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The Triplet-State Lifetime of Indole in Aqueous and Viscous Environments: Significance to the Interpretation of Room Temperature Phosphorescence in Proteins

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Abstract: The interpretation of room temperature phosphorescence studies of proteins requires an understanding of the mechanisms governing the tryptophan triplet-state lifetimes of residues fully exposed to solvent and those deeply buried in the hydrophobic core of proteins. Since solvents exposed tryptophans are expected to behave similarly to indole free in solution, it is important to have an accurate measure of the triplet state lifetime of indole in aqueous solution. Using photon counting techniques and low optical fluence (J/cm^2), we observed the triplet-state lifetime of aqueous, deoxygenated indole and several indole derivatives to be approximately 40 μs, closely matching the previous reports by Bent and Hayon based on flash photolysis (12 μs; Bent, D. V.; Hayon, E. *J. Am. Chem. Soc.* **1975**, *97*, 2612−2619) but much shorter than the 1.2 ms lifetime observed more recently (Strambini, G. B.; Gonnelli, M. *J. Am. Chem. Soc.* **1995**, *117*, 7646−7651). However, we have now been able to reproduce the long lifetime reported by the latter workers for aqueous indole solutions and show that it likely arises from geminate recombination of the indole radical cation and solvated electron, a conclusion based on studies of the indole radical cation in water (Bent and Hayon, 1975). The evidence for this comes from a fast rise in the phosphorescence emission and measurements of a corresponding enhanced quantum yield in unbuffered solutions. This species can be readily quenched, and the corresponding fast rise disappears, leaving a monoexponential 40 μs decay, which we argue is the true indole triplet lifetime. The work is put in the context of room temperature phosphorescence studies of proteins.

Introduction

Since the original discovery that room temperature phosphorescence (RTP) can be observed from proteins in aqueous solution,[1] this technique has been increasingly used in protein studies. RTP has been used to monitor many aspects of protein chemistry including changes in substrate binding,[2] protein denaturation,[3,4] and changes in the internal dynamics of proteins[5−10] (see reviews[11−13]).

The triplet-state lifetime of an aromatic chromophore is commonly believed to depend upon several processes that are distinguishable by their viscosity dependence. The triplet state

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is extremely susceptible to trace impurity quenching, and therefore in low-viscosity solvents the triplet-state lifetime is greatly shortened by residual impurities or trace amounts of oxygen.[14−16] The triplet-state characteristics of aromatic chromophores in high-viscosity media (such as frozen media or rigid glasses at higher temperature) have been studied for several decades.[17−23] Under these conditions the behavior is often described by the excess-energy model which assumes that the lifetime is predominantly determined by a temperature-dependent nonradiative process enabled by the thermal population of higher vibrational states that have more efficient singlet−triplet mixing.[24−28] The temperature dependence of this process is associated with the activation energy necessary to populate these vibrational modes.

Because both quenching and thermal activation to higher vibrational states can modulate the triplet-state lifetime, these measured lifetimes may reflect numerous complex processes in the immediate environment of tryptophan residues in proteins. Tryptophan residues which are deeply buried within the hydrophobic core of proteins are associated with lifetimes that can exceed 1−2 s[11] while solvent-exposed tryptophan residues are expected to have much shorter triplet-state lifetimes similar to that of free tryptophan in solution. Knowledge of the latter lifetime is thus of great importance for the interpretation of the results of time-resolved protein RTP, and any ambiguity regarding the lifetime of free indole in solution confounds the interpretation of the observed lifetime of surface-exposed tryptophan residues.

The pioneering work on the triplet-state lifetime of indole by Bent and Hayon in 1975[29] reported a value of 12 μs in aqueous solution at room temperature based upon triplet−triplet absorption following flashlamp excitation. Other indole derivatives yielded similar results (indole, 12 μs;[29] *N*-acetyl-L-tryptophanamide (NATA), 30 μs,[30] 22 μs;[31] 1-methylindole (1-MI), 29 μs;[32] NATA, 40 μs[33]). However, in more recent work using a time-resolved analogue technique,[34] an aqueous indole triplet-state lifetime of 1.2 ms was reported, approximately 40 times longer than in the previous measurements. It was suggested that low concentrations of unidentified quencher(s) in the aqueous solution were responsible for the short lifetimes previously reported, and that only with a more stringent control of the purity of the solvent can the true aqueous triplet-state lifetime of indole be attained. Since tryptophan residues in numerous proteins show sub-millisecond lifetimes,[11] it became unclear whether these lifetimes result from quenching, or reflect the fact that the limiting lifetime of free tryptophan in solution is also sub-millisecond as originally reported. Clearly, resolution of this apparent difference is of fundamental importance to the understanding of indole photophysics and for the interpretation of time-resolved RTP from proteins.

