Pulse Radiolysis Studies of Intramolecular Electron Transfer in Model Peptides and Proteins. 8. Trp[NH$^{\bullet+}$] → Tyr[O$^{\bullet}$] Radical Transformation in H-Trp-(Pro)$_n$-Tyr-OH, n = 3−5, Series of Peptides

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The kinetics of intramolecular long-range electron transfer (LRET) between the protonated tryptophan indolyl radical cation Trp[NH$^{\bullet+}$], remaining in equilibrium with its neutral Trp[N$^{\bullet}$] form, and tyrosine in aqueous H-Trp-(Pro)$_n$-Tyr-OH, n = 3−5, peptides (abbreviated **3, 4**, and **5)** has been studied by pulse radiolysis at pH 2−8 at 298 K and at pH 4 over the temperature range 283−328 K. LRET has been found to occur down to pH 2, contrary to expectations based on the electrochemical redox potentials for Trp and Tyr in the form of free amino acids. The first-order rate constants of LRET, k_{obs}, varied sigmoidally with pH, allowing evaluation of pK_{a1}'s for deprotonation of Trp[NH$^{\bullet+}$]: 3.7, 4.1, and 4.3 for **3**, **4**, and **5**, respectively, as well as the intrinsic rate constants, k_2, of LRET involving solely Trp[NH$^{\bullet+}$] radicals. Variation of pK_{a1} was attributed to electrostatic interactions between the indolyl radical and the COOH group of terminal Tyr, expected to decrease in the shown order, and taken as an indication of variation, in the same order, of the electrochemical driving force $\Delta G°$ of LRET. In **4** and **5**, k_2 proved to be about 60-fold larger than the corresponding k_1 values characteristic for Trp[N$^{\bullet}$], which indicates that the kinetics of LRET involving Trp[NH$^{\bullet+}$] exhibits similar through-bond distance dependence as that found earlier for the reaction with Trp[N$^{\bullet}$]. For **3** the ratio of k_2/k_1 was found to be about 3-fold lower than for its two longer-bridged analogues, **4** and **5**. Arrhenius activation energies, E_{a2}, of LRET involving Trp[NH$^{\bullet+}$] proved to be rather low for **4** and **5**, 8.6 and 7.2 kJ mol^{-1}, respectively. In the case of **3**, however, k_{obs} varied nonlinearly with temperature. A nonlinear fit of Arrhenius type function to $k_{obs}(T)$ data under the assumption $\delta E_{a2}/\delta T = 0$ and $\delta pK_{a1}/\delta T \neq 0$ gave E_{a2} of 31.6 kJ mol^{-1} and indicated a decrease in pK_{a1} of Trp[NH$^{\bullet+}$] by about one unit in the studied temperature range. Conformational preferences of **3** were thus studied by means of molecular dynamics modeling to understand why the parameters of LRET in this peptide were so different from those of **4** and **5**. It has been shown that a $\beta \rightarrow \alpha$ transition at the central ψ(Pro$_3$) dihedral angle, which could bring NH (indole) and COOH groups into a close contact compatible with formation of a hydrogen bond, may occur in **3** on the time scale of the observed electron-transfer reaction. In longer-bridged analogues two such concerted transitions would be necessary to perturb LRET measurably, an event of extremely low probability. It is thus argued that this transition may explain the kinetic and energetic peculiarities of LRET in **3**. Analysis of thermodynamic parameters indicated that in reactions involving both Trp[NH$^{\bullet+}$] as well as Trp[N$^{\bullet}$] radicals the free energy barrier of activation of LRET, $\Delta G^{\#}$, is for the most part entropic in nature. A more detailed analysis of thermodynamics of these reactions must await experimental determination of $\Delta G°$ for both reactions in the peptides studied.

Introduction

Intramolecular one-electron oxidation of tyrosine, Tyr[OH], to phenoxyl radical, Tyr[O$^{\bullet}$], by the tryptophan indolyl radical, Trp[N$^{\bullet}$], involving long-range electron transfer (LRET), has been intensively studied by pulse radiolysis in model peptides[1−11] and proteins[12−16] because of the importance of the mechanism of free radical damage to proteins for understanding of free-radical-induced cell injury[17] and growing evidence for participation of Trp[N$^{\bullet}$] and Tyr[O$^{\bullet}$] radicals in the functioning of photosystem II and redox proteins.[18−26] The pulse radiolysis method allows us to trigger this reaction in a very short time (<1 μs) by selective oxidation of tryptophan, Trp[NH], to Trp-[N$^{\bullet}$] by $N_3^{\bullet}$ (in neutral solution) or $Br_2^{\bullet-}$ (in acidic solution) radicals.[27] LRET accompanying this reaction has been thoroughly studied in model proline-bridged peptide systems,[5−11,28] whose conformational properties are well-known.[29−31] These studies allowed us to establish kinetic and activation parameters characteristic of a single LRET through-bond pathway along a helical PLPII peptide backbone.[7−8,31]

Oxidation of Trp[NH] in acidic aqueous solution results in formation of the tryptophyl radical cation Trp[NH $^{\bullet+}$] remaining in equilibrium with its neutral form Trp[N$^{\bullet}$] (pK_a = 4.3 for free amino acid[32,33] and between 4.6 and 5.4 when in a

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dipeptide[34]). It has been shown[6,11,35] that the rate of oxidation of Tyr[OH] by Trp[NH$^{\bullet+}$] is much faster than by Trp[N$^{\bullet}$]. Therefore, the radical transformation reaction can be formulated for each radical species separately, as envisaged below:

$$\text{H-Trp[N}^{\bullet}\text{]-(Pro)}_n\text{-Tyr[OH]-OH} \rightarrow \text{H-Trp[N]}^{-}\text{-(Pro)}_n\text{-Tyr[OH}^{\bullet+}\text{]-OH} \quad (1a)$$

$$\text{H-Trp[N}^{-}\text{]-(Pro)}_n\text{-Tyr[OH}^{\bullet+}\text{]-OH} \rightarrow \text{H-Trp[NH]-(Pro)}_n\text{-Tyr[O}^{\bullet}\text{]-OH} \quad (1b)$$

Reactions 1a and 1b are accompanied by a net proton transfer with fast protonation in the transition state of the incipient tryptophan indolyl anion, Trp[N^{-}] (pK_a = 16.8 for deprotonation of the indole nitrogen[35]) and deprotonation of the phenoxy radical cation, Tyr[OH$^{\bullet+}$] (pK_a = −1[36,37]). The protonation and deprotonation processes are fast on the microsecond time scale of the overall reaction 1; electron transfer thus can be regarded as the rate-determining step.[7,10] In reaction 2,

$$\text{H-Trp[NH}^{\bullet+}\text{]-(Pro)}_n\text{-Tyr[OH]-OH} \rightarrow \text{H-Trp[NH]-(Pro)}_n\text{-Tyr[OH}^{\bullet+}\text{]-OH} \quad (2a)$$

$$\text{H-Trp[NH]-(Pro)}_n\text{-Tyr[OH}^{\bullet+}\text{]} \rightarrow \text{H-Trp[NH]-(Pro)}_n\text{-Tyr[O}^{\bullet}\text{]} + \text{H}^{+} \quad (2b)$$

such a net proton transfer does not occur, since it has been recently demonstrated by the similarity of kinetic parameters for this reaction and an analogous one,

$$\text{H-Trp[NMe}^{\bullet+}\text{]-(Pro)}_n\text{-Tyr[OH]-OH} \rightarrow \text{H-Trp[NMe]-(Pro)}_n\text{-Tyr[O}^{\bullet}\text{]} + \text{H}^{+} \quad (3)$$

induced by 1-*N*-methyltryptophyl radical cations, Trp[NMe$^{\bullet+}$] (Metrp$^{\bullet+}$ in the original formulation), formed by one-electron oxidation of 1-*N*-methyl-tryptophan.[11]

