Fluorescence Quenching in Indoles by Excited-State Proton Transfer

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Abstract: Indoles undergo two isotopically sensitive temperature-dependent fluorescence quenching processes: solvent quenching and excited-state proton transfer. Fluorescence quantum yields of simple indoles in protium and deuterium solvents were measured in the absence and presence of glycine. Photochemical H-D exchange was monitored by ^{1}H NMR and mass spectrometry. Although the fluorescence quantum yield and lifetime of 2-methylindole show large deuterium isotope effects in aqueous solutions, photochemical H-D exchange was not detected after extensive irradiation, whereas, H-D exchange is readily observed for 2- and 3-methylindole in solutions containing glycine. Stern-Volmer plots of glycine quenching data give bimolecular rate constants k_q from (0.5-3) $\times 10^8$ M^{-1} s^{-1} for indoles in water. The k_q values of 2- and 3-methylindole are faster in protium than in deuterium solvents. The isotope effect on k_q implicates excited-state proton transfer in the collisional quenching mechanism. This contrasts with iodide quenching which has no isotope effect on k_q. A glycine derivative lacking the ammonium protons, *N,N,N*-trimethylglycine, does not quench indole fluorescence. The intermolecular excited-state reaction of 2- and 3-methylindole with 0.3 M glycine-d_5 in 50% D_2O/CD_3OD induces H-D exchange at three ring carbons. In 2-methylindole the exchange is fastest at C3 and occurs with similar rates at C4 and C7 on the indole ring. The temperature dependence of 3-methylindole fluorescence in 0.5 M glycine was also determined. The large difference in temperature dependence for solvent quenching and glycine quenching causes curvature in the Arrhenius plot. The frequency factor $A_2 = 7.2 \times 10^{10}$ s^{-1} and activation energy $E_2^* = 3.6$ kcal/mol for glycine quenching are similar to the values for intramolecular excited-state proton transfer in tryptamine. Possible mechanisms for the excited-state proton transfer reaction and the implications of this reaction for tryptophan fluorescence in proteins are discussed.

Tryptophan fluorescence is highly sensitive to environment. In proteins the emission maximum occurs anywhere between 308 and 360 nm, depending on polarity and perhaps also on rigidity of the surrounding matrix.[1,2] The fluorescence quantum yield and lifetime vary over a considerable range.[3] Likewise, the emission maximum or intensity of tryptophan in a particular protein may change in response to ligand binding and conformation transitions. The shifts in emission maxima are due to the presence of two overlapping excited electronic states, ^1L_a and ^1L_b, with different dipole moments.[4] The relative energies of these two states, and hence the energy and identity of the emitting state of the indole chromophore, are influenced by solvent interactions.[5] Quantum yield or lifetime differences are largely due to nonradiative decay processes that compete with emission for deactivation of the excited state. Thus, for example, the fluorescence intensity of a protein may increase or decrease upon unfolding, depending on whether a quenching mechanism is turned off or on in the denatured protein. The nonradiative pathways of tryptophan are discussed in the preceding paper.[6] Three excited-state reactions, proton transfer, electron transfer, and exciplex formation, are sensitive to local environment.

Excited-state proton transfer reactions have long been recognized to quench indole fluorescence,[7-14] although their role in tryptophan has been disputed.[15-20] There are two signatures of excited-state proton transfer reactions: deuterium isotope effect and H-D exchange of aromatic protons. As pointed out by Stryer,[8] the fluorescence quantum yield and lifetime increase in D_2O compared to H_2O because the transfer rate is slower for a deuteron than for a proton. The ubiquity of the deuterium isotope effect in indole and its derivatives sparked the controversy about excited-state proton transfer.[8,10,11,13,15,17,19,21] The fluorescence quantum yield of simple indoles is independent of pH in the range of about pH 3-10 but is quenched at both lower and higher pH.[7,9,10,16] The quenching below pH 3 and above pH 11 is due to two different excited-state proton transfer reactions: acid-catalyzed protonation of the indole ring[9] and base-catalyzed deprotonation of the indole N-H.[7] For indole derivatives with a pendant amino group, such as tryptophan and tryptamine, the quantum yield peaks in the alkaline pH range, coinciding with deprotonation of the ammonium.[7,10,16] Lehrer[10] proposed that the ammonium on the alanyl side chain quenchs the fluorescence at neutral pH by an intramolecular proton transfer reaction. The observation of photochemical H-D isotope exchange of aromatic hydrogens in tryptophan, tryptamine, and *N*-(aminoalkyl)tetrahydrocarbazoles by Shizuka and co-workers[14,22,23] provided direct evidence for intramolecular proton transfer. Iodide quenches the H-D exchange at the same rate as fluorescence, showing that proton transfer occurs in the excited singlet state.[14] Chelation of the ammonium with a crown ether lengthens the fluorescence lifetimes of these compounds, earmarking ammonium as the proton donor.[22,23] Detection of photochemical H-D exchange by ^{1}H NMR is a general method for studying excited-state proton

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transfer reactions in aromatic chromophores.[24]

In the preceding paper,[6] we proposed that the intrinsic isotope effect seen in all indoles is due either to "invisible" or incomplete proton transfer or to exciplex formation. These quenching mechanisms may or may not exhibit hydrogen exchange. In this paper we look for H–D exchange in 2-methylindole under conditions where there is a large isotope effect on the fluorescence quantum yield and lifetime. We also investigate an intermolecular version of the intramolecular proton transfer reaction of tryptophan.[10,16,25] Deuterium isotope effects and H–D exchange are measured in 2- and 3-methylindole solutions containing glycine. The temperature dependence of 3-methylindole fluorescence quenched by glycine is also determined. Finally, we discuss the implications of our findings for tryptophan fluorescence in proteins.

