Fluorescence Studies with Tryptophan Analogues: Excited State Interactions Involving the Side Chain Amino Group

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Received: September 23, 1994; In Final Form: January 26, 1995⊗

The fluorescence of a large set of tryptophan analogues, including several that are conformationally constrained, was studied. The constrained analogues include tetrahydrocarboline-3-carboxylic acid and 3-amino-3-carboxytetrahydrocarbazole. Steady state and time-resolved fluorescence measurements were made as a function of pH. The fluorescence quantum yields of the constrained analogues are higher than those for the unconstrained counterparts. The emission intensity of the constrained analogues, as well as 4-methyltryptophan, decreases with deprotonation of the side chain α-ammonium group; this is in contrast to the increase in fluorescence of tryptophan with deprotonation of this group. These results are consistent with the existence of excited state proton transfer to carbon 4 of the indole ring as a quenching mechanism, which is sterically prohibited in the constrained analogues and 4-methyltryptophan. From quantum yield and lifetime data (most decays are nonexponential), the effective rate constant for nonradiative depopulation of the excited state was calculated. For tryptophan analogues having two side chain functional groups, there is a synergistic effect; the presence of two side chain groups causes more quenching than expected from the sum of the individual contributions. For analogues having an α-ammonium group, this synergism appears to be correlated with an induced change in the pK_a of this group. Deprotonation of this α-ammonium group also causes a red shift in the emission of these compounds; this appears to be due to electrostatic repulsion between the α-NH_3^+ group and the excited indole dipole.

Introduction

The fluorescence of indole and tryptophan has been extensively studied because of the importance of this fluorophore as an intrinsic reporter group in proteins.[1−3] Indole has two nearly degenerate and orthogonal electronic oscillators, ^1L_a and ^1L_b, which contribute to its ultraviolet absorption and emission spectra.[4−7] The ^1L_b transition is relatively structured and solvent independent and appears to be the lowest energy singlet state (S_1) in apolar media. The ^1L_a transition is broad and featureless, has a higher dipole moment, interacts more strongly with solvent molecules, and appears to be S_1 (or a precursor of S_1) in polar media.[8−11]

Much effort has gone to studying the factors that determine the fluorescence decay kinetics of indole and tryptophan and to studying the interaction (i.e., quenching) of the excited indole ring with functional groups typical of the protein milieu (e.g., amino, carboxylate, amide, sulfhydryl).[12,13] The fluorescence decay of indole and 3-methylindole is monoexponential, but the decay of tryptophan, in neutral aqueous solution, is biexponential with decay times of $\tau_1 \approx 0.5$ ns (maximum at 335 nm) and $\tau_2 \approx 3$ ns (maximum at 350 nm).[1,14−16] Since equilibration between the ^1L_a and ^1L_b oscillators occurs within about 2 ps,[17] such interconversions cannot account for the 0.5 ns component. Szabo and Rayner[14] proposed that slowly interconverting rotamers about the α−β bond, having different distances between the carboxylate and amino groups and the indole ring, can explain the biexponential decay. Since there is an increase in the fluorescence of tryptophan upon proton dissociation of the α-ammonium group,[13−15] the protonated ammonium group must act as an intramolecular quencher. A rotamer having this ammonium group in the g^- orientation with respect to the indole ring (see Figure 1) is proposed to be quenched, accounting for the 0.5 ns component.[14] Fleming and co-workers[15,17] elaborated on this rotamer model and argued that charge transfer from the excited indole to electrophilic side chain groups is the most important quenching process. They also proposed that rotamers about the β−γ bond may be more important as a basis for the biexponential fluorescence decay of tryptophan analogues. Studies of tryptophan in jet expansions have provided evidence for the rotamer model by showing different fluorescence lifetimes for different conformers of the cooled gas phase molecules.[18]

Our approach in this work is to test the rotamer model and other ideas concerning intramolecular quenching by studying a set of tryptophan analogues, including some that are conformationally constrained[19] and that lack either the side chain amino or carboxylic groups (see Figure 2 for structures and abbreviations). The decarboxylic acid series (tryptamine, β-THPI, γ-THPI, and 3-amino-THC) enables us to study the nature of intramolecular quenching by a side chain amino group. We will show that, while such quenching occurs in the flexible tryptamine, in the constrained analogues the interaction is different. Likewise, the deamino series (IPA and 3-carboxy-THC) enable us to study intramolecular quenching by the carboxylic acid group.[23] Other Trp analogues studied are 2-methyl-, 4-methyl-, and α-*N*-methyltryptophan. The first two were studied to test the mechanism of proton transfer quenching from the α-amino group to the C2 or C4 position on the indole ring. The presence of the methyl groups at these positions should block such a mechanism. We will present steady state and time-resolved fluorescence data for over 30 tryptophan

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1995.

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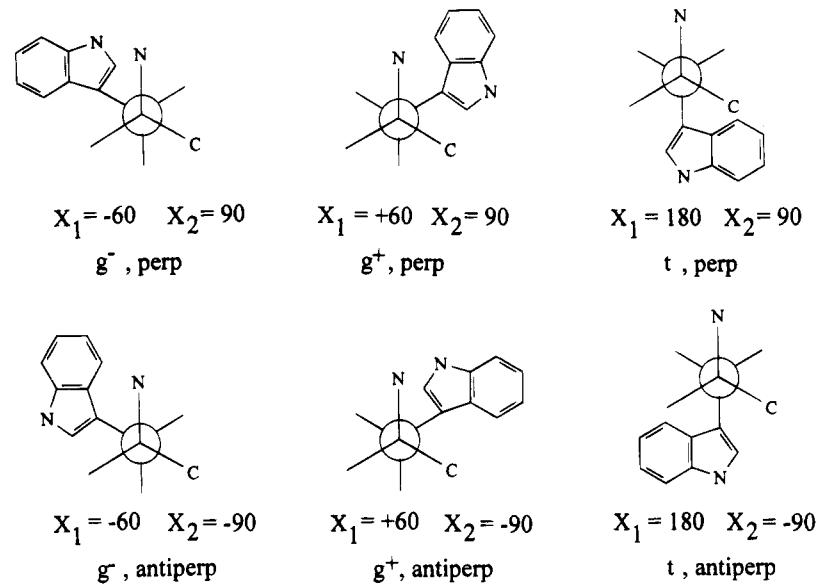

Figure 1. Newman projections of the rotamers of L-tryptophan, viewed down the α−β bond. N refers to α-NH_3^+; C to α-CO_2^-.

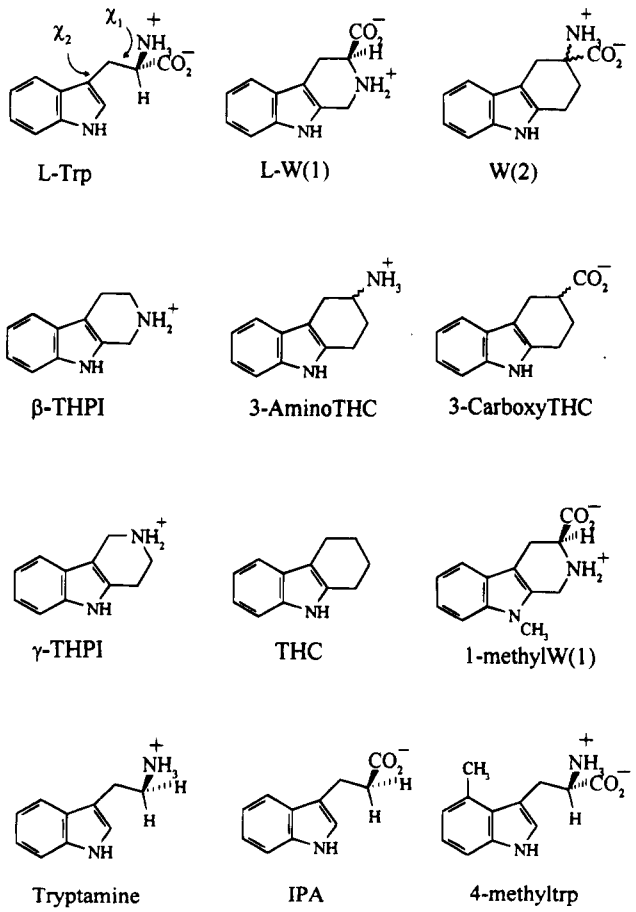

Figure 2. Structures and abbreviations for several of the tryptophan analogues in this study. 4-Methyltryptophan is shown to indicate the numbering system for the various methylindoles and methyltryptophans.

analogues. The intent is to present data on new Trp analogues and to test specific notions concerning the intramolecular quenching reactions. Some further understanding is achieved regarding the fluorescence of tryptophan, but a number of unresolved issues will remain.

