Long-Range Electron Transfer in Peptides. Tyrosine Reduction of the Indolyl Radical: Reaction Mechanism, Modulation of Reaction Rate, and Physiological Considerations

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Abstract: We have observed long-range electron transfer (LRET) across the oligoproline spacer from the tyrosine side chain to the indolyl cation radical derived from the 1-electron oxidation of 1-*N*-methyltryptophan (Metrp$^{\bullet+}$). On the basis of these results and measured bimolecular electron transfers in the model system *p*-cresol/*N*-methylindole, phenol O–H bond breaking can clearly accompany the 1-electron transfer under conditions in which H-atom transfer to the indole nitrogen is impossible. With Metrp$^{\bullet+}$ as the electron acceptor, the rate of the LRET process across the oligoproline spacer is an order of magnitude higher than with the tryptophanyl radical (Trp$^{\bullet}$) as electron acceptor. However, the apparent distance dependence, as measured by the exponential constant β, of the LRET process is the same with both radicals. We argue that these observations support our earlier conclusion, based on kinetic arguments, that proton transfer is not rate determining in the LRET between tyrosine and Trp$^{\bullet}$. We have also shown that LRET rates are enhanced considerably in the electron transfer from the tyrosine phenolate side chain to Metrp$^{\bullet+}$ and from tryptophan to Metrp$^{\bullet+}$. These rates are fast enough to allow speculation about the design of peptide "wires". Finally, the reported results are the basis of a discussion on a possible mechanism for protein structural control of an LRET process.

Long-range electron transfer (LRET) in proteins, peptides, and various other spacers over distances ≥20 Å is well documented.[1] Electron transfer between a donor and its distant acceptor in a protein (i) is known to be an obligatory event in photosynthesis, (ii) has been postulated as important in respiration and a variety of other enzyme-catalyzed redox reactions, (iii) may be important in mediating free radical damage initiated by ionizing radiation and oxygen assault, and (iv) may be part of the radical processes associated with chemical carcinogenesis and cellular aging. In addition to the biological importance of LRET, there are theoretical questions of interest. One in particular is how protein structure might control the LRET process, for there is the possibility that different proteins could have evolved so as to control the LRET rate and/or the path of the electron transfer. However, the study of LRET in proteins is complicated by the individual protein's complex structural features and those amino acid residues that could behave as electron-transfer mediators. Due to these complexities, we have turned our attentions to LRET in peptides, under the assumption that peptides will serve as simpler models for proteins.

Current understanding of the LRET mechanism is incomplete. The original theoretical discussions, couched in terms of Marcus theory, were based on the picture of a *through-space* tunneling process:[1] electron transfer because of orbital overlap between the electron donor and acceptor. However, there are also theories, developed in terms of superexchange coupling, that can be described as *through-bond* or *through-atom*,[2] with the orbitals of intervening atoms contributing to the tunneling process. And Reimers and Hush[3] have proposed a theory that encompasses both through-bond and through-space mechanisms. The experimental evidence to date has not resolved the question of the LRET mechanism(s) in proteins. For example, considering peptides (with proline residues as the sole spacer amino acid) as models for proteins, DeFelippis et al.[4a] and Wishart et al.[4b] have concluded that the LRET processes they studied involved a through-bond process, Cabana and Schanze[5a] and Inai et al.[5b] have argued for a through-space LRET, and Bobrowski et al.[6] have proposed that in shorter peptides ($n = 0$ to 2) electron transfer is predominantly through-space and in longer peptides ($n \geq 3$) through-bond.

There is also the related problem of constructing and utilizing molecular circuits. As Lehn[7] has discussed, molecular circuits require molecular wires, and the observation of LRET in peptides and proteins over distances that can be greater than 20 Å makes these compounds attractive candidates for molecular wires. In the case of peptides, there is already a substantial synthetic chemistry and technology background upon which to draw, and peptides currently are produced commercially on a large scale. In the peptides with which we have been working, the value of β is relatively low;[4a] a low β[8] would be essential for efficient electron transfer over long distances. However, LRET in these

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same peptides is relatively slow. For example, in TyrOH-$(Pro)_4$-Trp•, where LRET occurs because the Trp indolyl side chain neutral radical (Trp•) oxidizes the tyrosine side chain phenol (TyrOH), the k_{et} is 860 s^{-1} over a nominal distance of ca. 15 Å. This rate compares poorly with those reported by Oevering et al.[9] and Calcaterra et al.[10]—10^8 s^{-1} and higher over rigid saturated spacers with center-to-center distances of ca. 15 Å. Thus, there is a question of whether it is possible to design peptide systems with both a low β value and an intrinsically fast LRET rate.

Our studies with peptides have utilized the pulse radiolytic initiated electron transfer between tyrosine (TyrOH) and tryptophan (TrpH), an experimental system developed by Land and co-workers.[11] Pulse radiolytically generated azide radical (N_3•) rapidly and preferentially oxidizes the TrpH indole side chain to the indolyl radical (Trp•) in peptides that contain both TyrOH and TrpH.

$$N_3^{\bullet} + \text{TrpH-X-TyrOH} \rightarrow \text{Trp}^{\bullet}\text{-X-TyrOH} + N_3^- + H^+ \quad (1)$$

where X represents a peptide spacer. Trp•, with a midpoint potential slightly greater than 1 V at pH 7 (ref 12 and references to the work of other laboratories therein), can oxidize the TyrOH side chain to the phenoxy radical (TyrO•) in the intramolecular reaction

$$\text{Trp}^{\bullet}\text{-X-TyrOH} \rightleftarrows \text{TrpH-X-TyrO}^{\bullet} \quad (2)$$

Since both TyrO• and Trp• absorb in the visible region, it is easily shown that reaction 2 is a one-step process with stoichiometric electron transfer from the TyrOH donor to the Trp• acceptor.[13] Parenthetically, both TyrO• and Trp• are physiologically significant. TyrO• is an intermediate in the photosystem II light reaction[14] and in at least two important enzyme-catalyzed reactions—those of ribonucleotide reductase[15] and prostaglandin synthase.[16] More recently a neutral Trp• has been observed in the H_2O_2/cytochrome *c* peroxidase complex.[17]

Our initial LRET experiments have been with peptides in which profile residues serve as the intervening spacer. Since oligoproline chains are extended in solution,[18] the Tyr and Trp residues at the two ends cannot touch one another. With these system we have observed the exponential distance dependence[4a] observed generally with LRET and specifically with other redox centers connected by the oligoproline spacer.[19] But in contrast with most other reports, the value we find for the constant β, the description of that exponential dependence, is low (for a discussion on the uncertainties associated with the calculation of an apparent β, see below): ca. 0.2 $Å^{-1}$ in Tyr-$(Pro)_n$-Trp and ca. 0.4 $Å^{-1}$ in Trp-$(Pro)_n$-Tyr.[4a,12] (The sequence order of Tyr and Trp are reversed in the two.) Both these values for β are much lower than had been previously suggested[21] or observed in other systems.[22] Marcus theory predicts that the value of β could depend on the nature of the donor and acceptor, and this is indeed the case with the different donors/acceptors joined by an oligoproline.[12,19] But Marcus theory does not predict that β would also depend on the (Tyr/Trp) sequence order. Because of the low β values which we have observed and which are consistent with a through-bond proposal[3] and due to the apparent sensitivity of β to sequence order, we have proposed[4a] that LRET between tyrosine and tryptophan with an oligoproline as the intervening spacer is a through-bond process.[6]

However, the electron transfer of reaction 2 differs from many of the LRET processes described in the literature; this reaction also involves a *net* proton transfer. We have already established from kinetic evidence that proton bond making/breaking independent of the actual electron transfer is not rate determining in the overall process of reaction 2; i.e., there is no mandatory requirement for a $TyrO^-$ and/or $TrpH^{\bullet+}$ intermediate before the electron transfer or for the accumulation of a $TyrOH^{\bullet+}$ and/or Trp^- intermediate after.[4a,12] However, phenol O–H bond cleavage and/or indole N–H bond formation could occur through the transition state and so could be a factor in any theoretical discussion of the observed rates and of the low β values.

