Steady-state and time-resolved fluorescence anisotropy of 7-azaindole and its derivatives

R. L. Rich
Iowa State University

Y. Chen
Iowa State University

D. Neven
Iowa State University

M. Negerie
Iowa State University

G. Gai
Iowa State University

See next page for additional authors
Follow this and additional works at: http://lib.dr.iastate.edu/chem_pubs

Part of the Physical Chemistry Commons

The complete bibliographic information for this item can be found at http://lib.dr.iastate.edu/chem_pubs/807. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.
Steady-state and time-resolved fluorescence anisotropy of 7-azaindole and its derivatives

Abstract
The fluorescence excitation and excitation anisotropy spectra at -60 OC in a propylene glycol glass are reported for 7-azaindole and three related derivatives: 7-azatryptophan, N1-methyl-7-azaindole (1 M1AI), and 7-methyl- 7H-pyrrolo[2,3-b]pyridine (7M7AI). At the reddest excitation wavelengths, steady-state anisotropy values are observed in the range 0.17 to 0.23, which is significantly less than the theoretical limiting anisotropy of 0.4. The anisotropy spectra indicate the presence of closely-spaced 'Laa nd ~LLba, nds as in indole. The low temperature anisotropies are compared with results from time-dependent measurements. An alternative method of collecting fluorescence depolarization data in time-correlated single-photon counting experiments is also presented. It is reliable, provides long-term stability, which is essential for weakly fluorescent samples, and obviates the need for "tail-matching" scaling procedures. This method is tested with the well-characterized fluorescein derivative, rose bengal, and is employed to compare the rotational diffusion times of the normal and the tautomer species of 7-azaindole in methanol and butanol with that of the fluorescent, "blocked," species of 7-azaindole in water.

Disciplines
Chemistry | Physical Chemistry

Comments

Authors
R. L. Rich, Y. Chen, D. Neven, M. Negerie, G. Gai, and Jacob W. Petrich
Steady-State and Time-Resolved Fluorescence Anisotropy of 7-Azaindole and Its Derivatives


Department of Chemistry, Iowa State University, Ames, Iowa 50011

Received: August 20, 1992; In Final Form: December 1, 1992

The fluorescence excitation and emission anisotropy spectra at −60 °C in a propylene glycol glass are reported for 7-azaindole and three related derivatives: 7-azatryptophan, N1-methyl-7-azaindole (1M7AI), and 7-methyl-7H-pyrrolo[2,3-b]pyridine (7M7AI). At the reddest excitation wavelengths, steady-state anisotropy values are observed in the range 0.17 to 0.23, which is significantly less than the theoretical limiting anisotropy of 0.4. The anisotropy spectra indicate the presence of closely-spaced 1Lg and 1Ls bands as in indole. The low temperature anisotropies are compared with results from time-dependent measurements. An alternative method of collecting fluorescence depolarization data in time-correlated single-photon counting experiments is also presented. It is reliable, provides long-term stability, which is essential for weakly fluorescent samples, and obviates the need for "tail-matching" scaling procedures. This method is tested with the well-characterized fluorescein derivative, ros bengal, and is employed to compare the rotational diffusion times of the normal and the tautomer species of 7-azaindole in methanol and butanol with that of the fluorescent, "blocked," species of 7-azaindole in water.

Introduction

7-Azaindole (Figure 1) is the chromophoric moiety of the nonnatural amino acid, 7-azatryptophan. We have proposed 7-azatryptophan as an alternate to tryptophan for use as an optical probe of protein structure and dynamics.¹⁻⁵ The suitability of 7-azatryptophan is based on its single-exponential (780-ps) fluorescence decay (when emission is collected over the entire band), its spectroscopic distinguishability from tryptophan in both absorption and emission, and its amenability to peptide synthesis and incorporation into bacterial protein.

To use 7-azatryptophan as a probe of protein structure and dynamics it is crucial to understand the photophysics of the 7-azaindole chromophore in water. We have obtained evidence for four nonradiative processes: photoionization, intersystem crossing, internal conversion, and excited-state tautomerization.³⁻⁵ Unlike its dimer⁶⁻¹⁰ or when it is in alcohols,¹¹⁻¹⁴ 7-azaindole in water exhibits only a minor amount, ≤20%, of excited-state tautomerization.³⁻⁵ (Figure 2). On the other hand, when emission is collected over the entire band, tautomerization is imperceptible because the fluorescence decay time of the population which can tautomerize, the "normal" species, is compensated for by the fluorescence rise time of the tautomer. Careful wavelength-resolved lifetime measurements reveal that a small fraction of tautomerization occurs in water. If fluorescence is collected over the entire band, however, the normal emission that decays as a result of tautomer formation is exactly canceled by the rising component of the tautomer emission. Hence, when emission is collected over the entire band, the lifetime is single exponential.³⁻⁵ Chou et al.¹⁵ have also discussed the inability of 7-azaindole in water to execute double-proton transfer.¹⁶ Because tautomerization of 7-azaindole in water is not facile, the fluorescence spectrum is smooth with a single maximum at 386 nm instead of bimodal as it is in alcohols (e.g., for methanol λmax = 374, 505 nm). The small fraction of molecules that do tautomerize are most likely rapidly protonated and have an emission maximum at ∼440 nm.³

