Nonexponential Fluorescence Decay of Tryptophan, Tryptophylglycine, and Glycyltryptophan

Mary C. Chang, Jacob W. Petrich, Daniel B. McDonald, and Graham R. Fleming*†

Contribution from the Department of Chemistry and James Franck Institute, The University of Chicago, Chicago, Illinois 60637. *Received May 3, 1982*

Abstract: The photophysics and spectroscopy of tryptophylglycine, tryptophylalanine, glycyltryptophan, alanyltryptophan, glycyltryptophylglycine, and tryptophan itself have been investigated by using steady-state and subnanosecond spectroscopy. For tryptophan and the peptides where the tryptophyl residue is N-terminal we demonstrate the involvement of the state of protonation on both the excited-state dynamics and the absorption and emission spectra. We show that the deprotonated amino group gives rise to red-shifted absorption and emission spectra and to a longer fluorescence decay time compared with the protonated form. pK_a values over a range of temperatures were determined for tryptophan (23 °C, 9.50), tryptophylglycine (23 °C, 7.84), and tryptophylalanine (23 °C, 7.79). The temperature dependence of the amplitude of the long-decay component in tryptophylglycine is attributable to the temperature dependence of the ground-state pK_a. Examination of the Arrhenius plots for tryptophan, tryptophylglycine, and glycyltryptophan clarifies the role of the protonated amino group in fluorescence quenching. We suggest that the role of the protonated amino group in the fluorescence quenching of the N-terminal tryptophyl compounds is not proton transfer to the indole ring but an enhancement of charge transfer from the indole ring to the adjacent carbonyl group.

Introduction

It is well-known that the fluorescence decay of tryptophan has great potential for use as a probe of the environments and motions of proteins and smaller peptides, and in fact, the fluorescence of the tryptophyl residue has been widely exploited in this regard.[1-3] For example, nonexponential decay of protein fluorescence has been attributed to fluorescence from either tryptophyl residues in different environments or to one tryptophyl residue and multiple conformations of the molecule.[3-6] Recently, however, it has been found that the nonexponential decay of isolated tryptophan in aqueous solution may be fit well to sums of two and three exponentially decaying components.[7] Thus, the intrinsic nonexponentiality of tryptophan makes the protein fluorescence more difficult to interpret.

In tryptophan, the nonexponential decay arises from two sources: one that is independent of pH from 4 to 8 and one that is strongly dependent on pH for pH >8. In this paper we devote special attention to this pH-dependent nonexponentiality. Such a study may prove useful in understanding such hormones as mellitin and glucagon which have been shown to form aggregates at a given pH.[8] In these cases we may ask whether the nonexponentiality[9,10] is due to a particular quaternary or tertiary structure induced by the pH or the effect of pH on the indole moiety.

In order to understand this pH-dependent nonexponentiality in detail, we have undertaken an investigation of the absorption and emission properties of di- and tripeptides containing tryptophan as well as tryptophan itself as a function of pH. The specific questions we have addressed are:

(1) Using steady-state and time-resolved emission data, Szabo and Rayner[11] have observed spectral shifts associated with the pH-independent nonexponential decay of tryptophan. For tryptophan, it is known that the weight of the third lifetime component increases with pH.[7,12,13] This component has been associated with anionic as opposed to zwitterionic tryptophan. Is there a spectral shift of the anionic species with respect to the zwitterionic species?

(2) What relationships exist among the observed spectra, the components of the time-resolved fluorescence emission, and the ionic forms? More specifically, can we generate a steady-state emission spectrum from the fluorescence decay parameters?

(3) Is there a large pK_a change for the N-terminal amino group of these compounds upon excitation? For example, given the fact that at pH 7 tryptophan is completely zwitterionic and has a double-exponential fluorescence decay, a large change in pK_a upon excitation could give rise to the anionic form and hence a third lifetime component in the decay law.

(4) Finally, and most importantly, what nonradiative processes give rise to pH-dependent and -independent nonexponentiality? Are there different pathways of nonradiative decay present in Trp-Gly and in Gly-Trp, or do these two molecules share a common nonradiative pathway that in certain cases is accentuated under conditions of low pH?

Experimental Section

Tryptophan, tryptophylalanine (Trp-Ala), tryptophylglycine (Trp-Gly), alanyltryptophan (Ala-Trp), glycyltryptophan (Gly-Trp), and glycyltryptophylglycine (Gly-Trp-Gly) were obtained from the Sigma Chemical Co. Their purity was checked by HPLC (Waters Associates 6000A). Aqueous samples of various pHs were prepared by adding solid sample or a concentrated stock solution to buffers prepared either from commercially available (Hydrion from Carolina Biological Supply) solid buffer material or from KH_2PO_4. The concentrations of the buffers were kept at or below 0.05 M to prevent quenching from occurring. For pH values between the integer intervals, mixtures of the adjacent pH buffers were prepd. by using a Beckman 3500 digital pH meter. Buffers were used without preservative but were frequently checked for pH stability and mold. Fluorescence from the buffers was not a problem.