One possible way to evaluate the contribution of O–H bond cleavage and/or N–H bond formation would be to study electron transfer in the absence of net proton transfer, i.e.

$$\text{TrpH}^{\bullet+}\text{-X-TyrO}^- \rightleftarrows \text{TrpH-X-TyrO}^{\bullet} \quad (3)$$

However, the electron transfer of reaction 3 is not easily arranged.

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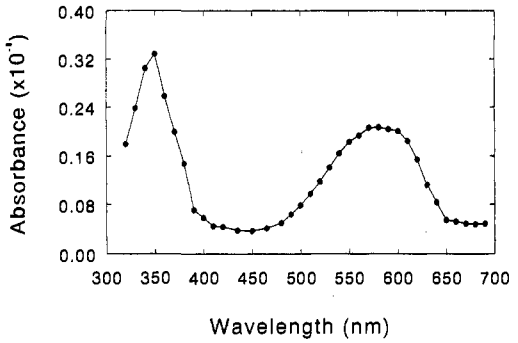

Figure 1. Transient spectrum of the 1-*N*-methyltryptophan cation radical obtained 5 μs after the electron pulse. The amino acid (0.500 mM) was dissolved in 5 mM phosphate buffer, pH 7, containing 0.1 M NaN_3 and saturated with N_2O. The initial $N_3^\bullet$ concentration was 2.4 μM.

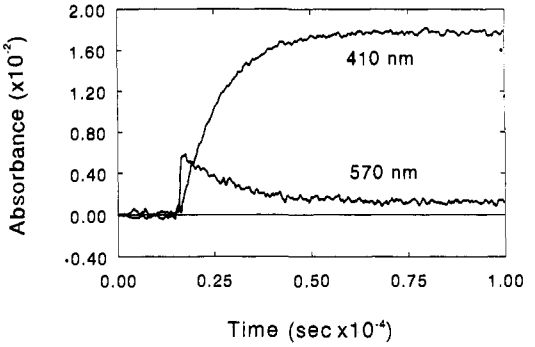

Figure 2. $N_3^\bullet$ oxidation of 1-*N*-methylindole followed by the bimolecular oxidation of *p*-cresol. The absorbance at 570 nm traces the change in indolyl cation radical as a function of time. The initial absorbance increase is due to $N_3^\bullet$ oxidation of the indole ring to form the cation radical. The subsequent decline is due to the reduction of this radical with concomitant oxidation of *p*-cresol to its phenoxy radical as followed at 410 nm: 1-*N*-methylindole, 0.5 mM; *p*-cresol, 1 mM; 5 mM phosphate buffer, pH 7; NaN_3, 0.1 M; $N_3^\bullet$, $\leq$1 μM; solution saturated with N_2O.

Since the pK_a of $TrpH^{\bullet+}$ is somewhere between 4.3 and 5.4[23] and the pK_a of the tyrosine side chain near 10, there is no pH at which $TrpH^{\bullet+}$ and $TyrO^-$ coexist significantly in aqueous solution. We, therefore, considered an analogous reaction in which 1-*N*-methyltryptophan (Metrp) replaces Trp in the peptide.

Metrp, a derivative of Trp with a methyl group substitution on the indole nitrogen, is oxidized rapidly by $N_3^\bullet$ ($k_2 \sim 10^{10}$ M^{-1} s^{-1}) to yield the 1-electron oxidized 1-*N*-methyltryptophan cation radical ($Metrp^{\bullet+}$). With no dissociable proton at the ring nitrogen, the Metrp cation radical enjoys certain similarities with $TrpH^{\bullet+}$. $Metrp^{\bullet+}$ has a spectrum similar to that of $TrpH^{\bullet+}$ (ref 24 and Figure 1) with a λ_{max} at 570 nm. It has a reduction potential of 1.14 V,[23c] which is the same as that of $TrpH^{\bullet+}$ within experimental error.[23c,23d] And as does $TrpH^{\bullet+}$,[4,24,25] $MeTrp^{\bullet+}$ (see below) more rapidly oxidizes 1-electron donors—tyrosine in particular—than does $Trp^\bullet$. Thus, $Metrp^{\bullet+}$ appears to be an analog of the tryptophan radical cation, $TrpH^{\bullet+}$. On the basis of this close similarity, we designed the experiments described here. In addition to the Metrp peptide experiments, we have investigated the reaction between *p*-cresol and the radical cation of methylindole.

Methods and Materials

Beginning with commercially obtained and unpurified Trp, we synthesized Metrp by the method of Rajh et al.[26] After two crystallizations from 1/1 methanol/H_2O, the white Metrp had a mp of 247–249 °C (dec). When using Metrp to study the properties of its 1-electron radical cation form, one must be concerned with (i) Trp contamination, since there is a rapid reduction of $Metrp^{\bullet+}$ by Trp present even at a few percent contamination, and (ii) the iodide present as a counterion, since I^- can reduce the cation radical. When present, we separated away contaminating Trp on a C_{18} reversed-phase Dynamax column (Rainin Instrument, CA) with an acetonitrile/water gradient. We removed the iodide counterion by passage of the Metrp preparation, adjusted to ca. pH 3 with HCl, through a column containing Dowex 1X8 ion-exchange resin in the chloride form.

For the use of Metrp in peptide syntheses, we prepared its FMOC derivative by the method of Carpino and Han.[27] After recrystallization from 1/1 ethyl acetate/hexane, the Metrp FMOC derivative had a melting point of 151–153 °C. Peptides were custom synthesized in the Peptide Synthesis Laboratory of The Ohio State University Comprehensive Cancer Center by a solid-phase method using the FMOC protocol. The completed peptides were cleaved from the resin with 90% trifluoroacetic acid in dichloromethane, and the resultant solution was neutralized with 5–10% aqueous ammonium bicarbonate before lyophilization, since Metrp is acid sensitive. We purified the peptides from the dry powder by reverse-phase HPLC using a H_2O/acetonitrile gradient from 0–60% in the absence of the acid normally used. The peptides were characterized by their FAB mass spectra, in each of which we found the expected parent peak and a base peak of MW 154 corresponding to the *N*-methylindole fragment. The UV spectra of these peptides indicated the presence of both Try and Metrp on the basis of the extinction coefficients of the two free amino acids at 280 and 288 nm.

1-*N*-methylindole and *p*-cresol (Aldrich, Milwaukee, WI) were both distilled *in vacuo* (ca. 20 Torr) and stored under nitrogen in the refrigerator. We have described elsewhere the pulse radiolysis equipment and experiments with the azide radical.[13] All experiments were conducted at 25 °C, and apparent first-order rate constants were extracted from absorbance vs time curves with standard nonlinear analytic procedures.

Because reduction of Metrp by TyrOH in the peptides Metrp-$(Pro)_n$-Tyr occurs by both intramolecular first-order and intermolecular second-order processes, we measured apparent rate constants as functions of peptide concentration. In all cases, plots of k_{app} vs peptide concentration were linear and extrapolated to a finite value at zero concentration. The first-order rate constant obtained from the intercept is the intramolecular LRET rate constant. All rate constants have an associated 10–20% uncertainty unless stated otherwise.