Experimental Section

Materials. L-Tryptophan (99+%, recrystallized from water), D-tryptophan (99+%), tryptamine (98%), 1-methyl-D,L-tryptophan, α-*N*-methyl-L-tryptophan (99+%), indole (purified by sublimation), 4-methyl-D,L-tryptophan, α-methyl-D,L-tryptophan, and 5-methyl-D,L-tryptophan were obtained from Sigma Chemical Co. (St. Louis, MO). 2,3,4,9-Tetrahydro-1*H*-β-carboline-(β-THPI, 98%, purified by sublimation), 4-chloropyridine·HCl (97%), 4,4-ethylenedioxycyclohexanone, phenylhydrazine, *o*-phenylenediamine (98%), and the various methylindoles were obtained from Aldrich Chemical Co. (Milwaukee, WI). Pentanetricarboxylic acid (97%) was purchased from American Tokyo Kasei Inc. (Portland, OR).

Synthesis of L- *or* D-*Constrained Tryptophan (L-W(1) or D-W(1)), L-2,3,4,9-Tetrahydro-1H-β-carboline-3-carboxylic acid.* L-W(1) was prepared following the procedure of Harvey *et al*[24] with a yield of 83%; it was twice recrystallized from water, giving needles with a constant mp of 284−285 °C. Its ^{1}H NMR spectrum was consistent with that reported by Tilstra *et al.*[20] L-W(1) showed mass spectrum (*m/z*) 216 (M^+), 172, 171, 169, 168, 144, 143 (calculated: *m/z* 216 M^+), had $[\alpha]_D^{20} = -133°$, and a molar extinction coefficient of $\epsilon_{280} = 6030$ in methanol. TLC on silica gel, developed with ethanol/acetic acid/water (4:1:1), showed a single spot, distinct from L-tryptophan. HPLC on a C18 reverse phase column using an isocratic solvent (methanol/acetic acid/water, 59:1:40) gave a single peak (separate from tryptophan). In some fluorescence lifetime measurements, recrystallized L-W(1) was passed through this HPLC column, collected, dried with N_2, and redissolved in the buffer of interest. Samples thus prepared gave similar results as directly prepared samples. The same procedure was used to prepare D-W(1) and 1-methyl-W(1), starting with D−tryptophan and 1-methyl-D,L-tryptophan, respectively. D-W(1) has a mp of 284−286 °C and $[\alpha]_D^{20} = +135°$, and 1-methyl-W(1) has a mp of 260 °C (dec).

Synthesis of γ-THPI, 2,3,4,9-Tetrahydro-1H-γ-carboline. 4-Chloropyridine was first liberated from the commercially available HCl salt by dissolving 6 g in 5 mL of water (in an ice/salt bath), adding with stirring 6.96 mL of triethylamine (vacuum distilled), and then extracting the solution with 20 mL of ether. After evaporation of the ether, 4-chloropyridine was purified by vacuum distillation (22 mmHg, bp 52−55 °C).

Following the procedure of Robinson and Thornley,[25] a mixture of 3.34 g of 4-chloropyridine and 3.18 g of *o*-phenylenediamine was refluxed at 98 °C under vacuum for 4 h, yielding 3.78 g (69%) of the intermediate *N*-γ-pyridyl-*o*-phenylenediamine. The latter (1.88 g) was dissolved in 10%

HCl (70 mL). To this was slowly added (below 5 °C) a solution of $NaNO_2$ (1.24 g in 30 mL of water), until KI-starch paper turned black and a green precipitate formed. After a few hours this solid was collected, recrystallized from water, and air dried. This intermediate, 1-γ-pyridylbenzotriazole, was obtained in 58% yield and was stored in a vacuum over H_2SO_4. The triazole (1.24 g) was then dissolved in syrupy H_3PO_4 (12.5 mL) and rapidly heated with agitation over a flame to form γ-carboline. The completion of this reaction was judged by cessation of the evolution of gas. The resulting solution had a dull cherry red color; it was diluted with water and adjusted to pH 10 with 5 M NaOH. This basic solution was extracted three times with 25 mL portions of ether. After removal of the ether, the free base γ-carboline was dissolved into water by addition of dilute HCl. Subsequent addition of base gave the product as a cream-colored precipitate, which was recrystallized from water, yielding 0.49 g (46%) of γ-carboline. In the final step, 0.84 g of γ-carboline, dissolved in 44 mL of *n*-butanol, was reduced by adding 4.3 g of sodium in small chunks. After removal of the *n*-butanol with steam under vacuum, the crystalline precipitate was washed with water and was recrystallized from ethanol. The light tan solid was further recrystallized from water and sublimed to give γ-THPI as a white solid (0.2 g, 23% yield), with mp 217−218 °C (dec); *m/z* 172 (M^+), 144, 143, 115; *m/z* calculated, 172. TLC on silica gel, with ethanol/acetic acid (99:1), gave a single spot, as did reversed phase HPLC, using a H_2O to methanol/triethylamine (98:2) gradient.

Synthesis of 3-Carboxytetrahydrocarbazole (3-Carboxy-THC). Following the method of Perkin,[26] pentane-α,γ,ε-tricarboxylic acid (41 g) and sodium carbonate (33 g) were dissolved in water and then evaporated to dryness. The salt was powdered and heated with acetic anhydride at 165 °C for 4 h, with the evolution of CO_2. The cooled product (7 g) was dissolved in water, mixed with semicarbazide-HCl (5 g) and sodium acetate (5 g), and heated to boiling for a few minutes. Overnight the sparingly soluble semicarbazone precipitated. This was collected, washed with cold water, dissolved in hot 0.1 M HCl, and extracted into ether. After evaporation of the ether, a pale yellow oil was obtained. This oil, which is γ-ketohexahydrobenzoic acid, solidified. To form the title compound, the keto-acid (1 g) was dissolved in a minimum amount of water, to which was added phenylhydrazine (2 g) dissolved in dilute aetic acid. A pale yellow oil formed. This oil was dissolved in warm, concentrated HCl. Upon heating to boiling, and pouring into cold water, a tan solid appeared, which is crude 3-carboxytetrahydrocarbazole. It was dried and dissolved in benzene. The benzene was evaporated to yield a light tan solid, which was further washed with benzene, to give mp = 190 °C, *m/z* = 215 M^+.

Synthesis of 3-Aminotetrahydrocarbazole(3-Amino-THC). This compound was prepared by the reductive amination of tetrahydrocarbazol-3-one with $LiBH_3CN$ and ammonium acetate in absolute ethanol following the procedure of Borch *et al.*[27] The product was sublimed directly before being studied.

Synthesis of D,L-*Constrained Tryptophan(W(2)), 3-Amino-3'-carboxytetrahydrocarbazole.* This compound was synthesized according to Britten and Lockwood[28] and was rcrystallized from water acetic acid (50:50) to give white crystals with mp 320 °C.

*Synthesis of 2-Methyl-*D,L*-tryptophan.* The procedure of Snyder and MacDonald[29] was followed to synthesize *N*-acetyl-2-methyltryptophan. The product was washed with water and recrystallized. It was then hydrolyzed by refluxing over $Ba(OH)_2$ for 30 h to yield the title compound, which was then recrystallized from ethanol water to give mp 235 °C.

Methods. *Steady State Fluorescence Measurements.* Emission spectra were measured with a Perkin-Elmer MPF 44A fluorometer. An excitation wavelength of 280 or 295 nm was used, and temperature was maintained at 25 °C. Fluoroscence quantum yields, Φ, were determined relative to $\Phi_{Trp} = 0.14$ for L-tryptophan in water at neutral pH, using the relationship $\Phi/\Phi_{Trp} = (S/S_{Trp})(A_{Trp}/A)$, where S is the integrated fluorescence intensity of the compound and A is its absorbance at the excitation wavelength.

The pH dependence of the emission of compounds was measured with the above instrument. The fluorophores were dissolved (to an $A_{280} \approx 0.2$) in a 0.1 M NaCl, 0.001 M Na_2HPO_4 solution. This was titrated, directly in a 1 × 1 cm cuvette, with 0.1 M NaOH or 0.1 M HCl. The pH was measured with a London Radiometer pH meter. Correction was made for dilution of the sample. The pH dependence of fluorescence intensities was fitted with the following function using nonlinear least squares.

$$F = \frac{F_{NH_3^+} + F_{NH_2}K_{NH_3^+}/[H_3O^+]}{1 + K_{NH_3^+}/[H_3O^+]} \frac{1}{1 + K_H[H_3O^+] + K_{OH}[OH^-]} \quad (1)$$

In this equation $K_{NH_3^+}$ is the acid dissociation constant for the ammonium group of the tryptophan analogues and $F_{NH_3^+}$ and F_{NH_2} are the fluorescence intensities for species having this group protonated and unprotonated. Quenching by hydronium ions a low pH and hydroxide ions at high pH is also included as the last factor in this equation. (This equation uses a Stern−Volmer quenching constant to describe H_3O^+ and OH^- quenching; alternatively, alkali quenching can be described by a function having a second acid dissociating group, the deprotonation of which causes full quenching.) For the deamino compounds indolepropionic acid (IPA) and 3-carboxy-THC, the $K_{NH_3^+}$ in the above equation becomes the acid dissociation constant of the carboxylic acid group (i.e., K_{CO_2H}).