Experimental Section

2-Methylindole, 3-methylindole, and *N*,2-dimethylindole (Aldrich) were recrystallized from aqueous ethanol, sublimed under reduced pressure, and stored in an inert atmosphere at 4 °C covered by aluminum foil to exclude light. *N*-Methylindole (Aldrich) was purified by distillation. Glycine-d_5 (Aldrich, 98% D) and deuterated solvents (Aldrich, 99.9% D) were used as received. Some lots of D_2O were basic, so the pD of solutions containing D_2O was checked and adjusted to pD 7 with DCl. Other solvents were HPLC grade. Aqueous solutions contained 0.01 M phosphate buffer, pH(D) 7.

Steady-state and time-resolved fluorescence measurements were made as described in the preceding paper.[6] Quantum yields were measured relative to tryptophan at 280-nm excitation wavelength, 25 °C. Lifetimes were measured at the emission maximum with excitation at 296 nm. Stern–Volmer quenching constants were determined from relative quantum yield or lifetime data.

Photochemical isotope exchange was monitored on a Bruker AC-200 FT-NMR spectrometer at ambient temperature. 2- and 3-Methylindole were dissolved in D_2O/CD_3OD (1.5 mg/0.5 mL) in a thin wall Pyrex NMR tube. The solvent contained 30% methanol or 50% methanol and 0.3 M $^+D_3NCD_2CO_2^-$. Solutions were irradiated with a 200-W Hg–Xe lamp filtered by a Corning UV transmitting/visible absorbing filter (No. 7-54), which cuts off light below 260 nm. A slight precipitate was visible after 1 h of irradiation. A saturated solution of 2-methylindole in D_2O (1.3 mg/mL) was irradiated in a 1-cm quartz cell for 4 h. The solution was filtered to remove the precipitate formed after extensive irradiation and was transferred to an NMR tube. H–D exchange was determined from integrals of aromatic proton resonances relative to methyl proton resonances. The same instrument settings were used throughout an experiment. Percentage of H–D exchange is calculated relative to an unirradiated sample. Proton assignments (referenced to HDO at 4.8 ppm) were taken from the literature:[14,26,27] 2-methylindole (1:1 D_2O/CD_3OD) δ 2.4 (s, CH_3), 6.13 (s, HC3), 7.45 (d, HC4), 6.93–7.04 (m, HC5 + HC6), 7.33 (d, HC7); 3-methylindole (1:1 D_2O/CD_3OD) δ 2.25 (s, CH_3), 7.06 (s, HC2), 7.53 (d, HC4), 7.0–7.2 (m, HC5 + HC6), 7.37 (d, HC7).

Iodide quenching of photochemical isotope exchange was monitored on a Hewlett-Packard 5971A MSD interfaced to a 5890 Series II GC. A saturated solution of 3-methylindole in D_2O was prepared by sonication for 1 h. The solution was made 0.5 M in glycine by adding solid $D_2NCD_2CO_2D$. One-milliliter samples were placed in 1-cm quartz cells containing various amounts of solid KI. Samples were irradiated as described above, except the lamp was defocused to reduce light intensity. Minor amounts of iodide oxidation product were detected by absorbance under these irradiation conditions: $<4 \times 10^{-6}$ M I_3^- after 20 min of irradiation of 0.3 M KI in H_2O, and about 2×10^{-5} M I_3^- after 3 h. Next, 0.1-mL aliquots were removed and extracted with 2 mL of D_2O-saturated ether containing 0.1 mM naphthalene as internal standard. A portion of the ether phase was separated on a Carbowax 20-m capillary column. Mass spectra were obtained by EI ionization at a 500-V threshold. Molecular ions of naphthalene at m/z 128 and 3-methylindole-d_2 at m/z 133 were used to quantify H–D exchange. 3-Methylindole-*d* with a deuteron on the indole nitrogen gives ions at m/z (relative intensity) 130 (82 ± 10) $[M - D]^+$, 131 (100) $[M - H]^+$, 132 (35 ± 6) M^+, and ^{13}C isotope of M^+ at 133 (3.5 ± 0.7). H–D exchange of one ring carbon proton in 3-methylindole-*d* gives 3-methylindole-d_2 with ions $[M - D]^+$ at 131, $[M - H]^+$ at 132, M^+ at 133, and ^{13}C isotope of M^+ at 134. In a mixture of 3-methylindole-*d* and 3-methylindole-d_2, the peak at m/z 133 comprises the molecular ion of 3-methylindole-d_2 as well as the ^{13}C isotope of the molecular ion of 3-methylindole-*d*. At early times in the photochemical reaction, only one H–D exchange occurs per molecule and the only product is 3-methylindole-d_2. The concentration of 3-methylindole-d_2 is proportional to X,

$$X = I_{133}/I_{128} - y \quad (1)$$

where I_{133} is the total intensity at m/z 133, I_{128} is the intensity of M^+ of the naphthalene standard, and y

$$y = I^0_{133}/I^0_{128} - I_{134}/I_{128}$$

is the fractional contribution of the ^{13}C isotope of M^+ of 3-methylindole-*d*. I^0_{133} is the intensity of the ^{13}C isotope of M^+ of pure 3-methylindole-*d* in an unirradiated sample, and I_{134} is the intensity of the ^{13}C isotope of M^+ of 3-methylindole-d_2. For pure 3-methylindole-*d*, $I^0_{133}/I^0_{128} = 0.015 \pm 0.003$.