The much faster rate of radical transformation in reaction 2, compared with reaction 1 involving Trp[N$^{\bullet}$], could not be explained in terms of the thermodynamic driving force[6] because the difference, ΔE_m, between electrochemical reduction potentials of the Trp[N$^{\bullet}$],H^{+}/Trp[NH] and Tyr[OH],H^{+}/Tyr[O$^{\bullet}$] couples,[38,39]measured for free amino acids, begins to diminish at a pH close to the pK_a of Trp[NH$^{\bullet+}$] dissociation and, moreover, was claimed to reverse its sign below pH 3.[39] Therefore, the explanation of the effect of protonation on the rate of LRET was sought[34] in a lower value of the free energy of reorganization being necessary for attainment of the transition state in reaction 2 than in reaction 1. The latter point of view seemed to be supported by lower values of the observed Arrhenius activation energy at pH 4 in short-bridged (n = 2−3) peptides than those found for these peptides in reaction 1.[6,7]

So far, reaction 2 has been studied only in weakly acidic solutions (pH ≥ 4) and in short-bridged peptides[6] so that neither corresponding intrinsic rate constants k_2 nor activation energies $E_{a,2}$ have been determined. Therefore, in this work we extended our earlier pulse radiolysis investigations of this reaction to a lower pH range, using $Br_2^{\bullet-}$ instead of $N_3^{\bullet}$ for oxidation of Trp-[NH] to Trp[NH$^{\bullet+}$] and to peptides with longer proline bridges (n_{Pro} = 3−5). The pH dependence of kinetic and activation parameters obtained for reactions 1 and 2 and the distance dependence of kinetics of LRET in reaction 2 are interpreted within the framework of the semiclassical Marcus theory[40] of electron transfer and our model of distance dependence of LRET kinetics in proline-bridged peptides.[7−8,31]

Materials and Methods

All the peptides H-Trp-(Pro)$_n$-Tyr-H, n = 3, 4, 5, abbreviated henceforth as **3**, **4**, **5**, respectively, prepared and purified according to standard methods, were of the same stock as used in our earlier studies.[5,7] All other chemicals were commercial products of the highest analytical purity available.

The pulse radiolysis experiments were performed with a 10 MeV HRC linear accelerator at Risø National Laboratory[41] using 500 ns electron pulses with low doses up to 2 Gy per pulse. Temperature studies were performed in a spectrophotometric cell equipped with a thermostated water jacket.

Solutions of the compound studied were prepared immediately before use in triply distilled water at a peptide concentration of ca. 0.2 mM and adjusted to a desired pH value by adding an appropriate amount of perchloric acid or sodium hydroxide. They were saturated with high-purity N_2O and contained also 0.1 mM KBr, necessary for generation of dibromide radical anions, $Br_2^{\bullet-}$. Intramolecular electron-transfer reactions 1 and 2 were induced by selective oxidation of the indole side chain of tryptophan to Trp[N$^{\bullet}$] and/or Trp[NH$^{\bullet+}$] radicals with $Br_2^{\bullet-}$, formed at ≤1 μs after the electron pulse as described earlier.[27,42] The kinetics of reactions 1 and 2 were followed spectrophotometrically at the absorption maxima of Trp[N$^{\bullet}$], Trp[NH$^{\bullet+}$], and Tyr[O$^{\bullet}$] radical species located at λ = 510, 580, and 405 nm, respectively. Wherever possible, the kinetic traces for tryptophyl radicals were determined simultaneously at 510 and 580 nm. Time-resolved transient absorption spectra were obtained from separate pulse radiolysis records taken at 10 nm intervals in the spectral range 350−650 nm.

Results and Discussion

1. Trp[NH$^{\bullet+}$] → TyrO$^{\bullet}$ Transformation at pH 2. In view of the claimed change at about pH 3 of the sign of the difference between electrochemical redox potentials of Trp[NH$^{\bullet+}$]/Trp-[NH] and Tyr[O$^{\bullet}$]/TyrOH couples,[39] and thus of the direction of LRET, transient absorption spectra were recorded on pulse radiolysis of H-Trp-(Pro)$_3$-Tyr-OH in 0.01 M $HClO_4$ solution (pH 2) at early and late times after the electron pulse. The spectrum obtained at 20 μs after the pulse (Figure 1a) was characterized by an absorption band with λ_{max} at 580 nm, corresponding closely to that of the Trp[NH$^{\bullet+}$] radical[43] formed in a reaction of **3** with $Br_2^{\bullet-}$. In the spectrum recorded at 100 μs after pulse (Figure 1b), the absorption band at λ_{max} = 580 nm has almost disappeared and a new band was present with λ_{max} at about 400 nm, characteristic of Tyr[O$^{\bullet}$] absorption.[1] Moreover, the rate of disappearance of the 580 nm absorption proved to be similar to the rate of growth of that at 405 nm (cf. insets in Figure 1) when the latter was properly corrected for a fast decay of $Br_2^{\bullet-}$ absorbance in this region. Similar time-dependent changes in the absorbance at 580 and 405 nm were also observed for the other two peptides studied, **4** and **5**. These findings are evidently consistent with the occurrence of reaction 2 in acidic solutions down to pH 2 and indicate that under these pH conditions the difference between standard redox potentials of the donor and acceptor is still negative, $\Delta G^{\circ} < 0$, contrary to predictions based on Harriman's experimental redox potentials determined for the two redox couples in question in the form of free amino acids. This discrepancy can be more apparent than real due to the claimed dependence of the reduction potentials considered on the location of Trp and Tyr residues in peptides.[9,34] This point will be discussed later.

