Fluorescence Properties of Benz[*f*]indole, a Wavelength and Quenching Selective Tryptophan Analog

Bo Liu and Mary D. Barkley*

Department of Chemistry, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

Guillermo A. Morales and Mark L. McLaughlin

Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803

Patrik R. Callis

Department of Chemistry & Biochemistry, Montana State University, Bozeman, Montana 59717

Received: July 23, 1999; In Final Form: December 20, 1999

Tryptophan analogues with unique spectral and photophysical properties offer intrinsic fluorescent probes for studying peptide−protein and protein−protein interactions. Two benzannulated indole derivatives, benz[*f*]indole and 3-methylbenz[*f*]indole, were synthesized and their fluorescence was characterized. The absorption and fluorescence emission spectra are red shifted about 75 nm to the red of the spectra of indole and 3-methylindole. INDO/S−CIS computations indicate two nearly degenerate lowest excited singlet states, analogous to the ^1L_a and ^1L_b transitions of indoles but with almost collinear transition moments. The limiting excitation anisotropy spectra increase and the emission anisotropy spectra decrease with increasing wavelength, probably due to vibronic coupling of the ^1L_a state with higher energy B states. Solvent and temperature effects on the wavelength of maximum emission argue that emission occurs from ^1L_a in polar environments. The fluorescence quantum yields and lifetimes are high and essentially independent of temperature and solvent isotope. Quantum yields are 0.80 for both compounds. Lifetimes are 19 ns for benz[*f*]indole and 25 ns for 3-methylbenz[*f*]indole. The fluorescence is not quenched by most protein functional groups in Stern−Volmer experiments, though it is quenched weakly by strong electron acceptors, such as histidine and cysteine. These results indicate that two nonradiative processes of indoles, solvent quenching and excited-state proton transfer, are not important decay pathways of benzannulated indoles. A third nonradiative process, excited-state electron transfer, occurs in benzannulated indoles under more limited conditions than in indoles.

Introduction

Being an intrinsic fluorescent probe in peptides and proteins, tryptophan has attracted extensive study. The fluorescence wavelength maximum, quantum yield, and lifetime are highly sensitive to the local environment of the indole chromophore. The first absorption band of indole around 280 nm consists of two overlapping $\pi \rightarrow \pi^*$ electronic transitions, ^1L_a and ^1L_b. In most proteins and polar solvents, fluorescence occurs from ^1L_a.[1] The large increase in permanent dipole moment in the ^1L_a state compared to the ground state accounts for the large Stokes' shift in polar solvent due to inhomogeneous broadening and solvent relaxation. Consequently, in proteins the fluorescence wavelength maximum reports the local electrostatic environment due to an internal Stark effect.[2] Environmental sensitivity of the fluorescence quantum yield and lifetime comes from the multiple nonradiative decay pathways that compete with emission for deactivation of excited indole: intersystem crossing, solvent quenching, and excited-state proton and electron transfer reactions. The latter three quenching processes depend on type and proximity of solvent molecules, proton donors, and electron acceptors. Water quenching refers to an isotopically sensitive temperature-dependent nonradiative process that occurs in all indoles.[3] In proteins, six amino acid functional groups comprising eight amino acid side chains and the peptide bond quench indole fluorescence: two by excited-state proton transfer and six by excited-state electron transfer.[4] Given the complex photophysics, structural interpretation of fluorescence data from even a single tryptophan in proteins remains a daunting task.

Spectrally enhanced proteins produced by biosynthetic incorporation of tryptophan analogues are useful to study protein−protein and protein−nucleic acid interactions.[5,6] Analogues with minor modifications to the indole ring, such as 5-hydroxytryptophan and 7-azatryptophan, can be selectively excited at 310 nm in the presence of natural tryptophans. Like tryptophan, the absorption spectrum of 5-hydroxytryptophan has a maximum at 280 nm, but the shoulder on the red edge extends to about 320 nm. The absorption spectrum of 7-azatryptophan is less structured and is red-shifted about 10 nm compared to tryptophan. In benzannulated indole derivatives, such as the benzindoles and carbazoles, the long wavelength absorption maximum is shifted even further to the red.[7] Moreover, the smaller energy gap between the ground and first excited singlet states may simplify the photophysics of benzannulated tryptophan derivatives.

This paper reports the spectroscopic and photophysical properties of benz[*f*]indole (BzIn) and 3-methylbenz[*f*]indole (3-MeBzIn). Fluorescence emission, excitation, and limiting anisotropy spectra are presented and interpreted in terms of two

* Corresponding author e-mail: mdb4@po.cwru.edu

10.1021/jp9925536 CCC: $19.00
Published on Web 02/08/2000

nearly degenerate low-lying excited states, which are shown to be closely related to the ^1L_a and ^1L_b states of indole and tryptophan by INDO/S−CIS computations.[8] As before,[4] we identify the nonradiative processes which these chromophores undergo from the dependence of fluorescence quantum yield and lifetime on temperature, solvent isotope, and solute quencher. The unique fluorescence properties and potential utility of benzannulated tryptophans as probes of peptide structure and peptide−protein interactions are discussed.

Experimental Section

Chemicals. 3-Methylcarbazole was prepared according to a literature procedure.[9] The final product was recrystallized twice from warm methanol. Indole (Sigma) was recrystallized twice from aqueous ethanol. 3-Methylindole (Aldrich, 99+%) was used as received. All indole derivatives were stored in an inert atmosphere at 4 °C in the dark. Aqueous solutions of benzannulated indoles contain 10% methanol (vol/vol) to increase solubility. Acetone (Fisher), 3-methyl-2-butanone (Aldrich), and 3,3-dimethyl-2-butanone (Aldrich) were distilled twice. Amino acids and *N*-acetyl derivatives were purchased from Sigma. Malonamide (Aldrich), succinamide (Aldrich), *N*-acetyltyrosine, histidine, *N*-acetylphenylalanine, *N*-acetylcysteine, and lysine were recrystallized from water. Glutamic and aspartic acids were recrystallized by carefully adjusting monosodium glutamate and aspartate solutions to pH 3.0 with 6 N HCl. *N*-Acetylglutamine and *N*-acetylasparagine were recrystallized from methanol/ether mixture. Glycerol (glass distilled) was from EM Science. Other chemicals from Sigma and Aldrich were highest grade available.

