5 of tyrosine. Therefore, the polarization of these protons is used for illustration of the kinetic behavior of CIDNP, unless otherwise stated.

The sign of nuclear polarization formed for DP depends on the partner radical in the radical pair. The *g*-factors of the radicals increase in the sequence g(Trp) < g(DP) < g(Tyr).[42−44]

According to the Kaptein rules, the sign of polarization is determined by the sign of the difference between the g-factors of the paired radicals.[45] For the pair consisting of dipyridyl and tyrosine radicals, this difference is negative (g(DP) − g(Tyr) < 0), whereas for the pair of dipyridyl and tryptophan radicals it is positive (g(DP) − g(Trp) > 0). Accordingly, for DP, enhanced absorption is formed in the radical pair with tyrosine[22] and emission is formed in the radical pair with tryptophan.[21] The resulting CIDNP detected for DP is sensitive to the relative concentration of tryptophan and tyrosine radicals, making it convenient to follow intramolecular transformations of the peptide radical by analyzing DP polarization. The most intensive polarization in DP is observed for the H3,4 protons.

At each value of pH, the absolute value of NMR signal intensity of H3,4 of dipyridyl detected immediately after the laser pulse (the first point in the kinetics) is taken as unity. The polarization of the other protons is scaled accordingly. For tryptophan and tyrosine, the magnitude of line intensities is plotted; for DP, the sign of polarization is also shown.

Acidic Solution. Figure 1 shows CIDNP spectra, obtained immediately after the laser pulse for the dipeptide and for comparison, for an equimolar mixture of *N*-acetyl derivatives of tryptophan and tyrosine at pH 3.0. These spectra are very similar, despite some deviations in chemical shift of the corresponding protons and in the shape of the NMR signal of the β-CH_2 protons of tryptophan. In the dipeptide, this signal appears as a doublet due to splitting with the α-CH proton indicating magnetic equivalency of the β-CH_2 protons of tryptophan, while in *N*-acetyl tryptophan it has a multiplet pattern formed from a quartet of an AB system of nonequivalent β-CH_2 protons with an additional splitting due to spin−spin interaction of each β-CH_2 proton with the proton in α-position. The close similarity of absolute and relative intensities in the geminate CIDNP for the dipeptide system and for the solution of DP with the mixture of amino acids is good evidence that the primary photochemical processes are the same for both cases. In acidic solution, the protonated triplet dipyridyl $^{T}DPH^{+}$ (pK_a = 5.8) is quenched by *N*-acetyl tryptophan and *N*-acetyl tyrosine via electron transfer with the rate constants 4.7×10^9 M^{-1} s^{-1} [21] and 3.5×10^9 M^{-1} s^{-1},[22] respectively. The reaction of the quenching of the protonated dipyridyl triplet by the dipeptide proceeds in the following ways:

$$^{T}DPH^{+} + TrpH - TyrOH \xrightarrow{k_q^{Trp}} DPH^{\bullet} + TrpH^{+\bullet} - TyrOH \quad (2)$$

$$^{T}DPH^{+} + TrpH - TyrOH \xrightarrow{k_q^{Tyr}} DPH^{\bullet} + TrpH - TyrO^{\bullet} + H^{+} \quad (3)$$

The tyrosine-centered cation radical $TrpH - TyrOH^{+\bullet}$ of the dipeptide undergoes fast deprotonation with the formation of $TrpH - TyrO^{\bullet}$ ($pK_a = -1$ for the tyrosine radical[46]), as it is shown in eq 3. The neutral radical $DPH^{\bullet}$ ($pK_a = 8.5$[23]) is subsequently protonated in the bulk to $DPH_2^{+\bullet}$ in the acid (pH 3.0) and in the neutral (pH 7.4) solutions.

The negative sign of polarization of DP in the geminate product results from the concerted action of two factors: The first is the predominance of the radical pairs with tryptophan-centered radicals due to the higher value of the corresponding quenching rate constant. At our experimental conditions (pH 3), $k_q^{Trp}/k_q^{Tyr} \approx 1.3$ for free amino acids. The second is the slightly higher CIDNP enhancement factor in the pair with the tryptophan radical.[24]

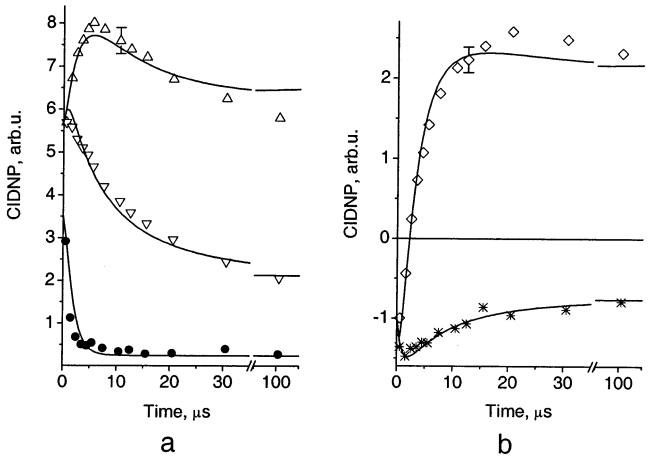


Figure 2. CIDNP kinetics at pH 3.0 for (a) H3,5 of tyrosine residue in peptide (△), H3,5 of *N*-acetyl tyrosine (▽), and for H2,6 of the tryptophan residue in peptide (●) obtained in photoreactions of the corresponding compounds with DP and for (b) H3,4 of DP obtained in its photoreaction with the dipeptide tryptophan−tyrosine (◇) and with a 1:1 mixture of *N*-acetyl tryptophan and *N*-acetyl tyrosine (*). Solid lines, calculations (see text). Parameters of simulation: $\alpha = 0.56$; for the peptide: $k_t \times R_0 = 3 \times 10^5$ s^{-1}, $k_1 = 6 \times 10^5$ s^{-1}; for the amino acid mixture: $k_t \times R_0 = 5 \times 10^5$ s^{-1}, $k_1 = 0$; and in (a) $T_{1Trp} = 26$ μs, $T_{1Tyr} = 33$ μs (in peptide), $T_{1Tyr} = 63$ μs (*N*-acetyl tyrosine), $\gamma = 1.4$ (Tyr), $\gamma = 2.8$ (Trp), $k_{ex} = 3.3 \times 10^7$ M^{-1} s^{-1} (in the pair $TrpH^{+\bullet}$−TyrOH/TrpH−TyrOH); in (b) $T_{1DP} = 44$ μs, $\gamma = 2.8$, P^G(Trp)/P^G(Tyr) = −1.7.