In this work we use high-speed photon counting with fast temporal resolution and careful solvent preparation techniques to measure the phosphorescence lifetime of indole and several indole derivatives in aqueous solution. On the basis of previous work characterizing the photoexcitation products of indole,[29,32,35−40] we conclude that the long lifetimes recently reported[34] are most likely a consequence of the photogeneration of the longer-lived indole radical cation and solvated electron from the excited singlet state rather than a reflection of the intrinsic indole triplet-state lifetime. A likely hypothesis is that geminate recombination of the indole radical cation and the solvated electron, a first-order process, creates an additional slower pathway for the generation of the indole triplet state, thus creating the longer decay times. This model is consistent with the long lifetime of the solvated electron and indole radical cation (several hundred microseconds to milliseconds[29,32,38]), their susceptibility to quenching, and our determination of a negative preexponential term indicative of an excited-state photophysical process. Under conditions that quench the indole radical cation, we observe an indole emission lifetime of ∼40 μs, which is consistent with the value cited in most other laboratories.

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Material and Methods

Phosphorescence measurements were conduced with 10 ns pulsed UV excitation and photon-counting acquisition techniques.[41] The frequency-doubled pulses of a Spectra-Physics model DCR-11 Nd:YAG laser with a wavelength of 532 nm and a pulse width of 8 ns (fwhm) are used to pump a Spectra-Physics model PDL-3 dye laser emitting at 560 nm. This light is then frequency-doubled in a BBO crystal to produce 280 nm radiation. The excess 560 nm light is removed by a Schott glass UG-11 filter, and the remaining 280 nm light is directed into the sample. Cuvette luminescence was substantially masked with a spatial filter, and phosphorescence emission was passed through a 418 cutoff filter (KV418, Shott) before being detected by a Hamamatsu R928P photomultiplier tube (PMT). Pulses from the PMT are sent into a Pacific Instruments model AD6 amplifier/discriminator whose output is collected by an ACEMCS multichannel scaler (acquiring in photon-counting mode) from EG&G Ortec, capable of collecting counts at up to 100 MHz.

The phosphorescence signal was derived by subtracting, at each time point, the intensity of total luminescence of a fully oxygenated sample of indole from the intensity of a fully deoxygenated sample of indole. Fluorescence from the sample and cuvette are gated out by ignoring the initial 18 μs of emission. Typically, 5000 laser shots at 10 Hz were used to acquire a decay, and at least 5 decays were averaged together for analysis; the error bars presented in our analysis are derived from this averaging. Laser intensity between samples was normalized by using a phosphorescence standard (typically *Escherichia coli* alkaline phosphatase due to its high stability and low susceptibility to photobleaching). It should be noted that previous studies[34] of the phosphorescence of free indole had used matched PMTs in a T-configuration, however, we found it experimentally problematic to balance analogue signals from two PMTs experiencing different low incident intensities. In order to evaluate the effect of the large fluorescence emission on the PMT/detection apparatus, we provided a steady background photon input (1−2 MHz) in addition to providing 10 Hz laser pulses of a 1−5 μM indole sample. The large fluorescence emission from the indole sample resulted in an attenuation of the steady background photon-counting rate. The delay between the laser input/fluorescence emission and the reattainment of the normal counting rate was determined to assess PMT saturation. In this study, recovery of normal counting rates occurred within 15 μs following the fluorescence pulse. Photon-counting systems can reattain normal counting rates before complete recovery of the PMT from large excitation pulses with the proper choice of PMT base hardware, discriminator, and PMT voltage settings. While the gain of an analogue signal is dependent upon recovery of all PMT dynode voltages, photon-counting systems need only to recover the gain that amplifies the signal beyond a discriminator setting. Hence photon-counting systems can easily be adapted to evaluate low light levels even after large saturating excitation pulses. Furthermore, photon-counting techniques are more sensitive than analogue techniques at low light levels.[42]

A detailed description of our deoxygenation system appears in the literature[43] and was only slightly modified for these experiments. Specifically, the prepurified nitrogen gas was further purified by passing through an OxiClear oxygen-trapping filter (from LabClear, Oakland, CA), and the cuvette was mechanically rocked gently during deoxygenation to increase the mixing of the solution and the nitrogen. The cuvette was made of high-quality quartz (purchased from Starna) and modified by the University of Michigan glassblower for use in our deoxygenation system. All cuvettes and glassware were cleaned according to the methods described in the literature,[34] and all reagents used were purchased from Sigma Chemical Co. (St. Louis, MO). The W220Y mutant of alkaline phosphatase (AP) was used as an intensity control and was prepared as previously described;[43] the mutant W220Y was utilized to provide only a single phosphorescent tryptophan (W109).

Indole (Aldrich) was triple recrystallized in purified 3-methylpentane (3-methylpentane was treated with Al_2O_3 (Aldrich) and refluxed with potassium for 3 h at 50 °C). Polymethyl methacrylate (PMMA) from Microchem Corp (Newton, MA) was used as is. Indole in PMMA was annealed under vacuum for 48 h prior to phosphorescence measurements. HPLC grade water was purchased from Fisher Scientific (Pittsburgh, PA). Milli-Q water was purified with a Milli-Q model UF/plus cartridge system producing 18.2 MΩ water (Millipore, Bedford MA).