2-Methyl-3-nitro-naphthalene-bis(hexachlorocyclopentadiene)-adduct. 2-Methylnaphthalene-bis(hexachlorocyclopentadiene)-adduct (68.77 g, technical grade, Aldrich) was dissolved in 1.1 L of dichloromethane. The solution was filtered and 990 g of fuming nitric acid was added while stirring. After 30 min the reaction mixture was poured into 4 L of ice−water. The organic layer was washed and evaporated and the residue was recrystallized from toluene (62.23 g, 84.9% yield): mp 249−250 °C. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.41 (s, 1H), 7.73 (s, 1H), 4.03−3.96 (t, 1H), 3.57−3.54 (d, 1H), 2.65 (s, 3H). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ 147.75, 133.86, 133.80, 133.01, 132.80, 129.52, 129.40, 127.95, 125.23, 101.14, 84.52, 84.46, 82.15, 46.89, 46.67, 41.58, 41.45, 20.43.

2-Methyl-3-nitronaphthalene. 2-Methyl-3-nitro-naphthalene-bis(hexachlorocyclopentadiene)adduct (82.21 g) was pyrolyzed at 350 °C under vacuum. The resulting slurry was cooled in an ice−water bath, washed with cold hexanes, and crystallized from hexanes (20.8 g, 99.3% yield): mp 119−121 °C. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.50 (s, 1H), 7.91−7.88 (d, 1H), 7.82−7.78 (d, 1H), 7.72 (s, 1H), 7.65−7.51 (m, 2H), 2.71 (s, 3H). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ 147.75, 135.01, 131.22, 130.77, 129.28, 128.98, 127.05, 125.13, 20.44.

Benz[f]indole. 2-Methyl-3-nitronaphthalene (0.37 g), *N*,*N*-dimethylformamide dimethyl acetal (2.38 g), and pyrrolidine (1.42 g) were mixed in 10 mL of dry DMF and refluxed for 6 h under argon. After removing the solvent the dark residue was dissolved in methanol, mixed with Pd−C (10%), and hydrogenated at 30 psi for 9 h at room temperature. The mixture was filtered, evaporated, and separated by flash chromatography on a silica gel column (1:1 hexanes/CH_2Cl_2) to afford white flakes (0.04 g, 12% yield): mp 185−186 °C. ^{1}H NMR (CD_3OD, 400 MHz): δ 8.02 (s, 1H), 7.86−7.79 (m, 3H), 7.44 (t, 1H), 7.27−7.19 (m, 3H), 6.56 (s, 1H). ^{13}C NMR (CD_3OD, 100 MHz): δ 138.49, 131.42, 131.30, 130.09, 129.78, 128.88, 128.16, 123.96, 122.87, 118.06, 106.99, 101.46. GC−MS: *m*/*z* 167, 139, 83, 70.

3-Methylbenz[f]indole. 3-Bromo-2-aminonaphthalene (0.221 g), 1-trimethylsilylpropyne (0.224 g), *n*-Bu_4NCl (0.278 g), Na_2CO_3 (0.265 g), $Pd(OAc)_2$ (22 mg), and PPh_3 (26 mg) were placed in a 100 mL three-necked round-bottom flask under argon, and 30 mL of anhydrous acetonitrile was cannulated into the flask with argon. The mixture was refluxed under argon at 100 °C for 4 h. Hydrogen chloride was bubbled through the solution for 2 min and the solution was refluxed for 1 h and then poured into 250 mL of 3 N NH_4OH. The final solution was extracted by ethyl acetate. The combined organic extract was washed and evaporated. The brown residue was taken up in CH_2Cl_2 and separated by silica gel chromatography (1:1 hexanes/CH_2Cl_2) (0.146 g, 80.8% yield). The final product was sublimed twice at 80−90 °C under reduced pressure: mp 192−193 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.03 (s, 1H), 7.98−7.94 (dd, 1H), 7.88−7.85 (dd, 1H), 7.71 (s, 2H), 7.39−7.23 (m, 2H), 7.11 (s, 1H), 2.42 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 137.36, 130.67, 130.52, 128.46, 127.47, 125.63, 123.87, 122.65, 111.26, 106.30, 10.08. GC−MS: *m*/*z* 181, 152, 139, 127, 90, 76, 63, 51. Anal. ($C_{13}H_{11}N$) H, N; C: calcd, C 86.15; found, C 85.33.

Absorbance and Fluorescence. Absorption spectra were measured at 25 °C on a Cary 3E UV−vis spectrophotometer. Extinction coefficients of benzindole and 3-methylbenzindole were determined from the absorbance of solutions containing 1.5 mg of benzannulated indole dissolved in 500 mL of 9:1 water/methanol: $\epsilon_{344} = 4.4 \times 10^3$ M^{-1} cm^{-1} for benzindole and $\epsilon_{353} = 4.5 \times 10^3$ M^{-1} cm^{-1} for 3-methylbenzindole. Sample absorbance was below 0.1 at the absorption maximum for steady-state fluorescence measurements. Fluorescence spectra were measured on an SLM 8000 photon counting spectrofluorometer with a single emission monochromator (1- or 4-nm band-pass). The excitation polarizer was set at 54.7°, and the emission polarizer was set at 0° to eliminate anisotropic effects. Background fluorescence from a solvent blank was subtracted. Between about 300 and 400 nm, excitation spectra were corrected by the internal quantum counter in the SLM operating in the ratio mode as judged by comparing the absorption and excitation spectra of anthracene in ethanol. Emission spectra were corrected for wavelength-dependent instrument response using correction factors determined with a standard lamp from Optronics. Quantum yields Φ were determined relative to quinine sulfate (Fisher) in 1 N sulfuric acid (GFS Chemicals, double distilled) at 365 nm excitation wavelength, 25 °C. The quantum yield of quinine sulfate was taken to be 0.546.[10]