The CIDNP kinetics for tryptophan and tyrosine at pH 3.0 is shown in Figure 2a. A fast decay of the tryptophan signal is observed, as in the case of nonlinked tryptophan, when the CIDNP kinetics is determined by degenerate electron exchange in the pair $TrpH^{+\bullet}$/TrpH.[21] For tyrosine, however, both kinetic behavior and stationary value of its CIDNP change upon linkage into the peptide. An even higher change is detected for the CIDNP kinetics of dipyridyl (Figure 2b). With the dipeptide as reaction partner, polarization becomes positive after 3 μs, and the stationary absorptive polarization is two times higher than the emissive CIDNP intensity immediately after the laser pulse. With the nonlinked amino acids, the CIDNP of dipyridyl remains negative, and the shape of the kinetic curve is similar to that obtained with individual free amino acids.

Neutral Solution. CIDNP spectra for the peptide and the mixture of amino acids, obtained at pH 7.4 with no delay between the laser pulse and the detecting rf pulse, are shown in Figure 3.

The measured quenching rate constants for *N*-acetyl tryptophan and *N*-acetyl tyrosine at this pH are 3.7×10^9 M^{-1} s^{-1} [21] and 7.9×10^7 M^{-1} s^{-1},[22] respectively. Because of the big difference in quenching rate constants, only weak signals of tyrosine are seen in the spectrum of the amino acid mixture. In contrast, the signals of tyrosine in the case of the peptide are much more pronounced. The intensities of tryptophan signals are nearly the same in both spectra. This leads us to the conclusion that the reactivity of tyrosine increases upon linkage into a peptide. The CIDNP kinetics for both peptide residues and for DP (in reaction with peptide and mixture) are shown in Figure 4.

At neutral pH, triplet dipyridyl (^{T}DP) is not charged. As it was found earlier, quenching of ^{T}DP by tyrosine proceeds via hydrogen transfer,[22] the rate constant of which is usually more than an order of magnitude lower than that of electron transfer. However, from CIDNP data, it is impossible to determine the mechanism of quenching of ^{T}DP by the tyrosine residue in the peptide. In the case of electron transfer, the primary-formed

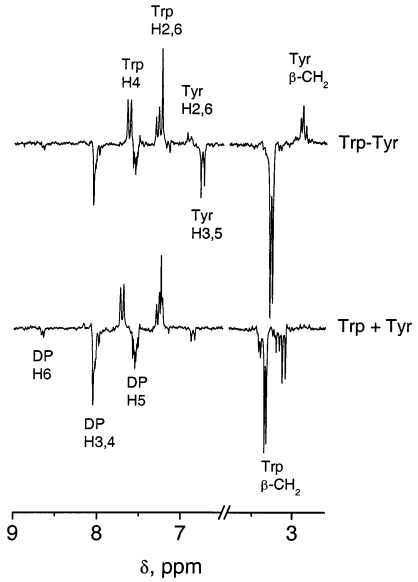


Figure 3. ^{1}H CIDNP spectra obtained immediately after the laser pulse for the solution containing DP with the tryptophan−tyrosine dipeptide (top) and with an equimolar mixture of *N*-acetyl derivatives of tryptophan and tyrosine (bottom) at pH 7.4

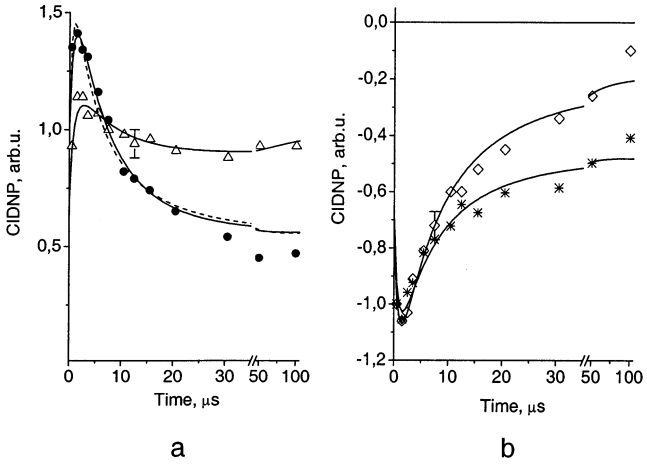


Figure 4. CIDNP kinetics at pH 7.4 for (a) H3,5 of tyrosine residue in peptide (△) and for H2,6 of the tryptophan residue in peptide (●) obtained in photoreactions of the corresponding compounds with DP and (b) H3,4 of DP obtained in its photoreaction with the dipeptide tryptophan−tyrosine (◇) and with a 1:1 mixture of *N*-acetyl tryptophan and *N*-acetyl tyrosine (*). Lines, calculations (see text). Parameters of simulation in (a): Solid lines: $k_t \times R_0 = 5 \times 10^5$ s^{-1}, $k_2 = 4 \times 10^4$ s^{-1}, $\alpha = 0.7$, $T_{1Tyr} = 26$ μs, $T_{1Tyr} = 33$ μs, $\gamma = 2.8$. Dashed line: $k_t \times R_0 = 8.7 \times 10^5$ s^{-1}, $T_{1Trp} = 44$ μs, $k_2 = 0$; in (b): for DP with peptide: $k_t \times R_0 = 5 \times 10^5$ s^{-1}, $k_2 = 4 \times 10^4$ s^{-1}, $\alpha = 0.7$, $T_{1DP} = 44$ μs, $\gamma = 2.8$, P^G(Trp)/P^G(Tyr) = −2.9; for DP with the mixture of the amino acids: $k_t \times R_0 = 6 \times 10^5$ s^{-1}, $k_2 = 0$, $\alpha = 1$, $T_{1DP} = 44$ μs, $\gamma = 2.8$.