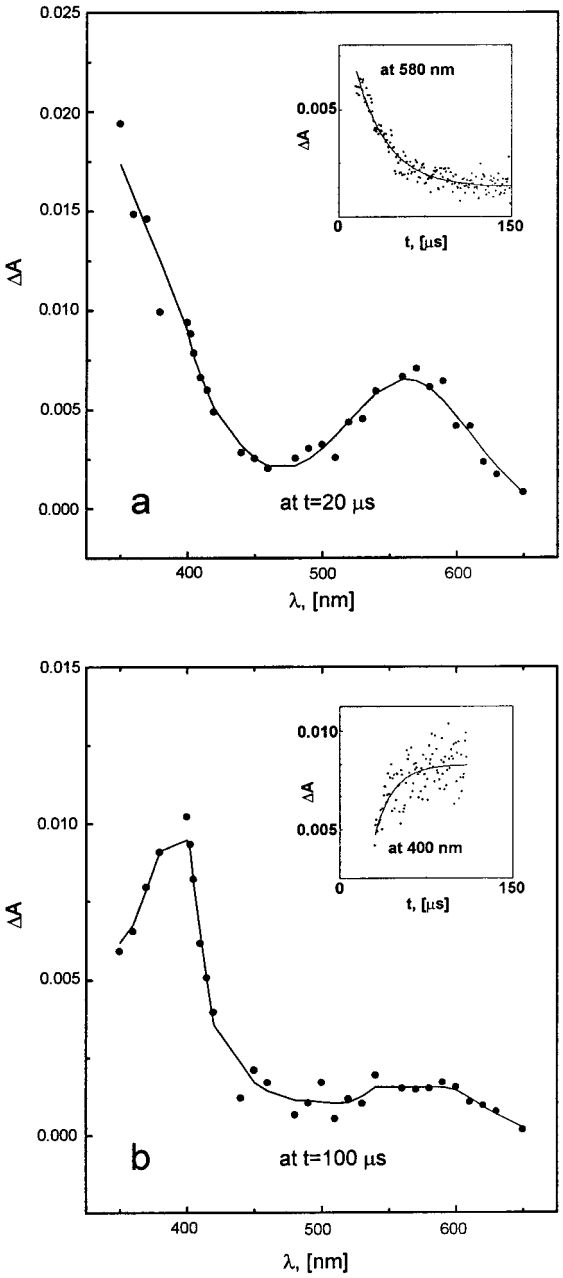


Figure 1. Transient absorption spectra of Trp[NH$^{\bullet+}$] (a) and Tyr[O$^{\bullet}$] (b) radicals recorded at 20 and 100 μs after the pulse for aqueous solution of H-Trp-(Pro)$_3$-Tyr-OH peptide at pH 2. The inset of (a) shows the decay of Trp[NH$^{\bullet+}$] absorption measured at 580 nm. The inset of (b) shows the growth of Tyr[O$^{\bullet}$] absorption measured at 405 nm (corrected for decay of $Br_2^{\bullet-}$ absorption).

2. Effect of Trp[N$^{\bullet}$] Protonation on Kinetics of LRET. The kinetics of the observed electron-transfer reaction in the three peptides studied proved to be first-order over the whole pH (2−8) and temperature (278−328 K) ranges investigated. The rate of LRET, measured under pH conditions in which both reactions 1 and 2 can occur simultaneously, was the same whether followed by the decay of Trp[N$^{\bullet}$] and Trp[NH$^{\bullet+}$] at 510 nm (isosbestic point) or solely by the decay of Trp[NH$^{\bullet+}$] at λ_{max} = 580 nm. The measured first-order rate constants were found to be pH-independent in the pH range 6−8, in which the tryptophan radical exists in its neutral form, Trp[N$^{\bullet}$]. Below pH 6 the rate of LRET began to increase with growing concentration of hydronium ions, and thus also the rate of Trp-[NH$^{\bullet+}$] in a way characteristic of a titration curve (cf. Figure 2). Since the observed rate of LRET conformed to a single-exponential function over the whole pH range investigated and was the same whether measured at the absorption maximum of Trp[N$^{\bullet}$] (510 nm) or of its protonated Trp[NH$^{\bullet+}$] form (580 nm), it can be assumed that the two species were in a fast equilibrium on the experimental time scale and coupled to acid−base equilibria, as shown in the simplified reaction scheme below:

$$\begin{array}{ccccc} & & k_2 & & \\ & \mathrm{Trp[NH^{\bullet+}]} & \rightarrow & \mathrm{Trp[NH]} & \\ K_{a1} = k_{a1}/k_{-a1} \approx 1 \times 10^{-4} & k_{a1} \downarrow\uparrow k_{-1a} & & k_{a2} \downarrow\uparrow k_{-a2} & K_{a2} = k_{a2}/k_{-a2} \approx 1 \times 10^{-17} \\ & \mathrm{Trp[N^{\bullet}] + H^{+}} & \rightarrow & \mathrm{Trp[N^{-}] + H^{+}} & \\ & & k_1 & & \end{array}$$

Hence, k_{obs} could be regarded as an average value of the two composite rates k_1 and k_2, weighted by populations of Trp[N$^{\bullet}$] and [TrpNH$^{\bullet+}$] species:

$$k_{obs} = (1 - f)k_2 + fk_1 \qquad (4)$$

where $f = 10^{-pK_{a1}}/(10^{-pK_{a1}} + 10^{-pH})$ is the molar fraction of Trp[N$^{\bullet}$] radicals. Fitting of function 4 to the experimental k_{obs}-(pH) data allowed us to evaluate the apparent pK_{a1} and k_2 values for **3**−**5** peptides (Table 1).

The apparent pK_{a1} values determined for peptides **3**−**5**, namely, 3.7, 4.1, and 4.3, respectively (Table 1), decrease as the length of the proline bridge becomes shorter. For the −(Pro)$_2$− bridged peptide a still lower value of p$K_{a1} \cong 3.5$ was estimated from measurements of k_{obs} in the limited pH range 3.5−7 (K. L. Wierzchowski and K. Bobrowski, unpublished results). Similar, although less accurate, pK_{a1} values were obtained when the ratio of amplitudes A_{580}/A_{510}(pH) = 1 − f, derived along with k_{obs}'s from the experimental dA/dt decay profiles, was used instead of k_{obs}(pH) (not shown).

A dependence of pK_{a1}(Trp[NH$^{\bullet+}$]) values on the location of Trp[NH] in peptides had been observed earlier in a differential pulse polarography study of electrochemical properties of tryptophan indolyl radicals.[34] In particular, when Trp[NH] was C-terminal or located at the C-terminus, as in Gly-Trp, the pK_{a1} exhibited a value lower by 0.8 units than that found in Trp-Gly. We have found that the pK_a of the N-terminal amino group of Trp[NH] in all-trans isomers of H-Trp-Pro-Met-OH and H-Trp-(Pro)$_3$-Met-OH peptides differs also by 0.1 unit.[44] The redox potentials for Trp[NH$^{\bullet+}$] were all found to be the same, within experimental error, while those for Trp[N$^{\bullet}$], E_m(pH 7), appeared to differ slightly (by ca. 20 mV), in parallel with differences in pK_{a1}'s.[34] Moreover, it has been estimated[9] (from the final equilibrium positions attained in the electron-transfer reaction in H-Trp-X$_n$-Tyr-OH and H-Tyr-X$_n$-Trp-OH peptides (n = 0−3, X = Pro or Gly) and differential pulse polarography traces) that at pH 7 the difference δE_m between one-electron redox potentials for the indolyl and phenol side chains depends on both the Trp[NH] location and the number of X residues in the bridge. When Trp[NH] was located N-terminally, δE_m was about 200 mV and increased by ca. 10 mV per X residue added. C-terminal location of Trp caused a strong decrease in δE_m to a value of ≤70 mV. In the case of tyrosine, the E_m of the Tyr-[OH],H$^+$/Tyr[O$^{\bullet}$] couple was found to vary also slightly with its position in the peptide.[34] It can thus be concluded that the actual value of E_m(Trp[N$^{\bullet}$]/Trp[NH]) depends on the distance between Trp[NH] and the COO$^-$ group and decreases as the strength of the electrostatic field of the latter at the indolyl side chain becomes larger.