Low temperature fluorescence spectra were measured using an Oxford model DN1704 liquid nitrogen cryostat with a model ITC-4 temperature controller. The sample cell was a quartz NMR tube cut to about 4 cm length. Solutions were placed in the cryostat at room temperature and the temperature was lowered to 130 K. Solutions in ethanol (AAPER) were prepared immediately prior to use and saturated with nitrogen. Limiting anisotropy spectra $r_o(\lambda)$ were calculated from

$$r_o(\lambda) = \{I_{VV}(\lambda) - G(\lambda)I_{VH}(\lambda)\}/\{I_{VV}(\lambda) + 2G(\lambda)I_{VH}(\lambda)\} \quad (1)$$

where the subscripts V and H denote vertically (0°) and horizontally (90°) polarized components of the intensity at wavelength λ, and $G(\lambda) = I_{HV}(\lambda)/I_{HH}(\lambda)$ is an instrumental correction factor.

Fluorescence decays were measured by time-correlated single photon counting and deconvolved as described elsewhere.[11] The laser system was a Coherent Antares 76-S mode-locked Nd:YAG laser and a model 701 dye laser. For experiments on benzannulated indoles, the laser dye was DCM, excitation

wavelength was 332 nm; emission wavelength was 450 or 470 nm (8-nm band-pass), and the instrument response was about 350 ps full width half-maximum (fwhm). For experiments on indoles, the laser dye was R6G, excitation wavelength was 290 nm, emission wavelength was 350 or 370 nm (8-nm band-pass), and the instrument response was about 100 ps fwhm. All decays gave good fits to monoexponential functions with reduced chi-square values of 1.1−1.6.

Stern−Volmer quenching rate constants k_q were determined from fluorescence quantum yield Φ and lifetime τ data in the absence (subscript 0) and presence of various concentrations of quencher Q in 9:1 methanol/water at 25 °C.

$$\Phi_0/\Phi = \tau_0/\tau = 1 + k_q\tau_0[Q] \qquad (2)$$

Computations. Absorbing and emitting states of benzindole and 3-methylbenzindole were modeled using the same programs, parameters, and analysis previously applied to indole[12] and 3-methylindole. The INDO/S−CIS method was used with 196 singly excited configurations. The Mataga−Nishimoto electron repulsion parameterization and the default interaction factors appropriate to that scheme were used. Geometries were obtained by MOPAC[13,14] using the AM1 Hamiltonian for the ground state.

Results and Discussion

Synthesis. Benzindole synthesis was first attempted more than a century ago by Fischer synthesis with acetone 2-naphthylhydrazone and was later improved.[15,16] Among the three isomeric benzindoles, benz[*e*]-, benz[*f*]- and benz[*g*]-indoles, the linear benz[*f*]indole is the most difficult to synthesize, because the Fischer indole synthesis methods are not effective in this case. When incorporated in place of indole in tryptophan, the linear benz[*f*]indole moiety offers the best mimic of the side-chain conformation and planar ring system of tryptophan. Recent syntheses of benz[*f*]indole are long synthetic routes.[17,18] Batcho and Leimgruber[19] reported a general, mild, two-step process for indole synthesis from 2-methylnitrobenzenes by condensation with formamide acetals followed by reduction. We have adapted this method to synthesize benz[*f*]indole. The required starting material, 2-methyl-3-nitronaphthalene, is prepared from the commercially available 2-methylnaphthalene-bis(hexachlorocyclopentadiene)adduct via nitration and high vacuum pyrolysis to unmask the naphthalene ring. The condensation reaction gave poor, but acceptable yields of benz[*f*]indole as other routes gave good yields of 3-substituted derivatives. For instance, the palladium-catalyzed hydroarylation of 1-trimethylsilylpropyne with 3-bromo-2-aminonaphthalene provides a good yield of 3-methylbenz[*f*]indoles.[17,20,21]

Absorption and Fluorescence Spectra. Figure 1 shows the absorption and fluorescence emission spectra of benzindole and 3-methylbenzindole in 10% CH_3OH. The absorption spectrum of benzindole is structured with a maximum at 344 nm and peaks at 330 and 358 nm. The spectrum of 3-methylbenzindole is less structured and red-shifted about 10 nm. The emission spectra of both compounds are broad and featureless with the 3-methylbenzindole spectrum about 25 nm to the red of the benzindole spectrum. Both compounds show large Stokes' shifts: about 70 nm for benzindole and 86 nm for 3-methylbenzindole. As temperature decreases from 298 to 150 K, the emission spectrum of benzindole in glycerol narrows from 57 to 45 nm fwhm and shifts to the blue (Figure 2). Vibrational structure begins to emerge below 253 K. In cyclohexane, the absorption and emission spectra of benzindole are structured