tyrosine cation radical undergoes very fast deprotonation resulting in the same TyrO$^\bullet$ radical as in the case of quenching via the hydrogen atom transfer, with the result that the CIDNP kinetics of dipyridyl in neutral solution on the microsecond time scale is independent of the quenching mechanism.[22]

Basic Solution. Figure 5 shows TR-CIDNP spectra obtained at irradiation under basic conditions (pH 11.4) for the amino acids mixture (top) and the dipeptide (bottom). As in the case of acid and neutral solutions, the spectra are taken at zero delay between laser pulse and rf detection pulse.

At this pH, the tyrosine phenol ring is negatively charged (pK_a = 10.1 for the phenol proton[26]): TyrO$^-$ in the free amino acid and TprH − TyrO$^-$ in the dipeptide. For this molecular

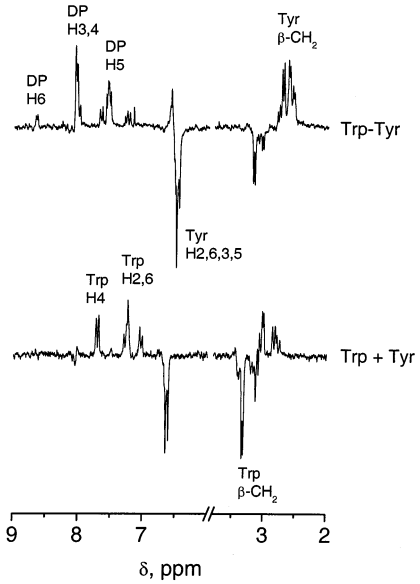


Figure 5. ^{1}H CIDNP spectra obtained immediately after the laser pulse for the solution containing DP with the tryptophan−tyrosine dipeptide (top) and with an equimolar mixture of *N*-acetyl derivatives of tryptophan and tyrosine (bottom) at pH 11.4.

structure, the aromatic 2,6 and 3,5 protons of tyrosine in the dipeptide appear as an A_2B_2 system in the NMR spectra. Because the hyperfine interaction constants for H2,6 and H3,5 in the intermediate radical differ in magnitude and sign, an antiphase multiplet structure is observed around 6.7 ppm. In basic solution, the β-CH$_2$ protons of the tryptophan residue become inequivalent after removal of the proton from the N-end (p$K_a \approx 9$ for the proton of the amino group)[26] with the result that the corresponding NMR signal is changed from a doublet (as it was in neutral and in acid solution) to a multiplet, which is an AB quartet split into eight lines due to interaction with the α-CH proton.

In basic solution, quenching of the triplet excited dipyridyl proceeds via electron transfer from both tryptophan and tyrosine.[21,22] For nonlinked amino acids, the quenching rate constants measured at this pH are 3.3×10^9 M^{-1} s^{-1} (*N*-acetyl tryptophan)[21] and 1.7×10^9 M^{-1} s^{-1} (*N*-acetyl tyrosine).[22] From the analysis of the geminate CIDNP pattern, we can conclude that the following primary quenching reactions take place between the triplet dipyridyl ^{T}DP and the dipeptide TrpH − TyrO$^-$:

$$^T\mathrm{DP} + \mathrm{TrpH} - \mathrm{TyrO}^- \xrightarrow{k_q^{Trp}} \mathrm{DP}^{-\bullet} + \mathrm{TrpH}^{+\bullet} - \mathrm{TyrO}^- \quad (4)$$

$$^T\mathrm{DP} + \mathrm{TrpH} - \mathrm{TyrO}^- \xrightarrow{k_q^{Tyr}} \mathrm{DP}^{-\bullet} + \mathrm{TrpH} - \mathrm{TyrO}^\bullet \quad (5)$$

The quenching reactions 4 and 5 are followed by a fast protonation of the dipyridyl anion radical (pK_a = 14 for DPH$^\bullet$),[47] which is not shown. Deprotonation of the tryptophan cation radical is discussed below in conjunction with eq 19.

Two major differences are seen from the comparison of CIDNP of linked and nonlinked amino acids. The first one is the relatively low intensity of the tryptophan signals in the spectrum of the dipeptide. The second difference is the CIDNP pattern of dipyridyl. In the case of the amino acid mixture, polarization of dipyridyl is close to zero, whereas in the case of the peptide enhanced absorption is observed. It means that for the product of geminate termination in the reaction with the peptide the polarization of dipyridyl protons in the pair with tyrosine radical (reaction 5) predominates, while in the reaction