Table I. Fluorescence Quantum Yields and Lifetimes in Various Solvents at 25 °C

solvent	Φ^a	τ,[b] ns	$10^{-7}\ k_r$, s^{-1}	$10^{-7}\ k_{nr}$, s^{-1}
		2-Methylindole		
H_2O	0.12 ± 0.01	1.9 ± 0.02	6.3 ± 0.5	46 ± 1
D_2O	0.25 ± 0.02	3.7 ± 0.05	6.8 ± 0.3	20 ± 1
CH_3OH	0.32	3.6 ± 0.05	8.9	19
CH_3OD	0.42	4.0 ± 0.1	10.5	15
CH_3CN	0.30 ± 0.03	3.7	8.1 ± 0.8	19 ± 1
1:1 H_2O/CH_3OH	0.43 ± 0.03	4.8 ± 0.02	9.0 ± 0.7	12 ± 1
1:1 D_2O/CH_3OD	0.55 ± 0.04	5.9 ± 0.2	9.3 ± 0.7	7.7 ± 0.9
		3-Methylindole		
H_2O	0.34 ± 0.02	8.2 ± 0.1	4.2 ± 0.3	8.2 ± 0.3
D_2O	0.50 ± 0.03	12.0	4.2 ± 0.3	4.2 ± 0.3
1:1 H_2O/CH_3OH	0.43	9.9	4.4	5.7
1:1 D_2O/CH_3OD	0.54	12.1	4.5	3.8
		N-Methylindole		
H_2O	0.46	8.10 ± 0.05	5.7	6.8
		N,2-Dimethylindole		
H_2O	0.36	5.5	6.5	12

[a] 280-nm excitation wavelength. Errors from 2 to 5 experiments. [b] 296-nm excitation wavelength. Errors from 2 to 5 experiments.

Results and Discussion

Solvent Quenching. Nonradiative processes in aqueous solutions of simple indoles include internal conversion, intersystem crossing, and solvent isotope effect. The fluorescence quantum yield Φ and lifetime τ are given by

$$\Phi = k_r/(k_r + k_{nr}) \quad (2)$$

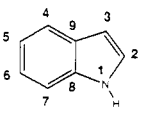
$$\tau^{-1} = k_r + k_{nr} \quad (3)$$

where k_r and k_{nr} are the radiative and nonradiative rates

$$k_{nr} = k_{ic} + k_{isc} + k_{si} \quad (4)$$

Here k_{ic} is the internal conversion rate, k_{isc} is the intersystem crossing rate, and k_{si} is the isotopically sensitive quenching rate. Excited-state proton transfer reactions leading to exchange of ring carbon protons in aromatic compounds can be monitored by ^1H NMR.[24] Detection of solvent-induced H–D exchange on the indole ring requires that the exchange reaction compete efficiently with other deactivation channels and that the compound be sufficiently soluble for NMR experiments. These conditions can be met in aqueous methanol solutions where simple indoles have large deuterium isotope effects.

Methyl substitution of ring carbons enhances the deuterium isotope effect in indoles at room temperature. For example, the quantum yield ratio in D_2O and H_2O is about 1.3 for indole, 1.5 for 3-methylindole, and 2.1 for 2,3-dimethylindole.[11,17] Table



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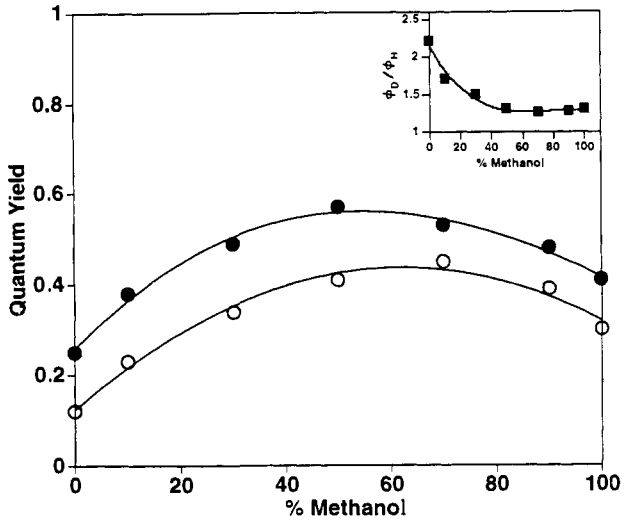

Figure 1. Deuterium isotope effect on quantum yield of 2-methylindole in aqueous methanol solutions at 25 °C: (○) protium solvent, (●) deuterium solvent. Inset shows quantum yield ratio in deuterium and protium solvents.

I shows quantum yield and lifetime data for 2- and 3-methylindole in different isotopic solvents at 25 °C. The quantum yield and lifetime ratios in D_2O and H_2O are about 2.0 for 2-methylindole, dropping to 1.3 in methanol. The radiative rates do not change from protium to deuterium solvent, indicating that the increases in quantum yield and lifetime in deuterium solvents are due to decreases in the nonradiative rates. Internal conversion and intersystem crossing rates are expected to depend little, if at all, on solvent isotope. In deuterated solvents the indole N–H proton exchanges within seconds.[28,29] Two facts argue that the isotope effect is not due to deuterium substitution on the indole nitrogen. First, in stopped-flow experiments the isotope effect on the fluorescence of simple indoles reaches its maximal value within 1 ms and remains constant,[12] while absorbance changes due to N–H exchange go on for about 500 ms.[29] Second, tryptophan and tryptophan-d_4 have the same lifetimes in dimethyl sulfoxide.[19] Internal conversion and intersystem crossing rates are governed by Franck–Condon factors,[30] which may be influenced by solvent.[31] The lifetime of 2-methylindole was measured in cyclohexane and perdeuteriocyclohexane. The values 3.45 ns for C_6H_{12} and 3.50 ns for C_6D_{12} are the same within experimental error. This implies that internal conversion and intersystem crossing do not involve strong coupling of indole vibrations to the solvent.