Table 1. The Triplet-State Lifetime of Free Indole, NATA, and 1-MI in H_2O and D_2O at Various Concentrations at 20 °C

concn (μM)		lifetime (μs) H_2O	lifetime (μs) D_2O
		Indole	
2		41.5 ± 0.5	66.3 ± 1.6
10		40.7 ± 0.9	64.8 ± 2.2
50		41.1 ± 0.8	66.0 ± 2.4
	av:	41.3 ± 0.4	65.8 ± 1.1
		NATA	
2		40.5 ± 0.7	51.3 ± 1.6
10		40.2 ± 0.7	50.5 ± 1.7
50		37.5 ± 0.6	52.0 ± 1.3
	av:	39.2 ± 0.4	51.4 ± 0.9
		1-MI	
2		43.3 ± 0.8	41.2 ± 0.8
10		41.2 ± 4.0	42.1 ± 0.7
50		40.9 ± 0.8	44.0 ± 4.1
	av:	42.0 ± 0.6	41.7 ± 0.5

Results

Utilizing sensitive photon-counting techniques, the luminescence decay of 10 μM indole in deoxygenated water was measured. The luminescence decay from the cuvette with the sample reoxygenated was subtracted, and the resulting corrected curve was then analyzed using discrete component analysis[41,42] to give a lifetime of 40.7 μs at 20 °C (Table 1). In the presence of atmospheric oxygen (240 μM, in air equilibrated water), the emission is quenched to less than 4 μs (the minimum observable with our experimental apparatus) consistent with the sensitivity of the triplet state to oxygen. Without the availability of an intensified CCD, phosphorescence emission was too weak to allow for measurement of a detailed spectrum. However, using a series of interference filters we determined that the emission is restricted to between 400 and 550 nm, as expected for Trp phosphorescence.

The indole triplet-state lifetime presented here is somewhat longer than that observed by Bent and Hayon[29] (12 μs) using an absorption technique, but much shorter than more recently reported[34] (1.2 ms) under similar conditions. Furthermore, we found the phosphorescence lifetime to be independent of the concentration of indole in the 2−50 μM range (Table 1).

The addition of ultrapure TRIS buffer (40 mM, pH 8.0) in Fisher HPLC water had little effect on the indole emission lifetimes compared to values in pure Fisher HPLC water (Figure 1). However, when Milli-Q water was used before it attained the maximum resistance (<18.2 MΩ), long lifetimes were observed (as shown in Figure 2) that were extremely susceptible

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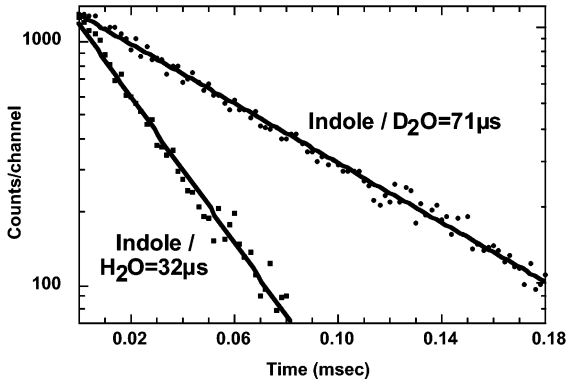

Figure 1. Phosphorescence decay of 10 μM indole in 40 mM TRIS, pH 8.0 in H_2O and D_2O at 20 °C. Similar lifetimes of 30−40 μs are observed for unbuffered solutions for well-purged (40 min) Milli-Q purified water, Fisher HPLC grade water (Lot 885083), and 60−75 μs for Aldrich D_2O (Lot 03409CU).

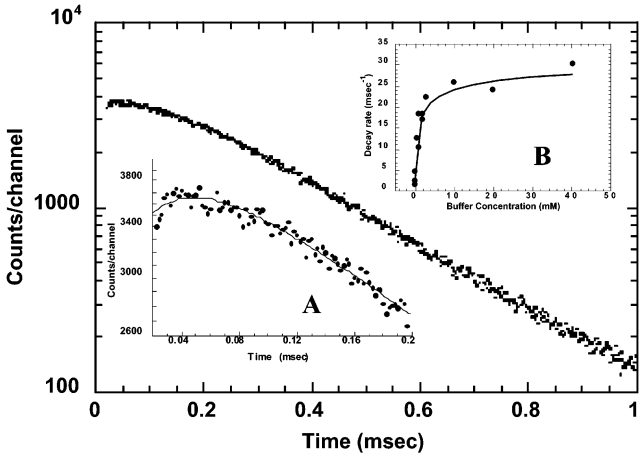

Figure 2. The long-lived emission of indole in some water sources can be observed, but with a clearly pronounced negative preexponential component. This decay was observed with incompletely purged Milli-Q purified water (15.5 MΩ conductance). Inset A: an expanded view of the first 200 μs of the decay is fit with a 2 discrete component analysis yielding a1 = −0.31, t1 = 36 μs; A2 = 1.0, t2 = 182 μs. An additional component of 620 μs is needed to fit the full decay to 1 ms. Inset B: quenching plot of long-lived component with TRIS, pH 8.0 buffer in H_2O. The long-lived lifetime and negative preexponential component in the emission decay were similarly susceptible to buffer quenching. The spectrum of the unquenched long-lived emission is given in Figure 3.