Absorption measurements were made on a Cary dual-beam spectrophotometer equipped with temperature-controlled sample chamber. The pK_a values for N-terminal tryptophyl residues were determined by a method similar to the spectrophotometric titration of Hermans et al.[14] Samples of optical density about 1.0 at the 279-nm absorption maximum were prepared by weighing the amounts of stock solution and buffer added to cuvettes of known relative path length. This, combined with the relative densities of the buffer solutions, allowed corrections for the differences in concentration and path lengths. Steady-state fluorescence

† Alfred P. Sloan Foundation Fellow.

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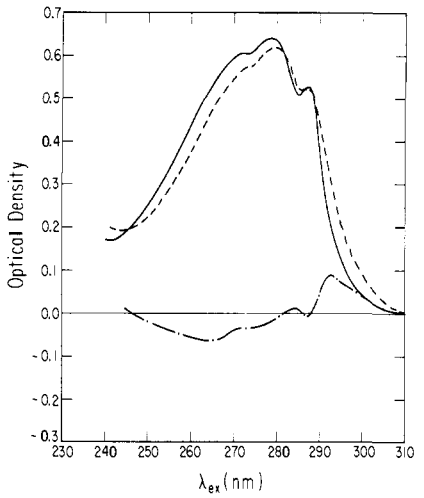

Figure 1. Absorption spectra of equal concentrations of tryptophylglycine at pH 5.0 (—) and pH 10.0 (- - -) and difference spectrum (- · -) with lower pH sample as the reference (T = 20 °C).

spectra were recorded on a Perkin-Elmer MPF4 corrected fluorimeter.

Fluorescence decay profiles were recorded on a subnanosecond time-correlated single-photon counting apparatus similar to one described elsewhere.[13] Fluorescence was collected through a polarizer oriented 54.7° relative to the vertically polarized excitation; this eliminated possible distortion due to the reorientation of the emitting dipoles.[15] Resonant laser scatter was eliminated with a cutoff filter, $\lambda_{em} \geq 320$ nm, and 10-nm bandpass interference filters were used to resolve the emission. Except in cases where the sample concentration was fixed by some other constraint, the samples were adjusted to an optical density of approximately 0.3 at the exciting wavelength. Instrument response functions of about 340-ps fwhm were recorded by collecting resonant scatter from nondairy creamer in water. The fluorescence decays were fit to single-, double-, or triple-exponential functions by the method of iterative convolution. The quality of fit was judged by the χ^2 criterion and by visual inspection for systematic deviations in the weighted residuals.

Results

(I) Determination of the pK_a Values of Tryptophyl Ammonium. Figure 1 shows the absorption spectra of Trp-Gly at pH 5.0 and 10.0 and the difference spectrum obtained with the lower pH sample as the reference. A similar spectral shift was observed with Trp-Ala and tryptophan, but no shift was observed with Gly-Trp, Ala-Trp, or Gly-Trp-Gly. The difference in peak absorbance of the two samples is reproducible. This difference in the absorption spectra of zwitterionic and anionic forms of tryptophan has been detected by Scheraga[14] but was unnoticed by Jameson and Weber.[16] To our knowledge, this spectral shift has not been observed in Trp-Gly, and in no case has the shift been correlated with the long- or short-lifetime components of the fluorescence decay. Since the zwitterion and the anion possess different absorption spectra, we would expect to see different preexponential factors for the short- and the long-lifetime components of the fluorescence decay as a function of excitation wavelength. Furthermore, we should be able to predict the value of these preexponential factors. This indeed is the case (see Table I and the discussion below). This is in contrast to the results of Szabo and Rayner,[11] who have demonstrated that the two decay components of tryptophan at intermediate pH have different emission spectra but identical absorption spectra since the weights of the two components are independent of excitation wavelength.

This spectral shift we have observed has also been verified by comparing the absorbance of two halves of a pH 7.9 sample titrated with equal volumes of acid and base to points well removed from the estimated pK_a. This procedure served as an alternate method for ensuring that the concentrations of chromophore in both samples were equal. Repeated measurement gave statistically reliable values for the relative absorptions $A(\gg pK_a)$ and $A(\ll pK_a)$ at the extreme pHs of the acid–base equilibrium.

Table I. Excitation Wavelength Dependence of Preexponential Factors for Tryptophylglycine

pH	λ_{ex}	a_1/a_2	$f(\lambda)$	$A_{1,calcd}$	$A_{1,obsd}$
7.5	290	0.86	1.92	0.71	0.67
	295	0.62	1.38	0.63	0.61
	300	0.45	1.00	0.56	0.55
	305	0.33	0.74	0.48	0.44
7.8	290	0.86	0.96	0.55	0.51
	295	0.62	0.69	0.47	0.43
	300	0.45	0.50	0.39	0.38
	305	0.33	0.37	0.32	0.30

Table II. pK_a and ΔH Values for the Tryptophyl Amino Group

compd	pK_a[a] 4 °C	23 °C	42 °C	52 °C	ΔH, kcal/mol
tryptophan	10.01	9.50	9.03	8.80	10.4 ± 0.1
Trp-Gly	8.31	7.86	7.56	7.27	8.49 ± 0.6
Trp-Ala	8.18	7.79	7.35	7.13	8.93 ± 0.4

[a] The error in pK_a is ±0.03.