While much has been learned about the photophysics of 7-azaindole, there are outstanding questions that must be resolved in order to appreciate fully its use as a biological probe: how does the electronic structure of 7-azaindole differ from that of indole and how do these differences result in the "well-behaved" photophysics of 7-azatryptophan, which are exemplified by its single-exponential fluorescence lifetime as opposed to the non-exponential fluorescence lifetime of tryptophan?¹⁷

In order to provide answers to these questions, we have performed fluorescence excitation anisotropy measurements of 7-azaindole in order to compare its excited-state level structure with that of indole. Valeur and Weber¹⁸ resolved the fluorescence excitation spectrum of indole into overlapping 1Lg and 1Ls bands at −58 °C in propylene glycol and reported the 1Ls transition to be quite structured with maxima at 282.5 and 289.5 nm. They reported the 1Lg transition to be broader and to absorb farther to the red than the 1Ls transition. An important question is whether 7-azaindole and related compounds share these features in common with indole or whether the nitrogen at the 7 position is sufficient to perturb significantly the overlap of these two bands.

Footnotes

¹ To whom correspondence should be addressed.
² Present address: Laboratoire d'Optique Appliquée, Ecole Polytechnique—ENSTA, INSERM U275, 91120 Palaiseau Cedex, France.
The purpose of this article is thus to investigate, through fluorescence excitation spectra and fluorescence excitation anisotropy spectra, the lowest lying electronic transitions of 7-azaindole and its analogs. In addition to 7-azaindole and 7-azatryptophan, we investigate derivatives that mimic untautomered and tautomered 7-azaindole: N1-methyl-7-azaindole (1M7AI) and 7-methyl-7H-pyrrolo[2,3-b]pyridine (7M7AI) (Figure 1). We reproduce the results of Valeur and Weber for indole. The fluorescence excitation spectra for indole and 7-azaindole are decomposed into $I_L$ and $I_S$ components using the conventional analysis.\textsuperscript{18,19} We shall discuss in detail when such an analysis may be inappropriate. For 7-azaindole, we compare the steady-state low temperature anisotropy, $r_0$, with the limiting anisotropy, $r(0)$, obtained in time-dependent measurements in the liquid phase.

**Experimental Section**

I. Materials. Indole and d,L-7-azatryptophan (Sigma Chemical Co.) were used without further purification. 7-Aza-indole (Sigma) was purified via flash chromatography\textsuperscript{3-5} using ethyl acetate as a solvent. Detailed methods of synthesis and purification for 1M7AI and 7M7AI have been described elsewhere.\textsuperscript{5-9} For the steady-state measurements, compounds were dissolved in propylene glycol to make 1-50 pM solutions. Solutions of low concentration are required to prevent aggregation of the solute during cooling.

II. Steady-State Measurements. Steady-state measurements were performed using a Spex Fluoromax (error of ±0.5 nm) adapted to hold a quartz-windowed Dewar flask containing a 5 mm-diameter quartz sample tube. A methanol/dry ice slurry maintained a sample temperature of less than −60 °C. All spectra were corrected. A 1-nm band-pass was used for excitation and emission.

We follow the procedure outlined by Valeur and Weber\textsuperscript{18} and utilized by Eftink et al.\textsuperscript{19} for the resolution of the excitation anisotropy spectra into two bands arising from $I_L$ and $I_S$ electronic transitions.

The steady-state anisotropy, $r_0$, of a system is given by

$$r_0 = \frac{I_0 - I_{-1}}{I_0 + 2I_{-1}}$$  \hspace{1cm} (1)

where $I_0$ is the emission polarized parallel to the excitation source and $I_{-1} = gI_0$. $g$ is a correction factor for the polarization dependence of the excitation monochromator; and $I_0$ is the uncorrected intensity of emission polarized perpendicular to the excitation source. $g$ is obtained for a sample at room temperature, where the steady-state emission is expected to be completely depolarized. For our experiments, we define $g = I_1/I_{-1}$, where the excitation source is polarized vertically in the laboratory frame for both emission measurements.

III. Time-Resolved Measurements. The time-correlated single-photon counting apparatus\textsuperscript{5} and the data analysis for fluorescence anisotropy measurements are described elsewhere.\textsuperscript{22,23} The time-dependent anisotropy, $r(t)$, is constructed in a manner similar to that of its steady-state counterpart. It is related to the correlation function of the transition dipole moment for absorption to state $i$ at time zero, $\mu_{im}(0)$, with the transition dipole moment for emission from state $i$ at subsequent times, $t$, $\mu_{em}(t)$. For a sphere undergoing rotational diffusion by Brownian motion:\textsuperscript{24}

$$r(t) = \frac{I(t) - I_{-1}(t)}{I(t) + 2I_{-1}} = \frac{1}{2}\left\{P_2[\mu_{bs}(0)\mu_{em}(t)]\right\} = \frac{2}{2}P_2[\mu_{bs}(\cos \theta)\mu_{em}(t)]e^{-\gamma/\tau_0}$$  \hspace{1cm} (2)