Figure 1 plots the dependence of the fluorescence quantum yield of 2-methylindole on methanol concentration. The quantum yield reaches a maximum at about 70% methanol in H_2O/CH_3OH mixtures and at about 50% methanol in D_2O/CH_3OD mixtures. Indole gives a similar curve with a maximum at 40% methanol in H_2O/CH_3OH mixtures at 25 °C;[17] the solvent composition at the maximum depends on temperature.[32] The quantum yield ratio for 2-methylindole in deuterium and protium solvent decreases from 2.1 in water to 1.3 in 50% methanol, leveling off at higher methanol concentration. In 30% methanol, the quantum yield ratio is about 1.5 and the solubility of 2-methylindole (3 mg/mL) is sufficient for NMR studies.

Photochemical isotope exchange experiments were performed on 2-methylindole in 30% methanol. At pD 7.0, only 10 ± 5% H–D exchange was observed at the C3 position after 1.5 h of irradiation. A control experiment without irradiation shows that this is ground-state exchange.[33] Under the same irradiation conditions, a tryptophan solution in 90% methanol at pD 7.0 showed about 50% exchange at the C4 position after 0.5 h. A photochemical isotope exchange experiment using more intense irradiation was performed on a saturated solution of 2-methylindole in D_2O. No H–D exchange beyond ground-state exchange was detected after 4 h of irradiation. These experiments show no signs of excited-state H–D exchange at positions C3, C4, C5, C6, and C7 in 2-methylindole. They do not address exchange at the indole nitrogen or at C2, C8, and C9. Excited-state exchange on the indole nitrogen is not detectable because of the rapid ground-state exchange. Exchange at the quaternary carbons may be reversible before isotope scrambling occurs through hydride (deuteride) shifts with adjacent carbon centers.

Table II. Stern–Volmer Quenching Constants in Various Solvents at 25 °C

solvent	quencher[a]	K_{SV}, M^{-1}	10^{-8} k_q[b], M^{-1} s^{-1}
	2-Methylindole		
H_2O	$^+H_3NCH_2CO_2^-$	0.091[c]	0.47
D_2O	$^+D_3NCD_2CO_2^-$	0.029	0.078
1:1 H_2O/CH_3OH	$^+H_3NCH_2CO_2^-$	2.1	4.4
1:1 D_2O/CH_3OD	$^+D_3NCD_2CO_2^-$	1.1	1.9
1:1 H_2O/CH_3OH	$^+(CH_3)_3NCH_2CO_2^-$	nd[d]	nd[d]
1:1 H_2O/CH_3OH	KI	11	23
1:1 D_2O/CH_3OD	KI	13	23
	3-Methylindole		
H_2O	$^+H_3NCH_2CO_2^-$	2.1	2.6
D_2O	$^+D_3NCD_2CO_2^-$	0.67	0.60
H_2O	$^+(CH_3)_3NCH_2CO_2^-$	0.033	0.040
H_2O	$^+(CH_3)_3NCH_2CH_2OH$	enh[e]	enh[e]
1:1 H_2O/CH_3OH	$^+H_3NCH_2CO_2^-$	3.6	3.6
1:1 D_2O/CH_3OD	$^+D_3NCD_2CO_2^-$	1.6	1.4
H_2O	KI	13	16
D_2O	KI	18	15
	Indole		
H_2O	$^+H_3NCH_2CO_2^-$	0.59	1.5[f]
H_2O	$^+(CH_3)_3NCH_2CO_2^-$	0.025	0.063[f]
H_2O	$CH_3CO_2^-$		<0.1[g]
H_2O	$CNCH_2CO_2^-$		0.6[g]
	N-Methylindole		
H_2O	$^+H_3NCH_2CO_2^-$	1.8	2.2
	N,2-Dimethylindole		
H_2O	$^+H_3NCH_2CO_2^-$	1.6	2.9
	3-Carboxy-1,2,3,4-tetrahydro-2-carboline		
H_2O	$^+H_3NCH_2CO_2^-$	0.87	1.5

[a] pH(D) 6.5 for glycine; pH 7.0 for other quenchers. [b] Lifetimes used to compute k_q from Table I and ref 6. [c] Lifetime quenching experiment. [d] Not detected. [e] Fluorescence increased about 30% in 0.3 M choline, 7% in 0.3 M choline plus 0.7 M $NaClO_4$. [f] Lifetime used to compute k_q from ref 17. [g] Data from ref 41.