Results and Discussion

Mechanism of 1-Electron Transfer: N–H and O–H Bond Making/Breaking. This discussion begins with the 1-electron transfer between the *N*-methylindole cation radical and *p*-cresol. To initiate this reaction, we utilized the 1-electron oxidant $N_3^\bullet$, which oxidizes *N*-methylindole (1.3×10^{10} M^{-1} s^{-1}, pH 7)[28] more rapidly than it oxidizes *p*-cresol (6×10^8 M^{-1} s^{-1}, pH 7). Thus at sufficiently high indole to phenol ratios, $N_3^\bullet$ preferentially oxidizes the indole ring as observed by an absorbance increase at 570 nm,[24] which is an absorption maximum of the methylindole cation radical as well as of $Metrp^{\bullet+}$. The subsequent cation radical oxidation of *p*-cresol to the phenoxy radical can then be observed at 410 nm, where phenoxy radicals absorb. With the concentrations of *N*-methylindole and *p*-cresol much greater than that of $N_3^\bullet$, all observed reactions are pseudo-first-order. In Figure 2 is an example of the absorbance–time profiles we obtained. After the electron pulse, there is a rapid absorbance rise at 570 nm due to indolyl cation radical formation, and then a slower absorbance decline with an accompanying absorbance increase at 410 nm. This slower process is the 1-electron transfer from *p*-cresol to the methylindole radical cation. The products of this 1-electron transfer are the *p*-cresol neutral radical (the 410-nm

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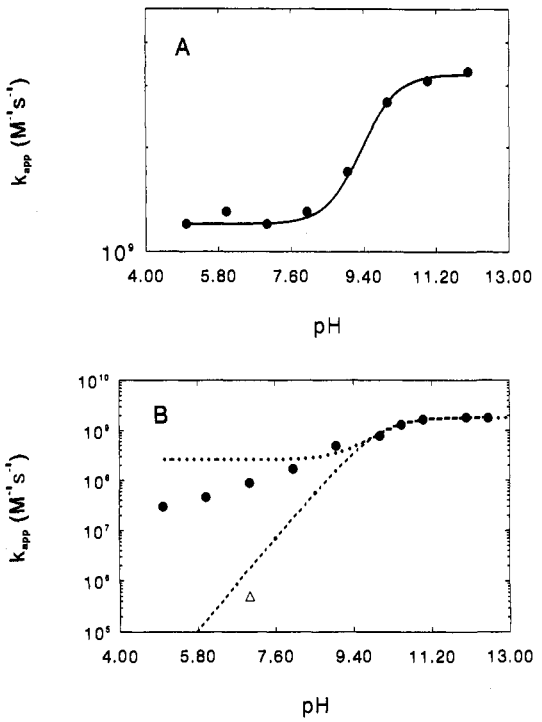
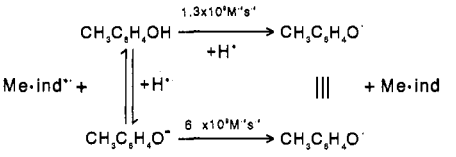


Figure 3. pH dependence of phenol to indolyl radical electron transfer. (A) *p*-cresol oxidation by the *N*-methylindole cation radical. Points are measured second-order rate constants. The solid line is calculated from a best-fit computation assuming that phenol and phenolate species, in rapid equilibrium with one another, both react with the cation radical. The equation on which the fit was based is $k_{app} = [k_1 + k_2(H^+)/K]/[1 + (H^+)/K]$, where k_1 and k_2 are the second-order rate constants associated with the reaction of the phenolate and phenol species, respectively, and K is the phenol dissociation constant. Conditions: *N*-methylindole, 0.5 mM; *p*-cresol, 5–50 μM; phosphate buffer, 5 mM; NaN_3, 0.1 M; $N_3^•$, ≤1 μM; solutions saturated with N_2O. All reactions were carried out at 25 °C. (B) Tyrosine oxidation by 1-*N*-methyltryptophan cation radical. Points are measured second-order rate constants: ●, 1-*N*-methyltryptophan cation radical; △, tryptophan radical. The upper dashed line is calculated from a best-fit (pH ≥ 9) computation assuming that phenol and phenolate species in rapid equilibrium with one another both react with the cation radical; i.e., $k_{app} = [k_1 + k_2(H^+)/K]/[1 + (H^+)/K]$. The lower dashed line is calculated from a best-fit (pH ≥ 9) computation assuming that only the phenolate species, while in rapid equilibrium with phenol, reacts with the cation radical; i.e., $k_{app} = k_1/[1 + (H^+)/K]$. Conditions are similar to those for Figure 3a, except that 1-*N*-methyltryptophan, 0.5 mM, and tyrosine, 0.05–2 mM, replace *N*-methylindole and *p*-cresol, respectively.

absorbing species) and methylindole. We obtained second-order electron-transfer rate constants associated with this slower phase from the dependence of the apparent first-order rate constants on the *p*-cresol concentration. These second-order constants are dependent on the pH as shown in Figure 3a.[29] The observed pH dependence is consistent with a model in which the *p*-cresol phenolate anion reacts more rapidly with the cation radical ($k_2 = 6 \times 10^9$ M^{-1} s^{-1}) than does the neutral phenol species ($k_2 = 1.3 \times 10^9$ M^{-1} s^{-1}). Nonlinear fitting, based on this model, to the observed data extracts a pK_a of 9.8, a value expected of a *p*-cresol proton dissociation.

The results of Figure 3a indicate that the indole radical can oxidize the neutral protonated *p*-cresol species; i.e., electron transfer can occur together with phenol O–H bond cleavage. At pH 7, where *p*-cresol is fully protonated, there is a solvent $^2H_2O/^1H_2O$ kinetic isotope effect (kie) of 2.4 (±0.1). Since the phenolic proton is fully exchangeable into the deuterated solvent, the observed kie is consistent with proton involvement in the transition state for electron transfer from the phenol to the indole cation radical.[30] At pH 12, there is, within experimental error, no observable kie, a result expected when the reductant is the phenolate anion and O–H bond cleavage cannot be part of the overall electron transfer. Thus, the indole cation radical directly oxidizes both the phenolate anion and the phenol species (see scheme below). Moreover, since the methylindole radical cannot



be protonated, direct hydrogen atom transfer from the phenol to the indole ring nitrogen is not possible, a suggestion that had been made for the oxidation of tyrosine by Trp•.[31]

Metrp•+ oxidation of the tyrosine side chain shows a more complex pH dependence (Figure 3b). The observed rate constant also declines with decreasing pH to suggest different reactivities for the protonated and unprotonated phenol side chain species. However, unlike the methylindole cation radical oxidation of *p*-cresol, at lower pH the electron-transfer rate constant continues to decline slowly with decreasing pH. A simple model for the observed pH dependence is that only the phenolate form of tyrosine reacts with Metrp•+. However, significant electron transfer does occur at a pH much lower than the phenol side chain pK_a, a result inconsistent with the data extrapolation based on this simple model (the lower dashed line in Figure 3b obtained from nonlinear fits of the observed data above pH 7). A second simple model—in which Metrp•+ also oxidizes the tyrosine phenol species but at a slower rate than the phenolate species—results in an extrapolation (the upper ultimately dashed line in Figure 3b) that is similar to the observed pH dependence in the reaction between the methylindole cation radical and *p*-cresol, but different from the results obtained with the amino acids. That the experimental points lie on neither of these hypothetical dashed lines indicates that neither model is adequate. However, because the experimental curve lies clearly between these two extrapolations and because of the analogy with the methylindole/*p*-cresol reaction, Metrp•+ must be able to oxidize the tyrosine phenol species. There is at least one possible explanation for the continued slow decline in k_{app} at lower pH—an electrostatic effect associated with the increased positive charge of both tyrosine and Metrp•+ as the amino and carboxylate groups become protonated. In support of that explanation, we have found that increasing the phosphate buffer concentration (ionic strength) also increases the electron-transfer rate. This increase is independent of the pH between 5 and 8 and, therefore, is not due to phosphate acid/base catalysis. Finally, we note an important comparison based on the data of Figure 3b: at pH 7, the rate of tyrosine oxidation by Metrp•+ is significantly greater than the rate of tyrosine oxidation by Trp•.