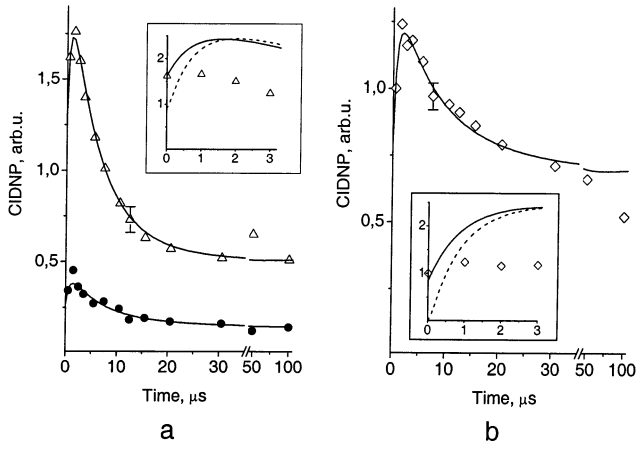


Figure 6. CIDNP kinetics at pH 11.4 obtained in photoreactions of tryptophan−tyrosine dipeptide with DP for (a) H3,5 of tyrosine residue in peptide (△) and for H2,6 of the tryptophan residue in peptide (●) and (b) H3,4 of DP (◇). Lines, simulations (see text) with the parameters: $\alpha = 0.38$, $\gamma = 2.8$, $k_t \times R_0 = 5.5 \times 10^5\ s^{-1}$, $k_4 = 4 \times 10^4\ s^{-1}$. In (a), $T_{1Trp} = 26\ \mu s$, $T_{1Tyr} = 33\ \mu s$, $k_{ex} = 4 \times 10^7\ M^{-1}\ s^{-1}$ (in the pair TrpH−Tyr$^{\bullet}$/TrpH−TyO$^-$); in (b), $T_{1DP} = 44\ \mu s$, $P^G(Trp)/P^G(Tyr) = -0.12$. Inserts (solid and dashed lines, simulations with and without taking into account rf pulse lengths, respectively; see text): $k_3 = 1.2 \times 10^7\ s^{-1}$, $k_d = 4 \times 10^6\ s^{-1}$, $\alpha = 0.7$, $T_{1Tyr} = 33\ \mu s$, and $P^G(Trp)/P^G(Tyr) = -1$.

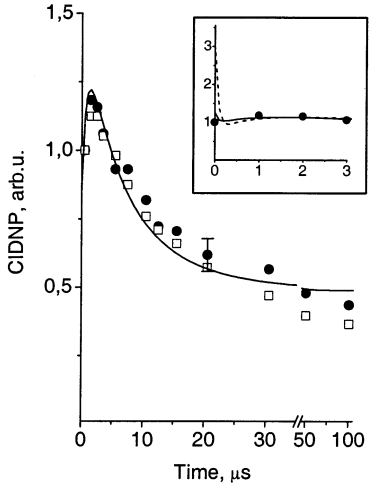


Figure 7. CIDNP kinetics for β-CH_2 protons of the tryptophan residue in the dipeptide: (●) pH 11.4; (□) pH 7.4. Lines, calculation (see text) with the parameters: $k_t \times R_0 = 5.5 \times 10^5\ s^{-1}$, $k_4 = 4 \times 10^4\ s^{-1}$, $T_{1Trp} = 25\ \mu s$, $\gamma = 2.8$. Insert: $k_t \times R_0 = 5 \times 10^5\ s^{-1}$, $k_3 = 1.2 \times 10^7\ s^{-1}$, $k_d = 4 \times 10^6\ s^{-1}$, $T_{1Trp} = 25\ \mu s$, and $\gamma = 2.8$.

with the amino acid mixture polarization of roughly equal magnitude but of opposite sign is formed in the two pairs, with tyrosine and with tryptophan radical.

Figure 6a shows the CIDNP kinetics for the H3,5 protons of the tyrosine residue and H2,6 of the tryptophan residue in the dipeptide. The CIDNP kinetics for H3,4 of DP obtained in its photoreaction with the dipeptide is shown in Figure 6b.

A remarkable fact is that a very good coincidence of the CIDNP kinetics in basic and in the neutral solution is found for the protons of the tryptophan residue, as it is shown in Figure 7 for the β-CH_2.

Discussion

CIDNP in Reversible Radical Reactions. The studied reactions of amino acids with photoexcited triplet dye molecules are reversible; that is, radical termination restores the initial compounds. In these reactions, transient triplet geminate radical pairs are formed via electron or hydrogen atom transfer from the amino acid molecules to the triplet excited dye. Intersystem crossing in the geminate radical pair and subsequent back electron or hydrogen transfer out of the singlet state results in restoration of the reactants. The probability of triplet−singlet conversion in the radical pair is sensitive to the spin configuration of nuclei having hyperfine coupling with the electron spin. Thus, in the geminate products, nuclear polarization is formed. Radicals that escape geminate termination carry nuclear polarization equal in magnitude and opposite in sign with respect to the geminate one. This process is known as the spin-sorting S-T_0 mechanism of CIDNP formation in high magnetic fields.[12,13] When radicals decay in the bulk, such "escape" polarization is transferred from radicals to products, leading to so-called CIDNP cancellation.[11−13] The cancellation is incomplete because of nuclear spin−lattice relaxation in the radicals. Processes that lead to polarization transfer from radicals to products in the bulk are (i) second-order radical termination reactions (rate constant k_t) and (ii) degenerate electron exchange with corresponding ground state molecules (rate constant k_{ex})

$$*RH^{\bullet+} + RH \rightarrow *RH + RH^{\bullet+}$$

where the asterisk denotes nuclear polarization. A difference between the two pathways is that in bimolecular reactions in randomly colliding radical pairs (pathway i), additional polarization can be formed (so-called F pair polarization). In the case of a triplet precursor, it has the same sign as geminate polarization.[19,20]

The evolution of the radical concentration R and of spin polarization in radicals, P^R, and in diamagnetic products, P, can be described by the following set of differential equations:[19,20]

$$R(t) = \frac{R_0}{1 + k_t R_0 t} \tag{6}$$

$$\frac{dP^R}{dt} = -k_t P^R R - k_t \beta R^2 - \frac{P^R}{T_1} - k_{ex} P^R \tag{7}$$

$$\frac{dP}{dt} = k_t P^R R + k_t \beta R^2 + k_{ex} P^R \tag{8}$$

where R_0 is the initial radical concentration, k_t is the second-order radical termination rate constant, T_1 is the nuclear spin−lattice relaxation time in the paramagnetic state, and parameter α represents the efficiency of polarization formation in F pairs, $\beta = \gamma P^G/R_0$, where P^G is the geminate polarization. In the case of a triplet precursor, the upper limit of γ is 3. In previous simulations,[21−25] we used in most cases $\gamma = 2.8$ as suggested by Vollenweider.[19] Degenerate electron exchange is usually treated as irreversible with a pseudo-first-order rate constant k_{ex}, which is a product of a second-order exchange rate constant and the concentration of the molecules, participating in the exchange reaction. Initial conditions are $P(Pr) = -P(R) = P^G$.

