Using 7-Azatryptophan To Probe Small Molecule-Protein Interactions on the Picosecond Time Scale: The Complex of Avidin and Biotinylated 7-Azatryptophan

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Abstract: The utility of 7-azatryptophan as an alternative to tryptophan for optically probing protein structure and dynamics is demonstrated by investigating the complex of egg-white avidin and biotinylated 7-azatryptophan. We report the synthesis of biotinylated 7-azatryptophan and optical measurements of its complex with avidin. Although there are four biotin binding sites, the emission from the 7-azatryptophan tagged to biotin decays by a single exponential, whereas the tryptophyl emission from avidin requires two exponentials in order to be adequately fit. Fluorescence depolarization measurements of the complex probed by emission from 7-azatryptophan reveal both rapid (~80 ps) and much longer-lived decay. The former component is attributable to the local motion of the probe with respect to the protein; the latter component represents overall protein tumbling. In addition, energy transfer from tryptophan to 7-azatryptophan and a blue-shift in the spectrum of biotinylated 7-azatryptophan are observed upon formation of the complex. Modified strategies of effecting optical selectivity are also discussed.

Introduction

We have proposed the nonnatural amino acid, 7-azatryptophan, as an alternative to tryptophan as an optical probe of protein structure and dynamics.[1-12] The merits of 7-azatryptophan lie in its intrinsic single exponential fluorescence decay in water[1,5,10] as compared to the nonexponential decay exhibited by tryptophan,[13-19] as well as in its spectroscopic distinguishability with respect to tryptophan in both absorption and emission.[3,5,11] Furthermore, 7-azatryptophan can be incorporated into bacterial protein and is amenable to peptide synthesis.[1,3,12] Important applications of 7-azatryptophan are its incorporation into small peptides, its binding to cofactors, and the subsequent investigation of the dynamics of these smaller, tagged molecules bound to the target protein of interest. In this article, we demonstrate the feasibility of this approach by studying biotinylated 7-azatryptophan (inset of Figure 1) bound to avidin.

Avidin is a tetrameric protein found in avian egg white. Each subunit contains 128 residues of which 4 are tryptophan. Avidin is believed to function as an antibacterial agent through its ability to reduce the free concentration of biotin. The dissociation constant of the avidin-biotin complex is about 10^{-15} M.[20-27] The essentially irreversible binding afforded by this complex, the specificity of its formation, and the ready modification of the carboxylate group of biotin have permitted the study of the interactions of several biotin adducts with avidin. Biotin-avidin species have found extensive use as analytical reagents.[24,26] Examples of other uses of biotin-avidin complexes include perturbation of rhodium hydrogenation catalyst activity,[27] formation of ordered protein monolayers,[22] and hybrid glycoproteins.[23] Recently, X-ray structures of egg-white avidin and its complex with biotin have appeared.[25] Biotin is shown to bind in a β-barrel constructed from eight antiparallel β sheets. The tryptophan residues 70 and 97 of one monomer and tryptophan 110 of an adjacent monomer form part of the avidin binding site and are anchored through hydrogen bonds to other residues, thus stabilizing the binding site. The ureido ring of biotin forms hydrogen bonds with Asn-12, Ser-16, Tyr-33, Thr-35, Asn-118, and possibly Thr-77.[25]

We have prepared a biotin-7-azatryptophan adduct in order to demonstrate further the spectroscopic distinguishability of

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7-azatryptophan from tryptophan and to investigate the mobility of the 7-azatryptophan moiety in the complex.