Time-Resolved Fluorescence Studies. The fluorescence decay of the tryptophan analogues was measured by multifrequency phase fluorometry. The instrument uses a 10−200 MHz Pockels cell modulation system from ISS Instruments (Urbana, IL). Most measurements were made with a 300 W xenon lamp and a 290 nm interference filter as the excitation source. More recent measurements were made with a Coherent 200 argon ion laser, which was excitation lines at 300−305 nm. Emission was collected through a Corning 7/60 filter. The temperature of the samples was 20 °C. Decay data were collected using either a glycogen scattering solution or *p*-terphenyl (τ = 1.0 ns) in ethanol as reference. Intensity decays were described as a sum of exponentials:

$$F(t) = F^0 \sum \alpha_i e^{-t/\tau_i} \quad (2)$$

where α_i is the amplitude associated with decay time, τ_i. This decay law was related to raw phase and modulation data via a nonlinear least squares procedure, as described elsewhere.[31]

Results

Fluorescence Quantum Yields. In Table 1 are listed the fluorescence quantum yields of the indoles and tryptophan analogues in this study. Table 1 is divided into six groups: indole and its alkyl derivatives; tryptophans; constrained tryptophan analogues; tryptamine and constrained amines; carboxylic acids; and other analogues. The quantum yields were

TABLE 1: Fluorescence Parameters for Indoles, Tryptophans, and Constrained Tryptophan Analogues[a]

	τ (ns)	Φ	k_r ($\times 10^{-7}$ s^{-1})	k_{nr} ($\times 10^{-7}$ s^{-1})	λ_{max} (nm)
		Indoles			
indole	4.8	0.318	7.07	13.8	346
1-methylindole[b]	8.7	0.33	3.8	7.7	
2-methylindole	2.1	0.166	7.9	4.0	357
3-methylindole	9.4	0.343	4.0	6.6	374
4-methylindole	0.38	0.042	11.0	253	333
5-methylindole	2.9	0.192	6.5	28	347
6-methylindole	1.6	0.107	6.7	56	350
7-methylindole	2.5	0.06	2.4	37.6	339
2,3-dimethylindole	4.1	0.12	2.9	21.5	384
tetrahydrocarbazole	3.4	0.17	5.0	24.5	382
		Tryptophans			
tryptophan	⟨2.6⟩	0.14	5.3	33.2	355
	8.2	0.32	3.9	8.3	364
1-methyltrptophan	⟨4.1⟩	0.24	5.8	18.6	362
	14.4	0.51	3.5	3.4	370
2-methyltryptophan	⟨2.7⟩	0.13	4.7	32.4	368
	4.3	0.20	4.7	18.6	375
4-methyltryptophan	1.2	0.10	8.6	75	341
	1.1	0.065	6.1	85	348
5-methyltryptophan	⟨2.2⟩	0.07	3.2	42.3	357
	⟨4.4⟩	0.11	2.5	21.1	366
α-methyltryptophan	⟨1.5⟩	0.125	8.7	60.8	351
	⟨4.6⟩	0.23	5.0	16.9	361
α-*N*-methyltryptophan	⟨1.5⟩	0.10	6.7	60	353
	⟨4.3⟩	0.18	4.2	19.1	363
		Constrained Analogues			
L-W(1)	⟨6.3⟩	0.49	7.8	8.1	349
	⟨4.8⟩	0.33	6.9	14	368
W(2)	⟨5.4⟩	0.33	6.1	12.5	366
	⟨4.2⟩	0.22	5.2	18.7	373
W(1)EE	⟨3.4⟩	0.34	10.1	19.5	349
1-methyl-W(1)	⟨8.8⟩	0.45	5.1	6.3	356
	⟨11.1⟩	0.40	3.6	5.4	378
		Amines			
tryptamine	5.5	0.30	5.4	12.8	356
	6.2[g]	0.33[g]	5.3	10.8	363
β-THPI	6.3	0.42	6.6	9.3	351
	5.3	0.28	5.5	13.4	367
γ-THPI	5.5	0.41	7.5	10.7	348
	4.6	0.33	7.3	14.5	357
3-amino-THC	⟨4.6⟩	0.24	5.2	16.6	367
	3.8	0.21	5.5	20.8	373
		Carboxylic Acids			
IPA	9.0	0.33	3.7	7.4	368
	4.9[c]	0.19	3.9	16.6	364
3-carboxy-THC	⟨4.5⟩	0.29	6.4	15.8	378
	4.0[c]	0.33	8.2	16.8	374
		Other Analogues			
NATA	2.9	0.14	4.9	29.6	362
acetyltryptophan	⟨4.3⟩	0.20	4.7	18.6	363
acetyltryptamine	5.1	0.22	4.3	15.3	
indolepropionamide	8.7[d]	0.39[d]	4.5	7.0	
tryptophanamide	⟨1.4⟩	0.085	6.0	64.9	348
	5.9	0.25	4.2	12.7	361
indole propionate ethyl ester	3.4[d]	0.15[d]	4.5	25.3	
tryptophan methyl ester	⟨0.50⟩	0.031	6.2	194	348
	⟨3.5⟩	0.13	3.7	25.0	361.5
tryptophan ethyl ester	⟨0.51⟩[e]	0.029	5.7	190	
acetyltryptophan methyl ester	⟨1.24⟩	0.055	4.4	76.2	
acetyltryptophan ethyl ester	⟨1.29⟩[e]	0.066	5.1	72.4	
Trp-Gly	⟨1.38⟩[f]	0.09[f]	4.7	65.9	
	⟨5.6⟩[f]	0.32[f]	4.0	12.2	

[a] All values for 20 °C, aqueous solution. For the tryptophans and constrained analogues, the upper data set is for the zwitterion form (pH ~ 5.0) and the lower data set is for the anionic form ($-NH_2$ form) at pH 9.5 or 10.5. Likewise, for the amines the upper data set for each entry is for the ammonium NH_3^+ form and the lower data set is for the neutral amine NH_2 form. The angle brackets (⟨ ⟩) indicate that the decay is biexponential; the value given is $\Sigma\tau_i\alpha_i$. [b] Taken from Meech *et al.* (1983). [c] The lower set of data is for the protonated, neutral carboxylic acid form at pH 3.0. The upper data set is for the anionic, carboxylate form at pH 7.0. [d] Taken from Szabo and Rayner (1980). [e] Taken from Petrich *et al.* (1983). [f] Taken from Chen *et al.* (1991). [g] Data for tryptamine measured at pH 10.5. This is approximately the pK_a of tryptamine, so the data are for a mixture of protonated and unprotonated states. On going to higher pH, an additional quenching reaction occurs (see Figure 3B) which makes impossible determination of fluorescence data for the fully unprotonated state of tryptamine.