Glycine Quenching. The ammonium group can act as an inter- as well as an intramolecular quencher of indole fluorescence.[10,16,25] Both excited-state proton[10] and electron[16,25] transfers have been invoked to explain the intermolecular quenching by amino acids, ^+H_3NOH, $^+H_3NNH_2$, and related compounds. The presence of external quencher Q adds another nonradiative process to eq 4

$$k_{nr} = k_{ic} + k_{isc} + k_{si} + k_q[Q] \quad (5)$$

where k_q is the bimolecular quenching rate constant. The value of k_q is determined from the Stern–Volmer constant $K_{SV} = k_q\tau_0$, where τ_0 is the lifetime in the absence of quencher.

$$\Phi_0/\Phi = 1 + K_{SV}[Q] \quad (6)$$

Φ_0 and Φ denote the quantum yield in the absence and presence of quencher. The quenching rate constant $k_q = \gamma k_d$ is proportional to the diffusion-limited rate constant k_d through the quenching efficiency γ.[34] Figure 2 shows a Stern–Volmer plot for the

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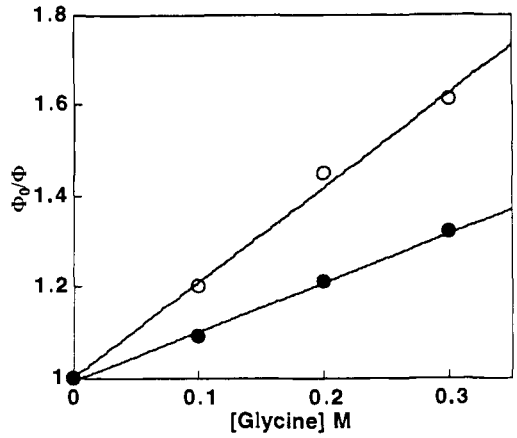

Figure 2. Stern–Volmer plot of 2-methylindole in 50% methanol quenched by glycine at 25 °C: (○) 50% H_2O/CH_3OH, (●) 50% D_2O/CH_3OD.

Table III. Quenching Rate Constants of 3-Substituted Indoles by Ammonium in H_2O at 25 °C

quencher	pH	pK_a	10^{-8} k_q, M^{-1} s^{-1}
3-Methylindole			
$NH_4CH_3CO_2$	7.0	9.7	0.1
NH_4Cl	7.0	9.7	0.2[a]
$^+NH_3CH_2CO_2^-$	6.5	9.6	2.6
3-(2′-Hydroxyethyl)indole[b]			
NH_4Cl	7	9.7	0.2
$^+NH_3CH_2CO_2^-$	7	9.6	4.2
Gly–Gly	6	8.6	13.5
$^+H_3NNH_2$	8.2	8.2	12.5
^+H_3NOH	4.2	6.2	22.7

[a] Ionic strength held constant at 1 M with $NaClO_4$. [b] Data from ref 16.

quenching of 2-methylindole fluorescence by glycine in 50% water/methanol mixture. Table II presents glycine quenching data for several indole derivatives in various solvents. The absorption spectra of the derivatives are the same in the absence and presence of 1 M glycine, indicating no ground-state complexation. The shape of the emission spectrum is not changed by glycine. The quenching rate constant k_q depends on the compound and the solvent. In water, k_q varies from 4.7×10^7 M^{-1} s^{-1} for 2-methylindole to 2.9×10^8 M^{-1} s^{-1} for *N*,2-dimethylindole. These values are 1–2 orders of magnitude slower than the diffusion-limited rate $k_d \approx 7 \times 10^9$ M^{-1} s^{-1}. The quenching rate is faster in 50% water/methanol mixtures than in water, almost 10-fold for 2-methylindole but only 1.4- to 2.4-fold for 3-methylindole. The diffusion rate is slower in 50% water/methanol, which has about 1.8-fold higher viscosity than water.

Quenching by glycine is faster in protium than in deuterium solvents (Table II). The ratio of the quenching rate constants $k_q(H)/k_q(D)$ is 2.3 and 2.6 for 2- and 3-methylindole in 50% water/methanol, increasing to 4.3 for 3-methylindole in water. The quantum yield ratios for 2- and 3-methylindole in 50% D_2O/CD_3OD and 50% H_2O/CH_3OH increase from about 1.3 in the absence of glycine to about 1.7 in the presence of 0.3 M glycine. The isotope effect on k_q implicates an excited-state proton transfer reaction in the collisional quenching mechanism. As in the case of the acid-catalyzed[9] and intramolecular proton transfers,[10] the intermolecular reaction yields a nonfluorescent product. There is no isotope effect on k_q for quenching by iodide, which presumably quenches by an external heavy atom effect on intersystem crossing rates.[30]

The intermolecular quenching of indole fluorescence by amino acids has been attributed to excited-state electron transfer.[16,25] Both the carboxylate and the ammonium of amino acids have been proposed to be electron acceptors. In general, carboxylate is a poor intermolecular quencher.[25] Formate, acetate, hydroxyacetate, propionate, and butyrate quench indole fluorescence with rate constants $k_q < 1 \times 10^7$ M^{-1} s^{-1}, and cyanoacetate quenches with $k_q = 6 \times 10^7$ M^{-1} s^{-1}. These values are low compared to $k_q = 1.5 \times 10^8$ M^{-1} s^{-1} for glycine. Ammonium is a better intermolecular quencher than carboxylate. The quenching rate constant increases with the acidity of the ammonium group (Table III). However, the pK_a of ammonium ion is about the same as glycine, while the quenching rates of ammonium acetate and ammonium chloride are lower by factors of 26 and 13, respectively. Acids with lower pK_as being better proton donors, the rough dependence of k_q values for ammonium on pK_a is consistent with a proton transfer quenching mechanism. Finally, derivatives lacking ammonium protons, *N*,*N*,*N*-trimethylglycine and (2-hydroxyethyl)-trimethylammonium (choline), do not quench the fluorescence of indoles.