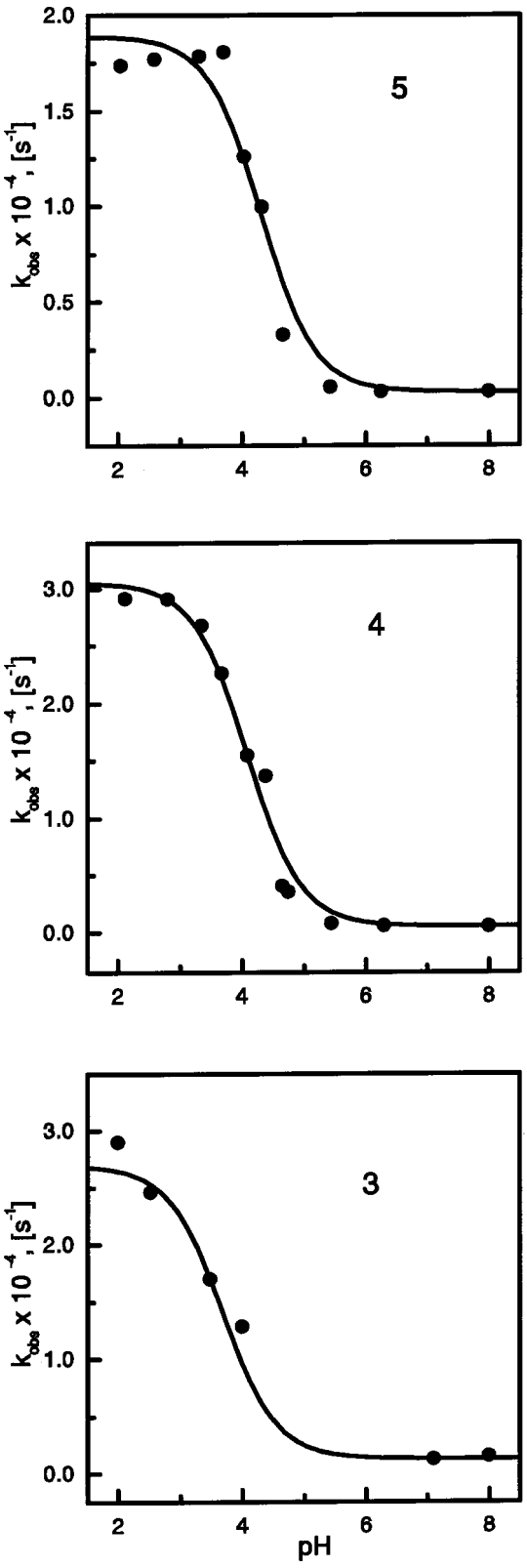


Figure 2. Dependence of k_{obs} on pH at 298 K for peptides **3**, **4**, and **5**. The solid symbols represent values of k_{obs} (from three to five independent decay experiments, individual rate constants were obtained by a nonlinear fit of a single-exponential decay function to the experimental $A_{580}(t)$ data). The solid lines are theoretical curves obtained by a nonlinear fit of function 4 to mean k_{obs}(pH) data with standard errors as statistical weights (cf. Table 1 for the values of the fitted parameters pK_{a1} and k_2). The rate constant k_1 was taken from our previous work[7] and kept constant during fitting.

TABLE 1: Kinetic Parameters of LRET in H-Trp-(Pro)$_n$-Tyr-OH Peptides and pK_{a1} for Trp[NH•+] Dissociation at 298 K[a]

peptide n	k_1[b] [s^{-1}]	k_2 [10^4 s^{-1}]	k_2/k_1	pK_{a1}
3	1500	2.67(0.08)	18	3.7(0.2)[c]
4	510	3.04(0.08)	59	4.1(0.1)
5	300	1.87(0.06)	61	4.3(0.1)

[a] Standard errors are in parentheses. [b] From ref 7. The standard error is ca. 10%. [c] Temperature dependent. The value apparently varies from 4.2 at 283 K to 3.85 at 298 K and 3.2 at 328 K (see section 4).

TABLE 2: Temperature Dependence of First-Order Rate Constants[a] for LRET in H-Trp-(Pro)$_n$-Tyr-OH Peptides

	k_{obs} [10^3 s^{-1}]		
T [K]	**3** (pH 4.0)	**4** (pH 4.5)	**5** (pH 4.6)
283	9.13	8.43	6.04
288		8.83	6.40
293	11.40	9.31	6.69
298	12.83	9.82	6.80
303		10.50	7.36
308	14.67	11.10	7.58
313		11.70	8.17
318	16.23	12.50	8.18
323		13.00	9.08
328	17.15	13.70	9.11

[a] Mean values. The standard error is ∼10%.

In view of the rather relatively small variation in E_m of the two amino acids, the difference between standard redox potentials, $\Delta G°$, of the two couples, i.e., electrochemical driving force for reaction 1, was until recently considered to be independent of the length of the oligoproline bridge.[7,25]

In light of the earlier and present findings, the variation of pK_{a1}(Trp[NH•+]) with n_{Pro} in the bridge (Table 1) can be attributed to an electrostatic effect of the COO^- group exerted on the indolyl radical cation. It can thus be postulated that the decrease in pK_{a1} is accompanied by a decrease in electrochemical reduction potentials of Trp[NH] and Trp[NH•+]. This point will be taken into consideration in a further discussion of the effect of Trp[N•] protonation on the observed rate of LRET in reactions 1 and 2.

The apparent values of k_2 estimated for reaction 2 in peptides **4** and **5** are about 60-fold larger than those of the corresponding k_1 (cf. Table 1). The effect of Trp[N•] protonation on the kinetics of LRET in **3**, measured as the ratio of $k_2/k_1 \approx 20$, appeared to be 3-fold smaller than that found for the two longer-bridged peptides. The observed protonation-induced enhancement of tryptophan radical reactivity in the intramolecular one-electron-transfer reaction with tyrosine is, in general, similar to that found[35] in a number of bimolecular reactions involving tryptophan neutral and cation radicals. Trp[NH•+] has been found[34,35] to be a better oxidant than Trp[N•] by ca. 100 mV at pH 3. Nevertheless, the increased reactivity of the radical cation form with phenols was considered unlikely to be due only to its higher reduction potential and sought in a lower reorganization energy involved in the electron-transfer reaction.[35] The large difference in reactivity of Trp[NH•+] toward Tyr[OH] in reaction 2 between **3** and its two longer-bridged analogues **4** and **5** seemed, however, surprising and deserved elucidation. In this connection it is worth noting that the first-order rate constant of intramolecular LRET accompanying reaction 3 found for the H-Trp-[NMe]-(Pro)$_3$-Tyr-OH peptide,[11] $k_3 = 2.1 \times 10^4$ s $^{-1}$, is of similar magnitude as k_2 for **3** determined in this work (cf. Table 2). Since redox potentials of Trp[NMe•+] and Trp[NH•+] were found also similar,[11,34] the rather low rates of reaction 2 in **3**

and reaction 3 in its Trp[NMe] analogue seem to be connected with some common conformational properties of the two peptides.

3. Distance Dependence of the Rate of LRET in Reaction 2. The similarity of k_2/k_1 ratios determined for peptides **4** and **5** indicates that LRET accompanying reaction 2 exhibits the same distance dependence as it does in reaction 1. This similarity supports our earlier conclusion, based on comparison between Trp[N•],Tyr[OH] and various metallic redox couples, that electron transferability of the helical PLPII type $-(\text{Pro})_n-$ bridge is independent of the nature of the attached donor−acceptor pair.[8] This conclusion does not seem to apply, however, to peptide **3** for which the k_2/k_1 ratio was found to be 3-fold lower.