$I(t)$ and $I_{-1}(t)$ are the time dependent fluorescence intensities parallel and perpendicular to the excitation polarization. $P_2$ is the second Legendre polynomial, $\theta$ is the angle formed by $\mu_{bs}$ and $\mu_{em}(0)$, and $\tau_0$ is the diffusion relaxation time for a sphere (i.e., $\tau_0 = 1/6D$, $D$ being the diffusion coefficient). Accurate construction of the fluorescence anisotropy decay function demands that the sample be exposed to equivalent excitation intensity during the collection of the parallel and perpendicular emission profiles. Numerous methods have been proposed for the normalized collection of $I(t)$ and $I_{-1}(t)$. They have been summarized by Cross and Fleming.\textsuperscript{23} In general, these methods fall into two categories: genuine simultaneous collection or alternate sampling procedures. If these methods are not adequate, the two curves must be scaled, "tail-matched," to have equal intensity at times where the fluorescence emission is expected to be depolarized. In the following, we present a method for acquiring fluorescence depolarization data without recourse to scaling procedures. This technique is based upon alternate detection of $I(t)$ and $I_{-1}(t)$, obviates the need for scaling procedures, and permits very precise measurements of fast reorientation times.

Automation of both polarizer movement and multichannel analyzer (MCA) operation provides our apparatus with the capability of sampling fluorescence at multiple orientations of the analyzer polarizer. A polarizer (Polaroid, HNP,B) is mounted on a modified motorized rotation stage (RSA-1TM, Newport, Corp.) and is synchronously controlled in conjunction with the MCA (Norland 5500), making possible alternate readings of $I(t)$ and $I_{-1}(t)$. Polarization bias in our system is negligible. By alternately acquiring $I(t)$ and $I_{-1}(t)$, one may compensate for drift in the laser system over long periods of time, at least up to seven hours, thereby allowing for the collection of fluorescence depolarization data without recourse to scaling procedures. (Long-term drift in electronic components may still present a problem that is more difficult to compensate for.) Such compensation is crucial if the sample is weakly fluorescent and many hours of data accumulation are required. An IBM 386 clone controls both the rotation stage and the MCA under the direction of Asyst software, an advanced fourth-generation data acquisition and analysis language. Data transfer and MCA control are achieved over an RS-232 serial link connecting the computer to the MCA. The motorized rotation stage is interfaced to the computer via a parallel port connection to a manual-stage controller (Newport 860 SC-C). The operator specifies the data names in which to store the $I(t)$ and $I_{-1}(t)$ data, the maximum count of any channel that will terminate the experiment, the length of the $I(t)$ and $I_{-1}(t)$ acquisition times (usually 1–5 min), and the present orientation of the analyzer polarizer. Approaches similar to ours have been presented.\textsuperscript{25-32} For example, Fayer and coworkers use a Pockels cell to rotate the polarization of their excitation beam by 90° every 20 s.\textsuperscript{30,31} Millar et al.\textsuperscript{32} describe an apparatus for sampling parallel and perpendicular emission intensities; but they do not discuss the long-term reliability of their system; nor are the results calibrated against a standard.

One of the most thoroughly investigated dye molecules is the fluorescein derivative, rose bengal.\textsuperscript{33,34} Figure 3 presents the data obtained for rose bengal in methanol at 20 °C using an acquisition time of 1 min for the $I(t)$ and $I_{-1}(t)$ curves. These data yield results that are in excellent agreement with literature values: ten measurements of $I(t)$ and ten measurements of $I_{-1}(t)$ yield a limiting anisotropy, $r(0) = 0.360 ± 0.003$, and a rotational diffusion time, $\tau_r = 173 ± 5$ ps. Above Figure 3 are displayed three sets of residuals corresponding, from top to bottom, to sampling times of 15, 2, and 1 min. As the sampling time is decreased, the fit of the data to a monoeponential anisotropy decay progressively improves, as measured by the $\chi^2$ criterion, from $\chi^2 = 1.60, 1.26$, and 1.05. More importantly, attendant to this improvement in the fit is an increasingly more accurate value of the rotational diffusion time: 166 ps for a 15-min sampling time and 179 ps for a 1-min sampling time. The deviation in the residuals for the 15-min sampling clearly indicates drift in the
I. Resolution of the Steady-State Fluorescence Excitation Spectrum into $1L_e$ and $1L_a$ Bands. Valeur and Weber\cite{ref18} and Eftink et al.\cite{ref19} have used the results of steady-state fluorescence excitation anisotropy spectra to resolve the fluorescence excitation spectra of indole and its analogs into $1L_e$ and $1L_a$ spectra. The procedure is as follows. The measured steady-state anisotropy, $r_0(\lambda)$, is considered to be the sum of the contributions of the transitions connecting $1L_e$ and $1L_a$ with the ground state:

$$r_0(\lambda) = f^a(\lambda) r_{aa} + f^b(\lambda) r_{ob}$$

where $r_{aa}$ and $r_{ob}$ are the anisotropies of each transition and $f^a(\lambda) + f^b(\lambda) = 1$. $r_{ob}$ is obtained from $r_{aa}$ by the relation $r_{ob} = r_{aa} P_{2}(\mu_{ab}^{\perp} + \mu_{ab}^{\parallel})$, assuming $r_{aa} = r_{a}$ at the reddest excitation wavelengths. For indole, the angle formed by the emission dipole moments of $1L_e$ and $1L_a$ is believed to be very close to 90°.\cite{ref35} It is also assumed that $(\mu_{aa}^{\parallel} - \mu_{aa}^{\perp}) = 1$ and that $(\mu_{ab}^{\parallel} + \mu_{ab}^{\perp}) = 1$. The $1L_e$ and $1L_a$ fluorescence excitation spectra, $I_L(\lambda)$ and $I_b(\lambda)$, are resolved using the following relations. $r_{aa}$ and $r_{ob}$ are defined as the limiting steady-state anisotropies of a single emissive transition:

$$f^a(\lambda) = \frac{r_{0a}(\lambda) - r_{ob}}{r_{aa} - r_{ob}}$$

$$f^b(\lambda) = \frac{r_{0b}(\lambda) - r_{ob}}{r_{aa} - r_{ob}}$$

$$I_L(\lambda) = f^a(\lambda) I(\lambda)$$

$$I_b(\lambda) = f^b(\lambda) I(\lambda)$$

involving either $1L_e$ or $1L_a$, respectively. (Eftink et al.\cite{ref19} describe some empirical criteria for determining whether a single state is responsible for the observed steady-state anisotropy spectrum.)

The assumptions underlying the spectral decomposition indicated by eqs 4 and 5 are quite severe and must be stated explicitly and commented upon.

1. In their original analysis of the steady-state anisotropy spectrum of indole, Valeur and Weber\cite{ref18} assumed that at the very reddest excitation wavelengths the steady-state anisotropy arose entirely from $1L_a$, which was known to lie below $1L_e$ in polar solvents.\cite{ref36, ref37} Their assignment, however, of $r_{aa} = 0.3$ still required rationalization since this value is less than 0.4, the theoretical limiting value of the anisotropy (eq 2). The diminished anisotropy has been attributed to either an angle between $\mu_{aa}^{\parallel}$ and $\mu_{ab}^{\perp}$, that was greater than 0° or, which is less likely, to exceedingly rapid depolarizing motion that could occur even in a glass.\cite{ref18, ref19, ref38}

2. The assumption that at the reddest excitation wavelengths the steady-state anisotropy arises entirely from $1L_a$ implies that at these wavelengths only $1L_a$ absorbs and emits radiation and that it is kinetically isolated from the $1L_e$ state. This assignment seems increasingly less valid as the measured values become lower. Eftink et al.\cite{ref19} have arbitrarily picked 0.3 as the value below which the assumption is no longer valid.

3. The $I_L(\lambda)$ and $I_b(\lambda)$ excitation spectra resulting from the spectral decomposition must be carefully interpreted. It has been tacitly assumed that $I_L(\lambda)$ and $I_b(\lambda)$ represent the absorption spectra connecting $1L_a$ and $1L_e$ to the ground electronic state. But the fluorescence intensity is proportional to $I_F \cdot e^{2 \phi}$, where $I_F$ is the excitation intensity, $e$ is the extinction coefficient of the absorbing species, and $\phi$ is the fluorescence quantum yield. If $\phi$ varies with excitation wavelength, the fluorescence excitation spectra cannot be identified with absorption spectra. Such an excitation wavelength dependence of the quantum yield has been cited for...
Section I of the Discussion, the resolution of the fluorescence similar to that of indole, the varying change in the proportions of the thow of 7-azaindole and 7-azatryptophan, showing only a slowly is broad, absorbing over a much larger wavelength region than

\[ \text{at 298.0 nm for 7-azaindole and} \]

methylated derivatives (not shown) lack the structure observed change in the anisotropy and the resolved measurements of Bulska et ale\(^4\) For indole, a strong spectrum with respect to those earlier work, we have resolved the excitation anisotropy spectra by \( \text{IL} \) and \( \text{IL}_b \) bands. \( r_{\text{ex}} = 0.31 \) and \( r_{\text{em}} = -0.16 \). The values for \( r_{\text{ex}} \) and \( r_{\text{em}} \) reported here and in Figures 5–8 were determined using the methods of Valeur and Weber\(^18\) and Eftink et al.\(^19\) This technique requires the assumption that at the reddest wavelengths only \( \text{IL}_a \) absorbs and emits radiation. Refer to the text for further discussion of these calculations.

We have also observed this dependence for both indole and 7-azaindole.\(^40\) This will be discussed in greater detail elsewhere.

With these caveats in mind, we tested our experimental procedure by resolving the fluorescence excitation anisotropy spectrum of indole in propylene glycol at \(-60^\circ\text{C}\) into contributions from the \( \text{IL}_a \) and \( \text{IL}_b \) electronic transitions (Figure 4). For purposes of comparison with earlier work, we have resolved the excitation anisotropy spectrum by means of eqs 3–5 and have assumed that for 7-azaindole \( \mu_{\text{em}} = \mu_{\text{em}} - \mu_{\text{em}} = 0 \). Our results are in excellent agreement with those of Valeur and Weber,\(^18\) except that our spectrum is blue-shifted by 2 nm with respect to theirs. We find anisotropy minima at 280.5 and 287.5 nm and a local maximum at 284.0 nm.