What can we now say about the mechanism of the Trp• LRET reduction by the tyrosine side chain in peptides? The following

(29) Reactions of the 1-*N*-methylindole cation radical with OH^- and with 1-*N*-methylindole are known to occur with respective rate constants of $k_2 \approx 6 \times 10^6$ and 6×10^5 M^{-1} s^{-1} (ref 39). The methylindole cation radical undergoes a bimolecular recombination reaction with a k_2 of 6.7×10^8 M^{-1} s^{-1}.[24] (Metrp•+ decays with a rate constant of 4×10^8 M^{-1} s^{-1}, this study). Under our experimental conditions, these reactions are much slower than, and hence do not interfere with, the pseudo-first-order 1-electron transfer from *p*-cresol to the methylindole radical.

(30) Solar et al.[24] have reported that there is no kie associated with the $N_3^•$ oxidation of both indole and tryptophan. From this result, they concluded that oxidation of both involves electron rather than hydrogen atom transfer. Taken together, their conclusions and ours are consistent and suggest that the proposal[31] of direct hydrogen transfer in the Trp^j oxidation of tyrosine is not reasonable. DeFelippis[33] has found a $^2H_2O/^1H_2O$ kie of approximately 2 associated with the intramolecular electron transfer in Trp•-Pro-TyrOH. The kie we have observed here together with the results of Solar et al. are consistent with a simple intramolecular electron transfer in which the kie arises entirely from the phenol O–H bond cleavage.

(31) Jovanovic, S. V.; Harriman, A.; Simic, M. G. *J. Phys. Chem.* **1986**, *90*, 1935–1939.

speculative argument is consistent with our results to date. We start with the following two conclusions: (i) indole N–H bond formation cannot be rate limiting in the intramolecular LRET from TyrOH to Trp• [32] and (ii) both the bimolecular *p*-cresol reduction of the 1-*N*-methylindole cation radical (see above) and the intramolecular TyrOH reduction of Trp• (see note 30) have associated solvent $^1H_2O/^2H_2O$ kie's of similar magnitude. We propose that the observed kie in the intramolecular oxidation of TyrOH by Trp• is due solely to tyrosine O–H bond cleavage and not to tryptophan indole N–H bond formation. This proposal is equivalent to the suggestion that indole N–H bond formation, with water as the possible proton donor, either does not occur during or is very late through the transition state for the 1-electron reduction of Trp• by tyrosine. Thus, the electron-transfer mechanism would be essentially the same with or without a methyl group substituted onto the indolyl radical nitrogen; i.e., the mechanisms of Trp• and Metrp•+ reduction are the same.

But, if the two 1-electron reductions have the same mechanism, then why does Metrp•+ (and TrpH•+) oxidize tyrosine faster than does Trp•? There are two possible explanations. First, Metrp•+ (and TrpH•+) is a better oxidant than Trp• by ca. 100 mV. Second, the putative initial product in the case of Trp• reduction would be the tryptophan anion, Trp⁻, which should have a significantly higher free energy than Metrp (or Trp). Thus, the activation energy to reach the transition state associated with Trp• as the oxidant may be greater than that associated with Metrp•+ (or TrpH•+); i.e., the faster oxidation rates obtained with an indole cation radical as compared with the indole neutral radical may be due to differences in $\Delta G°$ and/or in the nuclear rearrangement term, λ.

Table I. Comparison of Rate Constants for Intramolecular LRET in TrpH-$(Pro)_n$-TyrOH and Metrp-$(Pro)_n$-TyrOH

no. of prolines	Metrp-$(Pro)_n$-TyrOH (M^{-1} s^{-1})[a]	TrpH-$(Pro)_n$-TyrOH (M^{-1} s^{-1})[a,b]
1	16×10^4	2.4×10^4
2	6.3×10^4	0.67×10^4
3	2.1×10^4	0.22×10^4

[a] Reaction conditions are those of Figure 5, except that the pH was 7. All rate constants have an associated uncertainty of 10–20%. [b] Taken from ref 4a.

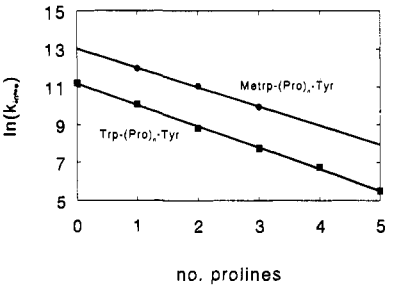


Figure 4. Distance depencence of LRET across te oligoproline spacer: ●–●, Metrp-$(Pro)_n$-Tyr; ■–■, Trp-$(Pro)_n$-Tyr; data taken from ref 4a. Reaction conditions are those of Figure 5 except that the pH was 7.0. The second-order rate constant for intramolecular LRET was obtained from the intercept of the linear plot of the apparent first-order rate constant vs peptide concentration.

We had mentioned in the introduction that N–H bond formation is part of the overall 1-electron transfer from tyrosine to Trp•. In contrast, there is no possibility of indole N–H bond formation as part of the overall 1-electron reduction of a 1-*N*-methylindolyl cation radical. Thus, by replacing Trp with Metrp and comparing the intramolecular LRET processes in the two peptide series, we can investigate whether indole N–H formation is an agent in setting the observed low β value for the Trp-$(Pro)_n$-Tyr series.[4a,13] From the results of this comparison (Table I and Figure 4) we conclude that (i) the plot of $\ln(k_{et})$ vs the number of intervening proline residues is linear for both the Metrp and Trp series, (ii) intramolecular LRET is uniformly faster in those peptides with Metrp•+ than in those with Trp•, which is consistent with our earlier studies on the bimolecular Tyr reductions of Trp• and Metrp•+, and (iii) the values of β in both systems are similar: 0.33 ± 0.02 Å^{-1} for Metrp-$(Pro)_n$-Tyr and 0.37 ± 0.01 Å^{-1} for Trp-$(Pro)_n$-Tyr (assuming an additional 3.1 Å per added Pro residue in both series). Thus, proton attachment to the indole side chain during or after the tyrosine phenol (these experiments were at pH 7) side chain reduction of the indolyl radical does not affect the LRET rate distance dependence, as reflected in the value of the parameter β. This conclusion is consistent with our earlier one that indole N–H bond formation is not crucial to the tyrosine reduction of the indolyl radical.

How Long Is the Oligoproline Chain? The apparent values that we have reported for the distance parameter β are relatively low and depend on the sequence order of the Tyr and Trp end groups.[4a] On the basis of these results, we had concluded that LRET in these pptides is a through-bond process. But the value that one estimates for β depends on the value one chooses for the distances between the indole and phenol side chains. The advantage to the use of oligoproline as a spacer is that the five-member ring of this imino acid imparts sufficient rigidity that the distance between chain ends should increase linearly with the number of prolines in the chain.[18a] However, dihedral bond angles are not totally fixed and so there is some flexibility in the oligoproline chains used for our studies. Therefore, we consider here whether the values of β that we have calculated are

(32) This conclusion is based on discussions we presented in refs 4a and 13. In those discussions, we argued that (i) rapid electron transfer followed by slower proton transfer would require an observable intermediate that is not observed, (ii) slow initial deprotonation of TyrOH to $TyrO^-$ or slow initial protonation of Trp• to TrpH•+ followed by rapid electron transfer is inconsistent with the magnitudes of the observed LRET rate constants, and (iii) in the TrpH-$(Pro)_n$-TyrOH series (n = 0 to 5), the observed rate constant at pH 7 decreases by a factor of approximately 300 from dipeptide to heptapeptide.