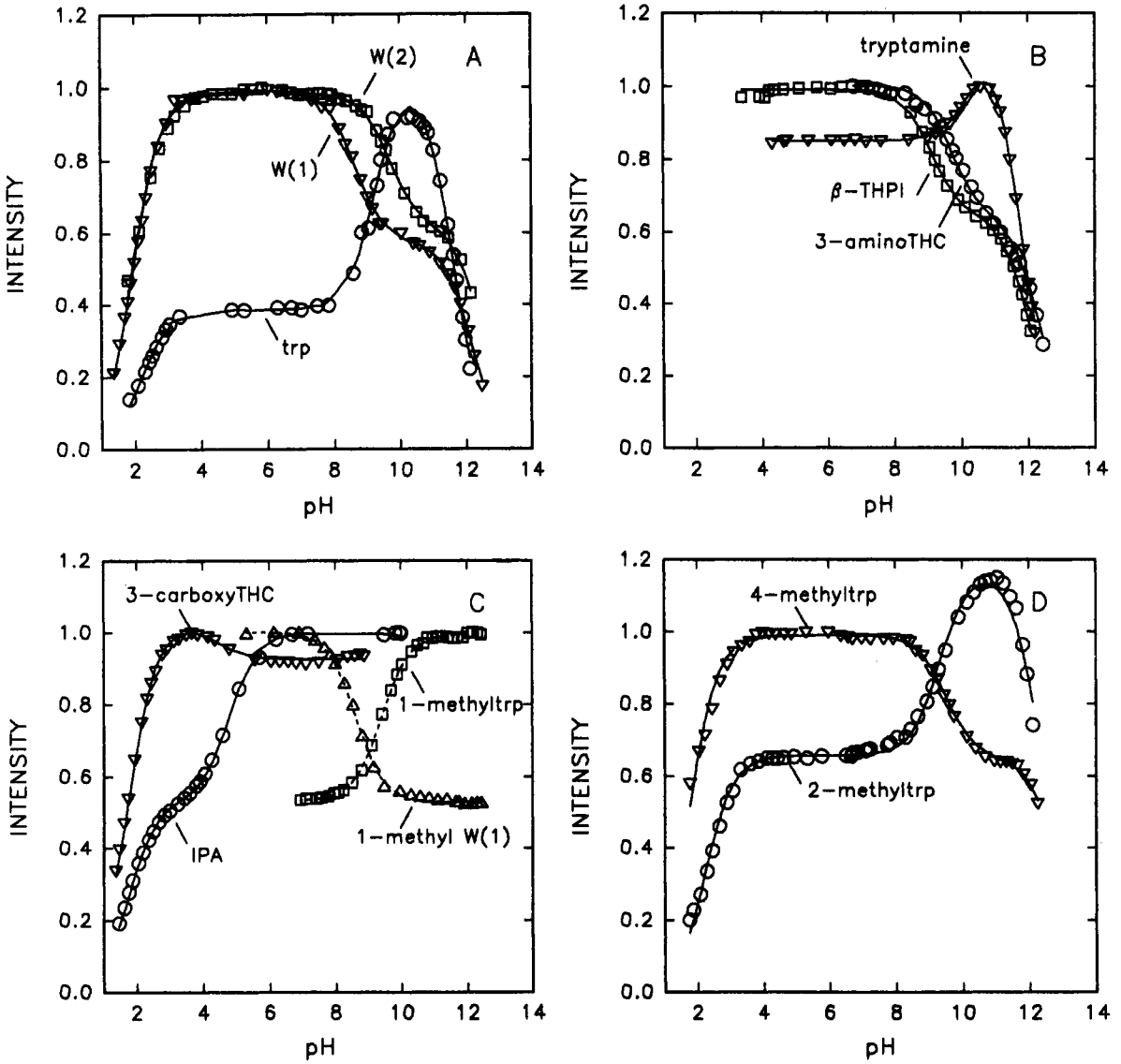

Figure 3. pH dependence of the steady state fluorescence intensity of various tryptophan analogues: (A) L-tryptophan (○), L-W(1) (▽), and W(2) (□); (B) 3-amino-THC (○), tryptamine (▽), and β-THPI (□); (C) indole propionic acid (○), 3-carboxy-THC (▽), 1-methyl-W(1) (□), and 1-methyltryptophan (△); (D) 2-methyltryptophan (○) and 4-methyltryptophan (▽). Excitation is at 280 nm; emission is observed at the λ_{max} for the compound. No correction was made for changes in absorbance and the λ_{max} during the titration. Data were collected at 20 °C, 0.1 M NaCl. The lines are fits of eq 1 with pK_a values in Table 2.

determined at pH 5.0, where the amino groups will be protonated and the carboxylate groups will be unprotonated, and at pH 9.5 or 10.5, where the amino groups will be unprotonated (pH 7.0 was used for indolepropionic acid and 3-carboxy-THC). Also listed are the emission λ_{max}, the mean lifetime, $\langle\tau\rangle(\equiv\sum\alpha_i\tau_i)$, the effective radiative rate constant, k_r, and the nonradiative rate constant, k_{nr}.

The constrained Trp analogues, W(1) and W(2), have quantum yields that are much larger than that for tryptophan itself (at pH 5). The quantum yields of the amine analogues (tryptamine, βTHPI, etc.) and the carboxy analogues (IPA, 3-carboxy-THC) are all higher than that for the zwitterion of Trp. Also note that, whereas the quantum yield of Trp increases on going to pH 10.5, the quantum yield of W(1), W(2), β-THPI, γ-THPI, 3-amino-THC, and 4-methyltryptophan decreases on going from pH 5 to pH 10.5.

pH Dependence of Fluorescence. Shown in Figure 3 are the pH dependencies of the steady state fluorescence of various molecules. The solid lines are fits of eq 1 to the data; the fitted acid dissociation constants are given in Table 2.

Trytophan (Figure 3A) shows the familiar pattern, in which the fluorescence intensity increases over 2-fold upon deprotonation of its α-amino group. Also, tryptophan (and all the molecules, including indole, methylindoles, and THC) shows quenching at low and high pH. Acid and alkaline quenching may involve diffusional quenching by H_3O^+ and OH^- ions or may be at least partially due to protonation of the α-carboxylic acid group (which is a better quencher than the carboxylate group) and deprotonation of the indole imino NH group (for which the excited state pK_a* is ~12).[32] The equation used to fit the data assumes that complete quenching occurs at low and high pH, regardless of the mechanism. Note that 1-methyltryptophan and 1-methyl-W(1), which lack an imino NH group, do not show quenching at high pH (see Figure 3C). Such a lack of quenching in the range of pH 11−12 has previously been observed for *N*-methylindole.[32a]

The fluorescence of W(1) drops between pH 8 and 10 with deprotonation of its amino group.[33] This is opposite to the pattern seen for tryptophan, where the protonated amino group is a quencher. Also, constrained W(2), 1-methyl-W(1) (Figure 3C), β-THPI (Figure 3B), γ-THPI (not shown), 3-amino-THC (Figure 3B), and 4-methyltryptophan (Figure 3D) show a pattern opposite to that for tryptophan. The fluorescence intensity of IPA and 3-carboxy-THC (Figure 3C) is constant from pH 10

TABLE 2: Apparent pK_a Values[49] for the Side Chain Amino Group of Tryptophan Analogues in the Ground and Excited (*) States[a]

compound	pK_a (fluorescence)	pK_a (absorbance)	ΔpK^*_a
tryptophan	9.30	9.32	1.5
1-methyltryptophan	9.40	9.25	1.3
2-methyltryptophan	9.43	9.54	1.1
4-methyltryptophan	9.50	9.35	1.2
5-methyltryptophan	ND	9.35	1.5
α-methyltryptophan	ND	9.63	1.7
α-*N*-methyltryptophan	ND	9.59	1.7
L-W(1)	8.65	8.78	3.1
W(2)	9.7	9.44	1.1
1-methyl-W(1)	8.61	8.53	3.5
tryptamine	10.35	10.18	1.1
β-THPI	9.14	9.27	2.6
γ-THPI	ND	9.74	1.5
3-amino-THC	9.86	9.83	0.9
tryptophanamide	7.7	7.60	2.2
tryptophan methyl ester	7.2	7.43	2.3
tryptophan ethyl ester	ND	7.46	ND
Trp-Gly	ND	7.53[b]	ND
IPA	4.76[c]	ND	−0.6
3-carboxy-THC	4.42[c]	ND	−0.6

[a] All values at 20 °C in 0.1 M NaCl or buffer solution. pK_a values obtained from nonlinear least squares fits of eq 1 to steady state fluorescence or absorbance data. The excited state ΔpK^*_a is calculated from the emission λ_{max} shift using eq 3. [b] Taken from Chen *et al.* (1991). [c] Represent pK_a values of carboxylic acid group.

down to 6 (since they have no amino groups). Below pH 6 the protonation of the carboxylate group of IPA leads to a partial quenching, whereas for 3-carboxy-THC, protonation of the carboxylate group results in a small increase in intensity. The solid lines in Figure 3C are a fit of eq 1, in which the pK_a refers to the carboxylate groups.

The pK_a values given in Table 2 are apparent values. Since there is a shift in the emission spectrum of most of these compounds as the functional group is titrated (and there is a small shift in the absorbance spectrum as well), these pK_a values are not directly attributable to either the ground state or the excited singlet state and should be used only for comparative purposes within the series. We see, for example, that the pK_a for the amino group of β-THPI is almost half a pH unit lower than that of γ-THPI. Also listed in Table 2 are pK_a values determined from changes in the absorbance in the 290−300 nm range; the latter pK_a values should be equal to the ground state pK_a of the amino groups.