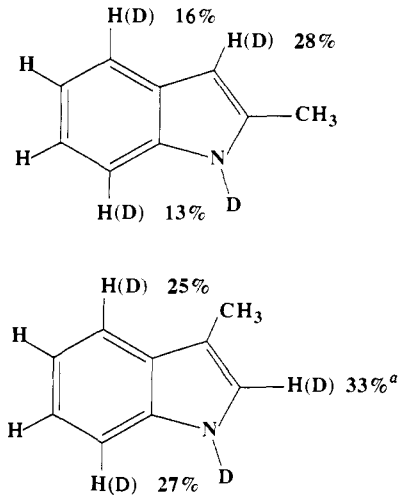

Figure 3. Photochemical H–D exchange of 2- and 3-methylindole in 50% D_2O/CD_3OD containing 0.3 M glycine-d_5 at 30 min of irradiation. Superscript indicates HC2, HC5, and HC6 resonances overlap. Exchange at HC2 is estimated by integration of expanded spectrum.

Table IV. Glycine-Induced Photochemical H–D Exchange in 2-Methylindole[a]

irradiation time, min	exchange, %		
	C3[b]	C4	C7
3	9	5	4
10	18	10	6
30	28	16	13
60	40	22	18

[a] 50% D_2O/CD_3OD, 0.3 M $^+D_3NCD_2CO_2^-$. [b] Ground-state exchange subtracted.

Glycine-Induced Photochemical Isotope Exchange. The intramolecular proton transfer reaction in tryptophan and tryptamine results in a highly selective H–D exchange at the C4 position on the indole ring.[14,22,23] Figure 3 shows that the intermolecular reaction of 2- and 3-methylindole with 0.3 M glycine-d_5 in 50% D_2O/CD_3OD induced H–D exchange at three positions on the ring. In 2-methylindole the exchange is fastest at C3 and occurs with similar rates at C4 and C7 (Table IV). As before, we are unable to detect exchange at the methyl-substituted carbons and other "invisible sites": N1, C8, and C9.

The photochemical H–D exchange reaction in tryptophan is suppressed by KI with the same Stern–Volmer constant as fluorescence quenching.[14] In order to confirm that the intermolecular proton transfer reaction also occurs in the excited singlet state, total H–D exchange was monitored by mass spectrometry in the absence and presence of KI. The dependence of the reaction quantum yield for H–D exchange on iodide concentration should obey the Stern–Volmer equation

$$\Phi_{R_0}/\Phi_R = 1 + K_{SV}[I^-] \quad (7)$$

where Φ_{R_0} and Φ_R are the reaction quantum yields in the absence

(34) Eftink, M. R.; Ghiron, C. A. *Anal. Biochem.* **1981**, *114*, 199–227.

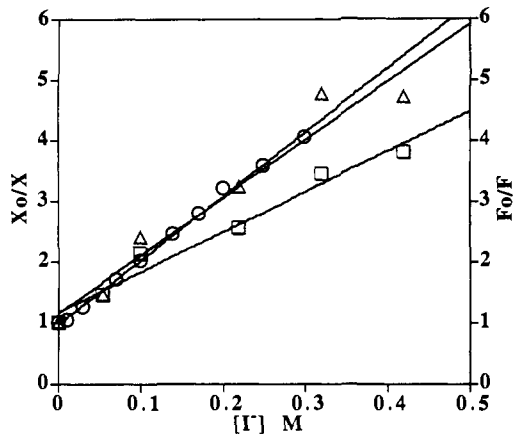

Figure 4. Stern–Volmer plot of iodide quenching of H–D exchange and fluorescence of 3-methylindole in D_2O containing 0.5 M glycine-d_5. H–D exchange neglecting correction for ^{13}C isotope after (□) 10 min and (△) 20 min of irradiation; (○) fluorescence quenching.

and presence of KI. If the light source and irradiation times are the same for all samples, then the reaction quantum yield ratio Φ_{R_0}/Φ_R equals the exchange ratio X_0/X, where X_0 and X represent exchange in the absence and presence of KI as defined in eq 1. Samples of 3-methylindole in D_2O containing 0.5 M glycine-d_5 and various concentrations of KI were irradiated for 10- and 20-min time intervals. The total amount of exchange in the absence of KI was estimated by ^1H NMR to be 16 and 26%, respectively. Figure 4 shows Stern–Volmer plots of the iodide quenching data. The Stern–Volmer constants for H–D exchange without correction for ^{13}C isotope are $K_{SV} = 6.6\ M^{-1}$ for 10 min and $K_{SV} = 9.5\ M^{-1}$ for 20 min of irradiation. These values are somewhat lower than the $K_{SV} = 11\ M^{-1}$ for fluorescence quenching in the presence of 0.5 M glycine. Correcting for the ^{13}C isotope contribution using eq 1 gives $K_{SV} = 19\ M^{-1}$ for 10 min and $K_{SV} = 21\ M^{-1}$ for 20 min of irradiation.