to electrolytes (inset: Figure 1) and always accompanied by a fast rise. A curve fit gives a negative preexponential associated with the fast rise and a positive decay component associated with the long lifetime (hundreds of microseconds). We also observed some dependence of the lifetime on input laser intensity, with longer lifetimes observable at higher intensities, but when excited with fluences below approximately 20 μJ/cm^2 the measured lifetime was constant. The long-lived emission in Figure 2 is comparable to that reported in ref 34, though, in that study, the displayed resolution was not adequate to observe the fast rise. In addition, as reported by the latter group, the lifetimes for this species varied substantially among samples. When using Milli-Q water that was extensively purged (>40min of 18.2 MΩ water), we found only short-lived species with no negative preexponential terms.

Interestingly, indole solutions in HPLC grade water from Fisher produced no long-lived species, while it was possible to observe long-lived components from solutions in HPLC water obtained from Aldrich. However, it was possible to add

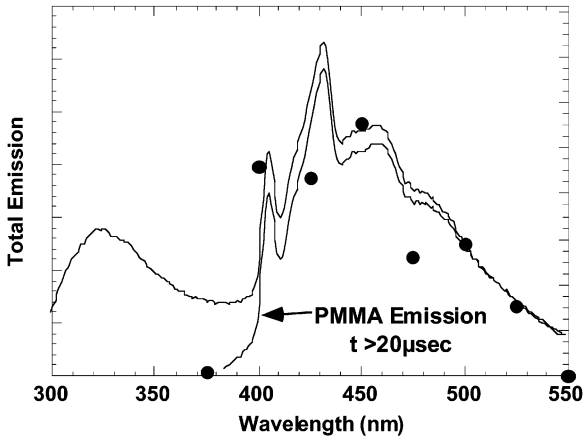

Figure 3. Emission spectra of indole in PMMA at 135 K, including time-resolved emission (20 μs to 500 ms after excitation) showing clearly resolved indole triplet emission at low temperatures. Points are emission from indole in unbuffered H_2O with long lifetime and negative preexponential curve (from Figure 2).

moderate amounts of TRIS buffer (up to 40 mM, pH 8.0) to quench simultaneously the long-lived components and the negative preexponential terms to arrive at an emission decay lifetime of 30−40 μs regardless of the source of water (inset: Figure 2). Since TRIS buffer is known not to quench the triplet state, we conclude that the quenching behavior is associated with the depletion of the indole radical cation and/or solvated electron. It is known that the indole radical cation and solvated electron remain associated following photoexcitation,[35] and this caged species has been postulated to be reactive.[44] A low-resolution emission spectrum of the long-lived species is included in Figure 3 and confirms that the emission is indeed from indole. The long-lived emission from the partially purged Milli-Q water may suggest that some indigenous impurities may indeed stabilize the indole photoproducts, while others may quench. The identity of impurities such as dissolved gases or solutes released from resins used in water purification processes has not been further characterized by this lab. It should be noted that the negative preexponential term for indole emission is independent of the counting rates showing the absence of any PMT saturation, which could complicate the analysis of the negative preexponential term.

As a further test of our system we compared the phosphorescence decay of a 2 μM sample of indole (in purged Milli-Q water) with a 1 μM sample of W220Y mutant of dimeric alkaline phosphatase (W109 concentration of 2 μM, cuvette luminescence subtracted from deoxygenated W220Y; Figure 4). The observed lifetime from this protein corresponded well to previously published values.[43,45] Furthermore, the extrapolated initial phosphorescence intensity for equal concentrations of emitters of both protein and free indole (under conditions where no negative preexponential is observed) were similar, thus showing that the 40 μs indole species has a quantum efficiency of intersystem crossing (ISC) that is roughly comparable with that of tryptophan-109 of alkaline phosphatase (the other Trp in W220Y AP is known to be quenched at the singlet level by a nearby disulfide). On the basis of total photon counts at equal excitation energies, and with adjustment for the increased

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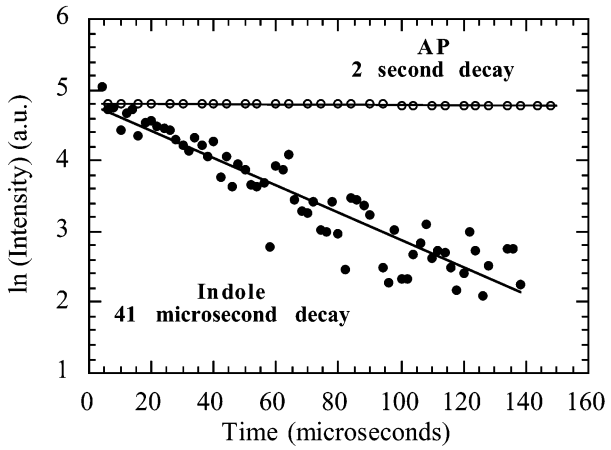

Figure 4. Phosphorescence decay of equal concentrations (2 μM) of indole and the single phosphorescent tryptophan W220Y mutant of alkaline phosphatase in H_2O at 20 °C. The contribution from background cuvette luminescence was subtracted from the phosphorescence decay of each sample. The initial phosphorescence intensities from equal concentrations of emitters in the two samples are nearly identical.

lifetime, *the long-lived emission shown in Figure 2 has an ISC quantum efficiency 2−3-fold higher than the 40 μs indole species when no negative preexponential is present.* This suggests that an additional pathway distinct from the $S_1 \rightarrow T_1$ intersystem crossing process may be implicated to populate the triplet state from the excited singlet state.