Time-Resolved Fluorescence of L-Tryptophan and L-W(1). Figure 4 shows multifrequency phase/modulation data for L-tryptophan and L-W(1). The tryptophan data are fitted by a biexponential decay law with $\tau_1 = 0.78$ and $\tau_2 = 3.16$ ns. The reduced chi squared (χ_R^2) for a monoexponential fit is a factor of 2 larger than that for the biexponential fit. These τ_i agree with literature values of $\tau_1 \approx 0.5$ ns and $\tau_2 = 3.1$ ns.[1,14−16] For L-W(1) the biexponential fit ($\chi_R^2 = 1.49$) is slightly better than the monoexponential fit ($\chi_R^2 = 2.0$) at 20 °C.

Since the distinction between a mono- and biexponential decay is less clear for L-W(1) than for L-Trp, we used several strategies to characterize the decay kinetics. These were to (1) measure the fluorescence decays as a function of temperature from 2 to 60 °C and to perform a global analysis in terms of activation energies for the components, (2) to measure the decay as a function of emission wavelength and to perform a global analysis, and (3) to measure the decay with a time-correlated single photon counting instrument, for comparison with data from our phase fluorometer. Temperature dependence lifetime data will be presented elsewhere, but are consistent with a biexponential model. A global analysis of data obtained at different emission wavelengths yields a two-component fit with $\tau_1 = 0.97$ ns, $\tau_2 = 6.69$ ns, and α_1 decreasing from 320 to 400 nm. Using the time-correlated single photon counting method, a biexponential decay was also observed for L-W(1), as listed in Table 3.

Time-Resolved Fluorescence of Other Analogues. Fluorescence decay parameters for other Trp analogues are given in Table 3. The decay of W(2) is a biexponential with lifetimes similar to those for W(1). The decays of β-THPI and γ-THPI are a monoexponential (at the indicated pH), but the decays of 3-amino-THC and 3-carboxy-THC appear to be best described as biexponential. The other methylated Trp analogues, including 2-methyl-, 4-methyl-, 5-methyl-, and α-methyltryptophan, show biexponential decays, with the decay of 4-methyltryptophan being very rapid.

Discussion

The results presented here deal with the following interrelated questions regarding the photophysics of indole and tryptophan. What are the mechanisms of intramolecular quenching (e.g., proton transfer or electron transfer) by functional groups in tryptophan analogues? How do these quenching reactions change with the state of protonation of the α-amino and α-carboxylate groups? How do these reactions vary with intramolecular distances (e.g., rotameric conformers), and can the nonexponential fluorescence decay of many tryptophan analogues be rationalized? Does the radiative rate constant, k_r, as well as the sum of nonradiative rate constants, k_{nr}, vary with side chain groups and their state of protonation? What is the basis for the very large Stokes shift seen in tryptophan analogues, and why does deprotonation of side chain ammonium groups cause a further red shift in emission? This Discussion will comment on these questions. First, we recap the fluorescence characteristics of the conformationally restricted tryptophan analogues studied here. Second, we review the prevailing ideas about intramolecular quenching reactions and how they relate to the biexponential decay kinetics of tryptophan. Third, we discuss what we consider to be nagging questions and we offer some speculations regarding the fluorescence of flexible and constrained tryptophan analogues.

A. Fluorescence Properties of Conformationally Constrained Tryptophan Analogues. The fluorescence properties of some of the molecules in Table 1 and Figure 2 are reported here for the first time. Studies with L-W(1), W(2), THC, and 3-carboxy-THC have only recently been reported by Barkley's lab.[20−22] For these reasons we will briefly discuss the emission of these novel analogues.

The constrained tryptophan analogue, L-W(1), has a relatively high fluorescence quantum yield; its quantum yield is higher than that of the model compound THC, which has no side chain functional groups. The zwitterionic form of L-W(1) shows a slightly nonexponential fluorescence decay (see Table 3). Qualitatively similar results for L-W(1) have been reported by Tilstra *et al.*[20] for the decay of L-W(1). Also, there is essentially no deuterium isotope effect for the decay of L-W(1), and there is no photoinduced exchange of solvent deuterium into position C4 of the indole ring of L-W(1), as is found for tryptophan.[20,34,35] A striking difference between L-W(1) and tryptophan is seen in the pH dependence of the intensity of these molecules. In contrast to the familiar pattern for tryptophan, the fluorescence of L-W(1) decreases between pH 8 and 10 with deprotonation of the side chain α-ammonium. Deprotonation of the ammonium group of L-W(1) leads to a large emission red shift. As discussed below, this red shift can be interpreted, as a decrease in the pK_a in the excited state.

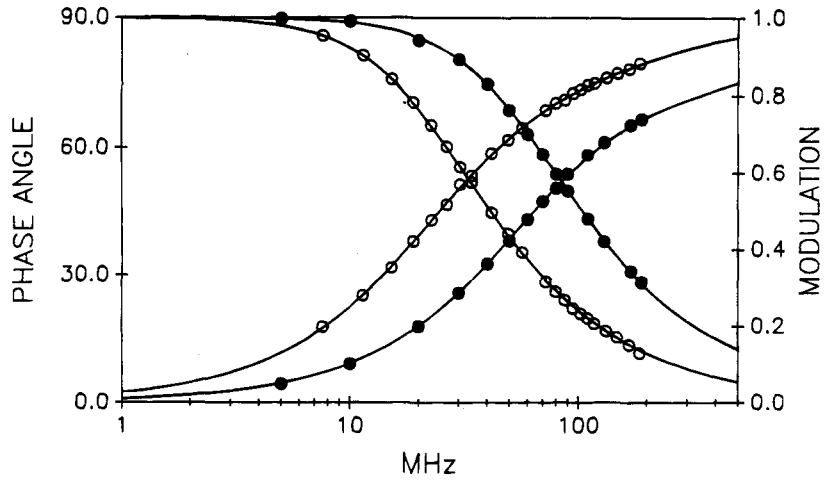

Figure 4. Phase/modulation fluorescence lifetime data for L-tryptophan (○) and L-W(1) (●) at pH 5, 20 °C. The solid lines are fits of eq 2 with τ_i and α_i values in Table 3.

TABLE 3: Time-Resolved Fluorescence Data for L-W(1), Tryptophan, and Other Constrained Analogues[a]

samples	τ_1 (ns)	α_1	τ_2 (ns)	χ_R^2
L-W(1)	1.05	0.098	6.65	1.49
L-W(1)[b]	0.97		6.69	3.13
L-W(1)[c]	2.05	0.047	6.35	1.27
L-W(1), pH 10	2.58	0.142	5.20	1.67
L-W(1), 5 °C	3.11	0.201	7.66	1.25
tryptophan	0.78	0.30	3.16	1.17
1-methyl-W(1)	0.79	0.10	9.69	0.34
W(2)	1.39	0.087	5.79	1.03
β-THPI			6.28	1.27
γ-THPI			5.45	1.06
3-amino-THC	1.46	0.204	5.15	3.06
tryptamine			6.41	1.14
IPA[a]			9.00	2.06
3-carboxy-THC[d]	1.63	0.196	4.99	2.44
1-methyltryptophan	0.61	0.73	5.40	3.80
2-methyltryptophan	0.74	0.465	3.94	1.46
4-methyltryptophan	0.94	0.515	1.46	1.36
5-methyltryptophan	0.70	0.330	2.69	2.13
α-methyltryptophan	0.39	0.541	2.77	3.58

[a] Data at pH 5.0−5.5, 20 °C, unless stated otherwise. The reduced χ_R^2 values were calculated using errors of 0.2° and 0.004 for the phase angle and modulation. [b] Global analysis of data at five emission wavelengths from 320 to 400 nm. [c] Obtained with a time-correlated single photon counting apparatus. [d] Data at pH 7.0.

The fluorescence of the second constrained tryptophan analogue, W(2), is similar to that of L-W(1). The zwitterion of W(2) also has a relatively high quantum yield and a biexponential decay. The pH dependence of its fluorescence decreases with deprotonation of the α-ammonium group.

Three constrained analogues, β-THPI, γ-THPI, and 3-amino-THC, have only a side chain ammonium group and have similar fluorescence properties. They have high quantum yields in their cationic form, and they show a decrease in intensity with deprotonation of their ammonium group, in contrast to the increase upon deprotonation for tryptophan and tryptamine. The three constrained amino analogues show a red shift upon ammonium group deprotonation; the largest shift is seen for β-THPI. The decay of the cationic form of β-THPI and γ-THPI is monoexponential; the decay of 3-amino-THC is nonexponential.

3-Carboxy-THC is a constrained analogue of L-W(1) and W(2) without an amino group. It also has a high quantum yield (higher than THC). At neutral pH (carboxylate form) its decay is slightly nonexponential, and at pH 3 (carboxylic acid form) it is monoexponential. 3-Carboxy-THC shows a slight red shift upon deprotonation of its carboxylic acid side chain.