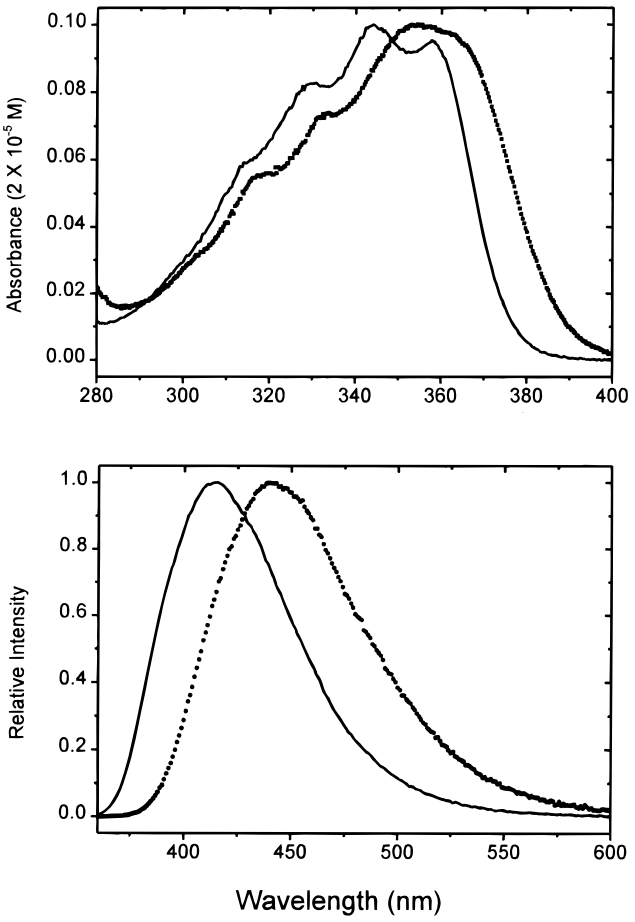

Figure 1. Absorption and emission spectra of benz[*f*]indole (—) and 3-methylbenz[*f*]indole (···) in 9:1 H_2O/CH_3OH, 25 °C. Upper panel: 2.3×10^{-5} M BzIn, 2.2×10^{-5} M 3-MeBzIn. Lower panel: excitation wavelength 348 nm.

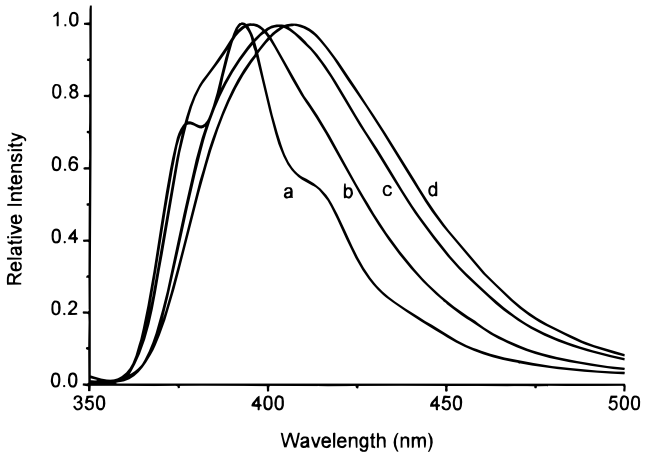

Figure 2. Temperature dependence of emission spectrum of benz[*f*]-indole in glycerol. 150 K (a), 253 K (b), 273 K (c), 298 K (d). Excitation wavelength 340 nm.

mirror images with almost no Stokes' shift (Figure 3). The absorption spectrum has a maximum at 354 nm, a sharp peak at 338 nm, and a peak at 323 nm. The emission spectrum has three well-resolved peaks: a maximum at 357 nm, a sharp peak at 377, and a peak at 396 nm. The solvent and temperature dependence of the emission spectrum of benzindole is characteristic of indole and its derivatives. Indeed, INDO/S−CIS computations reveal a strong parallel between indole and benzindole and the corresponding 3-methyl derivatives.[1,12] The

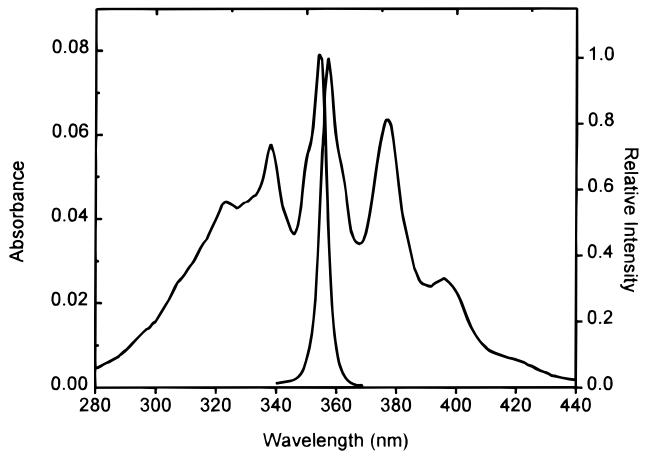

Figure 3. Absorption (left curve) and emission (right curve) spectra of benz[*f*]indole in cyclohexane, 25 °C. Excitation wavelength 330 nm.

two lowest excited singlet states are nearly degenerate, and their transition densities are characteristic of the ^1L_a and ^1L_b transitions of indoles.[1] As with indole, ^1L_a has slightly lower energy, the energy gap between ^1L_a and ^1L_b narrows upon methylation at the 3 position, and the transition to ^1L_a is several times more intense than to ^1L_b. The permanent dipole of ^1L_a is several Debyes larger than those of ^1L_b and the ground state. More specifically, the ground and ^1L_b state permanent dipoles are about 2 D, pointing toward the nitrogen from the center of the molecule, with the positive end pointing to the N. There is more electron transfer for ^1L_a, almost entirely along the long axis, in a direction away from the pyrrole end. The sensitivity of the fluorescence of the benzindoles to solvent and temperature is consistent with emission from ^1L_a.

Anisotropy Spectra. Figure 4 shows the excitation and limiting excitation anisotropy spectra of benzindole and 3-methylbenzindole in ethanol glass at 130 K observed at 390 nm emission wavelength. For both compounds the vibrational structure is better resolved in the glass than in ethanol solution at room temperature. The limiting anisotropy $r_o(\lambda)$ drops from a maximum value of 0.33 for benzindole and 0.4 for 3-methylbenzindole on the red edge of the excitation spectrum to around zero at 300 nm. The apparent rise in $r_o(\lambda)$ values of 3-methylbenzindole above the theoretical limit of 0.4 is due to errors in measurements below 375 nm including light scattering. Figure 5 shows the emission and limiting emission anisotropy spectra excited at 360 nm for benzindole and at 370 nm for 3-methylbenzindole. The anisotropy decreases monotonically with increasing emission wavelength in both cases. For benzindole, $r_o(\lambda)$ drops from 0.27 near the 0−0 band to ∼0.1 at 490 nm. For 3-methylbenzindole, the corresponding drop is from 0.3 to ∼0.15. The anisotropy spectra of benzindole in glycerol at 298 K measured in a 1 cm cell were also wavelength dependent (not shown). The excitation anisotropy spectrum observed at 410 nm emission wavelength dropped from about 0.17 at 390 nm to 0.05 at 300 nm; the emission anisotropy spectrum excited at 360 nm dropped from about 0.15 at 370 nm to 0.08 at 500 nm.