Absorption spectra at intermediate pHs can be used to determine the pK_a with the relation

$$pK_a = pH - \log \frac{A(\gg pK_a) - A(pH)}{A(pH) - A(\ll pK_a)} = pH - \log \frac{f_2}{f_1} \quad (1)$$

where f_1 and f_2 are the fractions of protonated and unprotonated species. Because of the relatively small differences in the spectra of the high- and low-pH species, small errors in the concentrations or pathlengths of the intermediate pH samples can lead to large errors in the directly measured value of f_2/f_1. Most of this error can be eliminated by using measurements made at two different wavelengths corresponding to the peak negative and positive portions of the difference spectrum. Measured values of f_2/f_1 should be independent of excitation wavelength; hence

$$\frac{A_{293}(\gg pK_a) - xA_{293}(pH)}{xA_{293}(pH) - A_{293}(\ll pK_a)} = \frac{f_2}{f_1} = \frac{A_{264}(\gg pK_a) - xA_{264}(pH)}{xA_{264}(pH) - A_{264}(\ll pK_a)} \quad (2)$$

where x is the correction factor for the intermediate pH sample concentration and pathlength. Solving eq 2 for f_2/f_1 yields

$$\frac{f_2}{f_1} = \frac{A_{293}(pH)A_{264}(\gg pK_a) - A_{264}(pH)A_{293}(\gg pK_a)}{A_{264}(pH)A_{293}(\ll pK_a) - A_{293}(pH)A_{264}(\ll pK_a)} \quad (3)$$

Note that (3) is independent of inaccuracies in concentration.

Table II contains the measured pK_a values at various temperatures for the compounds studied here. The change in pK_a vs. the reciprocal of temperature for tryptophan implies a ΔH for protonation of −10.4 kcal/mol, in good agreement with Scheraga's value of −10.5 kcal/mol.[14] Similar agreement between our value at 23 °C for the pK_a of tryptophan (9.50) and Scheraga's value at 25 °C (9.41) attests to the consistency of the method. Note also that the correlation between pK_a shift and change of ΔH values at 23 °C is consistent with the predicted 1.35 kcal/mol change in ΔH per pH unit shift in pK_a, assuming that the ΔS of protonation is invariant over the compounds studied.

(II) pH Effect on Steady-State Emission. The steady-state emission spectra of Trp-Gly, Trp-Ala, and Trp show pH dependences that are traceable to a protonation equilibrium. For example, high pH samples exhibit a red shift in emission that is larger than the observed red shift in absorption (~900 vs. ~300 cm^{-1}). Using absorption data, emission data, or averages of the two to compute the excited state pK_a* for these compounds by means of the Förster cycle,[17,18] we obtain an excited state pK_a* smaller than the ground state pK_a by about 1.5 pH units.

Since, however, the determination of pK_a* is dependent upon knowledge of the position of the 0–0 transition, which can be very sensitive to solvation of the ground and excited states, a 4-nm error (at 300 nm) in its estimation can lead to an error of 1 unit in the

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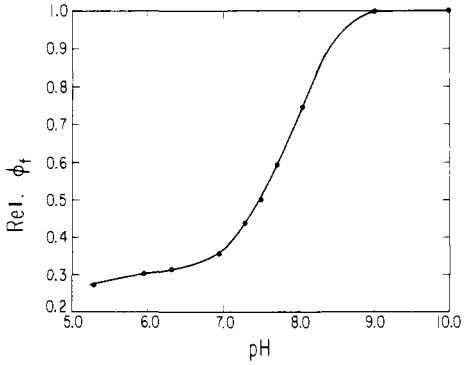

Figure 2. Peak fluorescence intensity of tryptophylglycine (λ_{em} = 353 nm) vs. pH (T = 20.4 °C, λ_{ex} = 279). All samples were matched to have the same absorbance at 279 nm.

Table III. Fluorescence Decay Data for Glycyltryptophan and Tryptophylglycine at 20 °C as a Function of pH[a]

compd	pH	A_1	τ_1	A_2	τ_2	A_3	τ_3	$A_{3,calcd}$
Gly-Trp	5.6	0.43	0.36	0.57	1.23			
Trp-Gly		0.14	0.64	0.87	1.84			
Gly-Trp	7.0	0.42	0.33	0.58	1.39			
Trp-Gly		0.16	0.80	0.71	1.92	0.13	7.49	0.11
Gly-Trp	7.5	0.49	0.54	0.51	1.55			
Trp-Gly		0.13	0.64	0.60	1.78	0.26	7.43	0.30
Gly-Trp	8.1	0.48	0.54	0.52	1.69			
Trp-Gly		0.06	0.66	0.38	1.84	0.56	7.94	0.56
Gly-Trp	10.0	0.37	0.86	0.63	2.10			
Trp-Gly				0.04	1.51	0.96	7.73	0.99

[a] A_1, τ, etc. are defined in eq 5. All decays were fit to a sum of three exponential components. In those reported as double exponential two decay times were identical in the best fit.

calculated pK_a*.[17] It is thus possible that pK_a may be equal to pK_a*. We must, however, consider the time-resolved emission in light of the steady-state emission before we can make any definite conclusions in this regard.

