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Photoinduced Electron Transfer for Pyrenesulfonamide Conjugates of Tryptophan-Containing Peptides. Mitigation of Fluoroprobe Behavior in N-terminal Labeling Experiments[1]

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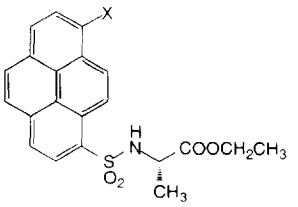
Abstract. The (nitro)pyrenesulfonamide group has been attached to the N-terminus of tryptophan and tryptophan-containing peptides. Intramolecular electron transfer between pyrene and Trp indole side chains, observed by fluorescence quenching and laser flash photolysis, depends on the spacing between Pyr and Trp moieties and the strength of the electron acceptor.

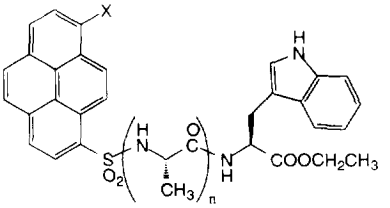
The oxidation of tryptophan (Trp) has been of interest for some years in a number of contexts, including the conversion of Trp to kynurenine by oxygenases, reaction with singlet oxygen, and one electron oxidation yielding radical(ions) via photolysis or pulse radiolysis.[2-4] The tryptophan residue is of special importance since, among the naturally occurring amino acids, it contains the most readily oxidized functional group (the indole side chain) at or near neutral pH [E^{o} ($Trp^{+\bullet}$ --> Trp) = 0.84 V vs SCE].[5] We have recently reported[6] on the *intramolecular* photoinduced electron transfer that occurs between Trp residues and an electron acceptor group (e.g., the dye chromophore, eosin Y) positioned remotely via linkage at an N-terminal position. Alternatively, Klapper and Faraggi have reported[7] on the "hole" transfer that occurs between tyrosine and Trp residues when the two electroactive amino residues are linked with inert spacers (e.g., glycine, alanine). These general findings are consistent with a through-bond mechanism of electronic interaction[8] that depends on the nature of the peptide backbone and attachment linkages (e.g., thiourea, peptide bonds). These recent papers constitute the only reference points regarding "long range" intramolecular electron transfer (LRET) for simple model systems that incorporate Trp as the primary reductant, where linkages and distance relationships for electron acceptor and donor groups are largely established.

A derivatization procedure that is widely utilized in protein chemistry involves the labeling of N-terminal residues. For a host of fluorescence probes (e.g., Dansyl),[10] the common linkage involves the sulfonylation of terminal amines.[11] A heretofore unresolved issue has to do with the extent to which fluorescence labels

Scheme



X = H PyrAla
X = NO_2 NPyrAla



X = H $Pyr(Ala)_nTrp$ (n = 0, 1, 2)
X = NO_2 $NPyr(Ala)_nTrp$ (n = 1, 2)

undergo photoinduced electron transfer with residues positioned along a peptide chain. According to a number of studies on proteins, LRET may occur over distances > 15 Å between reactive partners.[8,9] The distinction regarding possible *electron transfer* quenching is not a trivial one, since an apparently proven method of investigation of distance relationships in proteins involves a measure of the reduction of fluorescence intensity for a native or non-native chromophore as the result of intramolecular *energy transfer*, a process that may occur over distances up to 50 Å (the Forster, or dipole-dipole mechanism).[11,12]

As part of a study of electron transfer for Trp-containing peptides, we have sought to determine the inherent photochemical reactivity of selected chromophores and potentially electroactive Trp side chains. In order to investigate systems using laser flash photolysis (355 and 410 nm for Nd-YAG and Ti-sapphire excitation, respectively), the 1-pyrenesulfonyl group (Pyr), a reported fluoroprobe,[13] and a nitro-substituted analog (NPyr) were chosen for attachment to the N-termini of peptides. An amino acid incapable of ET, alanine (Ala), was incorporated in peptide oligomers, providing 0, 1, and 2 residues as spacers in order to establish a nominal distance relationship for attached chromophores and Trp residues. The results reveal two categories of reactivity: (1) in all cases examined, quenching of fluorescence of the N-terminal chromophore by attached Trp is important and dependent on the length of the intervening peptide chain and (2) for NPyr derivatives, the formation of chromophore triplets is efficient, so that triplet quenching via ET could also be inspected. In order to provide other reference points, the fluorescence properties of several amino acid sequences conjugated with the popular 5-N,N-dimethylamino-1-naphthalenesulfonyl (Dansyl) probe, were also investigated.