The value of the descriptor β of the exponential through-bond (TB) distance dependence of the rate of LRET between Tyr[OH] and Trp[N•] (reaction 1) separated by $n = 0-5$ Pro residues has been estimated previously as $\beta^{TB} = 2.5 \pm 0.1$ nm^{-1}, with the help of our model of LRET[7−8,31,45] based on the Marcus theory[40] and the knowledge of static and dynamic conformational properties of the peptides studied.[29−31] In short-bridged peptides, $n = 0-2$, LRET was shown to occur mainly through direct nonbonded contacts between aromatic side chains (called through-space TS pathway) of terminal Trp and Tyr amino acids, while in longer-bridged analogues ($n = 3-5$) involvement of only the TB pathway has been postulated.[7] The latter group of peptides, **3**−**5**, has a similar PLPII-like helical conformation both in neutral (zwitterionic form) and in acidic solution (cationic form) except for a small shift in trans ⇔ cis equilibrium at the Trp−Pro bond toward the trans isomer in the cationic form.[29] Also, the calculated electron density of the LUMO orbital was found to be similar in both the protonated and neutral forms of the indolyl radical.[31] On this basis, one could expect that also in reaction 2 all three members of this group of peptides ($n = 3-5$) would behave similarly. The much smaller than expected enhancement of the rate of LRET of **3** on protonation of Trp[N•] could result from two factors: (a) a higher experimental value of k_1 than that expected for the exclusively TB mechanism operating in **4** and **5**; (b) a much different electrochemical driving force for reactions 1 and 2 in peptide **3** due to the dependence of $\Delta G°$ on mutual location of Trp and Tyr residues, referred to above.[34] Linear extrapolation of the experimental k_1 data for **4** and **5** to shorter bridged peptides, according to the relationship $\ln k_1 = \beta n_{Pro}$, gave about a 2-fold lower value for **3** than that determined experimentally. This might indicate that LRET in **3** also involves, in addition to TB, a contribution from a TS pathway, like in short-bridged peptides **1** and **2**. To evaluate this possibility, we performed molecular dynamics (MD) simulations on selected conformers of **3**, the results of which are reported and discussed in section 5.

4. Effect of Temperature on the Rate of LRET. The first-order rate constants of LRET, k_{obs}, were determined for peptides **3**−**5** at pH ∼4 in the temperature range 278−338 K (Table 2). According to eq 5, the activation energy of LRET, $E_{a,obs}$, in the acidic pH range can be expected to be composed of contributions from reactions 1 and 2:

$$k_{obs} = A \exp\left(\frac{-E_{a,obs}}{RT}\right) = fA_1 \exp\left(\frac{-E_{a1}}{RT}\right) + (1-f)A_2 \exp\left(\frac{-E_{a2}}{RT}\right) \quad (5)$$

where parameters A and E_a marked with subscripts 1 and 2 correspond to reactions 1 and 2, respectively. A standard Arrhenius plot of $\ln k_{obs}$ vs T^{-1} should be linear, provided that

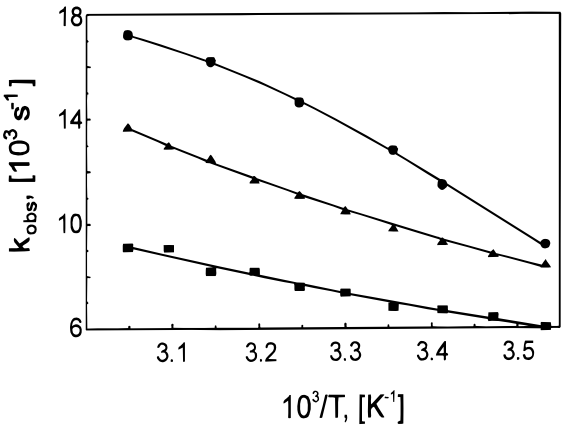


Figure 3. Temperature dependence of k_{obs}: (solid circles) peptide **3** at pH 4.0; (triangles) peptide **4** at pH 4.5; (rectangles) peptide **5** at pH 4.6; (solid lines) fitting functions (eq 4) obtained under the assumptions $\delta(pK_{a1})/\delta T \neq 0$ and $\delta E_a/\delta T = 0$ (cf. Table 2 for $k_{obs}(T)$ data and Table 3 for values of the fitted $E_{a,2}$ parameter). The values of pK_{a1} at 298 K and $\delta(pK_{a1})/\delta$T (in parentheses) obtained from a fit for **3**, **4**, and **5** are 3.85 (2.055×10^3), 4.1 (−1.08), and 4.3 (3.74), respectively.

both E_a's and pK_{a1} (thus also molar fractions of the two radical species) are temperature-independent in the studied temperature range. In fact, Arrhenius plots for **4** and **5** were linear, but that for **3** exhibited upward curvature (not shown), although in reaction 1, E_{a1} has been found to be temperature-independent for all three peptides.[7] This means that the conditions underlying linearity of the Arrhenius plot in both reactions 1 and 2 were fulfilled only in the cases of **4** and **5**. These conclusions were confirmed by nonlinear fitting to the experimental $k_{obs}(T)$ data of eq 5 under the assumption that either both or only one of the two parameters considered, pK_{a1} and E_a, may vary with temperature (cf. Figure 3). The thus derived $\delta(pK_{a1})/\delta T$ terms from the data of **4** and **5** appeared so small that fractions of the radical species could be considered practically constant over the studied temperature range (cf. legend to Figure 3). Moreover, the values of pK_{a1} and k_2 calculated for these two peptides at 298 K were in excellent agreement with those derived from analysis of the k_{obs}(pH) experimental data at 298 K with the help of eq 4. The sought values of E_{a2} proved to be similar to those of the corresponding $E_{a,obs}$ determined from the standard Arrhenius plots (Table 3). The practical equality of $E_{a,obs}$ and E_{a2} values in the acidic pH range stems from eq 5, according to which activation energies of reactions 1 and 2 are weighted by the corresponding rate constants so that contribution from reaction 1 to $E_{a,obs}$ is negligible, owing to its much smaller rate constant compared with that of reaction 2.

E_{a2} values for reaction 2 in **4** and **5** are in each case lower by only 2.1 kJ mol^{-1} than those determined for reaction 1. A similar but even smaller difference of $\delta E_a = 1.4$ kJ mol^{-1} is seen between activation energies of reactions 1 and 2 in both **4** and **5.** These observations indicate that activation of LRET in either of the two peptides involves a similar molecular mechanism irrespective of the protonation state of the indolyl radical. Low absolute values of E_a for both reactions suggest involvement of electron tunneling through a rather high free energy barrier of activation, evaluated and discussed in section 6.

In the case of peptide **3**, nonlinear fitting of eq 5 to the $k_{obs}(T)$ data indicated that both pK_{a1} and E_a parameters may vary with temperature. However, the thus derived $pK_{a1}(T)$ and $E_a(T)$ values (not shown) owing to strong correlation between the two parameters (dependency of ∼0.95) could not be considered as reliable. Assuming that in the narrow temperature range studied, $\delta E_a/\delta T = 0$, possible variation of pK_{a1} with temperature was

TABLE 3: Experimental Arrhenius Energies of Activation, $E_{a,obs}$, of LRET at Indicated pH Values and Calculated Thermodynamic Parameters[a] for Reactions 1 and 2 in H-Trp-(Pro)$_n$-Tyr-OH Peptides

n	$E_{a,obs}$[b] [kJ mol^{-1}]	E_a[c] [kJ mol^{-1}]	$\Delta H^{\#}_{298}$ [kJ mol^{-1}]	$\Delta S^{\#}_{298}$ [J mol^{-1} deg^{-1}]	$\Delta G^{\#}_{298}$ [kJ mol^{-1}]
			Reaction 2, pH ~4[d]		
3	16.4;[e] 6.5[f]	31.6	29.1	−62.5	47.7
4	8.6(0.2)	8.6	6.1	−138.6	47.4
5	7.2(0.4)	7.2	4.7	−147.4	48.6
			Reaction 1, pH 8[g]		
3	16.4(1.3)	16.4	13.9	−137.4	54.9
4	10.7(0.5)	10.7	8.2	−165.5	57.5
5	9.3(0.6)	9.3	6.8	−174.5	58.8