Figures 5–8 present the fluorescence excitation and emission spectra and the fluorescence excitation anisotropy spectra for 7-azaindole and its derivatives. Resolved \( \text{IL}_a \) and \( \text{IL}_b \) spectra are presented for 7-azaindole (Figure 5). Substitution of a nitrogen for a carbon at the 7 position of indole produces a significant change in the anisotropy and the resolved \( \text{IL}_a \) and \( \text{IL}_b \) excitation spectra with respect to those observed for indole. The presence of the \( \text{IL}_a \) and \( \text{IL}_b \) bands in 7-azaindole had been suggested by the measurements of Bulska et al.\(^41\) For indole, a strong \( \text{IL}_a 0-0 \) transition is apparent at 287.5 nm, yet the analogous transitions at 298.0 nm for 7-azaindole and 300.5 nm for 7-azatryptophan are much less pronounced. Resolved excitation spectra of the methylated derivatives (not shown) lack the structure observed in those of 7-azaindole and 7-azatryptophan, showing only a slowly varying change in the proportions of the \( \text{IL}_a \) and \( \text{IL}_b \) transitions. Similar to that of indole, the \( \text{IL}_a \) contribution of these compounds is broad, absorbing over a much larger wavelength region than that of \( \text{IL}_b \), and is less structured than the \( \text{IL}_b \) contribution.

For reasons that are listed above and elaborated in detail in Section I of the Discussion, the resolution of the fluorescence excitation spectrum of 7-azaindole \( \lambda_{\text{ex}} = 295.5 \text{ nm} \). At wavelengths less than 330 nm and greater than 425 nm, the emission anisotropy became too noisy to resolve. (b) The fluorescence excitation anisotropy of indole \( \lambda_{\text{ex}} = 372 \text{ nm} \). \( r_{\text{ex}} = 0.19 \) at \( \lambda_{\text{ex}} = 305 \text{ nm} \); \( r_{\text{ex}} = 0.14 \) at \( \lambda_{\text{ex}} = 285 \text{ nm} \). (c) The excitation spectrum of 7-azaindole resolved into contributions from the \( \text{IL}_a \) and \( \text{IL}_b \) bands. \( r_{\text{ex}} = 0.25 \) and \( r_{\text{ex}} = -0.13 \).

II. Fluorescence Anisotropy Decay. For 7-azaindole in water, the limiting anisotropy, \( r(0) \), was examined at 285 and 305 nm. At each wavelength, the time-resolved measurement agrees closely with that determined via steady-state techniques (Table I and Figure 5). Table II demonstrates that the fluorescence excitation spectra at 20 and \(-60^\circ\text{C}\) are essentially the same for all the compounds considered here. The fluorescence anisotropy decay of the normal and the tautomer bands of 7-azaindole were measured in butanol and in methanol (Table I and Figure 9). A notable result is that in methanol at \( \eta = 0.93 \text{ cP (}-9.0^\circ\text{C})\), the parallel and perpendicular emission curves of the tautomer are superposable and hence the tautomer appears to be completely depolarized. On the other hand, the parallel and perpendicular emission of the normal band is distinct and may be fit to yield \( r(0) = 0.11 \) when \( \lambda_{\text{ex}} = 285 \text{ and } r(0) = 0.21 \) when \( \lambda_{\text{ex}} = 305 \text{ nm} \). A rotational diffusion time, \( \tau_r \), of 34 ps is obtained in both cases (Table I).

For 7-azaindole in the alcohols the limiting anisotropy, \( r(0) \), for the tautomer appears to be significantly less than that for the normal species: in fact, for viscosities such as \( \eta \leq 3 \text{ cP}, r(0) \)
Anisotropy of 7-Azaindole and Its Derivatives

Figure 6. (a) The emission anisotropy of 7-azatryptophan ($\lambda_{em} = 295$ nm). (b) The fluorescence excitation anisotropy of 7-azatryptophan ($\lambda_{em} = 362$ nm).

Figure 7. (a) The emission anisotropy of 1M7AI ($\lambda_{em} = 295$ nm). (b) The fluorescence excitation anisotropy of 1M7AI ($\lambda_{em} = 361$ nm).

Figure 8. (a) The emission anisotropy of 7M7AI ($\lambda_{em} = 300$ nm). (b) The fluorescence excitation anisotropy of 7M7AI ($\lambda_{em} = 420$ nm).

The power of the time-correlated single-photon counting technique, coupled with our method of data collection, is demonstrated by our ability to obtain consistent limiting anisotropy and rotational diffusion times (Table I) with parallel and perpendicular emission curves that differ only slightly on the rising edges and the maxima (Figures 9 and 10).

Discussion

I. Comparison of Steady-State and Time-Dependent Measurements. From a comparison of Table I and Figure 5b, it can be seen that, for the excitation wavelengths investigated, the low limiting anisotropy, $r(0)$, obtained in time-dependent measurements is the same, within experimental error, as that obtained in steady-state measurements, $r_0$. Because the same results are obtained in liquid butanol, methanol, and water on the one hand and in propylene glycol glass on the other hand, the low limiting anisotropy may be attributed to nonmotional factors rather than to a rapid component of rotational diffusion that lies beyond the time resolution of our apparatus and that is expected to be viscosity dependent.

Similarly, the limiting anisotropy extrapolated for tryptophan using data obtained on time scales >5 ps is less than 0.4, is wavelength dependent, and agrees with the steady-state results for indole. First Cross et al. and then Szabo demonstrated how the presence of two excited electronic states whose energy gap is close to $kT$ can influence the short time anisotropy decay and hence give rise to apparently anomalously low $r(0)$ values if the anisotropy measurement is not performed with sufficient time resolution. Subsequently, Fleming and co-workers experimentally observed these effects in tryptophan and in the single-tryptophan-containing hormone and protein, melittin and Pseudomonas aeruginosa azurin, respectively. Subpicosecond resolution reveals $r(0) = 0.4$ and rapid components of anisotropy decay in the range of 1–4 ps.