Measurements of triplet-state lifetimes of the indole derivatives NATA and 1-MI were also done in aqueous solvent; the results are similar to those observed for indole, as indicated in Table 1. The average phosphorescence lifetime of NATA at 20 °C was 39 μs, slightly longer than the value reported by Sudakar et al.[30] (30 μs). Our average lifetime of 42 μs for 1-MI is slightly longer than the 29 μs reported by Pepmiller et al.[32] The observed lifetimes were invariant in buffered H_2O from different sources, and moreover, the lifetime of 1-MI was the same in both H_2O and D_2O (Table 1). In contrast, the phosphorescence lifetime of indole and NATA in D_2O was consistently longer than the lifetime in H_2O (Table 1, Figure 1). This is in line with previous studies[46,47] and with our observation of a longer RTP lifetime for D_2O-equilibrated alkaline phosphatase.[43] It is also worth noting that, since the addition of TRIS buffer had no effect on the lifetimes in either D_2O or H_2O, the difference between the lifetimes in these two solvents is not related to their pH(D).

A primary concern in experiments described here is that short lifetimes may result from the presence of diffusional quenchers. This is easily checked, however, since the observed lifetime will then correlate with the viscosity of the solvent. The viscosity of water changes by a factor of 2.5 between 5 and 45 °C,[48] which would then cause a corresponding change in the triplet lifetime due to the change in quenching rate. The data in Table 2, however, shows that the phosphorescence lifetime remains constant throughout this change in viscosity. The addition of 8 μM acrylamide to our solutions did quench the RTP lifetime

Table 2. The Triplet-State Lifetime of 10 μM Indole in H_2O as a Function of Temperature and Viscosity

temp (°C)	lifetime (μs)	viscosity (cP)
5	41.0 ± 0.6	1.5108
10	39.8 ± 0.8	1.3039
15	41.6 ± 0.5	1.1374
20	40.7 ± 0.9	1.0019
25	41.2 ± 0.8	0.8903
45	43.9 ± 1.3	0.5972

Table 3. The Triplet-State Lifetime of 10 μM Indole and 8 μM Acrylamide in H_2O as a Function of Temperature and Viscosity[a]

temp (°C)	viscosity (cP)	lifetime (μs)	k_q (M^{-1} s^{-1})
5	1.5108	34.5 ± 1.0	(5.97 ± 0.18) × 10^8
30	0.7973	29.0 ± 1.5	(1.28 ± 0.25) × 10^9
change	47%		53%

[a] The quenching constant, k_q, was calculated from eq 4 with the nonquenched lifetime of indole taken to be 41.3 ± 0.4 μs. The change in solvent viscosity correlates with the change in k_q.

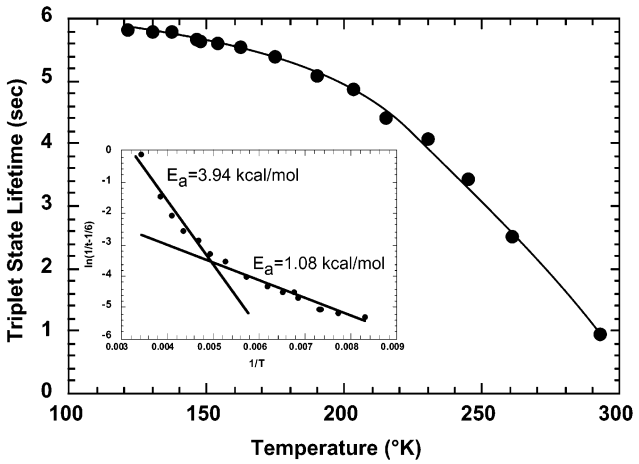

Figure 5. Phosphorescence lifetime of indole in PMMA glass as a function of temperature from 106 to 293 K. Inset: Arrhenius activation energy plot of the lifetime (assuming $k_0 = {}^1/_6$ s^{-1}), showing a break at approximately 200 K between the cold temperature limit of 1.08 kcal/mol (~400 cm^{-1}) and 3.94 kcal/mol (~1380 cm^{-1}) at higher temperatures.

of indole, and, more significantly, the extent of quenching was a function of the temperature (i.e., viscosity) of the solution (Table 3).