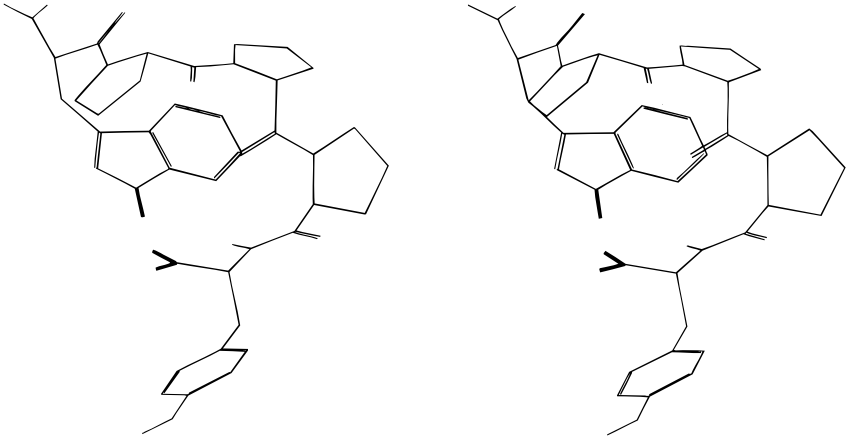
[a] According to relationships $E_a - RT = \Delta H^{\#}_T$, $\ln k_{obs} = 1 + \ln(kT/h) + \Delta S^{\#}/R - E_a/(RT)$, $\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$. [b] Standard error is in parentheses. [c] Fitted parameter from the nonlinear fit of eq 4 to the experimental $k_{obs}(T)$ data (Table 2) with use of experimental k_1 and $E_{a,1}$ values and under the assumption that $\delta(pK_{a1})/\delta T \neq 0$. [d] pH: 4.0, 4.5, and 4.6 for $n = 3$, 4, and 5, respectively. [e] Between 283 and 298 K. [f] Between 308 and 328 K. [g] Data (recalculated) from Bobrowski et al.[7]

evaluated, from 4.2 at 283 K to 3.2 at 328 K, while its value of 3.85 at 298 K closely corresponded to that obtained from the experimental dependence of LRET on pH (cf. Table 1 and Figure 3). The thus obtained $E_{a2} = 31.6$ kJ mol^{-1} proved to be twice as large as that estimated from the initial, quasi-linear part of the Arrhenius plot (Table 3). A similarly strong dependence of pK_a on temperature has been found experimentally for the N-terminal amino group in Trp-Glu and Trp-Ala dipeptides (p$K_a \approx 8$),[46,47]which dropped by about one unit between 277 and 325 K with a similar Arrhenius activation energy of about 36 kJ mol^{-1}. We thus felt justified to attribute the observed upward curvature of k_{obs} vs T^{-1} plot for reaction 2 in **3** (Figure 3) to similar thermal perturbation of acid−base equilibrium at the indolyl radical.

5. Molecular Dynamics of H-Trp-(Pro)$_3$-Tyr-OH. To understand the observed peculiarities of LRET in **3**, the results of previous molecular mechanics[7,31] and MD simulations[8,31] of conformational preferences of this peptide were reconsidered and supplemented. In general, the earlier simulations have indicated that peptides **3**−**5**, having a −(Pro)$_n$− bridge in PLPII conformation, are rather unlikely to attain low-energy conformations compatible with involvement of the TS pathway. The only conformational rearrangement that could allow the two terminal aromatic rings to come into a close contact within a microsecond-to-millisecond lifetime of indole radicals would be a $\beta \leftrightarrow \alpha$ transition at the ψ(Trp) and/or ψ(Pro) dihedral angles, as suggested originally by Sneddon and Brooks[48] on the basis of their MD simulations of oligoproline peptides in aqueous solution. We have subsequently performed similar potential-of-mean-force calculations with explicit aqueous environment and use of umbrella sampling for Trp-Pro, Pro-Pro, and Pro-Tyr dipeptide fragments.[8,31] The thus calculated energies of, and energy barriers between, the β and α ψ(Trp,Pro) conformers indicate that the $\psi(\beta)$ conformation is more stable than the $\psi(\alpha)$ one in the Pro-Pro fragment by $\Delta G^{\circ} \cong 12.5 \pm 4$ kJ mol^{-1}, whereas in the Trp-Pro fragment $\Delta G^{\circ} \cong 29 \pm 4$ kJ mol^{-1}. Thus, the population of $\psi(\alpha)$Trp conformers could be expected to be negligibly small. By use of the determined potentials of mean force, the frequency of the $\beta \rightarrow \alpha$ transition was also evaluated according to the transition-state theory as 1.5×10^3 and 8×10^5 s^{-1} for ψ(Pro) in Pro-Pro and Pro-Tyr fragments, respectively. On this basis, only the $\beta \rightarrow \alpha$ transition at the ψ(Pro)-Tyr fragment was included in the modeling of the TB pathway. In view of the similar order of $k_{\beta\rightarrow\alpha}$ at the Pro-Pro fragment and k_{obs} for reaction 1 in **3** at pH 8, we are now tempted to accept the possibility of occurrence of the $\beta \rightarrow \alpha$ transition at ψ(Pro$_3$) within the lifetime of the Trp[N•] radical. The kinetics of LRET accompanying reaction 1 in **3** would thus be under stronger nuclear control because of involvement of a TS pathway in addition to the TB one.

The TS pathway, consisting of direct contacts between the indole and phenol rings of aromatic side chains, has been shown to be dominant in short-bridged peptides **1** and **2** and characterized by much faster kinetics and higher energies of activation compared with those for the TB pathway through the −(Pro)$_n$− bridge.[7] Involvement of this pathway could explain the larger experimental values of k_{obs} and E_{a1} for reaction 1 in **3** compared with those expected for the TB pathway alone. Owing to the much faster kinetics of TB-LRET in reaction 2, dictated by the presence of Trp[NH•+], the TS-LRET pathway, mediated by the $\beta \leftrightarrow \alpha$ transition at the ψ(Pro$_3$), could be expected to be noncompetitive so that parameters k_2 and E_{a2} should attain values close to those extrapolated from experimental data for **4** and **5** in reaction 2. This is not the case, however, so other than kinetic consequences of the $\beta \leftrightarrow \alpha$ transition at the ψ(Pro$_3$) on LRET should also be considered.