The emission spectra exhibit a sigmoidal dependence on pH near the pK_a of the tryptophyl compound as is clearly demonstrated by plotting the relative quantum yield vs. pH (Figure 2), a phenomenon that was first observed for tryptophan and some of its analogues by White.[19] Most of this effect is due to the difference in the fluorescence lifetime (and thus quantum yield) of the protonated and deprotonated species. This will be discussed in more detail in the following sections. In obtaining the data for Figure 2, care was taken to match sample concentrations so that the absorbances at the excitation wavelength were the same for all samples.

(III) Time-Resolved Emission. The dependence of the fluorescence decay on pH is markedly different for the N-terminal tryptophyl compounds and the C-terminal tryptophyl compounds. The decays of Trp-Gly, Trp-Ala, and Trp are qualitatively the same as are those of Gly-Trp, Ala-Trp, and Gly-Trp-Gly. In the ensuing discussion we will consider Trp-Gly and Gly-Trp as being representative of their classes. Table III shows the results of fits to triple-exponential functions

$$F(t) = A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2} + A_3e^{-t/\tau_3} \quad (4)$$

to the fluorescence decays.

The Gly-Trp results are nearly pH independent with the nonexponential decay fitting well to a sum of two exponentials of nearly equal weight. In such cases the χ^2 values are almost always less than 1.1 and fits to a triple-exponential functional form converge such that two of the lifetimes are identical. The shortest lifetime component has a lifetime of about 0.4 ns, while the longer component lifetime is about 1.5 ns. As the pH approaches 8.0, the estimated pK_a of Gly-Trp,[20] the second component lifetime becomes slightly longer, but the relative weights do not change significantly. Similar behavior has been observed by Beddard et al. for Ala-Trp.[10]

At pH values well below its pK_a Trp-Gly exhibits a biexponential character similar to Gly-Trp, but with a lower weight of the shorter lifetime component. In the Trp-Gly class of compounds a third component appears as the pH increases toward the ground-state pK_a of the respective compound. At high pH this third component, with a lifetime approximately the same as 3-methylindole in aqueous solution, predominates. The fraction of the long-lived component has nearly the same pH dependence as the relative quantum efficiency does. At higher pH the fits seem to fuse the two shorter components into one. We believe that the longer time scale needed to fit the long component lessens the short-time resolution. It should also be noted that only a small portion of the total fluorescence emission is from the shortest lifetime species (for example, at pH 8.0 in Trp-Gly only about 1%).

The lifetimes of the three components remain nearly constant throughout the pH range of these experiments, except in marginal cases where the fitting procedure may introduce artifacts. This invariance supports the idea that the components correspond to three nearly independent species, possibly interconverting but at a rate slower than the fluorescence decay rates. The increase in the weight of the longest component in *N*-tryptophyl compounds as pH is increased implies that the long-lived component could arise from the presence of the unprotonated amine.

Table IV. Spectral Data for Tryptophylglycine at pH 8.5[a]

λ_{obsd}	$\gamma(\lambda)/\beta(\lambda)$	$X(\lambda)$	$b(\lambda)$	$c(\lambda)$
334	0.49	0.79	0.81	0.64
350	0.39	1	0.99	0.99
360	0.36	1.09	0.87	0.95
370	0.31	1.27	0.67	0.85
380	0.27	1.45	0.48	0.70
390	0.27	1.45	0.34	0.49
400	0.25	1.56	0.23	0.36
420	0.30	1.30	0.09	0.12

[a] The excitation wavelength is 295 nm.

Table IV contains data for the emission wavelength dependence of Trp-Gly at pH 8.5 and excitation wavelength of 295 nm. If we make the approximation that the long- and short-lifetime components are due solely to the emissions from the long- and short-wavelength emitting species, respectively, the fluorescence emission has the following functional form:

$$F(t,\lambda) = \beta(\lambda)e^{-t/\tau_S} + \gamma(\lambda)e^{-t/\tau_L} \quad (5)$$

where $\beta(\lambda) = b(\lambda)B$ and $\gamma(\lambda) = c(\lambda)C$. Here $b(\lambda)$ and $c(\lambda)$ are the steady-state emission spectra of the two species normalized to the same maximum value, and B and C are proportionality constants. If the radiative rates of the two species are equal (we will see that this is only approximately the case here), B and C are equal to the initially prepared populations of the short- and long-lifetime species. Independent of this assumption, however, is the following:

$$c(\lambda) = X(\lambda)b(\lambda) \quad (6)$$

where

$$X(\lambda) = \frac{\beta(\lambda_0)}{\gamma(\lambda_0)}\frac{\gamma(\lambda)}{\beta(\lambda)}$$

From steady-state emission spectra at pH 5.0 and 11.0, where we can be fairly sure that the emitting species is either protonated or unprotonated, $b(\lambda_0) = c(\lambda_0)$ at approximately λ_0 = 353 nm. Figure 3 shows the steady-state fluorescence emission spectra observed at pH 5.0 and 11.0 as well as the emission spectrum generated from eq 6 and the time-resolved emission at pH 8.5. The agreement is excellent.