A more fundamental understanding of indole triplet-state deactivation processes was gained by measuring the temperature dependence of indole phosphorescence lifetime in PMMA. The solid matrix of the PMMA polymer inhibits collisional triplet-state quenching processes and, therefore, provides an opportunity to observe the activation energy for the excess energy triplet-state deactivation mechanism. The temperature dependence of the indole triplet lifetime in the solid PMMA matrix is shown in Figure 5 (inset: Arrhenius plot), while the emission spectrum of indole in PMMA is shown in Figure 3 at low temperature (135 °K) with gated emission of the indole triplet. The limiting value of the Arrhenius activation energy at low temperatures is 1.1 kcal/mol, corresponding to an approximately ~400 cm^{-1} activation barrier. This value is lower than the tryptophan activation energy of 995 cm^{-1} determined by Smith and Melhuish[49] from 80 to 320 °K in frozen keratin. The biphasic

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activation energy observed for indole in PMMA, Figure 5, has also been observed for other chromophores in PMMA matrixes[23,50] and is generally attributed to quenching of the chromophore by residual impurities within the glass that are rendered more mobile by the melting of local motions within the glassing solvent.

Discussion

RTP has been used to study various structural and dynamic features of proteins.[11−13] These studies exploited the great sensitivity of RTP to the location of the emitting tryptophan in the protein since sequestering of tryptophan residues from the protein surface is known to increase their RTP lifetime.[51,52] Indeed, as tryptophan residues which are exposed on the surface of a protein become internalized, for example during folding, their RTP lifetime increases. An accurate knowledge of the RTP lifetime of surface-exposed tryptophans is critical for the correct interpretation of tryptophan RTP in proteins, especially in cases where changes in lifetime are used to monitor folding or other dynamic processes. Since the RTP lifetime of surface-exposed tryptophans is expected to approach that of free tryptophan in solution, it is clearly of interest to determine the lifetime of the latter.

A recent report[34] regarding the phosphorescence lifetime of indole and several indole derivatives in aqueous solution provides a lifetime that is almost 40 times longer than other reported lifetimes.[29,30,33] A number of factors were cited to explain the shorter lifetime reported in earlier studies, including triplet−triplet annihilation of the excited-state indole (leading to delayed fluorescence), the generation of photoproducts that cause additional quenching, and the presence of unidentified indigenous quenchers in the water. By using high-purity water and reducing the concentration of indole in aqueous solution to alleviate triplet−triplet annihilation and photoproduct generation, these authors reported the triplet-state lifetime of indole in solution to be 1.2 ms.

The results presented in this paper confirm the long emission lifetimes reported in ref 34 but show in fact that the emission follows a fast rise on a fast time scale (fast compared to the time scale displayed in the previous report). The present data indicates that the long lifetime likely arises from the generation of complex photophysical processes in unbuffered aqueous solutions. The clear negative preexponential term in the indole emission decay between 400 and 550 nm observed under the conditions associated with long-lived emission requires a model involving a long-lived nonemitting state that leads to a species with indole triplet-state-like emission characteristics. Our hypothesis of the existence of a geminate recombination between the indole radical cation and a solvated electron to produce an indole triplet state is consistent with the lifetime of the indole radical cation,[29,32,38] the increased quantum yield for the decay (factor 2−3), and the negative preexponential component. Kirby and Steiner propose that indole $S_1 \rightarrow T_1$ intersystem crossing efficiency is approximately 0.28 while photoejection quantum yields may be as high as 0.44.[53] This would obviously provide an explanation for the enhanced quantum yield if photoejected species are available to recombine to produce an additional pathway to the indole triplet state.

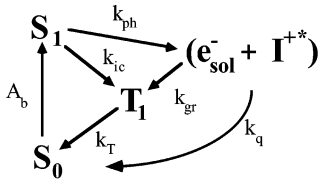

Figure 6. Schematic of dark reaction associated with long-lived (~milliseconds) triplet lifetime from indole in aqueous solution. The S_0 and S_1 refer to the ground and excited singlet states of indole, respectively, T_1 is the triplet state of indole, and e_{sol} and I^{+*} are respectively the solvated electron and indole radical cation states that are generated from the S_1 singlet state of indole.

A schematic for this dark-state process is outlined in Figure 6, with the singlet-state generation of the solvated electron and indole radical cation shown as terms within the parentheses. The $S_1 \rightarrow T_1$ intersystem crossing rate for aqueous indole is denoted by k_{ic} while the rate for generating the solvated electron/indole radical cation is shown by k_{ph}. The quenching of the indole radical cation to the ground state is shown by k_q, and the phosphorescence emission is shown by the k_T term. The geminate recombination of the solvated electron e_{sol} and indole radical cation I^{+*} occurs with the rate constant k_{gr}, which competes with the indole radical cation quenching process k_q.