The value and the sign of geminate polarization (per individual geminate pair) are determined by the magnetic parameters of the radicals in the pair: g-factors and hyperfine constants. The time evolution of CIDNP is determined by the rate and the mechanism of radical decay in the bulk, and the rate (T_1^{-1}) of nuclear paramagnetic relaxation. In particular, the stationary CIDNP intensity observed after completion of radical termination depends on the ratio of the radical lifetime and T_1 (spin−lattice relaxation in the diamagnetic products is negligible on the time scale of our experiment).

Also, when different quenchers compete for the excited triplet dye, the relative geminate CIDNP intensities are proportional to the ratio of the corresponding quenching rates.

In acid solution (pH 3), the observed effects are in accordance with the assumption of fast IET from the tyrosine residue to the tryptophan cation radical in the peptide:

$$\mathrm{TrpH}^{+\bullet} - \mathrm{TyrOH} \xrightarrow{k_1} \mathrm{TrpH} - \mathrm{TyrO}^{\bullet} + \mathrm{H}^{+} \qquad (9)$$

In this reaction step, tryptophan residue radicals that escape geminate termination and carry polarization opposite to the geminate one are converted into their diamagnetic ground state, thus contributing to CIDNP cancellation. Tyrosine radicals of the peptide formed via reaction 9 are initially not polarized. They can, however, acquire polarization during singlet−triplet transition in F pairs (vide supra) in subsequent bimolecular reaction with dipyridyl radicals. This explains why the rate of the polarization growth for tyrosine does not coincide with the rate of polarization decay of tryptophan but lags behind. The change of sign of CIDNP for DP with time also reflects CIDNP formation in the F pairs having a tyrosine radical as partner.

In the presence of IET, radical concentrations are described by the following equations:

$$R_{\mathrm{DP}}(t) = \frac{R_0}{1 + k_t R_0 t} \qquad (10)$$

$$R_{\mathrm{Trp}}(t) = \frac{\alpha R_0 \mathrm{e}^{-k_1 t}}{1 + k_t R_0 t} \qquad (11)$$

$$R_{\mathrm{Tyr}}(t) = \frac{R_0(1 - \alpha \mathrm{e}^{-k_1 t})}{1 + k_t R_0 t} \qquad (12)$$

Here, R_0 is the initial concentration of dipyridyl radicals, being equal to the sum of the concentrations of tryptophan- and tyrosine-centered peptide radicals; α is the fraction of tryptophan radicals, $\alpha = k_q^{\mathrm{Trp}}/(k_q^{\mathrm{Tyr}} + k_q^{\mathrm{Trp}})$. For simplicity, we presume that radical termination proceeds with the same rate constant for all radicals involved.

For simulation of the CIDNP kinetics, we use a combination of eqs 10−12 with the differential equations for polarization (6−8), modified for the case when radical interconversion via IET is present. This requires additional terms in the differential equations describing the polarization of tryptophan radical $P^{\mathrm{R}}_{\mathrm{Trp}}$ and ground state molecule P_{Trp}; for polarization of dipyridyl radical, $P^{\mathrm{R}}_{\mathrm{DP}}$, and ground state molecule, P_{DP}, we take into account that CIDNP of different sign in the two types of radical pairs (with tryptophan and tyrosine radicals, values β_1 and β_2, respectively) is formed

$$\frac{\mathrm{d}P^{\mathrm{R}}_{\mathrm{Trp}}}{\mathrm{d}t} = -k_t P^{\mathrm{R}}_{\mathrm{Trp}} R_{\mathrm{DP}} - k_t \beta R_{\mathrm{DP}} R_{\mathrm{Trp}} - \frac{P^{\mathrm{R}}_{\mathrm{Trp}}}{T_{1\mathrm{Trp}}} - k_{\mathrm{ex}} P^{\mathrm{R}}_{\mathrm{Trp}} - k_1 P^{\mathrm{R}}_{\mathrm{Trp}} \qquad (13)$$

$$\frac{\mathrm{d}P_{\mathrm{Trp}}}{\mathrm{d}t} = k_t P^{\mathrm{R}}_{\mathrm{Trp}} R_{\mathrm{DP}} + k_t \beta R_{\mathrm{DP}} R_{\mathrm{Trp}} + k_{\mathrm{ex}} P^{\mathrm{R}}_{\mathrm{Trp}} + k_1 P^{\mathrm{R}}_{\mathrm{Trp}} \qquad (14)$$

$$\frac{\mathrm{d}P^{\mathrm{R}}_{\mathrm{DP}}}{\mathrm{d}t} = k_t P^{\mathrm{R}}_{\mathrm{DP}}(R_{\mathrm{Trp}} + R_{\mathrm{Tyr}}) - k_t \beta_1 R_{\mathrm{DP}} R_{\mathrm{Trp}} - k_t \beta_2 R_{\mathrm{DP}} R_{\mathrm{Tyr}} - \frac{P^{\mathrm{R}}_{\mathrm{DP}}}{T_{1\mathrm{DP}}} \qquad (15)$$

$$\frac{\mathrm{d}P_{\mathrm{DP}}}{\mathrm{d}t} = k_t P^{\mathrm{R}}_{\mathrm{DP}}(R_{\mathrm{Trp}} + R_{\mathrm{Tyr}}) + k_t \beta_1 R_{\mathrm{DP}} R_{\mathrm{Trp}} + k_t \beta_2 R_{\mathrm{DP}} R_{\mathrm{Tyr}} \qquad (16)$$