Although many aspects of the spectral behavior of benzannulated indoles are similar to those of indoles, the anisotropy is qualitatively different. For indole and 3-methylindole, the excitation anisotropy spectra show a sharp dip at the position of the ^1L_b origin, followed by a considerable increase at shorter wavelengths. The emission anisotropy spectra are virtually independent of emission wavelength. Valeur and Weber[22] were therefore able to use the excitation anisotropy spectra of indole

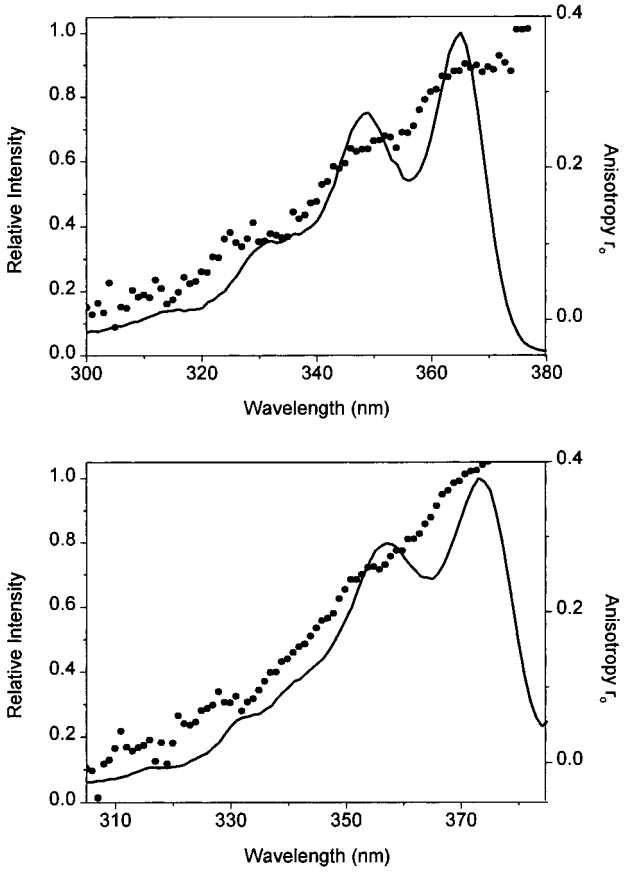

Figure 4. Excitation (—) and limiting excitation anisotropy (···) spectra of benz[*f*]indole (upper) and 3-methylbenz[*f*]indole (lower) in ethanol, 130 K. Emission wavelength 390 nm.

and tryptophan in propylene glycol at 245 K to resolve the indole absorption bands into ^1L_a and ^1L_b components by assuming a constant angle between the ^1L_a and ^1L_b transition moments. Their analysis was consistent with an angle of about 90° between the two moments, as also determined from polarized absorption spectra of single crystals.[23] An angle near 90° has been found by a variety of theoretical procedures, including the INDO/S−CIS method used in this study.[1] In contrast, the INDO/S−CIS computed transition dipole moment directions for benzindole and 3-methylbenzindole are seen in Figure 6 to be nearly parallel and approximately short-axis polarized. For naphthalene, ^1L_a is short-axis polarized by symmetry. The integrated absorptivity is computed to be an order of magnitude stronger for ^1L_a relative to ^1L_b, similar to the case of indole and naphthalene. Because of the wavelength dependence of the emission anisotropy, it is not justified to carry out the Valeur−Weber type of analysis for the benzindoles. In addition, an attempt to do so by us gave an angle of ∼45° between the moments, and worse, gave about equal integrated absorptivities for the two transitions.

We believe that naphthalene rather than indole is a better model for the anisotropy behavior of the benzindoles. It is known that the ^1L_a state of naphthalene shows strong vibronic coupling with intense B states lying at somewhat higher energies, both experimentally[24] and theoretically.[25] The experimental excitation anisotropy spectrum of naphthalene drops from 0.21 to zero across the ^1L_a band, with some modulation. When the naphthalene ^1L_a band is excited, the emission anisotropy spectrum drops from 0.21 to 0.13 with increasing wavlength, with considerable modulation because of the better resolved vibrational structure typical of nonpolar molecules. Similar large monotonic anisotropy changes across the ^1L_a band have been

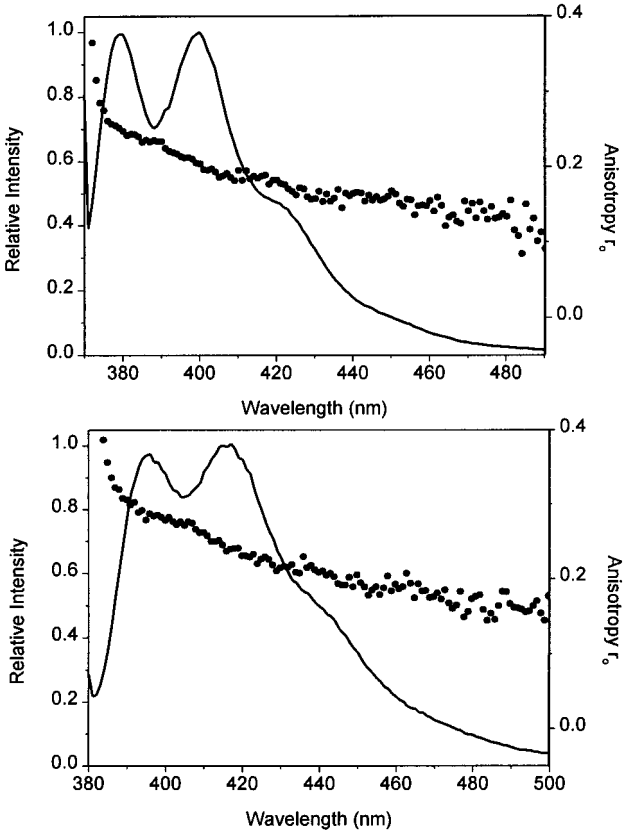

Figure 5. Emission (—) and limiting emission anisotropy (···) spectra of benz[*f*]indole (upper) and 3-methylbenz[*f*]indole (lower) in ethanol, 130 K. Excitation wavelength 360 nm for benz[*f*]indole and 370 nm for 3-methylbenz[*f*]indole.