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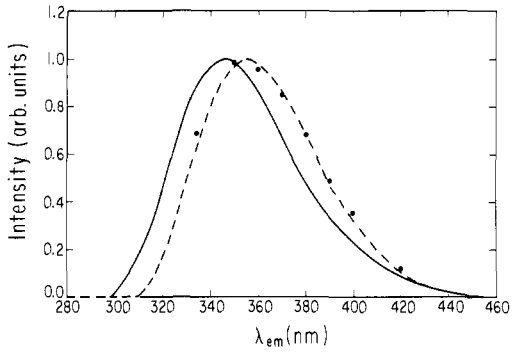

Figure 3. High-pH fluorescence spectrum of tryptophylglycine (●) generated from the ratio of time-resolved preexponential factors and steady-state fluorescence spectra observed at pH 5.0 (—) and pH 11.0 (---).

Although the spectra in Figure 3 are given relative to some arbitrary emission intensity, it is possible to give them the correct relative intensities. The long-component lifetime in Trp-Gly is about 4.4 times the weighted average of the short components. Thus, one would expect the quantum yields of the high and the low pH spectra to be in a similar ratio. Instead, however, the observed ratio is 3.5 (see Figure 2). The discrepancy could be due to a difference in the radiative rates for the protonated and unprotonated species. The radiative rate for the protonated one would have to be ~26% larger (ignoring the small red shift in the emission of the unprotonated species).

In an earlier investigation Ricci concluded that the radiative rate for a variety of tryptophan and indole analogues (none of which included the dipeptides in our study) was the same: $4.5 \pm 0.5 \times 10^7$ s^{-1}.[21] Our observation that the protonation of the amino group causes an increase in the radiative rate implies that similar changes in the radiative rate might occur upon interaction of the indole moiety with various side chains. Such possible changes in radiative rate should be taken into account when calculating populations of the different rotameric forms of tryptophan and its derivatives.[11,22-24]

The excitation-wavelength dependence of the short- and long-component weights can be predicted with a knowledge of the absorption spectra, the pK_a (or equivalently the absorption spectrum of the intermediate pH fluorescence sample), and the detected emission wavelength. Table I contains a_1/a_2, the relative absorption cross sections of the protonated and unprotonated species, with the relative populations f_1/f_2 determined by the equilibrium. The relative populations of protonated and unprotonated excited states is given by

$$f(\lambda) = \frac{a_1(\lambda)f_1(\mathrm{pH})}{a_2(\lambda)f_2(\mathrm{pH})} \quad (7)$$

The predicted value for the short-component preexponential factor is given by

$$A_{1,\mathrm{calcd}}(\lambda) = \frac{1.26f(\lambda)}{1 + 1.26f(\lambda)} \quad (8)$$

where the factor of 1.26 corrects for the difference in radiative rates between the two species. (Note that at 353 nm the emission strength of the two species is the same except for this factor.) The observed values of $A_{1,\mathrm{obsd}}$ agree fairly well with the calculated values considering the many possible sources of error. (The last column of Table III contains the predicted values of A_3 calculated in this manner. Again, agreement is good.) The fact that the preexponential factors can be predicted so well from the ground-state pK_a data further suggests the equality of pK_a and pK_a*—or a very slow proton transfer in the excited state.

Table V, Temperature Dependence of the Fluorescence Decay of Tryptophylglycine[a]

pH	T, °C	A_1	τ_1	A_2	τ_2	A_3	τ_3	$A_{3,\mathrm{calcd}}$
5.0	5	0.26	0.96	0.74	2.78			6×10^{-4}
	20	0.27	0.93	0.73	2.00			1.5×10^{-3}
	50	0.39	0.52	0.61	0.96			5.9×10^{-3}
7.0	5	0.25	0.96	0.70	2.71	0.05	9.51	0.06
	20	0.16	0.80	0.71	1.92	0.13	7.49	0.14
	50[b]			0.64	0.76	0.36	3.15	0.38
8.5	5	0.17	0.79	0.29	3.26	0.54	9.93	0.69
	20	0.07	0.92	0.23	2.28	0.70	8.10	0.83
	50[b]			0.14	0.95	0.86	3.37	0.95

[a] All decays were fit to sums of three exponentials (λ_{ex} = 295 nm, λ_{em} = 350 nm). In those reported as double exponential two decay times were identical in the best fit. [b] At high temperature the shortest lifetime component may be too short to be resolved.