INDO/S-CI calculations of the ^1L_a and ^1L_b excited states of indole show increased π-electron density on C4 and C7 compared to the ^1A_g ground state.[35] The increase in electron density is larger for the more polar ^1L_a state, which is the emitting state in polar solvents at room temperature. In the ^1L_a state, there is also a similar increase in electron density on C9, a smaller increase on C6, and large decreases in electron density on N1 and C3. The observation of photochemical H–D exchange at C4 and C7 in 2- and 3-methylindole is consistent with the theoretical calculations for indole. Electron donation by the methyl groups on the indole ring probably accounts for the excited-state exchange at C2 or C3. In the proposed mechanism for the intramolecular proton exchange in tryptophan zwitterion, the alanyl side chain loops back over the indole ring and delivers an ammonium proton to C4.[14] Intramolecular proton exchange has also been observed at C8 (equivalent to indole C7) in 3-[9-(1,2,3,4-tetrahydrocarbazolyl)]-propylamine.[22] Stopped-flow absorbance and fluorescence experiments confirm that the proton comes from the ammonium group, not the solvent.[12] In our case the ammonium group of glycine is the proton donor, which selectively attacks indole ring carbons having higher electron density in the excited state. These positions include C4, C7, C9, and presumably also C2 in 3-methylindole and C3 in 2-methylindole. (C3 protonation may also come from C2 protonation followed by 1,2-hydride rearrangement.) The indole nitrogen may also be an exchangeable site. Despite loss of electron density in the excited state, it is still more basic than any of the carbons. However, exchange at N1 is not detectable.

Temperature Dependence. In the presence of glycine, there will be at least two temperature-dependent terms in eq 5: solvent and collisional quenching. The preceding paper[6] associates the solvent quenching mechanism with large frequency factors A of about 10^{15}–$10^{17}\ s^{-1}$ and activation energies E^* of about 11–13 kcal/mol.

(35) Callis, P. R. *J. Chem. Phys.* **1991**, *95*, 4230–4240.

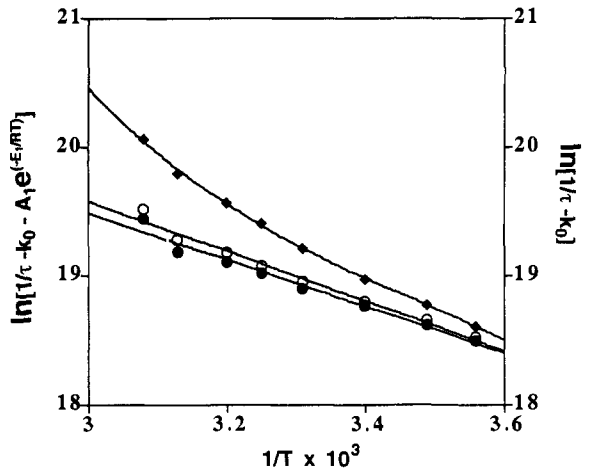

Figure 5. Temperature dependence of lifetime for 3-methylindole in H_2O containing 0.5 M glycine. (■) Plot of ln $(\tau^{-1} - k_0)$ with $k_0 = 7.5 \times 10^7\ s^{-1}$ assuming one temperature-dependent process. Plot of ln $(\tau^{-1} - k_0 - A_1 \exp[-E_1^*/RT])$ with $k_0 = 7.5 \times 10^7\ s^{-1}$ assuming two temperature-dependent processes. Arrhenius parameters for solvent quenching were used to construct the plots: (○) $A_1 = 7.8 \times 10^{16}\ s^{-1}$, $E_1^* = 12.7$ kcal/mol;[37] (●) $A_1 = 4 \times 10^{16}\ s^{-1}$, $E_1^* = 12.2$ kcal/mol at pH 11.[13]

Diffusional activation energies are typically 3–4 kcal/mol.[36] The presence of two Arrhenius factors with distinct activation energies will produce curvature in the Arrhenius plot. The fluorescence decay of 3-methylindole in H_2O containing 0.5 M glycine was measured in the range 8–52 °C. Figure 5 shows Arrhenius plots of the lifetime data assuming a temperature-independent rate $k_0 = k_r + k_{nr}^0$. The upper curved line is a plot of ln $(\tau^{-1} - k_0)$ vs $1/T$ with $k_0 = 7.5 \times 10^7\ s^{-1}$.[13] The nonlinearity is a sign of more than one temperature-dependent nonradiative process. The plot can be linearized by subtracting out the temperature-dependent contribution from solvent quenching. The lower lines plot ln $(\tau^{-1} - k_0 - A_1 \exp[-E_1^*/RT])$ vs $1/T$ for two different sets of Arrhenius parameters for solvent quenching in 3-methylindole: $A_1 = 7.8 \times 10^{16}\ s^{-1}$ and $E_1^* = 12.7$ kcal/mol in H_2O;[37] and $A_1 = 4 \times 10^{16}\ s^{-1}$ and $E_1^* = 12.2$ kcal/mol at pH 11.[13] The best straight lines through the points yield the respective Arrhenius parameters for glycine quenching of 3-methylindole fluorescence: $A_2 = 9.2 \times 10^{10}\ s^{-1}$, $E_2^* = 3.7$ kcal/mol and $A_2 = 5.3 \times 10^{10}\ s^{-1}$, $E_2^* = 3.5$ kcal/mol. These are close to the values of the Arrhenius parameters $A = 3.8 \times 10^{11}\ s^{-1}$ and $E^* = 4.1$ kcal/mol reported for the intramolecular proton transfer reaction in tryptamine.[22] Such small frequency factors and activation energies are typical of diffusion-controlled reactions with $A \approx 10^{11}\ s^{-1}$ and $E^* \approx 4$ kcal/mol.[36] By associating the collisional quenching term in eq 5 with the weakly temperature-dependent nonradiative process, so that $k_q[Q] = A_2 \exp[-E^*/RT]$, we calculate $k_q = (2.9\text{–}3.6) \times 10^8\ M^{-1}\ s^{-1}$ at 25 °C in reasonable agreement with the value of $2.6 \times 10^8\ M^{-1}\ s^{-1}$ obtained from the Stern–Volmer equation (Table II).