In our simulations, we took the values of nuclear paramagnetic spin−lattice relaxation times $T_{1\mathrm{DP}}$, $T_{1\mathrm{Trp}}$, and $T_{1\mathrm{Tyr}}$ found in previous investigations.[21,22,24] For the parameter γ, we used the value 2.8, except in the case of tyrosine, for which $\gamma = 1.4$ was found in the previous investigation.[22] The parameters $R_0 \times k_t$ and k_1 were varied to optimize the fit simultaneously for dipyridyl, tryptophan, and tyrosine. Besides, we varied the scaling factor (parameter P^{G}) for each polarized signal.

The results of the simulations are shown in Figures 2a and 3a by solid lines. Fitting parameters are shown in the figure captions. The best fit is obtained at $k_1 = 6 \times 10^5\ \mathrm{s}^{-1}$, in good agreement with the previously obtained value of $k_1 = 5.5 \times 10^5\ \mathrm{s}^{-1}$ at pH 3.8.[24] Although the number of fitting parameters used in the simulation procedure is fairly high, the range of their variability is rather constrained due to two reasons. The first reason is the necessity to obtain a satisfactory fit with common values of k_1 and $R_0 \times k_t$ simultaneously for tryptophan and tyrosine, both involved in the reaction of electron transfer, and for dipiridyl, the partner for tryptophan and tyrosine in the radical stage. The second reason is that the correlation among different parameters is low, since they affect the simulated curves in different, characteristic ways: the value of k_1 determines the decay rate of tryptophan CIDNP and the value of tyrosine polarization created in the F pairs; the rate of CIDNP amplitude change at early times for tyrosine and dipyridyl is sensitive to $R_0 \times k_t$; having k_1 and $R_0 \times k_t$ set the values of T_1 determines the stationary level of CIDNP with respect to the initial value. Altogether, 20% variation of the parameter values is the upper limit for staying in the frame of satisfactory simulations.

Neutral Solution. At neutral pH, the mechanism of quenching of $^{\mathrm{T}}$DP by tryptophan is electron transfer.[21] Because the tryptophan cation radical has p$K_a = 4.3$, in neutral solution, it undergoes deprotonation with the rate constant $1.5 \times 10^6\ \mathrm{s}^{-1}$:[48]

$$\mathrm{TrpH}^{+\bullet} \rightarrow \mathrm{Trp}^{\bullet} + \mathrm{H}^{+} \qquad (17)$$

Reaction 17 is faster than IET to the cation radical (9), hence electron transfer to the tryptophan cation radical does not manifest itself at neutral pH. The neutral tryptophan radical is also known to accept an electron from the tyrosine residue.[29] IET to the neutral tryptophan radical leads to formation of the tryptophan anion Trp^- with a p$K_a = 16.8$.[49] Thus, electron transfer is followed by fast protonation of the tryptophan anion and fast deprotonation of the tyrosine cation radical, as it was mentioned above. A second possibility is direct H^+ transfer from the tyrosine cation radical to the tryptophan anion. The total process of electron transfer and subsequent protonation/deprotonation in neutral pH can thus be written as:

$$\mathrm{Trp}^{\bullet} - \mathrm{TyrOH} \xrightarrow{k_2} \mathrm{TrpH} - \mathrm{TyrO}^{\bullet} \qquad (18)$$

The reported value of k_2 is $6 \times 10^4\ \mathrm{s}^{-1}$ in $\mathrm{H_2O}$[32] and $2 \times 10^4\ \mathrm{s}^{-1}$ in $\mathrm{D_2O}$,[32] i.e., much lower than the second-order termination rate under our conditions. To check whether IET has influence on CIDNP, we performed simulation of the CIDNP kinetics with and without this process.

Experimental data for the CIDNP kinetics for tryptophan in the dipeptide are almost the same as for nonlinked tryptophan (not shown): a small initial growth and decay to approximately

40% of the initial polarization. Without taking into account reaction step 18 in the model calculations, a reasonably good fit is in principle obtainable (Figure 4a, dashed line), but only if the same spin−lattice relaxation time in the paramagnetic state, T_1, as for nonlinked tryptophan (44 μs) is used, in contradiction to earlier results.[24] With taking into account IET and using $T_1 = 26$ μs as found earlier for tryptophan in the peptide,[24] we obtain the best fit at $k_2 = 4 \times 10^4$ s^{-1} (Figure 4a, solid line). The simulation of the CIDNP kinetics of dipyridyl and tyrosine is also sensitive to the parameter α, which is found from the best fit to be 0.7. Nuclear polarization of tyrosine rises higher than it would be without IET. In general, all of the features in the kinetic behavior caused by IET manifest themselves after 15 μs. It is illustrated by the comparison of the CIDNP kinetics for dipyridyl, obtained in the reaction with the peptide and with the amino acid mixture (Figure 4b). In the presence of slow IET, emissive polarization of dipyridyl is smaller at longer times, reflecting the growing contribution of absorptive F pair polarization with tyrosine radicals. Thus, although the value of k_2 is small in comparison with the rate of bimolecular radical termination, it affects the stationary plateau of polarization of DP; as a consequence, our simulation is sensitive to this value.

It is noteworthy that the initial and the stationary CIDNP values for tyrosine almost coincide in the cases of fast and slow IET (Figures 2a and 4a). To reproduce this observation, we have to use different values of γ in the simulations: 1.4 for acidic solution and 2.8 for neutral solution; that is, in acidic solution, polarization formation in F pairs is less effective. Earlier, we used the same γ values (1.4 for acidic solution and 2.8 for neutral solution) in studies of *N*-acetyl tyrosine.[22] We tentatively attribute the difference to the "pair substitution effect" due to fast deprotonation of the tyrosine radical in the geminate pair,[50] but so far, we have no exhaustive explanation of this difference.