Table VI. Summary of Data from Arrhenius Plots[a]

compd	E_a, kcal/mol	A, s^{-1}
indole[39]	12.5	1.2×10^{17}
3-methylindole[13]	12.6 ± 0.3	$1.1 \pm 0.6 \times 10^{17}$
indole-3-acetate[39]	12.8	1.7×10^{17}
indole-3-propanol[27]	12.8	
Trp (pH 11, τ_3)[13]	12.3 ± 0.4	$6.1 \pm 3.6 \times 10^{16}$
Trp-Gly (pH 10.9, τ_3)	11.5 ± 0.3	$1.3 \pm 0.7 \times 10^{16}$
Trp (pH 7, τ_1)	6.6 ± 0.7	$1.2 \pm 1.3 \times 10^{14}$
Trp (pH 7, τ_2)	6.7 ± 0.2	$2.2 \pm 0.6 \times 10^{13}$
Trp-Gly (pH 4.5, τ_1)	5.5 ± 0.5	$2.2 \pm 1.9 \times 10^{13}$
Trp-Gly (pH 4.5, τ_2)	5.4 ± 0.1	$4.6 \pm 0.9 \times 10^{12}$
Gly-Trp (pH 5.0, τ_1)	6.5 ± 0.2	$2.2 \pm 0.8 \times 10^{14}$
Gly-Trp (pH 5.0, τ_2)	5.5 ± 0.1	$9.2 \pm 2.2 \times 10^{12}$

[a] The activation energies and frequency factors correspond to the nonradiative rates associated with the various lifetime components which decrease in the order $\tau_3 > \tau_2 > \tau_1$.

The quantitative interpretation of the time-resolved fluorescence data may be complicated by the fact that the near-UV absorption band of tryptophan probably consists of two overlapping transitions. Valeur and Weber[25] have resolved the fluorescence excitation spectrum of indole and tryptophan in propylene glycol at −58 °C into L_a and L_b bands. Unfortunately no similar information is available in water, but judging from their data, only the L_a state is likely to fluoresce significantly, and even if there are small shifts of the relative energies our interpretation will be little affected. If the change in radiative rate between protonated and unprotonated species proposed above is accepted, it may arise from small shifts in relative energy of the L_a and L_b states and a consequent change in vibronic coupling between the two states.

(IV) Temperature Dependence of the Fluorescence Decays. Table V presents a summary of the temperature dependence of the fluorescence decay of Trp-Gly. An increase in temperature produces a striking change in the weights of the various components. The general increase of the weight of the long-lifetime component is easily attributable to the temperature dependence of the ground-state pK_a (Table II). Arrhenius plots of the nonradiative rate for all three components of the tryptophan and Trp-Gly fluorescence are shown in Figure 4, a and b, respectively. The data are collected in Table VI. We have calculated the nonradiative rate following Robbins et al.,[13] but we have used a radiative rate of 6.3×10^7 s^{-1} for all lifetime components except those that are due to an unprotonated N-terminal amino group, where we have used a rate of 5×10^7 s^{-1} (vide supra). The Arrhenius plots obtained from the two shorter lifetime components of Trp and Trp-Gly and from both lifetime components of Gly-Trp yield approximately the same activation energies (~6 kcal/mol) and frequency factors (~10^{13} s^{-1}). On the other hand, the activation energies obtained from the long-lifetime components (high-pH species) of Trp, Trp-Gly, and from 3-methylindole yield higher activation energies (~12.5 kcal/mol) and very large fre-

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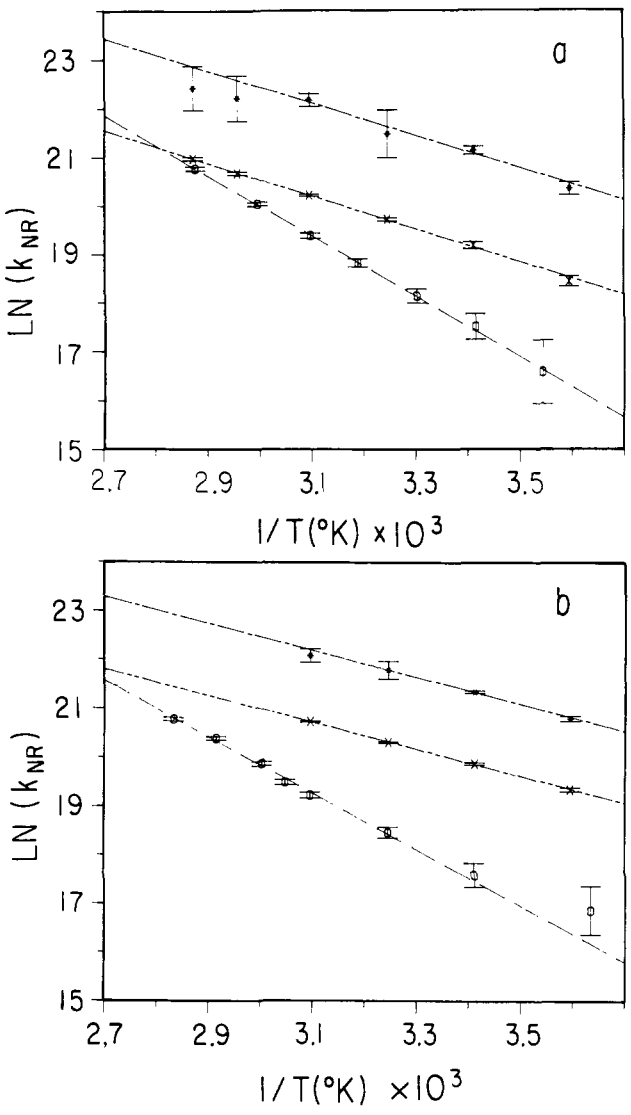