Conclusions

We have examined two isotopically sensitive nonradiative processes in simple indoles for evidence of excited-state proton transfer: water- and glycine-induced fluorescence quenching. No H–D exchange was detected in photochemical isotope exchange experiments on 2- and 3-methylindole in water or water/methanol mixtures. The absence of exchange is consistent with two quenching mechanisms discussed in the companion paper: "invisible" or incomplete proton transfer and exciplex formation.[6] On the other hand, H–D exchange was detected at several ring carbons in the presence of glycine. The intermolecular excited-state proton transfer reaction with glycine as proton donor has

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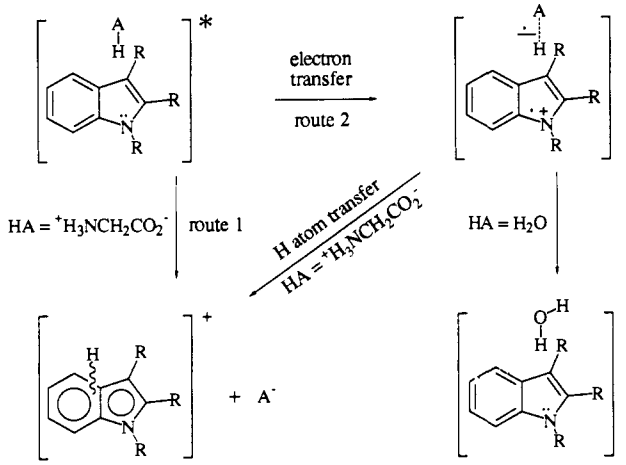
(37) Walker, M. S.; Bednar, T. W.; Lumry, R. In *Molecular Luminescence*; Lim, E. C., Ed.; Benjamin: New York, 1969; pp 135–152.

Scheme I



low activation energy E^* and frequency factor A in comparison to the intrinsic quenching by water, which has high activation energy and frequency factor.

We visualize these two quenching processes according to Scheme I. There is no detectable photochemical H–D exchange at indole ring carbons from solvent despite sizable deuterium isotope effects on the fluorescence quantum yield and lifetime. $H(D)_2O$ is too poor an acid to give complete proton transfer. In the presence of a better proton donor like glycine, photochemical H–D exchange is readily observed. The fact that *N,N,N*-trimethylglycine and choline do not quench indole fluorescence implies that at least one H–N bond is essential for quenching. Two explanations are consistent with the data. In route 1, the more acidic ammonium group of glycine quenches indole fluorescence by direct proton transfer. This is an intermolecular version of the intramolecular mechanism proposed for tryptophan.[14] Stopped-flow absorbance and fluorescence studies show that the amino protons interact directly with the excited indole ring.[12] In route 2, a charge-transfer exciplex forms with glycine as it does with solvent, except H atom transfer from the glycine radical cation to the indole radical anion competes with back electron transfer to give H–D exchange. Orientation effects in both mechanisms would account for bimolecular rate constants for glycine quenching below the diffusion limit. The electron–H atom transfer mechanism (route 2) is analogous to a fascinating theory for nucleophilic substitution reactions which postulates one electron transfer followed rapidly by group transfer.[38-40] A reviewer proposed a third explanation, in which the charge of the quencher distinguishes proton transfer from solvent quenching. In this mechanism the induced excited-state dipole of indole interacts with a charged proton donor to stabilize an intramolecular charge-transfer complex. The reviewer suggested that other cationic donors, such as choline, may be effective quenchers and proton donors. Our observation that choline slightly enhances indole fluorescence does not support this premise.

The two isotopically sensitive nonradiative processes in indoles have implications for protein fluorescence. Tryptophans that are accessible to solvent will show deuterium isotope effects on the fluorescence quantum yield and lifetime if the water quenching process is competitive with the other decay pathways. The magnitude of the isotope effect depends on solvent, being lower in methanol than in water. Therefore, the solvent isotope effect in proteins may depend on the local environment of surface tryptophans. The fluorescence of tryptophans in proximity to good proton donors may be quenched by excited-state proton transfer. Possible proton donors in proteins include histidine (pK_a = 6.0), the amino terminus ($pK_a \approx 7$), cysteine (pK_a = 8.4), tyrosine (pK_a = 10.5), lysine (pK_a = 10.7), and arginine (pK_a = 12.5). The free amino acids histidine, cysteine, lysine, and arginine quench the fluorescence of *N*-acetyltryptophan in water.[25] External tryptophans undergoing excited-state proton transfer would show additional deuterium isotope effects if the quenching group is readily exchanged. Buried tryptophans might also have isotope effects after unfolding and refolding the protein in deuterated solvent. In either case, excited-state proton transfer can be detected by photochemical isotope exchange experiments using ^1H NMR or mass spectrometry. A number of quenching mechanisms have been proposed for tryptophan fluorescence in proteins, including electron transfer, electron exchange, and energy transfer. Our work adds two isotopically sensitive processes to the list: water quenching and proton transfer. The latter quenching mechanism can be definitively established. Putative proton donors may then be identified by inspection of protein crystal structures.

Acknowledgment. This work was supported by NIH Grant GM42101.

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(39) Pross, A. *Adv. Chem. Soc.* **1987**, *215*, 331–338.
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