Two other tryptophan analogues studied are 2-methyl- and 4-methyltryptophan. The fluorescence of 2-methyltryptophan is similar to that for tryptophan. In contrast, the fluorescence of 4-methyltryptophan shows a pH dependence (see Figure 3D) that is more analogues to that of the constrained tryptophan analogues.

B. Intramolecular Quenching Mechanisms for Indole Derivatives. The fluorescence of indole, tryptophan, and their derivatives is thought to be quenched by the following excited state reactions in aqueous solution: (i) intersystem crossing (k_{isc}) to the triplet state, (ii) photoionization (k_{ion}), (iii) intramolecular proton transfer (k_{PT}) from a side chain (e.g., α-ammonium group) to the indole nucleus, and (iv) intramolecular charge transfer (k_{CT}) from indole to a side chain functional group.[14,15,22,34] The latter two intramolecular reactions do not apply, of course, to the bare indole (or methylindole) molecule. These various reactions will occur competitively in a fluorophore to determine its fluorescence quantum yield (i.e., the effective nonradiative rate constant will be $k_{nr} = k_{isc} + k_{ion} + k_{PT} + k_{CT}$, where $1/\tau = k_{nr} + k_r$). Depending on the nature of the side chain, a particular quenching reaction may dominate in a given fluorophore.

The rate constant for intersystem crossing, k_{isc}, and the rate constant for the radiative process, k_r, are considered to be independent of temperature and relatively independent of the presence of side chain functional groups (see below). The rate constant for photoionization, leading to the production of the indole cation and a hydrated electron, has been characterized for indole and 3-methylindole[36] and has an activation energy, E_a, of about 12−14 kcal/mol and a small solvent isotope effect. Other possible deactivation processes are photoinduced imino NH dissociation[37] and internal conversion to the ground singlet state; these are usually ignored or are lumped into k_{isc} or k_{ion}.

Evidence for the intramolecular proton transfer quenching mechanism comes from the observation of photoinduced exchange of solvent deuterium into the C4 position of tryptophan.[34,35] This exchange process is found in the zwitterionic form of tryptophan and the cationic form of tryptamine (i.e., requires a protonated ammonium group) and is not observed for L-W(1).[20] The rate constant for intramolecular proton transfer, k_{PT}, shows a large deuterium kinetic isotope effect and an E_a of ~5 kcal/mol.[13,22,34]

Intramolecular charge transfer, from the excited indole ring to an electrophilic side chain group, is believed to be an important quenching process but is difficult to show experimentally. Carboxylic acid, ester, ammonium, and amide groups are thought to act as electrophiles, in order of increasing

effectiveness.[12,14,15] The k_{CT} process is expected to have an E_a of 4−6 kcal/mol and to show no solvent isotope effect.[15,22]

The rate of charge transfer and proton transfer should be dependent on the distance between the indole ring (specifically, position C4 for proton transfer) and the quenching group. For this reason the existence of rotamers around the α−β and β−γ bonds is pertinent for these quenching processes.[14,15,22] Three primary rotamers (g^+, g^-, and t) exist about the α−β bond. The g^- rotamer is the most populated in solution (based on NMR studies[38−40]). There are two stable rotamers (perpendicular and antiperpendicular) about the β−γ bond.[15d,42b] Thus, there can exist six conformers of tryptophan and its unconstrained analogues (see Figure 1). Considering the evidence for proton transfer quenching involving the α-ammonium and C4 position, the g^- antiperpendicular and g^+ perpendicular conformers are ones in which these groups are closest and are likely candidates for rapid proton transfer quenching. These same two conformers also may be the most effective for charge transfer quenching, since they have the ammonium group closest to the indole ring. The g^- perpendicular and the g^+ antiperpendicular conformers have the ammonium group nearest to indole's pyrrole ring.

To rationalize the biexponential decay of tryptophan in terms of the rotamer model, rotation about the α−β and/or β−γ bonds must be slow or on the same time scale as the radiative process. Molecular dynamics calculations by Engh *et al.*[15d] show that β−γ bond rotation is much slower than that around the α−β bond. However, Gordon *et al.*[42b] have reported conflicting molecular dynamics calculations (with explicit modeling of all hydrogen atoms) showing that rotation about the β−γ bond is faster than about the α−β bond. The report of a negative preexponential for tryptophan fluorescence suggests that side chain rotations, or some other nonquenching excited state process, occur on the fluorescence time scale (see below).[42a] The conformationally constrained analogues are designed to limit the number of orientations between indole and the side chain groups, but these analogues are not actually rigid. Colucci *et al.*[21] have presented NMR and molecular mechanics calculations to show flexing of the cyclohexene ring of L−W(1) between two half-chair forms. These workers suggested that this flexing is slow on the nanosecond time scale, to account for the biexponential decay of L−W(1) and its ethyl ester (i.e., they proposed the existence of two slowly interconverting conformers, T and T′, which have different distances between indole and the quencher group).

Although emphasis has been placed here on quenching by the α-ammonium group in tryptophan, other side chain functional groups can act as quenchers. The compounds listed in Table 1 include several whose side chain has a single functional group. This allows comparison of the sum of the nonradiative rate constants, $k_{nr} = k_{isc} + k_{ion} + k_{CT} + k_{PT}$, for individual functional groups and for cases in which two groups are combined. The values of k_{nr} are calculated as $1/\tau - \Phi/\tau$. (The mean lifetime, $\langle\tau\rangle = \sum\alpha_i\tau_i$, is used, so that k_{nr} is an "average" rate constant.)

The value of k_{nr} is about $(6-7.5 \times 10^7\ s^{-1}$ for 3-methylindole (used as a reference for a nonquenching side chain) and for the α-carboxylate of indolepropionate and the carboxamide of indole propionamide. The latter two groups are essentially nonquenchers when by themselves. The α-ammonium of tryptamine gives some quenching ($k_{nr} = 12.8 \times 10^7\ s^{-1}$). The *N*-acetyl group of *N*-acetyltryptamine ($k_{nr} = 15 \times 10^7\ s^{-1}$) and the ester group of ethyl indolepropionate ($k_{nr} = 25 \times 10^7\ s^{-1}$) are the most effective of the common side chains. This order of ester > *N*-acetyl > ammonium > carboxamide ≈ amine ≈ carboxylate

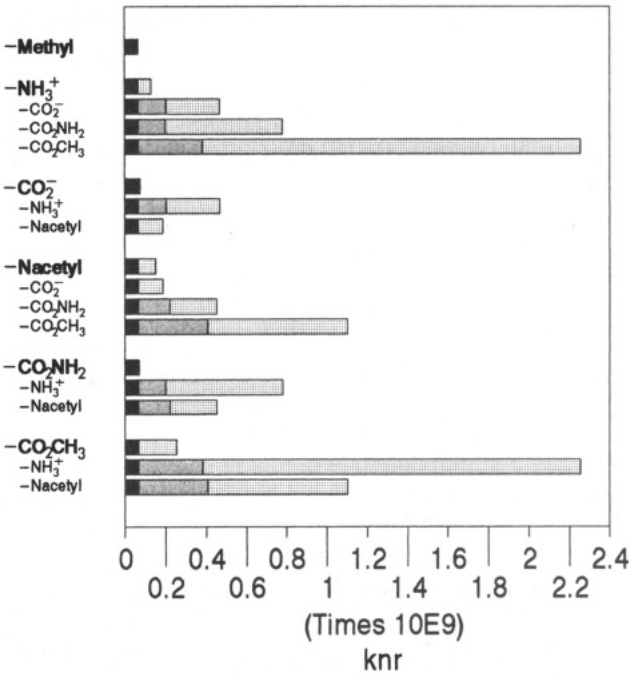

Figure 5. Values of the effective k_{nr} for indole/tryptophan analogues as a function of side chain. The group listed in smaller print on the ordinant is considered to be the secondary substituent to the group (directly above) in larger print. The darkly shaded areas represent the k_{nr} for 3-methylindole (i.e., the intrinsic quenching reactions in the absence of a side chain functional group). The medium shaded areas are the sum of the k_{nr} contributions expected for the individual side chain groups. The value of k_{nr} above the sum of these contributions (the lightest shading) is due to the synergistic effect between the pair of groups. As further explanation, the groups listed in larger print correspond to 3-methylindole and its monofunctional side chain analogues, tryptamine, indole propionic acid, *N*-acetyltryptamine, indolepropionamide, and indole propionate methyl ester. The first entry with smaller print is for tryptophan, for which the dark shaded area is the k_{nr} attributable to intrinsic quenching ($k_{isc} + k_{ion}$, seen in 3-methylindole), the medium shaded area is the expected contributions (additive, nonsynergistic) to k_{nr} from quenching by CO_2^- and NH_3^+ side chains, and the lightly shaded areas indicate the extra (synergistic) quenching that is seen when both functional groups are present.

was recognized in previous studies[14,15] and is throught to primarily reflect the ability of these groups to act as an electron acceptor.