In view of the observed dependence of pK_{a1} and E_m of Trp-[NH•+] on the spatial separation of the latter from the terminal COO$^-$ group of tyrosine, discussed earlier in this text, we searched with the help of MD simulations for a relatively stable conformer of **3** that would be characterized by a close approach between the indole nitrogen and oxygen of COO$^-$. The geometry of the main chain of the starting conformer was set, in agreement with NMR and CD[29,30] results, as an extended all-trans PLPII conformation with all the ψ angles in the β region. A series of 1 ps restrained MD runs were then performed with the distance between NH (indole) and OC (carboxyl) groups set to upper limits of 10, 7, 5, 3, and 1.5 Å while the ψ angles of Trp1, Pro2, and Pro3 were kept in the β region by extra dihedral constraints. In this way a conformer of **3** was selected, with a geometry corresponding to the transition-like state for the $\beta \rightarrow \alpha$ transition at the ψ(Pro3) dihedral, and was subjected subsequently to energy minimization (cf. Figure 4). This conformer is characterized by a short distance between the NH (indole) and OC (carboxyl) groups, compatible with the presence of a regular hydrogen bond. To analyze possible pathways of conformational evolution of **3** from this state, a 100 ps MD simulation was performed with only the dihedral restraints removed. Each of the 11 snapshots recorded at 10 ps intervals was then subjected to 100 ps MD runs with all conformational restraints removed. Generally, two types of conformational evolution were observed: (a) leading to reconstruction of the initial PLPII conformation characterized by a relatively large indole−carboxyl separation (cf. Figure 5, dotted curve) and (b) resulting in the formation of a thermodynamically stable main chain conformation with ψ(Pro$_3$) in the α region, allowing for short contacts between indole and carboxyl groups and for occasional reconstruction of the hydrogen bond between (cf. Figure 5, solid curve). Owing to the relatively free rotation of the phenol side chain of Tyr[OH] in **3**,[29] both aromatic rings in conformers of the second ensemble are able to assume a number of configurations compatible with formation of an efficient TS pathway for LRET**,** as postulated earlier in the preceding chapter. The postulated hydrogen bond should become much stronger when formed between the indolyl radical cation and the ionized carboxyl group. This would lead to an increase in the population of hydrogen-bonded species and to a stronger electrostatic



residue	ω	ϕ	ψ	χ1
Trp_1		52.15	140.97	161.25
Pro_2	179.47	-70.05	-167.44	14.60
Pro_3	177.21	-66.69	-178.34	9.15
Pro_4	-179.31	-47.64	104.72	-15.34
Tyr_5	-176.41	148.12	-168.40	-60.74 ,

Figure 4. Stereo drawing of a conformer of H-Trp-$(Pro)_3$-Tyr-OH exhibiting short contact (ca. 0.2 nm) between NH (indole, Trp) and CO (carboxyl, Tyr) groups. The geometry of this conformer, characterized by the conformational dihedral angles in the figure, was obtained by restrained molecular dynamics (with use of the TRIPOS force field,[49] AMBER all-atom charges,[50] and distance-dependent dielectric constant $\epsilon = 4r$).

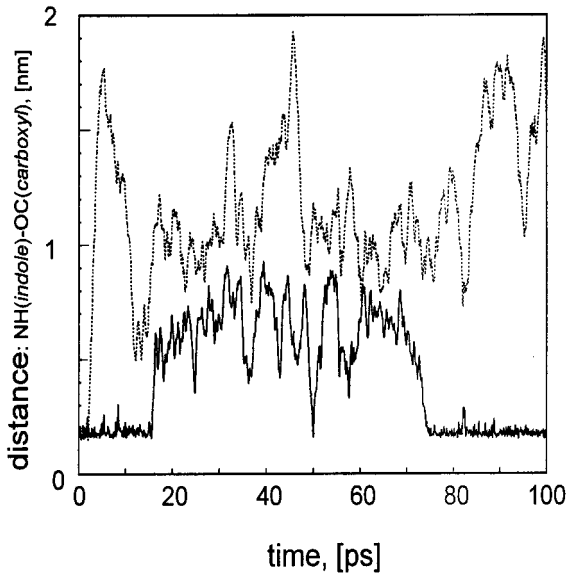


Figure 5. Evolution in time of the NH(indole)−CO(carboxyl) distance from the transition-state conformer of H-Trp-$(Pro)_3$-Tyr-OH shown in Figure 4, obtained by 100 ns MD simulation at 300 K: (solid line) conformers with $\psi(Pro_3)$ angle in the α region, where the NH···OC bond was broken at 18 ns and reconstructed at 60 ns; (broken line) conformers with $\psi(Pro_3)$ dihedral in the β region, where the NH···OC hydrogen bond was broken at 3 ns and the separation distance between NH (indole) and CO (carboxyl) fluctuated in a broad range of 0.5−2 nm.

perturbation of the radical cation, resulting in a lowering of its effective pK_{a1} and reduction potential. The lower reduction potential means also a smaller difference between reduction potentials of the radical redox couple in question, i.e., smaller electrochemical driving force for reaction 2 in **3.** Attainment by **3** of the transition state for the $\beta \rightarrow \alpha$ transition at the $\psi(Pro_3)$ dihedral would require additional energy of activation. On the other hand, a prerequisite for formation of an intramolecular hydrogen bond in **4** and **5** would be the simultaneous occurrence of at least two $\beta \rightarrow \alpha$ transitions, an event of extremely low probability. Thus, the observed differences in the rate and activation energy of LRET between **3** and its longer-bridged analogues can be reasonably explained in terms of the unique conformational properties of this peptide. The proposed temperature dependence of pK_{a1} in **3** could also be strengthened by the occurrence of the $\beta \rightarrow \alpha$ transition, since the thermally accelerated rate of this transition would increase the population of intramolecularly hydrogen-bonded Trp[$NH^{\bullet+}$] species of lower reduction potential.

6. Thermodynamics of LRET. The thermodynamic parameters of LRET activation at 298 K, $\Delta H^{\#}_{298}$, $\Delta S^{\#}_{298}$, and $\Delta G^{\#}_{298}$ calculated from activation energies and rate constants of reaction 2 with the use of known relationships, are collected in Table 3 along with those obtained previously at pH 8. Analysis of the data indicates that the free energy barrier of activation, $\Delta G^{\#}_{298}$, for both reactions 1 and 2 is for the most part entropic in nature, owing to the large and negative values of $\Delta S^{\#}_{298}$. The values of $\Delta S^{\#}_{298}$ for reaction 2 in **4** and **5** proved to be somewhat lower **(**by not more than ca. 20%) than those determined for reaction 1**.** This finding is not compatible with the earlier suggestion[35] that the higher reactivity of Trp[$NH^{\bullet+}$] compared with that of Trp[$N^{\bullet}$] in free amino acid is due to a high entropy barrier for activation of electron transfer involving the neutral form of the Trp radical, while for Trp[$NH^{\bullet+}$] $\Delta S^{\#} \cong 0$. Rather, it is that the larger value of $|\Delta G^{\circ}|$ for the Trp[$NH^{\bullet+}$],Tyr[OH] redox couple can be held responsible for the higher reactivity of the cationic form of the Trp[NH] indolyl radical. The values of $|\Delta S^{\#}_{298}|$ for both reactions increase on going from **3** to **5**, that is, in parallel with the growing thermodynamic stability of the $-(Pro)_n-$ bridge in these peptides. Both temperature CD investigations[30] and molecular mechanics modeling[31] have shown that the longer the $-(Pro)_n-$ bridge is the higher the thermodynamic stability exhibited by its PLPII-like helical conformation.

According to the semiclassical theory of one-electron-transfer reactions,[40] the activation free energy $\Delta G^{\#}$ includes contributions from the reorganization free energy λ and the standard free energy of the redox reaction ΔG°:

$$\Delta G^{\#} = \frac{(\lambda + \Delta G^{\circ})^2}{4\lambda} \qquad (6)$$

The reorganization energy is made of contributions due to the reorganization of inner (λ_{in}) and outer (λ_{out}) nuclear coordinates of the reactants: $\lambda = \lambda_{in} + \lambda_{out}$. In the case of an intramolecular LRET reaction, λ_{out} contains contributions from a bridge separating the reactants, λ_b, and the solvent, λ_s. λ_{out} is expected to grow the farther the electron moves because there must be greater nuclear movement to reequilibrate with the new charge distribution. Involvement of the TS pathway in **3** would additionally affect λ_{out}, as it has been discussed previously[7] in connection with LRET in short-bridged analogues.