Pyrenesulfonamide conjugates of various peptides (and nitropyrene analogs) were prepared from the 1-pyrenesulfonamides of Ala and Trp (as C-terminal ethyl esters), using established solution-phase peptide coupling procedures[14] as reported in detail elsewhere.[15,16] Absorption maxima typical of the respective chromophores (for Pyr, 280 and 350 nm, and for NPyr, 285 and 380 nm) were recorded; the presence of Trp led to small enhancements in absorptivity at about 280 nm. Peak fluorescence typical of the Pyr and NPyr chromophores appeared, respectively, at 385-400 and 440 nm with varying intensities for the conjugates. As shown in the Table, the quantum efficency of emission for PyrTrp was reduced substantially (100-fold) relative to the emission from a model conjugate, PyrAla. In less dramatic fashion the emission yield for the conjugates having one and two Ala spacers was also compromised.[17] For the NPyr series, the effects of Trp attachment

Table. Fluorescence quantum yields and phototransient properties of pyrenesulfonamide conjugates[a]

Conjugate	Φ_f	Phototransient		
		Solvent	λ_{max}[b]	(half-)life time[c]
PyrAla	0.31	-	-	-
PyrTrp	0.0033	CH_3CN	490 nm	τ = 0.79 ns
		DMF	490 nm	τ = 0.32 ns
PyrAlaTrp	0.014	CH_3CN	490 nm	τ = 0.76 ns
		DMF	490 nm	τ = 0.50 ns
PyrAlaAlaTrp	0.064	CH_3CN	490 nm	τ = 3.1 ns
		DMF	490 nm	τ = 1.3 ns
NPyrAla	0.0040	CH_3CN	450, 550 nm	$\tau_{1/2}$ = 12. μs
NPyrAlaTrp	0.00062	CH_3CN	440 nm	$\tau_{1/2}$ = 0.40 μs
NPyrAlaAlaTrp	0.00095	CH_3CN	440 nm	$\tau_{1/2}$ = 0.16 μs

[a] Argon-purged solutions (20° C), 10 μM in conjugate for fluorescence measurements with λ_{exc} = 330 nm and 0.1 mM in conjugate for Ti/sapphire laser phototransient measurements with λ_{exc} = 410 nm.

[b] Wavelength maxima for principal phototransient.

[c] Decay half-lives or lifetimes (τ = weighted average of two lifetimes from biexponential decay).

on fluorescence yield were small, since the singlet lifetime for the NPyr chromophores is short; formation of NPyr excited triplet states is important and competes strongly with fluorescence (vide infra).

Important verifications of mechanism for excited state quenching resulted from flash photolysis experiments. A femtosecond transient absorption apparatus that consisted of a self-mode-locked Ti/S oscillator capable of producing 100 fs pulses was used as an excitation source at 410-420 nm (the frequency doubled wavelength).[18] Using the ultrafast pump-probe technique the appearance and decay of a transient at 490 nm for Pyr derivatives was readily observed. This intermediate was assigned to the radical anion of the Pyr chromophore, in keeping with prior observations for reduction of pyrene and several derivatives.[19] The nature of the transient and the average decay time observed for various peptides are shown in the Table. Using the fs laser, the NPyr derivatives provided different results; a broad absorption at 450-550 nm grew in within 8.0 ps and persisted into the ns regime. The fate of this transient, identified as the NPyr triplet state, was further investigated by ns laser flash photolysis using a Nd-YAG laser apparatus previously described.[20] Interestingly, new intermediates were observed in the longer time domain: a dominant 440 nm transient similar to that observed as the radical anion of 1-nitropyrene[20] and a weaker signal assigned to the radical cation of Trp (ca. 540 nm).[3] The appearance of these transients and their decay times are also presented in the Table.[21] For both series, photobleaching did not appear to be very efficient and radical-ion phototransients returned to baseline (deaerated samples), consistent with a simple mechanism of excited state quenching involving forward and return electron transfer.

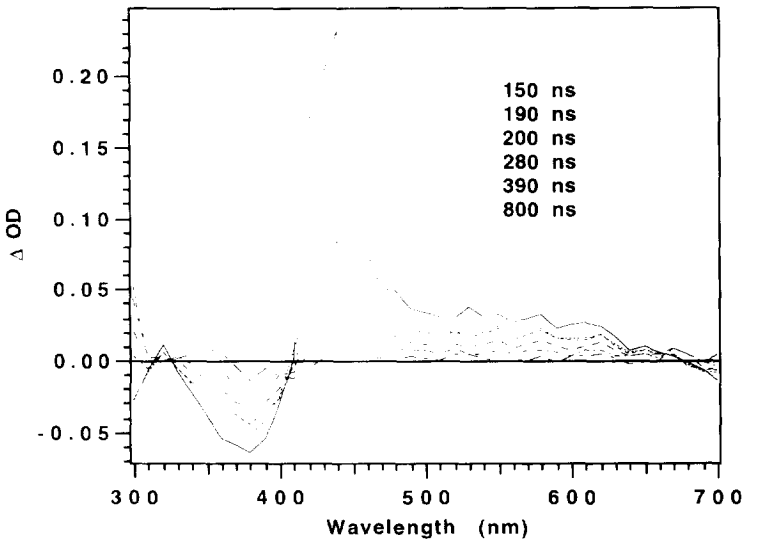


Figure. Transient absorption spectra for 20 μM NPyrAlaAlaTrp in Ar-purged acetonitrile (λ_{exc} = 355 nm, 60 mJ/pulse, 20° C)

Further details regarding mechanism for the observed electron transfers will be provided separately.[15] However, a number of trends in the data are important in the present context. While not perfectly uniform, there is a general pattern of falloff in both the degree of fluorescence quenching and the rate of return electron transfer for the radical-ion pair state for the Pyr conjugates. The data are consistent with the anticipated dependence of intramolecular electron transfer rates on the distance (through bonds) that separates electron donor and acceptor reactants (the Trp indole ring and the pyrenesulfonyl moiety). Molecular modeling (CHARMm) and 2D-NMR (NOE) results[15] are consistent with the notion that these short peptides display a random array of extended conformations. For example, average center-to-center (through-space) and edge-to-edge (through-bond) distances, computed from 500 energy minima in a conformational search were 8.8 and 12.4 (16.9) A, respectively, for PyrAlaTrp (PyrAlaAlaTrp).