Although the individual functional groups are not very strong intramolecular quenchers, when any two groups are together on the side chain, a synergistic effect usually exists. This is shown in Figure 5, where the k_{nr} for various mono- and bifunctional tryptophan analogues is plotted. The k_{nr} when two functional groups are present is usually greater than the sum of the k_{nr} for the two single groups. Considering only the values of k_{nr} above that for 3-methylindole (the value for which can be taken as $k_{isc} + k_{ion}$, which should be independent of the side chain), this synergism holds in essentially all cases and it can be very large. For example, the k_{nr} for the ester group is enhanced 4.5-fold by the presence of an α-ammonium group (as in tryptophan ethyl ester) and 2-fold by the presence of an *N*-acetyl group. The k_{nr} for the α-ammonium group is enhanced 5-fold by the presence of a carboxamide group (as in tryptophanamide) and 3-fold by the presence of a α-carboxylate group (as in tryptophan).

This synergistic effect between intramolecular quenching groups, particularly for tryptophan, has been noted before.[13−15] These activations may involve either (a) inductive effects that increase the electron affinity or proton-donating ability of the other functional group and/or (b) changes in the population of

various rotamers, due to steric effects, to favor quenching conformers. For tryptophan both effects are probably operating. The pK_a of the α-NH_3^+ group drops from 10.2 in tryptamine to 9.3 in tryptophan. This increase in acidity, induced by the α-CO_2^-, should enhance the rate of proton transfer to position C4 in tryptophan. (See below for further discussion on this point.) The presence of the α-CO_2^- also may change the $\alpha-\beta$ rotamer population in tryptophan, relative to that in tryptamine, to favor rotamers having the α-NH_3^+ group closer to the indole (i.e., the g^- and g^+ rotamers). In tryptamine the t rotamer is likely to be prevalent since it minimizes steric repulsion. In tryptophan the g^- rotamer should be favored since the CO_2^- group is larger than the NH_3^+ group; this would bring the α-NH_3^+ group nearer the indole ring for either proton or charge transfer quenching.

For the constrained analogues, the quenching patterns are altered considerably. The individual side chain functional groups, except the ester group, do not appear to act as intramolecular quenchers, when compared to the model compounds THC or 2,3-dimethylindole. The k_{nr} value for THC is 25×10^7 s^{-1}. The presence of carboxylate, ammonium, amino, or *N*-acetyl groups in the various constrained analogues lowers the value of k_{nr}. For the ethyl ester, W(1)EE, k_{nr} is increased slightly, presumably since the ester group is a strong electron acceptor. The conformational restriction by the additional ring must eliminate quenching reactions, particularly proton transfer to position C4.[20,21] Since k_{nr} is smaller for most constrained analogues than it is for THC, either k_{isc} or k_{ion} must be smaller in these analogues. Why this would be the case is not clear.[43]

Another reference compound for the constrained Trp analogues is 2-methyltryptophan, which, by having a methyl substituent at indole's C2, should model the effect of an alkyl substituent on the electronic properties. The k_{nr} for the constrained analogues are lower than that for 2-methyltryptophan, which can be attributed to the elimination of intramolecular quenching reactions by the ammonium groups in the constrained analogues.

C. Questions and Speculations. Here we discuss some nagging questions about the fluorescence of Trp and its analogues.

1. How and why does the radiative rate constant, k_r, vary with substitution on the indole ring and the nature of the side chain? For the series of methylindoles we find k_r to vary from 2.4 to 11 $\times 10^7$ s^{-1}. There is no obvious correlation of k_r with the L_a-L_b energy difference or the λ_{max} of these compounds.[7] From basic principles, k_r should be related to the transition probability and be proportional to a chromophore's molar extinction coefficient, ϵ. However, we find the integrated (from 240−310 nm) ϵ for 2-methylindole and 3-methylindole to be the same, within ±10%, whereas their k_r values differ by a factor of 2. Also the calculated (INDO) oscillator strengths for both the L_a and L_b transitions of 3-methylindole and 4-methylindole do not predict the large difference seen in k_r for these compounds.[7]

For both the flexible and constrained tryptophan analogues, the value of k_r drops by about 20% upon deprotonation of the α-ammonium group. This has been previously noted,[15] but the basis for this drop is unclear. As discussed below, the presence of a nearby charge perturbs indole's absorption and emission transitions; apparently this effect is seen in k_r as well.

2. What is the basis for the synergistic quenching effect of two side-chain functional groups? This synergism must be caused by either inductive (second group affects the electrophilicity or pK_a of the first group) or steric (change in the rotamer population) effects. Szabo and Rayner[14] rationalized the large synergism between the *N*-acetyl and ethyl ester groups in NATEE as being due to an effect on rotamer population; NMR studies on the analogous phenylalanine derivatives support this explanation.[47]

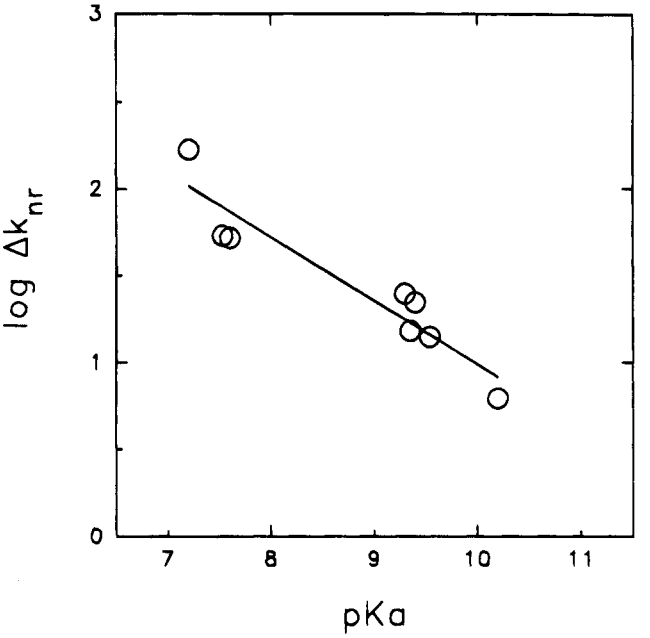

Figure 6. Dependence of the effective k_{nr} on the pK_a of tryptophan analogues. Entries include tryptamine, tryptophan, 1-methyltryptophan, 2-methyltryptophan, 5-methyltryptophan, tryptophanamide, tryptophanylglycine, and tryptophan methyl ester. The pK_as are the average values determined by absorbance and fluorescence (see Table 2); Δk_{nr} values are the difference between the k_{nr} for the protonated and unprotonated forms of the compound.

In examining the possibility of induced changes in the pK_a of the α-ammonium group by the second group, we find that a good correlation exists between changes in the pK_a and k_{nr}, as shown in Figure 6. Here Δk_{nr} is taken as either the k_{nr} value for the α-NH_3^+ minus the α-NH_2 form of the molecule or as the difference between the k_{nr} for the α-NH_3^+ form of a molecule minus the k_{nr} for the corresponding des-α-NH_3^+ molecule (i.e., k_{nr} for tryptophanamide minus k_{nr} for indolepropionamide). Thus, the Δk_{nr} and pK_a values induced by the secondary group are taken relative to values for tryptamine (α-NH_3^+ as a quencher with H atoms on the Cα). Figure 6 shows a good correlation. For example, an ester secondary group causes a downward shift in the pK_a of the α-NH_3^+ group from 10.3 in tryptamine to ~7.4 in tryptophan methyl ester. This correlation supports the hypothesis that the α-NH_3^+ acts as a proton transfer quencher, and it allows the prediction that the rate constant for photoinduced isotope exchange at the C4 position also should correlate with the pK_a for the series of molecules in Figure 6.

3. Why does *N*-acetyl-L-tryptophanamide show a monoexponential fluorescence decay? Although the rotamer model is generally successful in explaining the fluorescence of tryptophan analogues, it has been pointed out that there is difficulty in rationalizing the monoexponential decay of NATA and a few singly substituted analogues.[15d] We have no new comments on this matter beyond the explanations given by Petrich *et al.*[15c] and Engh *et al.*,[15d] but we list this as a slightly bothersome result.

4. What is the basis for the biexponential decays of constrained L-W(1) and W(2), and why do the side chain groups in the constrained analogues appear not to be quenchers? Except for the ester group in W(1)EE, side chain groups do not appear to quench the fluorescence of the constrained analogues.