(33) DeFelippis, M. R. *The Redox Potentials of the Tyrosine and Tryptophan Radicals and Long-Range Electron Transfer Between Tyrosine and Tryptophan in Peptides*, Ph.D. Thesis, The Ohio State University, Columbus, OH, 1990.

(34) $Br_2^{\bullet-}$ is produced under the same conditions used for the production of $N_3^•$, with the difference that 0.1 M Br^- replaces N_3^- in the reaction solution.

(35) One might argue for other schemes in which electron transfer depends on peptide bimolecular reactions and not on the intramolecular process of eq 4. However, these schemes would require either that the $N_3^•$ oxidation of the peptide is much slower than 10^9 M^{-1} s^{-1}, which is unlikely, or that a bimolecular reaction between peptide molecules is much faster than 6×10^9 M^{-1} s^{-1}, which is faster than diffusion controlled. A word on the observed small rapid rise at 570 nm (Figure 5) may also be in order here. An initial Trp side chain oxidation would cause an immediate absorbance increase at 570 nm, but that initial absorbance increase should be small as the cation radical is rapidly reduced in the second intramolecular electron transfer. As the reaction progresses to completion, the methylindole radical concentration and hence its absorbance at 570 nm should decrease. However, the phenoxy radical also absorbs weakly at this wavelength and thus as the reaction proceeds the absorbance at 570 nm due to the formation of the tyrosine phenoxy radical will increase. The overall signal as a function of time should be biphasic, but the net signal might be small by compensation of the contrary absorbance changes. The noise in the small signal we did obtain makes it impossible to attempt a differentiation between the decay of Metrp•+ and the formation of TyrO•.

(36) (a) Michel-Beyerle, M. E.; Plato, M.; Deisenhofer, J.; Michel, H.; Bixon, M.; Jortner, J. *Biochim. Biophys. Acta* **1988**, *932*, 52–70. (b) Gray, H. B.; Malmstrom, B. G. *Biochemistry* **1989**, *28*, 7499–7505. (c) Weinstein, M.; Alfassi, Z. B.; DeFelippis, M. R.; Klapper, M. H.; Faraggi, M. *Biochim. Biophys. Acta* **1990**, *1076*, 173–178.

(37) Tanford, C.; Hauenstein, J. D. *J. Am. Chem. Soc.* **1956**, *78*, 5287–5291.

(38) However, Bollinger and co-workers have reported an organic radical intermediate preceding the formation of the stable *E. coli* ribonucleotide reductase phenoxy radical. This unassigned radical has a reported visible spectrum that is similar to that of TrpH•+. Moreover, the X-ray crystal structure shows a Trp residue lying close to the protein's non-heme iron center and between that iron center and the Tyr phenoxy radical. Bollinger, J. M.; Edmondson, D. E.; Huynh, B. H.; Filley, J.; Norton, J. R.; Stubbe, J. *Science* **1991**, *253*, 292–298. Bollinger, J. M.; Stubbe, J.; Huynh, B. H.; Edmondson, D. E. *J. Am. Chem. Soc.* **1991**, *113*, 6289–6291.

(39) Neta, P.; Huie, R. E.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1986**, *17*, 1027–1284.

appropriate, i.e. whether for either thermodynamic or kinetic reasons the "true" value of β is greater than the apparent one we calculate. We begin the discussion with thermodynamic considerations.

First, the proline peptide bond exists in both the trans and the less favored energetically cis conformations. Were all the oligoproline peptide bonds cis, then the distance between the electron donor and acceptor would increase by 1.85 Å for each additional intervening proline residue; were all trans, then the distance would increase by 3.12 Å per residue. From our data of rate constant dependence upon chain length, this range of incremental distances implies a range of β from 0.37 to 0.63 Å^{-1}. However, Boulat et al.,[18c] on the basis of solution NMR studies, concluded that the peptide bonds in the pentaproline chain of Ala-Tyr-(Pro)$_5$-Thr-Leu-Ala are predominantly trans with an all-trans conformation in 75–80% of all molecules and the major cis conformer found with the first proline that follows Tyr.; i.e. beginning with the third proline residue, the "internal" proline amide bonds are overwhelmingly trans. Similarly, Bobrowski et al.[6] have estimated that 85–90% of the Trp-(Pro)$_n$-Tyr molecules are all-trans at the proline residues for the longer peptides. (Peptide bonds involving all natural amino acids other than proline are trans. Infrequent cis conformations appear in proteins and in otherwise strained peptide systems.) Thus, in the longer chains ($n > 2$), each added proline will overwhelmingly assume the trans conformation.

Because of a high activation energy, the peptide cis–trans isomerization is relatively slow, orders of magnitude slower than the LRET rates reported here.[20] Hence, during the LRET process, the peptide molecules are partitioned into four sets that are not interconvertible over the time course of the electron transfer: the predominant all-trans conformation, a small fraction with a cis peptide between the N-terminal amino acid and the first proline, an even smaller fraction with a single cis peptide between the first and second prolines, and a minor fraction with two cis peptide bonds. In all our electron-transfer studies with the oligoproline spacer, we have observed only single exponential transitions. We can conclude either that the electron-transfer rates are the same or closely similar in all four sets of molecules or that for some experimental reason (e.g., overlooking a much faster process that has only a smaller absorbance change) we do not identify an electron transfer associated with those molecules that contain one or more cis proline amide bonds. Moreover, from absorbance changes associated with formation of the tyrosine and loss of the tryptophan radicals, we can account for 100 ± 10% of the electron transfer.[13] Thus, we can conclude that the rate constants we have measured are those associated with intramolecular LRET in the set of all-trans amide bond molecules, the large majority of molecules in the collection.

However, there can be rapid (relative to the LRET process) conformational changes due to limited dihedral motions around the C^α, C^β, and C^γ carbons of the five-member proline ring and the dihedral rotations of the Tyr and Trp residues. Consider first motions in the proline ring. These conformational changes can result in stretching and compression of the oligoproline chain. On the basis of minimum energy calculations, Bobrowski et al.[6] have assigned an average inter-residue proline-C^β to proline-C^β distance of 2.7 Å. This compares with the inter-residue distance of 3.1 Å for the fully extended proline chain. The smaller 2.7-Å figure and our kinetic data yield an estimate for β of 0.43 Å^{-1}, the same value Bobrowski et al. calculated from their results.[6] The greater value of 3.1 Å yields an estimate for β of 0.37 Å^{-1}.

Dihedral rotations in the aromatic amino acids will certainly influence the side chain to side chain distance. Thus, these rotations would affect the LRET rate, especially if the electron transfer were through-space. But these rotations would most probably not be reflected in the estimated value of β. Consider the peptides with proline chain lengths of $n > 2$. Solution NMR data indicate that each additional proline inserted into the chain will have the trans amide conformation. Thus, unless one makes the additional assumption that the conformations of the terminal two amino acid residues affect the ring conformational fluctuations within the internal proline residue, the LRET rate decrease due to the insertion of an internal proline into the polypeptide is associated with a distance change of 2.7 or 3.1 Å (the average distance of Bobrowski et al., or the fully extended chain distance). In other words, the conformations of the two terminal amino acids should affect the absolute magnitudes of the LRET rate, but should not affect the incremental rate decrease associated with the internal prolines. Thus, the apparently low values of β calculated in these systems are not an artifact due to an incorrect estimate of the thermodynamically stable oligoproline distances. To emphasize this point, β is associated with the incremental distance due to the addition of an internal proline and should thus be insensitive to chain end Tyr and Trp conformations, unless one assumes that the distribution of Tyr and Trp conformers changes with each added proline. On the other hand, the absolute values of the LRET rate constants could be dependent on the conformations of Tyr and Trp, especially if the electron transfer were through-space.