Basic Solution. In basic solution, too, the observed intensity of tryptophan signals (relative to tyrosine) is approximately 3.5 times lower than it is expected when the reactivity of the amino acid residues in the peptide is the same as for free amino acids.

We see two explanations for the observed differences in the CIDNP spectra of the amino acid mixture and the peptide. The first one is that the reactivity of the amino acids toward the triplet dye changes upon linkage into the peptide. At neutral solution, we found evidence for this assumption.

At basic solution, the IET between the tryptophan radical and the tyrosine anion (reaction 19) is assumed to be fast;[30] therefore, it is possible that it competes with the deprotonation of the tryptophan cation radical (reaction 20):

$$\mathrm{TrpH^{+\bullet}} - \mathrm{TyrO^-} \xrightarrow{k_3} \mathrm{TrpH} - \mathrm{TyrO^\bullet} \qquad (19)$$

$$\mathrm{TrpH^{+\bullet}} - \mathrm{TyrO^-} \xrightarrow{k_d} \mathrm{Trp^\bullet} - \mathrm{TyrO^-} + \mathrm{H^+} \qquad (20)$$

To check the relative efficiencies of these processes, corresponding model calculation of CIDNP kinetics are performed. Because of the presumed high rates, it is necessary to use special care in the analysis, since the influence of the finite duration of the detecting rf pulse has to be considered.[51]

The experimental data for the kinetics of nuclear polarization result from a convolution of the evolution of magnetization under the influence of the rf pulse and the CIDNP kinetics in radical reactions. In our experiments, a detecting rf pulse of 1 μs duration is used. As long as the radical reactions and the CIDNP develop on the time scale of several microseconds, it is not necessary to invoke the convolution procedure for such a short detection pulse when model calculation and experimental data are compared. However, convolution becomes important when CIDNP changes significantly during application of the detecting rf pulse.

This is demonstrated for the case of fast electron transfer. The calculated time evolution of CIDNP is shown in the inserts in Figures 6a,b and 7 by dashed (simulated kinetics without convolution) and solid (after the convolution of the simulated data assuming a rectangular rf pulse of 1 μs duration) lines. At certain kinetic parameters, the decay of the tryptophan signal is so fast that the value of polarization detected during the first microsecond, i.e., with the delay time $\tau = 0$, is by more than a factor of three lower than the initial polarization, and this is in agreement with the low intensity of the experimentally detected signals of tryptophan. Thus, a consideration based only on the kinetic behavior of the CIDNP for the tryptophan residue does not rule out the importance of reaction 19. However, it is necessary to check the consistency of the model by considering the CIDNP kinetics of the tyrosine residue and of dipyridyl, too. The tyrosine radicals formed via IET are not polarized. They become polarized only in bimolecular reactions with the radicals of dipyridyl (F pair polarization). The rate of this process is much lower than the supposed rate of IET, and both simulated evolution curves, with and without convolution (dashed and solid lines in Figure 6a, insert) demonstrate significant growth, in contrast to observation. Qualitatively, the same is valid for the CIDNP kinetics of dipyridyl (Figure 6b, insert). If we use equal polarization from tryptophan and tyrosine radical pairs as initial conditions for the simulation, in the case of a fast IET, positive polarization during the first microsecond and its further growth are calculated, but this is not seen in the experiments. From the kinetic analysis we, therefore, conclude that the reaction of IET does not compete with deprotonation, and that it is the relatively high reactivity of the tyrosine residue in the dipeptide that is responsible for the observed CIDNP intensities. Indirect evidence in favor of increasing reactivity of tyrosine residue is the intensity of the geminate CIDNP signal observed for the tetrapeptide TrpH−Gly−Gly−TyrOH in basic solution.[52] For this peptide, we observed almost the same line intensities in the geminate CIDNP spectrum as for the peptide TrpH−TyrOH. The distance dependence of the IET rate was investigated for peptides with a varying number of glycine and proline spacers between tryptophan and tyrosine. As reported in neutral solution, the rate of IET drops by a factor of three when two glycine spacers are introduced between tyrosine and tryptophan.[30] If we presume that in basic solution the IET distance dependence is similar, the fact that the relative CIDNP intensities of tyrosine and tryptophan in basic solution are independent of the number of spacers also rules out the importance of the reaction 19.

For the estimation of the ratio between the quenching rates k_q^{Trp}:k_q^{Tyr} in the reactions of the triplet DP with the dipeptide, we compare the intensities of the lines H2,6 for the tryptophan moiety with the total signal of tyrosine (H3,5 and H2,6 protons together) in the aromatic region of the spectra in Figure 5. Taking into account that the ratio of quenching rate constants for *N*-acetyl derivatives of tryptophan and tyrosine k_q^{Trp}:k_q^{Tyr} = 1:0.5,[21,22] the corresponding ratio for the residues in the peptide is estimated to be 1:1.75.