In the specific case where there are two closely lying excited states, $1^{Aa}$ and $1^{Bb}$, the measured anisotropy decay function is a function of both wavelength and time.43

$$r(\lambda, t) = \frac{k_R b^a g^a(\lambda) K^a(t) + k_R b^b g^b(\lambda) K^b(t)}{k_R b^a g^a(\lambda) K^a(t) + k_R b^b g^b(\lambda) K^b(t)}$$

where $k_R$ are the radiative rate constants, $K^a,b(t)$ are the population (fluorescence) decay laws, and $g^a,b(\lambda)$ are the emission
TABLE I: Fluorescence Anisotropy Decay of 7-Azaindole

<table>
<thead>
<tr>
<th>solvent, °C</th>
<th>η (cP)</th>
<th>species, λem (nm)</th>
<th>τF (ps)</th>
<th>r(0)/r∞</th>
<th>λex = 305 nm</th>
<th>λem = 285 nm</th>
<th>τr (ps)/λ∞</th>
</tr>
</thead>
<tbody>
<tr>
<td>butanol, -9.0</td>
<td>6.96</td>
<td>normal, 320-460</td>
<td>358 ± 6</td>
<td>0.10 ± 0.01</td>
<td>191 ± 9</td>
<td>0.0044</td>
<td>174</td>
</tr>
<tr>
<td>butanol, 20.0</td>
<td>2.92</td>
<td>tautomer, &gt;505</td>
<td>254 ± 19; 1552 ± 36</td>
<td>0.10 ± 0.01</td>
<td>unsolved</td>
<td>unsolved</td>
<td></td>
</tr>
<tr>
<td>methanol, -9.0</td>
<td>0.93</td>
<td>normal, 320-460</td>
<td>211 ± 6</td>
<td>0.11 ± 0.02</td>
<td>unsolved</td>
<td>34 ± 8</td>
<td>34 ± 8</td>
</tr>
<tr>
<td>water, 2.0</td>
<td>1.67</td>
<td>entire band, &gt;320</td>
<td>1263 ± 14</td>
<td>0.21 ± 0.01</td>
<td>11 ± 0.01</td>
<td>41 ± 6</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>water, 23.5</td>
<td>0.92</td>
<td>entire band, &gt;320</td>
<td>886 ± 15</td>
<td>0.20 ± 0.05</td>
<td>12 ± 0.06</td>
<td>20 ± 2</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

*Temperatures are certain to within ± 0.5 °C. A From reference 50. 1 cP = 1.0 × 10¹³ N m⁻² s⁻¹. C There is no significant emission from the normal form when collecting data at λem ≥ 505 nm. If there were, we would detect either an instantaneous component in the rise time of the tautomer or an artificially shortened rise time of the tautomer. We observe neither. D The normal form in alcohols and the species in water are characterized by a single-exponential fluorescence decay time. E The resolved emission of the tautomer band in alcohols is fit to the function K(t) = A₁ exp(-t/τ₁) + A₂ exp(-t/τ₂), where A₁ < 0 for the rising component of the fluorescence, whose contribution to the emission is normalized to unity and is given in parentheses by |A₁|/(A₁ + A₂). F For the tautomer, the first number is the lifetime; the second, the decay time. * The absence of a value indicates that the measurement was not performed. ** Unresolved indicates that the emitting species was not sufficiently polarized at zero time to obtain a meaningful result, in other words r(0) = 0. * Where applicable, we present averages of experiments performed at both λem = 285 nm and λem = 305 nm. This low value reflects the similar time scales of tautomerization and rotational diffusion. If the transition dipole moment for emission from the excited-state tautomer were different from the transition dipole moment for the absorbing species by approximately 3°, the r(0) for 7-azaindole in methanol would be reduced by a factor of 10, thus rendering its measurements very difficult. G The anisotropy decay obtained in a glass, where the diffusion time and the rotational diffusion time are negligible, is more similar if the 'Lb-'La energy gap and if the internal conversion rate do not change significantly with temperature. H Very similar if the 'Lb-'La energy gap and if the internal conversion rate do not change significantly with temperature. I The majority of the 7-azaindole population in water is not capable of tautomerization; hence it is appropriate to refer to it as "normal". J Even though its fluorescence lifetime, quantum yield, and behavior with respect to deuterium substitution are tautomer-like, it is not a "tautomer". K Because of these distinctions, we have referred to this species as "blocked". L The quoted uncertainty is the standard deviation obtained from four independent measurements. M The reproducibility of the measurement is enhanced by the automated, alternate collection of I₁(t) and I₂(t), which obviates the necessity of determining a scaling factor. N Determination of a scaling factor would be quite difficult since most of the information concerning depolarization is contained in the rising edges of the curves. The best argument for confidence in this value is that it lies directly in the line obtained for the other τr obtained in water and that this line is parallel to that obtained in butanol (Figure 10).