This conclusion is also important with respect to our earlier proposal[4a] that there is a directional specificity for LRET in the two peptides series TrpH-(Pro)$_n$-TyrOH and TyrOH-(Pro)$_n$-TrpH based on the apparently different values of β we observed in these two peptide series, 0.37 and 0.23 Å^{-1}, respectively. Bobrowski et al.[6] conclude that "this difference is most likely due to somewhat different equilibrium populations of cis and trans isomers about the X–Trp and X–Tyr bonds and X_1 rotamers of C- and N-terminal Trp indole side chain, resulting in a different average separation distance between the aromatic rings in the two peptides series." This conclusion is, however, inconsistent with (i) their own argument that LRET is predominantly through-bond when $n \geq 3$, (ii) their calculations that the through-bond rates are almost insensitive to the cis/trans state for longer peptides, and (iii) our argument that conformational differences at the ends of the peptide chain could well raise or lower absolute LRET rates but would not affect the distance introduced by the addition of an internal proline into the peptide chain and hence not affect β. Thus, the observed difference in β between the two peptide series cannot be due to conformational differences at the two ends, and we continue to suggest that there is a directional specificity for LRET in these peptides.

Let us turn next to a consideration of kinetics and the question of "effective" distance. There has been a suggestion that electron transfer might occur primarily through a strained, low-probability conformation in which the electron donor and acceptor distances are shorter than estimated on the basis of the thermodynamically preponderant structures. Thus the "true" value of β would be larger than the "apparent" value, especially if LRET were through-space. It is unlikely that the energy of reaching such a highly strained conformation appears in the LRET activation energy, since reported activation energies for LRET in these peptides are relatively low—from ca. 10 to 20 kJ/mol.[4a,6] Thus, we pose the possibility that electron transfer occurs primarily within a small population of effective conformers in equilibrium with a much larger fraction of lower energy molecules. Since the magnitude of β depends on the incremental distance introduced with each added internal proline, a "true" β calculated on the basis of a smaller net "effective" distance requires simultaneously smaller "effective" distances for each of the prolines in the chain. From the available data, a distance of ca. 3 Å per additional proline yields an apparent β of ca. 0.4 Å^{-1}. Thus, to reach a β of ca. 0.8 Å^{-1} would require an effective distance of ca. 1.5 Å. We find it unlikely that there could be any significant fraction of peptide chains with all the proline five-member rings bent sufficiently to achieve anything close to this collapsed distance.

A reviewer has suggested another intriguing kinetic argument to explain the apparently low values of β observed with these peptides. According to this argument a diffusional pre-equilibrium is required to form an electron-transfer complex. If complex dissociation were to compete with electron transfer and electron transfer required the correct peptide conformation, then the complex of an incorrect conformer would dissociate, with electron transfer occurring only eventually upon $N_3^\bullet$ collision with conformationally correct (CC) molecules. However, the postulated electron-transfer complex could not exist for long since the oxidation of Trp by $N_3^\bullet$ occurs close to or at diffusion control. Thus, there could be little rapid hopping to ultimately find a CC molecule from that small fraction of the total peptide chains.

However, we can extend this suggestion. Let us consider that the reaction of eq 1 is a rapid equilibrium so that in an incorrect conformation there might be competitive reoxidation of N_3^- until the intramolecular electron transfer had been eventually effected only within the CC. There is good reason to reject this scheme as well. The redox potentials of Trp$^\bullet$ and $N_3^\bullet$ are ca. 1.05[23] and 1.33 V,[40] respectively. Thus, at equilibrium and under the conditions of our experiments, the ratio of $(N_3^\bullet)/(\text{Trp}^\bullet)$ is less than 10^{-10}. Since the total radical concentrations in our experiments rarely exceeded 1 μM, the equilibrium concentration of $N_3^\bullet$ would be far too low to support the rapid bimolecular reactions required for this hypothesis.

We suggest that the apparently low values of β reported by both us and Bobrowski et al. are real. We cannot at this time explain why they should be so low. We have, however, already concluded that a through-bond LRET might explain these data. Also the reader should keep in mind the fact that the peptide bond is an electron-delocalized grouping. This might result in a relatively insensitive LRET distance dependence.

Enhancing 1-Electron LRET in Oligoproline Peptides. As just reported, the 1-electron intramolecular LRET rate over the oligoproline spacer is faster when Metrp$^{\bullet+}$ replaces Trp$^\bullet$. Also, the bimolecular 1-electron reductions of both Metrp$^{\bullet+}$ and the *N*-methylindolyl cation radical are faster with the phenolate anion as compared with the phenol species. Both these observations led us to consider the 1-electron LRET from a phenolate anion to an indolyl cation radical, i.e., the intramolecular LRET of eq 3. However, as discussed in the introduction, the pK_a of TrpH$^{\bullet+}$ is near 5 while that of the tyrosine side chain phenol is ca. 10. Therefore, there is no pH at which the phenolate anion and the indolyl cation radical coexist in aqueous solution. To circumvent this difficulty we looked at the intramolecular electron transfer in the peptide series Metrp$^{\bullet+}$-(Pro)$_n$-TyrO$^-$. Our observation of the close analogy between Metrp$^{\bullet+}$ and TrpH$^{\bullet+}$ and the possibility of obtaining the Metrp cation radical when the solution pH is greater than the tyrosine pK_a were the basis for our use of this peptide species.

The kinetics we observed upon the 1-electron oxidation of Metrp-(Pro)$_n$-TyrO$^-$ (Figure 5) are unlike any we have previously with the other peptides of our LRET studies. Upon introduction of $N_3^\bullet$ at pH $\geq$ 11, there is rapid formation of the tyrosine phenoxy radical as measured at 410 nm. However, only a small nondescript absorbance increase at 570 nm precedes that TyrO$^-$ oxidation; i.e., unlike the results we have obtained with other related peptides, there is no clear absorbance rise and fall at 570 nm to indicate the intermediacy of the indolyl cation radical. Thus, for this particular case, there is the question as to whether $N_3^\bullet$ directly oxidizes TyrO$^-$ and not Metrp.

The apparent first-order rate constant for TyrO$^\bullet$ formation is linearly dependent on peptide concentration (Figure 5 inset), as would be expected for direct oxidation of the tyrosine side chain phenolate anion. From the slope of this straight line, we calculated an apparent second-order rate constant of 6×10^9 M^{-1} s^{-1}. (The intercept value obtained by linear extrapolation is too small for the rate constant of a first-order intramolecular oxidation of TyrO$^-$ by the Trp side chain indolyl cation radical and not significantly different than zero.) On the basis of the argument to follow, we propose that the primary peptide site of $N_3^\bullet$ oxidation is at Metrp not TyrO$^-$ and that the measured apparent second-order rate constant is that of the Metrp residue oxidation by $N_3^\bullet$.