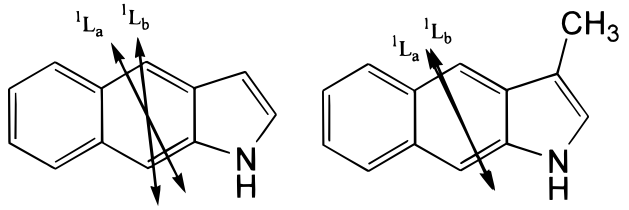

Figure 6. Transition moment directions calculated by INDO/S−CIS.

reported for quinoline,[24] isoquinoline,[24] tetraphene,[26] and carbazole.[27] The basis for such anisotropic behavior involving a single electronic state is well understood.[28] Zero point vibrational motion of one (or a few) normal modes of the right symmetry and form can mix strong, higher lying ^1B state character into the ^1L_a state with an amplitude proportional to the nuclear displacement. This, in turn, causes the electronic transition moment from the zero point vibrational wave function of the excited state to have a nonzero Herzberg−Teller integral with vibrational states of the ground electronic state that have one quantum of excitation in the active modes. The transition moment to the latter will have the polarization of the electronic state to which the mode couples the ^1L_a state.[28] Thus, in the simplified case of only one vibronically active mode coupling to a state polarized perpendicular to ^1L_a, the emission spectrum will have two progressions of the same shape: one beginning at the 0−0 having ^1L_a polarization; and the other displaced to longer wavelengths by one quantum of the coupling mode, having polarization perpendicular to ^1L_a. The ratio of the second to the first of these therefore increases with decreasing excitation wavelength or increasing emission wavelength, thereby resulting in decreasing anisotropy. The presence of strong vibronic coupling was confirmed by measuring the limiting emission anisotropy spectrum of benzindole at 320 nm excitation wavelength, where $r_o = 0.1$ when observed at 390 nm emission wavelength. The emission anisotropy spectrum was wavelength independent, as expected when equal amounts of two overlapped perpendicular oscillators are excited.

Thus, our interpretation of the limiting excitation anisotropy spectra in Figure 4 is that most of the drop at shorter wavelengths comes from vibronic coupling of ^1L_a with a higher lying B state having essentially long-axis polarization, and that this effect dominates depolarization caused by the weaker, and almost collinearly polarized, ^1L_b absorption. A possible explanation for why such vibronic coupling effects are much less noticeable for indole is that in the latter there is greater charge transfer and localized C2−C3 ethylenic nature for the ^1L_a transition, whereas for benzannulated indole the ^1L_a transition is more dominated by the naphthalene part of the molecule.

Photophysics. Benzindole and 3-methylbenzindole have high quantum yields and long fluorescence lifetimes in 10% methanol, which are independent of solvent isotope for benzindole and nearly so for 3-methylbenzindole (Table 1). The quantum yield and lifetime of 3-methylcarbazole are likewise independent of solvent isotope. The radiative rate $k_r = \Phi/\tau$ is 4.2×10^7 s^{-1} for benzindole, 3.2×10^7 s^{-1} for 3-methylbenzindole, and 2.4×10^7 s^{-1} for 3-methylcarbazole. The lifetimes of benzindole and 3-methylbenzindole are very slightly temperature dependent, decreasing with increasing temperature in the range 5−55 °C from 19.6 to 18.7 ns for benzindole and from 25.7 to 23.8 ns for 3-methylbenzindole. The lifetime of 3-methylcarbazole is independent of temperature from 5 to 55 °C. This behavior contrasts with simple indoles, whose quantum yield and lifetime in aqueous solution are isotopically sensitive and temperature dependent. Indoles have two isotopically sensitive temperature-dependent nonradiative processes: solvent or water quenching and excited-state proton transfer.[3,29] Water quenching occurs in aqueous solution at neutral pH in the absence of a strong proton donor. The mechanism of water quenching is unknown, perhaps involving an exciplex or incomplete proton transfer.

The fluorescence quantum yield of benzindole was measured from pH(D) 1.0 to 12.5 in 10% CH_3OH and in 10% CH_3OD at 25 °C (Figure 7). At the extremes of pH, the quantum yields show steep drops, which depend on solvent isotope. Isotopically sensitive drops in quantum yield at low and high pH are also exhibited by simple indoles and have been attributed to two excited-state proton-transfer reactions: acid-catalyzed protonation of the indole ring and base-catalyzed deprotonation of the indole nitrogen.[30] Assuming that analogous quenching mechanisms apply to benzindole, the excited-state pK*s in 10% CH_3OH are about 1.1 for protonation and 11.2 for deprotonation. The corresponding pK*s for indole in aqueous buffer are 2.1 and 12.3.[14]

Solute quenching experiments on benzindole, 3-methylindole, and 3-methylcarbazole in 10% CH_3OH using glycine, lysine, and phenol as quenchers showed that excited-state proton transfer does not occur in benzannulated indoles in aqueous solution at neutral pH. No change in quantum yield or lifetime was observed in the presence of 0.2 M quencher. In the presence of good proton donors, such as ammonium and phenol, simple indoles can undergo intramolecular[31,32] or intermolecular[4,29] photochemical H−D exchange at C2, C3, C4, and C7. The quenching rate constant and the proton exchange rate measured by mass spectrometry or NMR are the same, indicating that proton transfer is the fluorescence quenching mechanism.[4] Excited-state H−D exchange also occurs in simple indoles in