TABLE II: Steady-State Measurements of 7-Azaindole and Related Compounds

<table>
<thead>
<tr>
<th>compound</th>
<th>λexmax nm (20 °C)</th>
<th>λemmax nm (20 °C)</th>
<th>λexmax nm (−60 °C)</th>
<th>λemmax nm (−60 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>indole</td>
<td>272, 287 287</td>
<td>330</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td>7-azaindole</td>
<td>290.5, 297 290.5, 297</td>
<td>371</td>
<td>355</td>
<td></td>
</tr>
<tr>
<td>7-azatryptophan</td>
<td>287.5 287.5, 290.5 290.5</td>
<td>380</td>
<td>361</td>
<td></td>
</tr>
<tr>
<td>1M7Al</td>
<td>285</td>
<td>381</td>
<td>361</td>
<td></td>
</tr>
<tr>
<td>7M7Al</td>
<td>291</td>
<td>440</td>
<td>405</td>
<td></td>
</tr>
</tbody>
</table>

* All compounds were dissolved in propylene glycol and diluted to a final concentration of 10-50 μM. A Valeur and Weber report an emission maximum of ~330 nm for indole at −58 °C. This is the maximum we observe at room temperature. We observe a distinct blue-shift of the emission maximum as the sample is cooled. At −60 °C, the emission maximum is slightly less than 300 nm. We are unable to obtain fluorescence spectra below 300 nm that have been corrected for instrumental response. A A shoulder is apparent at 298 nm in the excitation spectrum of 7-azatryptophan. D The excitation spectra of each compound at 20 °C and −60 °C have similar shapes and maxima. When 7-azatryptophan is cooled, however, structure becomes apparent and two relative maxima are observed.

The steady-state anisotropy obtained in a glass, r₀, and the limiting anisotropy obtained at room temperature, r(0), are expected to be very similar if the 1Lb-1La energy gap and if the internal conversion rate do not change significantly with temperature.

The steady-state anisotropy obtained in a glass, r₀, and the limiting anisotropy obtained at room temperature, r(0), are expected to be very similar if the 1Lb-1La energy gap and if the internal conversion rate do not change significantly with temperature. r₀ can be determined from the approximation

\[ r₀ ≈ \int_0^\infty r(t) \, dt \]
Anisotropy of 7-Azaindole and Its Derivatives

Figure 10. Plots of the rotational diffusion (reorientation) time, $\tau_n$, against $\eta/T$ for the $a$ band of 7-azaindole in butanol (O) and for 7-azaindole in water (O). Emission wavelengths are noted in Table I. The $\tau_n$ data presented are averages from at least four measurements at a given viscosity using either an excitation wavelength of 285 or 305 nm. The data are fit to the relation, $\tau_n = cV + \tau_n$, where $c = V/kT$ if the diffusing species is a sphere. The butanol data yield $V = 93.8 \pm 3.2$ $\text{A}^2$; the water data, $V = 94.0 \pm 6.9$ $\text{A}^2$. The intercepts are the “free rotor” times and are $10.6 \pm 3.8$ and $-0.2 \pm 2.4$ ps for butanol and water, respectively. These are in good agreement with the estimated free rotor time of about 1 ps. The expression for the free rotor time is given by $\tau_n = (2\pi f/9)(1/kT)^{1/2}$, where the moment of inertia of 7-azaindole is $\sim 1 \times 10^{-44}$ kg m$^2$.

$r(0)$ is determined by extrapolating the long-time behavior of eq 6 back to $t = 0$.

We assume that $g'(\lambda) = g(\lambda)$ and that only $1_L$ is optically excited. For tryptophan, $k^b = k^b = 3.3 \times 10^9$ s$^{-1}$, $3.2 \times 10^9$ s$^{-1}$ and 500 cm$^{-1}$ are taken for the values of $K_{ab}$ and the $1_L - 1_L^*$ energy gap. For $-60^\circ$C and $20^\circ$C, $r_0$, $r_0$, and $r(0)$ are thus calculated to be 0.38 and 0.36, respectively.

This calculation indicates that thermal population of the $1_L$ level will reduce the limiting anisotropy. But the limiting anisotropy is still much larger than those obtained for 7-azaindole and its derivatives. In fact, in order to obtain $r(0) = 0.20$, it is necessary that for the same emission profiles, internal conversion rate, and energy gap, there is significant optical population of $1_L^*$. In other words, this requires the initial populations of the $1_L^*$ and $1_L$ states, $K^*(0)$ and $K(0)$, are such that $K^*(0)/K(0) = 2.4:1$ at $-60^\circ$C and $K^*(0)/K(0) = 2.1:1$ at $20^\circ$C. For simplicity, in these analyses we have not taken into consideration the depletion of the $1_L^*$ state by the production of solvated electrons.

These calculations lead us to conclude that the low $r(0)$ values observed for the normal form of 7-azaindole in alcohol and for the fluorescent species of 7-azaindole in water are a result of both level kinetics and of absorption by $1_L^*$ even at the reddest excitation wavelengths. We noted earlier that a standard assumption used in decomposing fluorescence excitation anisotropy spectra is that at the reddest excitation wavelengths only the lower of the two states is optically populated. This assumption may not be a good one for indole, where it has been asserted that $r_0 = r_0 = 0.31$; and it is most likely not appropriate for 7-azaindole and its derivatives. We have, nevertheless, employed this method of analysis because it indicates clearly, albeit only qualitatively, the presence of the $1_L^*$ and the $1_L$ states.