Due to the importance of the Dansyl (Dan) group in labeling studies,[10] we sought to establish reference points regarding the mitigation of fluorescence by Trp residues for this chromophore also. Fluorescence quantum yields were obtained for the amino acid derivatives, Dan-Gly, Dan-Trp, and Dan-Gly-Trp (commercial samples, Sigma), in which the glycine residue served as the inert spacer. For these experiments, solutions representing different states of protonation (for the terminal carboxyl) were inspected (5.0×10^{-5} M conjugates in 3:1 H_2O/CH_3CN with the aqueous component, respectively, 1.0 mM HCl, 1.0 mM NaOH, and 1.0 mM phosphate buffer, pH 6.8). The results showed that the emission from these conjugates did not vary in intensity by as much as ±5% in all three media.

The importance of excited state quenching for the Pyr conjugates and the contrasting failure to observe similar behavior for Dansyl derivatives are understood in terms of the differing driving forces for ET. The

relevant data for comparison were obtained using the Weller equation that relates the free energies for ET with redox potentials for oxidation and reduction of the respective donor and acceptor groups, the excited state energy, and a Coulombic term for stabilization of the radical-ion pair:[19,22,23] $\Delta G_{et} = 23.1\ [E_{ox} - E_{red}] - E_{oo} - e^2/\varepsilon r$. The values for excited singlet state quenching by Trp for Pyr and Dan groups (with one Ala spacer) are: ΔG_{et} = -13 and 0.5 kcal/mol, respectively, the latter estimate predicting a slow or negligible rate of intramolecular electron transfer.[24]

The degree to which there may be interference of electron transfer quenching by a neighboring tryptophan in fluorescence probe work will of course be highly variable in the general case. Within the present series, the diminution in fluorescence intensity is observed to depend on the ET driving force (basically, the lower reactivity of Dan as electron acceptor vs Pyr as indicated,[22] the length of the conjugating linkage (Pyr series, Table), and the importance of other processes that compete for decay of the fluorescent state (e.g., triplet formation for NPyr). For a group of common molecular probes such as the xanthene/rhodamine series that includes the familiar fluorescein isothiocyanate (FITC), an additional spacer (e.g., a phenyl group) intervenes between the chromophore and the primary link (e.g., a thiourea moiety). For these examples, the finding that about 75% of fluorescence is quenched on direct attachment of the eosin moiety to the N-terminus of Trp (thiourea derivative) offers an additional reference point.[6] With the photochemical data taken as a whole, the rough guide is that the quenching of N-terminal probes will be important (> 50%) for peptides/proteins with N-terminal Trp or with Trp residues removed by one or two spacer residues when the excited state reduction potential ($E_{red} + E_{oo}$) exceeds a rather common value of 1.5 V (vs SCE).

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16. Conjugates were new compounds, with structures assigned consistent with proton and carbon NMR and HRMS determinations. For the NPyr series, 1,6- and 1,8- isomers were distinguished by NMR; the latter (major) products were utilized.

17. Fluorescence lifetimes for the conjugates were also recorded using phase-shift fluorimetry [ref 6 and 11] with results that paralled the emission yields; for example, the average lifetimes from biexponential decays were 0.88 and 3.1 ns for PyrAlaTrp and PyrAlaAlaTrp, respectively, in acetonitrile.

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21. The transient decay kinetics from both fs and ns laser flash experiments were not cleanly exponential, as one might expect for systems in which back ET occurs at different rates for a multiplicity of peptide conformations. For the faster time scale, the decays could be fit nicely to double exponential functions for which weighted averages are presented (Table). For the NPyr series, the longer decay times that approach the μs range are under further investigation and reported here as half-lives.

22. New measurements of reduction potentials for PyrAla and DanGly electrophores were made using fast-scan cyclic voltammetry. Experiments carried out with 5.0 mM conjugates in dry CH_3CN with Bu_4NPF_6 supporting electrolyte and a Ag/AgCl working electrode (scans up to 15 V/s) yielded values of $E^{o'}$ = -1.69 and -2.15 V vs SCE, respectively (values referenced to the ferrocene couple and converted to SCE).

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24. The ET rate constants, according to the semi-classical model, will depend on a number of structure- and medium-dependent parameters in addition to driving force, ΔG_{et}, including the size of the solvent reorganization energy, and the attentuation of electronic coupling with the distance that separates reactant groups [ref 6, 9 and 23].

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