The kinetics for this process are analogous to a reaction scheme previously reported;[54] therefore, with the necessary modifications, the time dependence of the solvated electron and indole radical cation is given by

$$D(t) = D_0 \exp[-\Gamma t]$$

where $D = (e_{sol} + I^{+*})$, $\Gamma = k_{gr} + k_q$, and D_0 is the initial population of $(e_{sol} + I^{+*})$. The time dependence of the aqueous indole triplet state T_1 is then given by

$$T_1(t) = C \exp[-\Gamma t] + (A_0 - C) \exp[-k_T t]$$

where $C = (D_0 k_{gr})/(k_T - \Gamma)$and A_0 is the initial population of the T_1 species. The decays associated with k_{ph} and k_{ic} occur on the nanosecond time scale while the other processes occur on the microsecond to millisecond time scale; therefore, the rates observed in this work are dependent upon k_T, k_{gr}, and k_q, with only k_T associated with the emission of photons. This scheme accounts for the existence of the negative preexponential term that is required to fit the data in Figure 2. While the authors in ref 29 did not specifically report geminate recombination of these species to yield triplet-state molecules, their use of absorbance techniques to measure the lifetimes of the photochemically generated species would not be expected to be adequately sensitive to reveal this process.

Under conditions used in our experiments, low concentrations of indole and low photon flux, second-order recombination of the indole radical cation and solvated electron would be expected to be very slow compared to the decay rates we observe if the

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photoexcitation-generated species become freely diffusing. Indeed, in our data we saw no second-order recombination kinetics as evidenced by the absence of concentration or intensity dependence. Previous studies have shown that the indole radical cation/solvated electron remain as a caged pair and then undergo competing processes of geminate recombination and dissociation into freely diffusing species.[35] Our observation of the attenuation of the long-lived luminescence with increasing ionic strength may then be attributable to ionic interaction with the caged pair facilitating charge separation and reduced geminate recombination.

It is also of interest to consider why highly pure, purged, Milli-Q water and any buffered water produced shorter lifetimes, while the less "pure" unpurged Milli-Q water gave longer lifetimes. Since geminate recombination was previously observed on the microsecond time scale,[35] our detection of long-lived (hundreds of microseconds) species may be due to impurities that stabilize the caged pair against geminate recombination. Therefore the observation of long-lived luminescence may be attributable not to a lack of triplet-state quenchers but rather to conditions that increase the likelihood of indole radical cation/solvated electron remaining as a caged pair. Efficient quenching of the indole radical cation (k_q) and/or solvated electron, or dissociation of the caged pair under low photon fluxes, would result in the observation of the intrinsic indole lifetime of k_T.

Shorter lifetimes could result from triplet−triplet (T−T) annihilation. Indeed, work by Langelaar on several aromatic compounds[55] revealed an increase in triplet−triplet annihilation with monomer concentration, indicating self-quenching. Triplet-state quenching by ground-state monomers was also reported to occur in certain cases.[56] We did not observe a concentration or temperature dependence of the RTP lifetime in Fisher HPLC water or purged Milli-Q water using concentrations ranging from 0.5 to 50 μM. This is fully consistent with the report by Bent and Hayon, who did not observe triplet-state-lifetime changes up to a concentration of 1 mM.[29] Further evidence for T−T quenching was provided by Tsai and Robinson[14] who observed the production of photoproducts in their experiments with naphthalene resulting in significantly shorter lifetimes; the flash excitation technique they employed was particularly susceptible to this problem. In order to minimize similar difficulties in our experiments (and to avoid any other intensity-related photochemistry) we utilized an excitation pulse with energies <0.1 μJ/pulse corresponding to a fluence $<10^{12}$ photons/cm^2 (recent work[34] reported using pulse energies 5×10^{15} photons/cm^2 pulse). Therefore the lack of concentration dependence of indole triplet-state lifetimes reported in our work (with low concentration of buffer) suggests conditions not conducive to any of the second-order processes described above.

Another possible source of triplet-state quenching is molecular oxygen.[58] Two distinct deoxygenation systems were employed, and both systems led to phosphorescence lifetimes for proteins such as alkaline phosphatase, apoazurin, and LADH that are as long as or longer than those previously reported in the literature.[51] Furthermore, the indole triplet-state lifetimes measured here were independent of the deoxygenation method used; both the high-purity nitrogen (oxygen-free nitrogen) and the Englander method (oxygen removal by vanadate oxidation[59]) provided identical results.

To address the issue of other indigenous quenchers in solution, we measured the RTP lifetime of indole as a function of solvent viscosity in aqueous solution. The relationship between the dynamic quenching of an excited-state molecule and solvent viscosity is reviewed in the literature.[60] Specifically,

$$k = k_0 + k_q[Q] \tag{1}$$

where k is the measured triplet decay rate, k_0 is the triplet decay rate in the absence of quenchers, [Q] is the concentration of quencher, and k_q is the intrinsic quenching constant, which is defined by

$$k_q = \zeta 4\pi RDN/1000 \tag{2}$$

where R is the gas constant, ζ is the quenching efficiency, N is Avogadro's number, and the diffusion constant D is defined by

$$D = \frac{k_B T}{6\pi\eta R} \tag{3}$$

where k_B is Boltzmann's constant and η is the solvent viscosity. More succinctly:

$$k_q = \zeta \frac{k_B TN}{1500\eta} \tag{4}$$

Therefore there is an inverse relationship between the overall quenching rate and solvent viscosity. Since the viscosity of water changes from 1.5108 to 0.5972 cP between 5 and 45 °C,[48] one would expect a change in the indole's triplet-state lifetime with temperature if a dynamic quencher were present (eq 4). Indeed, in the presence of a known diffusional quencher, acrylamide, our data (Table 3) displays the expected viscosity dependence, consistent with the above analysis. However, in the absence of an added quencher, there is no change in the lifetime over this temperature range demonstrating the absence of quenchers and allowing us to conclude that the measured lifetime is not limited by diffusional quenching. Our observation that the RTP lifetime of indole in aqueous solution is viscosity independent (over a range of 2.5-fold change in viscosity) in the absence of a diffusional quencher is also interesting in light of work previously published[34,61] that suggested a correlation between solvent viscosity and RTP lifetime for the indole chromophore. Specifically, we change the viscosity by a factor of 2.5 which, on the basis of ref 34, would lead to a 2-fold change in the lifetime, which is not observed in this work. A correlation between viscosity and RTP lifetime has been observed for several aromatic molecules,[16,56,62,63] but this effect was generally

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attributed to the viscosity-determined diffusion of dynamic quenchers in solution. Therefore the effects of viscosity in determining triplet-state lifetimes are complicated by its effect on diffusional quenching rates in addition to potentially modulating other triplet-state deactivation mechanisms.

While the identification of the factors that determine the triplet-state lifetime remains a complex issue, it is useful to speculate on triplet-state deactivation mechanisms established for simpler systems. Indeed, it is clear that the lifetime is highly shortened by some process since the observed temperature-independent lifetime determined at cryogenic temperatures is 6.0 s.[64] In considering only the temperature-dependent attenuation of the indole triplet-state lifetime as described by the excess energy theory,[24−28] and on the basis of our determined activation energy of ∼400 cm^{-1}, one would expect that the indole phosphorescence lifetime should be in the vicinity of 4 s at room temperature. However, it is worth recalling that the excess energy model is interpreted as the thermal (Arrhenius) activation of certain excited triplet-state vibrational modes of the chromophore with large spin-vibronic coupling. In a condensed phase such as a rigid glass matrix, the chromophore's vibrational modes may be expected to have very different coupling with their environment than in media undergoing a temperature-dependent phase transition. Therefore, additional attenuation of the triplet-state lifetime in low-viscosity solvents may reflect predominantly the changes in the coupling of vibrational modes of the chromophore and the thermal bath.

In considering the relevance of these speculations to the interpretation of tryptophan phosphorescence lifetimes in proteins, we may view the internal environment of a protein as a polymer matrix. Since it is known that viscosity of polymer systems is a complex function of frequency,[65−67] it is questionable whether one can describe the rigidity of the tryptophan residue by a single scalar viscosity parameter. Rather the resistance to motion depends upon the frequency and amplitude of that motion, with certain vibrational frequencies being transmitted or dampened differentially depending upon the exact nature of interactions within the polymer matrix (see ref 68). In the context of the previous discussion of the role of temperature in determining the population of triplet deactivating modes of the indole chromophore, we must also consider how those modes might be actively coupled to the thermal bath of the protein interior. The 400 cm^{-1} vibrational modes that have low thermal accessibility at room temperature may be coupled with lower frequency (and higher amplitude) modes that are thermally excited through anharmonic coupling. Indeed, a perhaps relevant example of this relationship may be indicated by a change in the temperature dependence of the phosphorescence lifetime of glutamate dehydrogenase[6] which occurs at the same temperature as a dynamical transition from harmonic to anharmonic motion as shown by dynamic neutron scattering measurements.[69,70] As there is a great deal of interest and recent work in the functional effects of anharmonic coupling of low-frequency protein modes[71−74] and in DNA dynamics,[75] development of the relationship between phosphorescence lifetimes and protein dynamics may be of great interest.

We also note our observation that the triplet-state lifetime of indole is dependent upon the hydrogen or deuterium constituent of the aqueous solvent. The increase in the RTP lifetime of both indole and NATA, but not 1-MI, upon transfer between H_2O and D_2O (Table 1, Figure 1) is consistent with the well-known deuteration effect observed with many other molecules[14,25,46,47,76] and implies that the exchangeable enamine hydrogen is responsible for the deuteration sensitivity of indole's triplet-state lifetime. This observation supports the hypothesis presented in our previous report[43] that H/D exchange of the enamine proton of tryptophan-109 in alkaline phosphatase can be monitored by RTP.

In summary, the data presented above reports triplet-state lifetimes much shorter than the one reported in ref 34 but consistent with other studies.[29−33] However, our observation of the apparent triplet-state generation through geminate recombination of the indole radical species and solvated electrons may provide an explanation for the previous reports of indole triplet-state lifetimes. Our observation of the attenuation of the long-lived species with increasing ionic strength may then be attributable to ionic interaction with the caged pair facilitating charge separation and correspondingly less efficient geminate recombination.

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