In discussing the low values of $r(0)$, we have assumed that $\mu_{1L}^a = \mu_{1L}^b = 1$ and that $\mu_{1L^*}^a = \mu_{1L^*}^b = 1$. An angle between the absorption and emission moments for these respective transitions would, however, contribute to a lowering of $r(0)$ (eq 2). Fleming and coworkers have proposed a mechanism that provides such an angle. If there is vibronic mixing between the $1_L^*$ and $1_L$ states and if the rate of vibrational relaxation is much larger than the rates of interconversion between these two states, $k_{ab}$ and $k_{ab}$, then an angle is introduced between the initially excited Franck-Condon vibronic state and the vibronic state that is detected in emission.

II. Solvent Interactions with Normal, Tautomer, and “Blocked” 7-Azaindole Species. In alcohols, the fluorescence emission of 7-azaindole is characterized by two bands with distinct and widely separated maxima as well as different fluorescence lifetimes. The redder of the two bands observed in alcohols is attributed to an excited-state tautomer. Consequently, the bluer of the two bands is attributed to a “normal” species. An intriguing characteristic of the emission of 7-azaindole in water is that only a smooth band is detected and the fluorescence lifetime is single exponential when emission is collected over the entire band over most of the pH range. Previously we suggested that the fluorescent species in water was predominantly “tautomer-like” because it bears some similarities with respect to fluorescence quantum yield and the deuterium isotope effect of its fluorescence lifetime with the tautomer in alcohol. As mentioned in the Introduction and discussed in detail elsewhere, although a small fraction of the population in water is capable of tautomerization, the majority of the 7-azaindole molecules do not tautomerize. We have referred to this solute population as “blocked” in order to distinguish it from the “normal” and the “tautomer” species. We have suggested that tautomerization is not possible for the “blocked” species because of its inability to form the appropriate geometry with the solvent, the idealized cyclic intermediate (Figure 2), during the lifetime of the excited state. Chou et al. have commented on this phenomenon and have made the distinction between a 7-azaindole monohydrate and a 7-azaindole polyhydrate, the latter being incapable of tautomerization.

The blocked species possesses a short fluorescence lifetime and low quantum yield relative to the normal analog (1M7AI); 910 ps and 0.03 as compared with 21 ns and 0.55, respectively. These traits, along with the deuterium isotope effect, render the photophysics of the blocked species more like that of the tautomer species. On the other hand, in water the emission maximum is at 386 nm instead of 510 nm, as it is for the tautomer analog (7M7AI).

In the light of our recent results, we no longer consider it useful to discuss the “blocked” species as being “tautomer-like.” We thus feel that referring to the blocked species as either normal or tautomer is not appropriate when all the data are taken into account since it shares features of both.

The reorientation times obtained for 7-azaindole (Table I and Figure 10) allow us to compare the bulk solute-solvent interactions in different solvents. Figure 10 indicates that the normal form of 7-azaindole in butanol and the blocked species of 7-azaindole in water have, within experimental error, the same linear dependence of rotational diffusion time with respect to $\eta/T$. This indicates that the bulk interactions of 7-azaindole with butanol and water are the same.
determined. If the species is a sphere, c = V/kT; and the data presented in Figure 10 yield V = 94 Å³ for both butanol and water, (for other molecular shapes, however, a correction must be applied).47,48 This experimental volume is in very good agreement with the “theoretical volume” of 104 Å³ that may be determined from a consideration of van der Waals increments.49 This agreement is somewhat surprising since the experimental value is determined by assuming a spherical shape for the solute and by completely ignoring specific solute-solvent interactions, such as those depicted in Figure 2, for example.

Conclusions

Despite the single-exponential fluorescence decay of the nonnatural amino acid, 7-azatryptophan, compared with the nonexponential decay of tryptophan and the significant spectroscopic differences between 7-aza indole and indole,2-5 these two chromophores are much more similar to one another than might have been expected. Not only do they share the same pathways of nonradiative decay in water, photoionization and intersystem crossing,34,41,49 but they also possess similar excited-state structures as is manifested by closely-lying L1 and L2 states. The proximity of these two states is responsible for the wavelength dependence and the low value of the limiting anisotropy in steady-state structure as is manifested by closely-lying fluorescence depolarization measurements) on time scales of greater duration than the nonmotional depolarizing event (i.e., ≥5 ps). Absorption anisotropy must be performed if motion is to be detected unambiguously on a finer time scale.

Acknowledgment. We thank Professor G. R. Fleming for making available to us the simultaneous fitting programs developed by A. J. Cross. J.W.P is an Office of Naval Research Young Investigator. D.E.N. was supported by a NASA summer internship. Additional support was provided by the Iowa State University Biotechnology Council and IPRT. Travel support was provided to M.N. by NATO.

References and Notes

(16) While the conclusions of Chou et al.19 are qualitatively consistent with our results,20 another report of the photophysics of 7-azaindole in water has appeared by C. F. Chapman and M. Maroncelli (J. Phys. Chem. 1992, 96, 8430). In their study, Chapman and Maroncelli propose that all of the 7-aza indole populations tautomerizes in water with a rate constant of 1.2 x 10⁵ s⁻¹. We have discussed this proposal in detail.21
(24) Tao, T. Biopolymers 1969, 6, 609.
(46) The expressions in refs 42 and 43 for i and j need to be interchanged.