TABLE 1: Quantum Yield and Lifetime Data at 25 °C

compound	solvent	A_{max}, nm	F_{max}, nm	Φ^a	τ^a, ns
benz[*f*]indole	9:1 H_2O/CH_3OH	344	415	0.79 ± 0.03	19.1 ± 0.1
	9:1 D_2O/CD_3OD	344	415	0.80 ± 0.03	19.3 ± 0.1
	ethanol	344	395		
	glycerol	347	407		
	cyclohexane	354	357		
3-methylbenz[*f*]indole	9:1 H_2O/CH_3OH	353	439	0.80 ± 0.03	24.7 ± 0.1
	9:1 D_2O/CH_3OD	353	439	0.84 ± 0.03	25.9 ± 0.1
	ethanol	353	412		
3-methylcarbazole	9:1 H_2O/CH_3OH	329	368	0.23 ± 0.02	9.5 ± 0.1
	9:1 D_2O/CH_3OD	329	368	0.23 ± 0.02	9.8 ± 0.1
	ethanol	329	367		
indole	9:1 H_2O/CH_3OH	268	341	0.30 ± 0.01	4.7 ± 0.1
	9:1 D_2O/CH_3OD	268	341	0.37 ± 0.01	6.0 ± 0.1
	ethanol	280	326		
3-methylindole	9:1 H_2O/CH_3OH	277	364	0.45 ± 0.02	9.6 ± 0.1
	9:1 D_2O/CH_3OD			0.60 ± 0.02	12.7 ± 0.1
	ethanol	282	346		

[a] Values with standard deviation are from 3 to 5 experiments.

TABLE 2: Stern−Volmer Quenching Rate Constants

	k_q, 10^8 M^{-1} s^{-1}		
quencher[a]	benz[*f*]indole	3-methylbenz[*f*]indole	3-methylcarbazole
His, pH 4.7	0.72	0.52	
N-acetyl-cys, pH 6.0	0.54	0.35	0.82
KI	1.2	9.2	5.6
acrylamide, CH_2═$CHCONH_2$	54	51	28
CH_3CH═$CHCOOCH_3$	71		
$NCCOOCH_3$	57		
acetone	7.1	4.5	
3-methyl-2-butanone	4.7		
3,3-dimethyl-2-butanone	2.0		
$YbCl_3$	6.6		

[a] Solvent is 9:1 H_2O/CH_3OH.

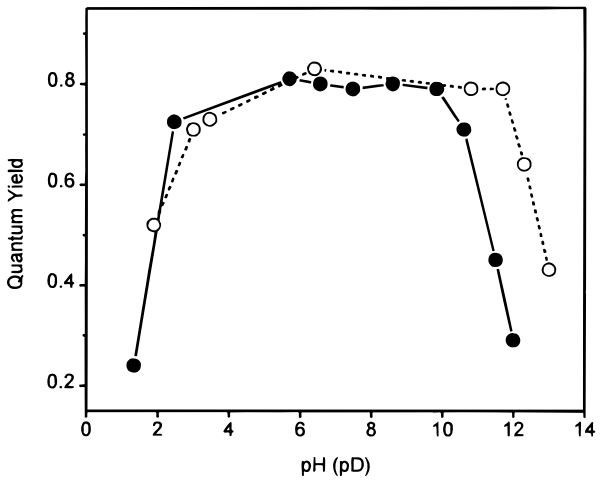

Figure 7. pH profile of fluorescence quantum yield of benz[*f*]indole in 9:1 H_2O/CH_3OH (- -●- -) and 9:1 D_2O/CH_3OD (- -○- -), 25 °C. Excitation wavelength 365 nm, pD = pH + 0.4. Lines drawn to connect the points.

trifluoroethanol, a solvent with higher acidity than water.[33] The quantum yield of benzindole drops in trifluoroethanol (Φ = 0.32 in CF_3CH_2OH) and shows a solvent isotope effect (Φ = 0.58 in CF_3CH_2OD), possibly due to excited-state proton transfer in this solvent.

Solute quenching experiments on benzannulated indoles in 10% CH_3OH using electrophilic compounds as quenchers suggest that excited-state electron transfer is an important fluorescence quenching mechanism (Table 2). Quenching by ester, amide, and carbonyl groups and by lanthanides was examined. No ground-state complexation was detected in the absorption spectra of benzannulated indoles in the presence of 0.2 M quencher. Only strong electron acceptors were effective quenchers. Weak quenchers of indole fluorescence, such as ethyl acetate, methylcyanoacetate, *N*-acetylglycineamide, malonamide, and $GdCl_3$, do not quench the fluorescence of benzannulated indoles. The lower limit of detection in the case of benzannulated indoles is $k_q \sim 10^6$ M^{-1} s^{-1}. The side chains of two amino acids, protonated histidine and cysteine, are weak quenchers with quenching rate constants $<10^8$ M^{-1} s^{-1}. Ketones are moderate quenchers with bulkier alkyl groups decreasing the quenching rate constant. However, compounds with double or triple bonds conjugated to an ester or amide group have quenching rates approaching the diffusion limit.

Conclusions

Benzannulation of the indole ring shifts the absorption spectrum about 75 nm to the red, allowing more selective excitation in the presence of tryptophan than tryptophan analogues, such as 5-hydroxytryptophan and 7-azatryptophan. It also raises the possibility of using tryptophan and benzindole derivatives as energy transfer donor/acceptor pairs. We have reported a synthesis of racemic *t*-Boc-benz[*f*]tryptophan.[21] Like tryptophan, the emission spectrum of benzindole is highly sensitive to solvent polarity with large Stokes' shifts in polar solvents. Our INDO/S computations say that there is about a 4 D increase in the permanent dipole of ^1L_a, with the pyrrole end becoming more positive. This means that if benz[*f*]tryptophan replaces a tryptophan in a protein and conserves the long axis orientation, the local protein field is predicted to shift the

fluorescence in the same direction and approximate magnitude as for tryptophan.[2]