In another possible IET reaction step, the tyrosine anion is converted to the neutral radical, and the reaction is accompanied

TABLE 1: Key Reaction Steps and Participating Molecules

pH	quenchers	initial reactants	transient radicals	radicals participating in DEE (with rate constants)	IET pathways (with rate constants)
3.0	dipeptide	$^{T}DPH^{+}$, TrpH − TyrOH	$DPH_2^{+\bullet}$ $TrpH^{+\bullet}$ − TyrOH TrpH − $TyrO^{\bullet}$	$TrpH^{+\bullet}$ − TyrOH $k_{ex} = 3.3 \times 10^7\ M^{-1}\ s^{-1}$	$TrpH^{+\bullet} - TyrOH \xrightarrow{k_1} TrpH - TyrO^{\bullet} + H^{+}$ $k_1 = 6 \times 10^5\ s^{-1}$
	1:1 mixture	$^{T}DPH^{+}$, TrpH + TyrOH	$DPH_2^{+\bullet}$ $TrpH^{+\bullet}$ $TyrO^{\bullet}$	$TrpH^{+\bullet}$ $k_{ex} = 9 \times 10^8\ M^{-1}\ s^{-1}$ [21]	
7.4	dipeptide	^{T}DP, TrpH − TyrOH	$DPH_2^{+\bullet}$, $DPH^{\bullet}$ $TrpH^{+\bullet}$ − TyrOH $Trp^{\bullet}$ − TyrOH TrpH − $TyrO^{\bullet}$		$Trp^{\bullet} - TyrOH \xrightarrow{k_2} TrpH - TyrO^{\bullet}$ $k_2 = 4 \times 10^4\ s^{-1}$
	1:1 mixture	^{T}DP, TrpH + TyrOH	$DPH_2^{+\bullet}$, $DPH^{\bullet}$ $TrpH^{+\bullet}$ $Trp^{\bullet}$ $TyrO^{\bullet}$		
11.4	dipeptide	^{T}DP, TrpH − $TyrO^{-}$	$DPH^{\bullet}$ $TrpH^{+\bullet}$ − $TyrO^{-}$ $Trp^{\bullet}$ − $TyrO^{-}$ TrpH − $TyrO^{\bullet}$	TrpH − $TyrO^{\bullet}$ $k_{ex} = 4 \times 10^7\ M^{-1}\ s^{-1}$	$TrpH^{+\bullet} - TyrO^{-} \xrightarrow{k_d} Trp^{\bullet} - TyrO^{-} + H^{+}$ $k_d = 4 \times 10^6\ s^{-1}$ $Trp^{\bullet} - TyrO^{-} \xrightarrow[H^{+}]{k_4} TrpH - TyrO^{\bullet}$
	1:1 mixture	^{T}DP + TrpH + $TyrO^{-}$	$DPH^{\bullet}$ $TyrO^{\bullet}$ $TrpH^{+\bullet}$ $Trp^{\bullet}$ $TyrO^{\bullet}$	$k_{ex} = 9 \times 10^7\ M^{-1}\ s^{-1}$ [22]	

by fast protonation of the tryptophan moiety:

$$Trp^{\bullet} - TyrO^{-} \xrightarrow[H^{+}]{k_4} TrpH - TyrO^{\bullet} \qquad (21)$$

From the analysis of the CIDNP kinetics, we made an attempt to reveal the importance of this process in basic solution.

For the tryptophan residue, the shape of CIDNP kinetics is the same for the tryptophan residue in neutral solution within experimental error. The intensity of polarization is the highest for β-CH_2 protons and thus most convenient for the analysis. For comparison, the normalized CIDNP kinetics for the β-CH_2 protons of tryptophan at neutral (circles) and basic pH (squares) are shown in Figure 7. The coincidence of the time evolutions leads to the conclusion that reaction 21 is not faster than reaction 18. For the tyrosine residue, we observe essentially a fast polarization decay, which is accelerated upon increase of the peptide concentration (not shown). Thus, for the tyrosine residue, the time evolution of nuclear polarization is determined mainly by the reaction of degenerate electron exchange, which makes quantitative extraction of the value of k_4 impossible. For dipyridyl, the detected CIDNP kinetics is practically the same as that in the case of *N*-acetyl tyrosine (cf. Figure 6 in ref 22). This is not surprising since the contribution from the pairs with a tyrosine radical predominates. The CIDNP kinetics of dipyridyl, too, does not allow a quantitative determination of the value of k_4. Thus, the only indication that reaction 21 takes place is the coincidence of the kinetics for the β-CH_2 protons of the tryptophan residue in neutral and basic solution. If we do not take reaction 21 into account, it is necessary for getting an acceptable fit to use in the simulations a value of T_{1Trp} for the protons of tryptophan radical in basic solution that is longer than in neutral solution and even longer than for free radical of *N*-acetyl tryptophan. We see no obvious reason for such a change in relaxation. On the basis of our data, a stricter analysis is impossible.

For the convenience of the readers, we list in Table 1 the data about the photochemical reactions under study, the transient radicals contributing to CIDNP formation, the radicals participating in the degenerate electron exchange with the rate constants of the exchange, and the pathway of the intramolecular electron transfer process in the dipeptide with the rate constants at different pH as determined in the present work.

Conclusion

From the comparison of CIDNP of linked and nonlinked amino acids, we have established that linkage into a peptide has a significant influence on the reactivity of amino acid residues toward triplet excited dye molecules. In particular, IET in the peptide has to be taken into account. In the case presented here, IET in acidic solution is fast and competes with bimolecular termination of the radicals. In neutral solution, IET is slower and competes with nuclear paramagnetic relaxation. In both cases, IET affects the ratio of CIDNP of tryptophan and tyrosine, detected after the laser pulse.

Although the IET was studied here for a small dipeptide, the results allow us to point out that CIDNP in proteins does not only depend on the accessibility of the amino acid residues and on the mechanism of photochemical reactions but also on the reactivity of the intermediates and the efficiency of IET. In the case of real time protein folding detection by CIDNP on the millisecond time scale, the spin polarization does not reflect the folding in a straightforward way, since it depends also on the change of the efficiency of IET upon the folding and on the dynamics of CIDNP formation on the microsecond time scale. In particular, we want to stress that only geminate, but not stationary CIDNP, should be used to draw conclusions about structural changes in denatured and molten globule states. However, with proper precaution taken, the combination of time-resolved CIDNP with the initiation of folding in the probe of the NMR spectrometer opens the promising possibility to follow the protein folding process by high-resolution NMR selectively enhanced by CIDNP.

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