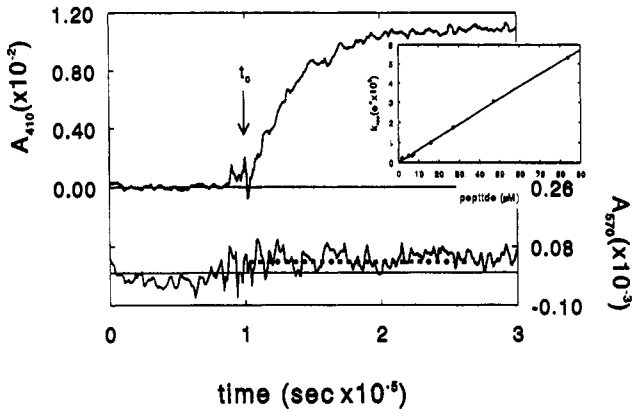


Figure 5. LRET in Metrp$^{\bullet+}$-(Pro)$_3$-TyrO$^-$. Inset: apparent first-order rate constant as a function of peptide concentration. The reaction initiated with $\leq$1 μM $N_3^\bullet$ in aqueous 5 mM phosphate buffer, pH 11, 25 °C: peptide, 47–175 μM; NaN_3, 0.1 M; solution saturated with N_2O.

Table II. Reaction of Tyr and Metrp with $Br_2^{\bullet-}$ at pH 12[a]

amino acid/peptide	k_2 (M^{-1} s^{-1})
Tyr	5×10^8 [b]
Gly-Tyr	5.2×10^8
Metrp	1.9×10^9
Metrp-Gly	1.7×10^9 [c]
Metrp-(Pro)$_3$-Tyr	1.3×10^9

[a] Reaction mixtures at room temperature contained 5 mM phosphate, 0.1 M KBr, saturating N_2O, $\leq$1 μM $N_3^\bullet$, and amino acid or peptide within the range 10–200 μM. All second-order rate constants were obtained from the slope of the linear k_{app} dependence on peptide concentration and have associated uncertainties of 10–20%. [b] Taken from ref 31. [c] pH 11.

The bimolecular rate constants for $N_3^\bullet$ oxidation of the amino acids TyrO$^-$ and Metrp are ca. 3×10^9 M^{-1} s^{-1} and 1×10^{10} M^{-1} s^{-1}, respectively. That the observed second-order rate constant obtained from the slope (Figure 5, inset) is approximately twice that observed for the $N_3^\bullet$ oxidation of the free tyrosine phenolate amino acid suggests that the azide radical oxidizes Metrp in the peptide and that Metrp$^{\bullet+}$ then rapidly oxidizes TyrO$^-$:

$$N_3^\bullet + \text{MeTrp-X-TyrO}^- \xrightarrow[6 \times 10^9\ \text{M}^{-1}\ \text{s}^{-1}]{k_2} \text{Metrp}^{\bullet+}\text{-X-TyrO}^- + N_3^-$$

$$\text{Metrp}^{\bullet+}\text{-X-TyrO}^- \xrightarrow{fast} \text{Metrp-X-TyrO}^\bullet \qquad (4)$$

According to this scheme, the 1-electron oxidation of Metrp by $N_3^\bullet$ is rate limiting and the observed second-order rate constant is that associated with Metrp oxidation. However, the three rate constants under comparison are all sufficiently close to diffusion controlled that this argument is not overly compelling. We, therefore, sought additional evidence against the direct $N_3^\bullet$ oxidation of the tyrosine phenolate anion side chain and for the suggestion that the rate constant of 6×10^9 M^{-1} s^{-1} is associated with *N*-methylindole oxidation. To obtain this additional evidence, we investigated dibromide anion radical ($Br_2^{\bullet-}$) oxidation of Metrp-Pro$_3$-TyrO$^-$.[34]

Gathered in Table II are the observed rate constants for the $Br_2^{\bullet-}$ oxidations of Tyr, Gly-Tyr, Metrp, and Metrp-Gly. $Br_2^{\bullet-}$ oxidizes Metrp approximately 3-fold faster than it oxidizes Tyr.

(40) DeFelippis, M. R.; Faraggi, M.; Klapper, M. H. *J. Phys. Chem.* **1990**, *94*, 2420–2424.

Table III. "Acid/Base" Modulation of LRET Rates

peptide radical	k_{et} (s^{-1})
Trp•-Pro-Pro-Pro-TyrOH[a]	2200
Metrp•+-Pro-Pro-Pro-TyrOH[b]	21 000
Metrp•+-Pro-Pro-Pro-TyrO⁻[c]	$>10^6$

[a] Taken from ref 4a. [b] Reaction conditions those of Figure 5, except the pH was 7. [c] Reaction conditions those of Figure 5.

The reactivity toward $Br_2^{\bullet-}$ of either aromatic amino acid when incorporated into a dipeptide is essentially unchanged. With this information in hand, we repeated the LRET peptide experiment of Figure 5 but with $Br_2^{\bullet-}$ rather than $N_3^\bullet$ as the initiating oxidizer. The observed kinetics of $Br_2^{\bullet-}$ oxidation were qualitatively similar to those of $N_3^\bullet$ oxidation; there is no indication at 570 nm for the intermediacy of the indolyl cation radical, and the pseudo-first-order rate constant associated with the formation of the absorbance at 410 nm (the phenoxy radical) depends linearly on the peptide concentration. In this case, the extracted apparent second-order rate constant was 1.3×10^9 M^{-1} s^{-1} (Table II), almost 3-fold greater than the rate constant for $Br_2^{\bullet-}$ oxidation of free Tyr phenolate and slightly less than the rate constant for $Br_2^{\bullet-}$ oxidation of Metrp. We have, therefore, concluded that in this peptide system $Br_2^{\bullet-}$ must be oxidizing Metrp predominately and that the subsequent oxidation of TyrO⁻ by Metrp•+ is too fast for us to measure. Hence, the observed second-order rate constant of 1.3×10^9 M^{-1} s^{-1} is associated with the 1-electron oxidation of the Metrp methylindole side chain by $Br_2^{\bullet-}$. These experiments support our previous suggestion that the scheme in eq 4 accounts for $N_3^\bullet$ oxidation of this peptide. Since the rate of the intramolecular electron transfer from TyrO⁻ to Metrp•+ is not dependent on whether $Br^{\bullet-}$ or $N_3^\bullet$ is the initiating oxidant, the intramolecular electron transfer in the $N_3^\bullet$-initiated reaction must also be too fast to measure, and as we have suggested, the observed second-order process of Figure 5 is not due to the direct oxidation of TyrO⁻.[35]

Thus, replacing Trp with Metrp and TyrOH with TyrO⁻ enhances the intramolecular electron transfer sufficiently that we cannot measure this LRET process in our laboratory; i.e., the LRET rate constant is $>10^6$ s^{-1}. This rate enhancement corresponds to an "acid/base" switching (going from the Trp•/TyrOH to the Metrp•+/TyrO⁻ pair and assuming Metrp•+ is a good analogue of TrpH•+) that increases the LRET rate by at least 3 orders of magnitude. There are two possible explanations for this observed enhancement. The first is the change in redox potential difference, going from ca. 140 mV for the first redox pair[23c] to ca. 300 mV for the second.[33] On the other hand, it is also true that the reaction is qualitatively different in the two cases since the side chain phenol species is the reductant in the first case and the phenolate anion the reductant in the second. Hence, the faster oxidation rate may be due to differences in $\Delta G°$ and/or in λ.

Further work is required to determine the rate constant of the intramolecular LRET in reaction 4 and to explain the cause of the observed rate enhancement. Nonetheless, it is clear that the electron-transfer rate in this peptide approaches those reported in some non-peptide systems[9,10] even though the redox potential difference is quite modest. We have achieved a fast intramolecular LRET rate by starting with observations in amino acids and simpler derivatives, and then altering the "acid/base" status of the redox centers in the peptide (Table III). We cannot yet claim the construction of a peptide wire, but our ability to increase LRET rates by the simple chemical manipulation of "acid/base" switching encourages us to believe that rational design of peptide wires may be possible.