Figure 4. Arrhenius plots obtained from the lifetime components of Trp and Trp-Gly (λ_{ex} = 295 nm, $\lambda_{em} \geq$ 320 nm). The lifetime components decrease in the order $\tau_3 > \tau_2 > \tau_1$. (a) Trp (– –) τ_3,[13] (– – –) τ_2, (– ·) τ_1; (b) Trp-Gly (– –) τ_3, (– – –) τ_2, (– ·) τ_1.

quency factors ($\sim 10^{17}$ s^{-1}). Such large frequency factors are suggestive of a process involving electronic rather than nuclear motion. These activation energies and frequency factors are discussed in greater detail in our companion paper.[26]

Discussion

(I) pH-Dependent Nonexponential Decay. There are two distinct sources of nonexponential fluorescence decay in tryptophan and small N-terminal tryptophyl peptides. In this work we have unambiguously identified one of these sources with the state of protonation of the N-terminal amino group by generating the steady-state emission spectrum of Trp-Gly at pH 11 from the fluorescence decay parameters and the steady-state emission spectrum of Trp-Gly at pH 8.5. We have also observed that the absorption spectrum of anionic Trp-Gly is red-shifted from that of zwitterionic Trp-Gly. Such a spectral shift has not been detected, to our knowledge, in any of the N-terminal tryptophyl peptides.

We have demonstrated that zwitterionic Trp-Gly has a larger radiative rate than anionic Trp-Gly, and we note as do Szabo and Rayner[11] that such a difference in radiative rate must be considered when using the conformer model to predict excited-state populations from the preexponential factors of the fluorescence decay.

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Furthermore, through Förster cycle calculations and accurate predictions of the preexponential factors in our fluorescence decays, we have shown that either there is not a significant pK_a change of the amino group of the N-terminal tryptophyl compounds upon excitation or if there is a large change in pK_a in the excited state, proton transfer is very slow. Such a result is important because it means that we need not consider an excited-state pK_a change as another complication in our analysis of the pH-dependent nonexponentiality of the N-terminal tryptophyl compounds. The increased magnitude of the "tryptophan fluorescence lifetime puzzle" referred to by Gudgin et al.[7] in their initial report of triple-exponential decay is thus restored to the problem of interpreting the double-exponential decays observed for Trp and Trp-Gly at pH values where the amino group is protonated and in, for example, Gly-Trp or NATE at all pH values.[11,27]

(II) Trp-Gly and Gly-Trp: Nonradiative Pathways. Time-resolved absorption measurements are necessary in order to determine the pathways of nonradiative deactivation for a given state. Such measurements indicate that all simple indole-containing species exhibit intersystem crossing and photoionization as nonradiative pathways.[28-31] These two processes alone are not able to explain the short lifetimes and low fluorescence quantum yields observed, for example, in tryptophan at pH 7. Many workers have thus considered the possibility of proton transfer from the protonated amino group[13,21,32,33] to the indole ring and charge transfer from the indole ring to an acceptor[34-37] as other modes of nonradiative decay.

The work of Bent and Hayon[28] has indicated that for Trp and Trp-Gly at pHs where the protonated amino group is present a transient species, T_1, is observed. T_1 is not present when the amino group is unprotonated. It is tempting to associate the intermediate pH nonexponentiality of Trp and Trp-Gly with the appearance of T_1 and to associate T_1 with the much discussed intramolecular proton-transfer mechanism.[13]

Two pieces of evidence, however, strongly call into question a quenching process based upon intramolecular proton transfer. First, Ware and co-workers have found that deuterated tryptophan and undeuterated tryptophan give rise to double-exponential decay with the same lifetime components (1.6 and 7.6 ns) and the same weights (12.2% and 87.8%) when placed in aprotic solvent such as Me_2–SO.[38] Such a result implies that the longer tryptophan lifetimes observed in D_2O[21,39] are not due to intramolecular proton transfer but to a decrease in the rate of another nonradiative pathway. The observation of a sizable solvent deuterium effect is not without precedent in cases where charge-transfer states are implicated. The (arylamino)naphthalenesulfonates (ANS derivatives) are believed to fluoresce from charge-transfer states in polar solvents,[40-43] and their fluorescence lifetimes are significantly enhanced in D_2O vs. H_2O[44] despite the absence of exchangeable

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protons. We believe that in the case of tryptophan D_2O is reducing the rate of charge transfer from the indole moiety to the side chain.