The photophysics of benzannulated indoles is simplified somewhat compared to indoles, presumably a consequence of the lower energy gap between ground and excited states. The weak, if any, dependence of quantum yield and lifetime on temperature and solvent isotope suggests that solvent quenching is negligible in benzannulated indoles. If intersystem crossing is the only nonradiative process, we can estimate the intersystem crossing rate k_{isc} from the nonradiative rate k_{nr}, where $k_{nr} = \tau^{-1} - k_r$. This gives k_{isc} values of 1.0×10^7 s^{-1} for benzindole, 8.5×10^6 s^{-1} for 3-methylbenzindole, and 8.1×10^7 s^{-1} for 3-methylcarbazole. Two other nonradiative processes, excited-state proton and electron-transfer reactions, can also occur in benzannulated indoles, but over a much narrower range of conditions than in indoles. For example, tryptophan fluorescence is quenched by six protein functional groups representing eight amino acid side chains plus the peptide bond.[4,34] However, only two amino acid functional groups quench benzannulated indole fluorescence in intermolecular quenching experiments. The relative insensitivity of quantum yield and lifetime of benzannulated indoles to solvent and protein functional groups make them potentially useful as probes of peptide−protein interactions. Their efficient quenching by acrylamide allows solute accessibility studies of the spectrally enhanced species.

Acknowledgment. This work was supported by NIH grants GM31824 and GM42101. We thank Mr. Paul Adams for conducting low-temperature fluorescence measurements, Dr. T. Scott Yokum for synthesizing 3-methylcarbazole, and Dr. Yu Chen for helpful discussions.

References and Notes

(1) Callis, P. R. *Methods Enzymol.* **1997**, *278*, 113.
(2) Callis, P. R.; Burgess, B. K. *J. Phys. Chem. B* **1997**, *101*, 9429.
(3) McMahon, L. P.; Colucci, W. J.; McLaughlin, M. L.; Barkley, M. D. *J. Am. Chem. Soc.* **1992**, *114*, 8442.
(4) Chen, Y.; Barkley, M. D. *Biochemistry* **1998**, *37*, 9976.
(5) Ross, J. B. A.; Senear, D. F.; Waxman, E.; Kombo, B. B.; Rusinova, E.; Huang, Y. T.; Laws, W. R.; Hasselbacher, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 12023.
(6) Ross, J. B. A.; Szabo, A. G.; Hogue, C. W. *Methods Enzymol.* **1997**, *278*, 151.
(7) Ahmad, A.; Durocher, G. *Photochem. Photobiol.* **1981**, *34*, 573.
(8) Ridley, J.; Zerner, M. *Theor. Chim. Acta (Berlin)* **1973**, *32*, 111.
(9) Lou, J.-K.; Castle, R. N.; Lee, M. *J. Heterocycl. Chem.* **1989**, *26*, 1213.
(10) Melhuish, W. H. *J. Phys. Chem.* **1961**, *65*, 229.
(11) Liu, W.; Chen, Y.; Watrob, H.; Bartlett, S. G.; Linda-Jen-Jacobson; Barkley, M. D. *Biochemistry* **1998**, *37*, 15457.
(12) Callis, P. R. *J. Chem. Phys.* **1991**, *95*, 4230.
(13) Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. *J. Am. Chem. Soc.* **1985**, *107*, 3902.
(14) Stewart, J. J. P. *QCPE Bull.* **1990**, *10*, 86.
(15) Robinson, B. *The Fischer Indole Synthesis*; Wiley: London, 1982.
(16) Rydon, H. N.; Siddappa, S. *J. Chem. Soc.* **1951**, 2462.
(17) Sakamoto, T.; Kondo, Y.; Yamanaka, H. *Heterocycles* **1986**, *24*, 1845.
(18) Watanabe, T.; Miyagi, C.; Murakami, Y. *J. Heterocycl. Chem.* **1993**, *30*, 217.
(19) Batcho, A. D.; Leimgruber, W. *Org. Synth.* **1985**, *63*, 214.
(20) Sakamoto, T.; Kondo, Y.; Yamanaka, H. *Heterocycles* **1986**, *24*, 31.
(21) Yokum, T. S.; Tungaturthi, P. K.; McLaughlin, M. L. *Tetrahedron Lett.* **1997**, *38*, 5111.
(22) Valeur, B.; Weber, G. *Photochem. Photobiol.* **1977**, *25*, 441.
(23) Yamamoto, Y.; Tanaka, J. *Bull. Chem. Soc. Jpn.* **1972**, *45*, 1362.
(24) Zimmerman, H.; Joop, N. *Z. Elektrochem.* **1961**, *65*, 61.
(25) Robey, M. J.; Ross, I. G.; Southwood-Jones, R. V.; Strickler, S. J. *Chem. Phys.* **1977**, *23*, 207.
(26) Zimmerman, H.; Joop, N. *Z. Elektrochem.* **1961**, *65*, 66.
(27) Schütt, H.-U.; Zimmerman, H. *Ber. Bunsen-Ges. Phys. Chem.* **1963**, *67*, 54.
(28) Fischer, G. *Vibronic Coupling: The Interaction Between the Electronic and Nuclear Motion*; Academic Press: New York, 1984.
(29) Yu, H.-T.; Colucci, W. J.; McLaughlin, M. L.; Barkley, M. D. *J. Am. Chem. Soc.* **1992**, *114*, 8449.
(30) Vander Donckt, E. *Bull. Soc. Chim. Belg.* **1969**, *78*, 69.
(31) Saito, I.; Sugiyama, H.; Yamamoto, A.; Muramatsu, S.; Matsuura, T. *J. Am. Chem. Soc.* **1984**, *106*, 4286.
(32) Shizuka, J.; Serizawa, M.; Kobayashi, J.; Kameta, K.; Suglyama, H.; Matsuura, T.; Saito, I. *J. Am. Chem. Soc.* **1988**, *110*, 1726.
(33) Chen, Y.; Liu, B.; Barkley, M. D. *J. Am. Chem. Soc.* **1995**, *117*, 5608.
(34) Chen, Y.; Liu, B.; Yu, H.-T.; Barkley, M. D. *J. Am. Chem. Soc.* **1996**, *118*, 9271.