Physiological Considerations. The observation of "acid/base" switching leads to speculation on feasible mechanisms for (i) structural control of the LRET process in proteins and (ii) linkage of electron transfer to the formation of a pH gradient. We start this discussion with a hypothetical bipolar element under the control of an environmental pH. For the construction of this bipolar element, consider that (i) TrpH•+, a weak acid, is a better oxidant than its conjugate base Trp• by approximately 100 mV and (ii) TrpH•+ absorbs in the visible region with a λ_{max} at ca. 570 nm, while Trp• absorbs with a λ_{max} at ca. 510 nm. Therefore, were the pH of the solution around TrpH•+ to be below its pK_a while the pH of the solution around a nearby unoxidized Trp above that same pK_a, then it should be both thermodynamically and spectrally possible to observe the following reaction:

$$\underset{\lambda_{max}=570\text{ nm}}{\overset{pH<pK_a}{TrpH^{\bullet+}}}\text{-(Pro)}_3\text{-TrpH} \rightleftarrows \text{TrpH-(Pro)}_3\text{-}\underset{\lambda_{max}=510\text{ nm}}{\overset{pH>pK_a}{Trp^\bullet}} + H^+ \quad (5)$$

Note that the location of the radical hole on the peptide depends on the pH at the two ends. Under the conditions shown in reaction scheme 5, the reaction would be pulled spontaneously to the right; i.e., the Trp radical would exist on the high pH side since TrpH•+ is a better oxidant than Trp•. Reverse the pH gradient and the position of the electron would also reverse from one end of the peptide to the other.

However, to achieve both a high pH at one end of the chain and simultaneously a low pH at the other requires that the two Trp residues be in two different, physically separated environments, for example, on two sides of a membrane. Rather than starting with an attempt at such a physical separation, we have tested for the possibility of reaction 5 by looking for the analogous oxidation of Trp by Metrp•+. Once again, the analogy between the two cation radicals is based on the similarities of their visible spectra and redox potentials. The advantage in using the N-terminal Metrp•+ as the analog "acid" conjugate radical is that it exists at pH 7 over the time course of our experiments while TrpH•+ does not. Thus, it should be possible to see the oxidation of the C-terminal Trp to the base conjugate neutral radical with the peptide in solution of pH 7, a pH above the TrpH•+ pK_a.

$$\underset{\lambda_{max}=570\text{ nm}}{Metrp^{\bullet+}}\text{-(Pro)}_3\text{-TrpH} \rightleftarrows \text{Metrp-(Pro)}_3\text{-}\underset{\lambda_{max}=510\text{ mm}}{\overset{pH>pK_a}{Trp^\bullet}} \quad (6)$$

It was first necessary to establish that the electron-transfer reaction of eq 6 is possible. To this end, we looked for the bimolecular reduction of Metrp•+ by Trp by utilizing the different spectra of the Metrp•+ and Trp• radicals. This bimolecular reaction does occur and is relatively fast with a second-order rate constant at pH 7 of 0.77×10^9 M^{-1} s^{-1}—an average from measurements at 350 nm, 510 nm (buildup of Trp•), and 570 nm (loss of Metrp•+). Thus, we conclude that the intramolecular reaction of eq 6 should also be possible. As an aside, we note the comparison of second-order reaction rate constants presented in Table IV. The reduction potential differences between Metrp and the three electron donors decrease in the order TyrO⁻ > TyrOH > TrpH. But this is not the order of reaction rate constants. This difference in ordering is consistent with our earlier discussion of reaction mechanism. Namely, oxidation of the

Table IV. Bimolecular Reactions of 1-*N*-Methyltryptophanyl Radical Cation

reductant	ΔE_m (mV)[a]	k_2 (M^{-1} s^{-1})[b]
TrpH	90	0.77×10^9
TyrOH	200	0.08×10^9
TyrO⁻	380	2.1×10^9

[a] Reduction potential differences were calculated from the potentials reported in refs 23c and 33. The values used for the E_m of TrpH and TyrOH were determined at pH 7, that for TyrO⁻ at pH 11. [b] Bimolecular rate constants were determined under the conditions indicated for Figure 3b. The rate constants with TrpH and TyrOH as reductants were obtained at pH 7, that with TyrO⁻ at pH 12.

phenolic side chain involves O–H bond cleavage in the transition state, while N–H bond cleavage does not occur or is late during the transition state associated with oxidation of the indole side chain. Additionally, we would argue that, if, as concluded earlier, the oxidations of Trp and Metrp do occur by similar or perhaps identical mechanisms, then the fast rate for TrpH reduction of $Metrp^{\bullet+}$ and a measured reduction potential difference of only 90 mV suggest this reaction as a model for electron self-exchange between indole and the indolyl radical.

Because $N_3^\bullet$ oxidizes Metrp (ca. 1×10^{10} M^{-1} s^{-1}) faster than it oxidizes Trp and various Trp derivatives ($4–5 \times 10^9$ M^{-1} s^{-1}),[33] it is possible to initiate the intramolecular electron transfer of reaction 6 with a selective $N_3^\bullet$ oxidation. (Even if the azide radical oxidizes one-third of the peptide directly at the C-terminal Trp, there should still be electron transfer from Trp to $Metrp^{\bullet+}$ in the remaining two-thirds of the peptides that are oxidized at the N-terminal residue. Moreover, the azide radical concentration can be kept sufficiently low so that the probability of two radical oxidations on one peptide molecule is very low.) The experimental results obtained with Metrp-$(Pro)_3$-Trp were similar to those shown in Figure 5 for Metrp-$(Pro)_3$-$TyrO^-$. While an absorbance increase at 510 nm indicated oxidation of the C-terminal Trp, there was no absorbance buildup and decay at 570 nm to indicate the intermediacy of $Metrp^{\bullet+}$ under conditions in which it should be the major site of $N_3^\bullet$ oxidation. Therefore, the rate constant for reaction 6 is also too great for us to measure ($k_{et} > 10^6$ s^{-1}), a result which suggests that reaction 5 could occur under the proper conditions. One such proper condition would be a pH gradient established across a membrane into which is embedded a peptide with Trp at both C- and N-terminal ends. Hence, a membrane-embedded peptide with tryptophans at both ends of the chain might permit linking electron transfer with a pH gradient across the membrane. Or conversely (as indicated in eq 5), the electron transfer could be used to establish a pH gradient.

Together with the observation of LRET in proteins, there have also been suggestions of and evidence for the structural control of both the LRET pathway and rate in proteins.[36] The results we have presented here suggest one possible mechanism for achieving such control. The phenolate anion is both a faster and a more thermodynamically favorable reductant than its conjugate acid, the phenol species. Therefore, by controlling the pK_a of its tyrosine side chains, a protein could control the location and velocity of 1-electron transfers. Moreover, were the indolyl radical the oxidant side chain, then the LRET rate could be further modified by controlling the pK_a of the tryptophan cation radical. We have known for decades[37] that protein structure can modulate the tyrosine side chain pK_a; to postulate a similar modulation of the $TrpH^{\bullet+}$ pK_a would be reasonable. While the oxidation of tyrosine by the indolyl radical in proteins has been seen in pulse radiolysis experiments (ref 36c and references therein), there is still no evidence for such an electron transfer *in vivo*. Moreover, while both $TyrO^\bullet$ and $Trp^\bullet$ have been proposed as intermediates in protein electron-transfer reactions, we know of no report for a protein stabilized $TrpH^{\bullet+}$.[38] Thus, the feasibility of both suggestions must await experimental verification.

In summary, we have shown that the oxidation of the tyrosine phenolate anion by the indolyl cation radical can lead to very fast LRET rates. From the properties of these oxidations, we can speculate upon mechanisms for controlling both the specificity and the rate of LRET processes in proteins. Moreover, we have experimental evidence for the possibility of fast electron transfer between tryptophan and its cation radical.

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