The values of λ_{298} could not be calculated from the respective experimental $\Delta G^{\#}$ data (Table 3), since ΔG° is not known.

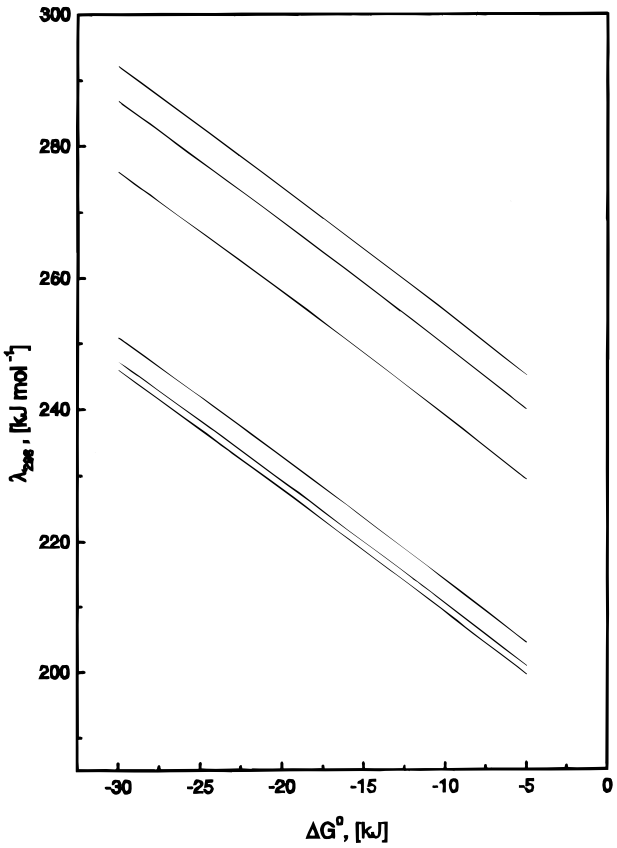


Figure 6. Calculated dependence of reorganization energy λ_{298} on electrochemical driving force $\Delta G°$ in reactions 1 and 2 for peptides **3**, **4**, and **5**.

Therefore, we could only calculate the dependence of λ_{298} on $\Delta G°$ with the help of eq 6. In the range of $\Delta G°$ from -10 to -30 kJ, corresponding approximately to 0.1−0.3 eV, in which values of $\Delta G°$ for both reactions 1 and 2 in **3**−**5** could be expected,[11,15,35,39] λ_{298} assumes large values weakly dependent on $\Delta G°$ (cf. Figure 6). At any selected $\Delta G°$ value, $\lambda_{298,1}$ for reaction 1 increases in the order **3** < **4** < **5**, in agreement with theoretical expectations. Since $\Delta G°$ can be expected to increase with the number, n_{Pro}, of Pro residues in the bridge, as discussed above, the differences in λ_{298} in **3**−**5** can be even larger. Assuming that $|\Delta G°|$ increases by 2 kJ mol^{-1} (ca. 0.02 eV) per Pro residue added,[34] it can be shown by linear correlation of $\lambda_{298,2}$ with n_{Pro} that in the selected range of $\Delta G°$, $\delta\lambda_{298,1} = \delta(\lambda_b + \lambda_s) \approx 12$ kJ (mol n_{Pro})$^{-1}$ and at $n_{Pro} = 0$ (H-Trp-Tyr-OH) $\lambda_{298,1}$ reaches a value in the range 200−224 kJ mol^{-1}. The latter can be regarded as the sum of λ_{in} and λ_s (at $n_{Pro} = 0$) contributions to λ_{out} for the TB pathway. Owing to the postulated stronger effect of unique conformational properties of **3** in reaction 2, the calculated $\lambda_{298,2}(\Delta G°)$ values are slightly higher than those for **4**, and thus, the $\lambda_{298,2}$'s do not correlate linearly with n_{Pro}. Extrapolation from the data for **4** and **5** to $n_{Pro} = 0$ gave values $\lambda_{298,1} \approx 175$ kJ mol^{-1} at $n_{Pro} = 0$ and $\delta\lambda_{298,1} \approx 9$ kJ (mol n_{Pro})$^{-1}$, which are somewhat lower than those evaluated for reaction 1.

Concluding Remarks

We have demonstrated in this work that the intramolecular radical transformation reaction 2, Trp[NH•+] → Tyr[O•] in H-Trp[NH]-(Pro)$_n$-Tyr[OH]-OH, $n = 3-5$, peptides occurs in acidic solutions to as low as pH 2 with an intrinsic rate constant that is 20-fold to 60-fold higher than that characteristic of reaction 1, Trp[N•] → Tyr[O•] in the corresponding peptides. This indicates that the electrochemical driving potential $\Delta G°$ for this reaction in the systems studied is negative in the whole pH range in which the Trp[NH•+] radical cation exists. Moreover, it varies most probably with the length of the oligoproline bridge, since pK_{a1} for Trp[NH•+] dissociation has been shown to decrease systematically from **5** to **3.** We argued that variation of pK_{a1}, and consequently also of $\Delta G°$, with the length of the $-(Pro)_n-$ bridge may result from electrostatic perturbation of the electronic wave function of indolyl radicals by the terminal COO$^-$ group of tyrosine. This effect seems to be particularly enhanced in **3** by the postulated occurrence of the $\beta \rightarrow \alpha$ transition at the ψ(Pro3) dihedral angle during the lifetime of indolyl radicals, leading to formation of a hydrogen bond between NH (indole) and OC (carboxyl) groups.

The demonstrated occurrence of reaction 2 at pH 2 in peptides **3**−**5** and the suggested variation of the electrochemical driving force with location of Trp in the peptide chain requires further investigation of the pH dependence of the electrochemical reduction potentials of Trp[N•],H/Trp[NH] and Tyr[O•],H/Tyr-[OH] couples. Exact knowledge of $\Delta G°$ would then allow us to estimate actual values of the reorganization energy and various contributions.

The observed variation of pK_{a1} for Trp[NH•+] dissociation and its interpretation in terms of the electrostatic field effect should also be subject to further investigation in view of the expected importance of electrostatic control over LRET involving Trp indolyl radicals in redox proteins and protein−membrane systems.[11]

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(45) According to this model, the distance-dependent rate of LRET for a single conformer of a peptide can be written as $k = k_0^{TB} \exp(-\beta^{TB} r^{\beta\alpha\beta}) + k_0^{TS}([I_{AB}]^2$, where k_0^{TB} and k_0^{TS} are respectively through-bond and through-space rate constants at a separation distance $r^{\beta\alpha\beta} = r_0$ corresponding to the closest approach of the redox centers and parametrized as the distance between the C^β atoms of the terminal amino acid residues along a line connecting C^α backbone atoms, β^{TB} is the descriptor of the exponential distance dependence of k, and I_{AB} is overlap integral between LUMO's of the indolyl radical and HOMO's of phenol rings. The application of this model consists of (i) calculation of relative rate constants, k_{ij}, for individual conformers of a peptide along an assumed pathway and calculation of average values for each trans and cis peptide isomer about the Trp-Pro bond, $\langle k \rangle_i = \sum_{ij} k_{ij} w_{ij}$ (w_{ij} is the Boltzman probability of occurrence of the jth conformer of the ith isomer) and (ii) fitting the latter to the experimental k_{et} data with use of the above function.
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