Deprotonation of the ammonium group in L-W(1), W(2), β-THPI, γ-THPI, and 3-amino-THC leads to a decrease in fluorescence. Proton transfer quenching cannot occur between their ammonium groups and the indole C4 position. Surprisingly, electron transfer to the charged ammonium groups also does not seem to occur in the constrained analogues. If anything, the neutral amino group acts as a weak quencher. This behavior also is seen in 4-methyltryptophan, for which proton transfer to C4 is blocked. We are not able to rationalize satisfactorily these observations. Clearly, the side chain functional groups are affecting the fluorescence of the constrained analogues, as is evident from the biexponential decay of L-W(1) and W(2), from the pH dependence of the lifetime, intensity, and emission spectra. Below we discuss a possible clue to understanding these effects.

5. What is the basis for the shift in the emission maximum of tryptophan analogues upon deprotonation of the side chain ammonium group? The red shift in the fluorescence of tryptophan upon deprotonation of its α-ammonium group has been commented upon infrequently.[15b,20,46] Gudgin-Templeton and Ware[44] noted the rather large red shift of 13 nm for the deprotonaton of 5-methoxytryptophan. For tryptophan, this red shift is about 9 nm, but for some analogues studied here the red shift is larger (e.g., 19 nm for L-W(1)). There is also a red shift in the absorbance of tryptophan and the analogues upon deprotonation, but this shift is small or more difficult to discern because of overlap between the L_a and L_b absorption transitions (i.e., the L_a appears to be the more shifted).[45,46]

The fluorescence λ_{max} shift with deprotonation of the NH_3^+ group can be interpreted in terms of the Förster cycle to estimate the apparent excited state pK_a^* via the following equation:[48,49]

$$pK_a - pK_a^* = Nhc(1/\lambda_{NH_3^+} - 1/\lambda_{NH_2})/(2.303RT) \quad (3)$$

where N, h, c, and R are Avogadro's number, Planck's constant, the speed of light, and the gas constant and where $\lambda_{NH_3^+}$ and λ_{NH_2} are the emission maxima of the protonated and unprotonated forms of the fluorophore. Table 2 lists the ground state pK_a and the apparent ΔpK_a ($=pK_a - pK_a^*$) for tryptophan an the various analogues.

The apparent ΔpK_a value is about 1.5 pH units for tryptophan and is as large as 3.5 pH units for 1-methyl-W(1). What is the basis for these pK_a shifts? We first note that the largest ΔpK_a are seen for 1-methyl-W(1), L-W(1) and β-THPI, which have the ammonium group restricted to be close to the pyrrole portion of the indole ring; the ΔpK_a is small for tryptamine, which may favor a rotamer having the α-NH_3^+ group as far as possible from the indole ring. Thus, the magnitude of the ΔpK_a values seems to be related to the closeness of the protonated ammonium group to the pyrrole ring. In support of this idea we note that James and Ware[52] have reported that homotryptophan, which has an additional methylene group between the α-NH_3^+ group and the indole ring, shows a smaller red shift upon deprotonation (3 nm), as compared to the 9−10 nm red shift upon deprotonation of the α-NH_3^+ of tryptophan. Also, we find that deprotonation of the side chain carboxylic acid group in IPA and 3-carboxy-THC results in a red shift in the emission of these compounds. These results indicate that the excited indole ring destabilizes a positively charged side chain NH_3^+ group and stabilizes a negatively charged CO_2^- group. This suggests that the indole ring, particularly the pyrrole portion that is nearest to the side chain groups, develops an increased positive charge in the excited state. Molecular orbital calculations on indole support this notion by showing a decrease in charge densities in the pyrrole ring and an increase in charge in the benzenoid ring upon going from the ground state to the excited state.[50,51] Callis[51] has calculated that there is a transfer of approximately 0.33 electrons from the pyrrole ring to the benzenoid ring upon excitation. Thus, the excited indole ring has a large dipole with its positive pole pointing through the C2 atom of the pyrrole ring. Consequently, the pK_a of side chain groups should be perturbed. Using the equation $\Delta pK_a = 24.25q_1q_2/(\epsilon r)$,[53] with point charges of $q_1 = 0.33$ for the pyrrole ring and $q_2 = 1.0$ for the protonated α-NH_3^+ and an effective dielectric constant of 10, we calculate ΔpK_a values to range from 1.33 to 3.2 for distances, r, of 6.0−2.5 Å between the charges. These distances correspond to the farthest (extended chain in tryptamine) and nearest (in ring of β-THPI) positions of the ammonium group from C2 of the pyrrole ring, and these ΔpK_a values are within the range of experimentally estimated values.

The red shift upon deprotonation of the ammonium groups and the ΔpK_a values thus can be rationalized in terms of the distance of the charged NH_3^+ from the C2 atom. These results and interpretation also suggest that, upon excitation, the side chain NH_3^+ and CO_2^- groups will be influenced to reorient in response to the newly created large dipole. That is, we suggest that this excited state dipole−charge interaction drives a relaxation process involving rotation about the α−β bond in the flexibile tryptophans or flexing of the ring structure in the constrained analogues.

Even if this reorientation is slow, the above dipole−charge interaction provides an initial framework for understanding the biexponential decay of the constrained analogues, L-W(1) and W(2). The two half-chair conformers of the extra ring of L-W(1) will both have the NH_2^+ group at the same distance from indole's C2 position. However, the CO_2^- group is nearer to the C2 position in its axial (T) conformer and is further from the C2 position in its equatorial (T′) conformer.[22] Since the above postulated dipole−charge interaction will involve both the NH_2^+ and CO_2^- groups, we suggest that the T conformer will be stabilized by the stronger Coulombic interaction between the excited state indole dipole and the CO_2^- group and will be associated with the redder emission. This model does not include a mechanism for quenching in the T′ or T conformers, but a biexponential decay could arise if the excited state T′ → T relaxation process (i.e., flexing of extra ring) occurs on the fluorescence time scale.

6. What is the nature of the excited state reaction that causes the negative preexponential in the decay of tryptophan? Willis *et al.*[42a] have reported that the short decay time ($\tau_1 \approx 0.5$ ns) of tryptophan has a negative pre-exponential at the red emission range. This negative preexponential is more pronounced in D_2O and must be due to an excited state reaction that "builds up" the emission of redder species. The nature of this reaction is of obvious interest, and we suggest that reorientation about the α−β in response to the excited state dipole may be involved.

Summary

We have studied steady state and time-resolved fluorescence of a large set of tryptophan analogues, including several that are conformationally restricted. Our studies lead us to draw the following conclusions and speculations.

1. The fluorescence patterns of the constrained analogues are significantly different from that of tryptophan. The pH dependence of the constrained analogues shows that the protonated ammonium group is not acting as an intramolecular quencher. The high quantum yield of these constrained analogues, in comparison with what should be suitable model compounds, is consistent with the inability of side chain groups to act as quenchers.

2. From a comparison of the nonradiative rate constant for various side chain groups (i.e., for flexible analogues) and the ground state pK_a of the side chain ammonium group, we speculate that the synergistic effect of two quenching groups on the α-carbon can be caused by an induced change in the proton-transferring ability of the α-ammonium group.

3. There is a decrease in the pK_a of the side chain ammonium group upon excitation of the indole chromophore. We speculate that this is caused by an electrostatic repulsion between the excited indole dipole and the point charge on the ammonium group.

4. We speculate that the biexponential fluorescence decay of some of the constrained tryptophan analogues is due to an excited state relaxation driven by the electrostatic interaction between the indole dipole and side chain charged groups.

From these considerations, the α-ammonium group affects the fluorescence of indole in two ways: (1) by acting as a proton transfer quencher, with interaction at C4 of the indole ring and (2) by repulsively interacting with the excited state dipole of the indole ring, thus causing a blue shift in the emission and possibly driving a side chain rotation/relaxation process.

Acknowledgment. This research was supported by NSF Grant DMB 91-06377, NSF Mississippi EPSCoR Grant OSR 91-08767, and American Heart Association Grant 93009000.

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(19) M. D. Barkley and co-workers, Louisiana State University, have recently published extensive fluorescence and structural studies with one of these conformationally constrained analogues, referred to as W(1).[20,21] They have also published fluorescence quantum yields and mean lifetimes for constained analogues referred to in this paper as W(2), β-THPI, 3-Carboxy-THC, and THC.[22] Where there is overlap, our values are in general agreement. We have also reported preliminary fluorescence yield and lifetime data for several of the constrained and flexible analogues in the following: Eftink, M. R.; Hu, D.; Jia, Y. Time-Resolved Laser Spectroscopy in Biochemistry. *Proc. SPIE* **1992**, *1640*, 31−36.

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JP9425833