Second, the activation energies obtained from the low-pH lifetime components of Trp and Trp-Gly are, within experimental error, the same as those obtained from the lifetime components of Gly-Trp, which does not produce the T_1 transient of Bent and Hayon.[28]

If the same primary nonradiative process is occurring in Gly-Trp and in Trp and Trp-Gly (which is implied by the Arrhenius parameters in Table VI), then T_1 must not be a primary product of the nonradiative decay. It could arise, for example, by a rapid proton transfer after the initial charge-transfer process and thus be only observable in those systems with labile protons close to the indole ring.

(III) Self-Consistent Tryptophyl Photophysics. If charge transfer is the nonradiative process involved in Trp, Trp-Gly, and Gly-Trp, then one must be able to invoke it when dealing with the following questions:

(1) Why does Gly-Trp have a lower quantum yield than Trp-Gly if the quenching mechanism in both cases is charge transfer from indole to peptide bond?

(2) Why does zwitterionic Gly-Trp have a lower quantum yield than anionic Gly-Trp? Are the nonradiative processes the same in the two species and one merely faster in the zwitterion, or is a new nonradiative process introduced in the zwitterion?

(3) What is the role of the protonated amino group in the fluorescence quenching of tryptophan?

(4) How does this charge-transfer interaction give rise to nonexponential decay?

Werner and Forster[36] have provided an answer to the first question through their examination of space-filling models. In Gly-Trp, the peptide bond which is the charge acceptor is able to make much better contact with the indole ring than in Trp-Gly, and thus charge transfer is enhanced. One might expect that such an orientational effect would decrease the observed activation energy. This is not necessarily so as Hopfield[45] has proposed models for electron transfer in which the orientation of the donor with respect to the acceptor affects the rate only through the frequency factor and not necessarily through the activation energy.

Ricci and Nesta[35] have argued that a given carbonyl group is able to accept electrons to the extent that there is an adjacent group capable of delocalizing the electron density in the carbonyl group. Thus we may argue, as do Werner and Forster,[36] that zwitterionic Gly-Trp has a lower quantum yield than anionic Gly-Trp because the protonated amino group is able to reduce the electron density in the peptide bond, making it a better charge acceptor. Such reasoning can be used to explain the fluorescence quantum yields of the following zwitterions studied by Weinryb and Steiner:[33] Gly-Trp < Gly-Gly-Trp < Gly-Gly-Gly-Trp. That is, the farther away the protonated amino group is from the peptide bond adjacent to the Trp the less able it is to delocalize the electron density in the peptide bond. In this way, the peptide bond becomes a less efficient quencher.

The protonated amino group plays a similar role in tryptophan. The COO^- group may not be an efficient electron acceptor[26] unless an adjacent group such as NH_3^+ is present to decrease its electron density. Thus, at low pHs we observe the lower quantum yield in tryptophan and the associated nonexponential decay.

Finally, we must point out that while this charge-transfer mechanism seems to be quite adequate in explaining the quantum yields of these compounds, it does not *in itself* explain why they exhibit nonexponential fluorescence decay. A current explanation of the behavior has been provided by Szabo and Rayner's[11] application of Wahl and co-workers'[22,23] rotamer model. In this model, during the excited-state lifetime of the molecule there exist conformations around the C^α–C^β bond that do not interconvert. The different lifetimes of the rotamers arise from the different distances of the acceptor group from the indole ring in the charge-transfer interaction.

Although the rotamer model in tandem with the charge-transfer quenching mechanism seems to be adequate in explaining the fluorescence properties of the di- and tripeptides, it has not been given an exhaustive test by being applied to a wide range of tryptophan analogues that exhibit nonexponential decay and some of which do not. Such a test is the subject of our companion paper.[26]

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Registry No. Trp-Ala, 24046-71-7; Gly-Trp, 2390-74-1; Trp-Gly, 7360-09-0; Ala-Trp, 16305-75-2; Gly-Trp-Gly, 23067-32-5; Trp, 73-22-3.

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On the Origin of Nonexponential Fluorescence Decay in Tryptophan and Its Derivatives

J. W. Petrich, M. C. Chang, D. B. McDonald, and G. R. Fleming*†

Contribution from the Department of Chemistry and James Franck Institute, The University of Chicago, Chicago, Illinois 60637. *Received October 21, 1982*

Abstract: The nonexponential fluorescence decay of tryptophan and its derivatives is discussed in terms of a simple model based on conformers about the C^α–C^β bond and the relative rates of charge transfer from indole to various electrophiles. Accurate predictions concerning the relative fluorescence lifetimes and the form of the fluorescence decay law are made for tryptophan and 17 of its derivatives, including three new derivatives synthesized specifically to test the model.

Introduction

In our previous paper,[1] we discussed the quenching processes in the N- and C-terminal tryptophyl compounds and suggested that in both classes of compounds it is charge transfer that competes with fluorescence. As we noted, however, while such a proposal can explain the relative quantum yields of the N- and C-terminal tryptophyl compounds and the relative fluorescence quantum yields of anionic and zwitterionic N-terminal tryptophyl

† Alfred P. Sloan Foundation Fellow.

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