Experimental Section

N-α-Biotinoyl-D,L-7-azatryptophan Methyl Ester. Biotin *N*-hydroxysuccinimide ester (100 mg, 0.302 mmol) and D,L-7-azatryptophan methyl ester dihydrochloride (71.2 mg, 0.245 mmol) in 3.0 mL of pyridine (distilled from ninhydrin) were stirred under N_2 for 19 h. The pyridine was removed under vacuum at 40 °C and the residue was suspended in approximately 30 mL of ethyl acetate and extracted three times with 50% saturated aqueous $NaHCO_3$, and once with saturated NaCl. The ethyl acetate layer was then dried over Na_2SO_4 and decanted; the solvent was removed to yield 90 mg (83% yield) of product as a white solid. An analytical sample was obtained by recrystallization from methanol and chloroform (53 mg, 49%, mp 220–222 °C): ^{1}H NMR (300 MHz, CD_3OD) δ 8.07 (d, 4.78 Hz, 1 H), 7.92 (d, 7.87 Hz, 1 H), 7.81 (s, 1 H), 7.01 (dd, 4.84 Hz, 7.83 Hz, 1 H), 4.66 (m, 1 H), 4.39 (m, 1 H), 4.12 (m, 1 H), 3.59 (s, 3 H), 3.09–2.97 (m, 2 H), 2.82 (dd, 4.89 Hz, 12.72 Hz, 1 H), 2.59 (d, 12.73 Hz, 1 H), 2.09 (t, 6.93 Hz, 2 H), 1.62–1.39 (m, 6 H); IR (KBr) 1735, 1699, 1536, 1536, 1655 cm^{-1}. Calcd for $C_{21}H_{27}N_5O_4S(CHCl_3)_1(CH_3OH)_1$: C, 46.28; H, 5.40; N, 11.73. Found: C, 46.54; H, 5.39; N, 11.55.

Samples used in fluorescence studies were dissolved in a 95:5 water/methanol mixture. The small amount of methanol was necessary to dissolve the biotinylated 7-azatryptophan (7ATB). No degradation of avidin was observed at this concentration of methanol. Affinity-purified, egg-white avidin was obtained from Sigma and used without further purification. Since avidin has four biotin binding sites, the complex was prepared in the ratio of four biotinylated 7-azatryptophan molecules to one avidin molecule. The concentrations of avidin and biotinylated 7-azatryptophan were determined spectrophotometrically using ϵ_{avidin} (282 nm) = 96 000 cm^{-1} M^{-1} [20] and $\epsilon_{biotin\text{-}7\text{-}azatrp}$ (288 nm) = 6200 cm^{-1} M^{-1}. Thin-layer chromatography confirms the lack of free biotinylated 7-azatryptophan in solutions of the complex and consequently demonstrates that attachment of 7-azatryptophan to biotin does not affect the ability of the latter to bind to avidin.

Spectroscopic Measurements. Fluorescence lifetimes, $K(t)$, and fluorescence anisotropy decays, $r(t)$, were obtained using the time-correlated, single-photon counting technique.[5,6] Parallel and perpendicular emission intensities for measurements of anisotropy decays were collected alternately with a rotating analyzer polarizer in order to obviate scaling procedures.[6] Unless otherwise indicated, fluorescence decays were collected to a maximum of 10 000 counts. Fluorescence anisotropy decays were collected so that a maximum of 16 000 counts were obtained in the parallel curve. Data were analyzed as usual[5,6] by an iterative comparison with the convolution of the instrument response function with trial functions for $K(t)$ or for $I_{\parallel}(t)$ and $I_{\perp}(t)$, simultaneously. A nonlinear least-squares fitting procedure was employed, and the quality of fit was determined by the χ^2 criterion. The full-scale time base for all lifetime and anisotropy measurements was 3 ns in order to measure accurately the rapid component of the anisotropy decay. Consequently, long-time depolarizing events (which are not the primary concern of this work) were not fully characterized. All measurements of the 4:1 biotinylated 7-azatryptophan–avidin complex were performed with λ_{ex} = 310 nm and λ_{em} > 400 nm to minimize the detection of emission from tryptophan residues within the protein. All other measurements were performed with λ_{ex} = 285 nm and λ_{em} > 320 nm. The values reported for the time-resolved measurements are the average of three to seven measurements.

The dependence of the fluorescence quantum yield on excitation wavelength was determined for indole, 5-methoxyindole, 7-azatryptophan, and the 4:1 complex of biotinylated 7-azatryptophan–avidin. Indole, 5-methoxyindole, and D,L-7-azatryptophan were purchased from Sigma Chemical Co. and used as supplied. Experiments involving biotinylated 7-azatryptophan were performed in 95/5 water/methanol solutions; all other samples were dissolved in pure water.

Absorbance measurements were made using a Shimadzu UV-2101PC double-beam spectrometer. Fluorescence measurements were obtained with a Spex Fluoromax fluorimeter whose excitation and emission bandpasses were set to 1 nm. All measurements were conducted at room temperature. Calibration of the absorption spectrometer and of the fluorimeter was performed using indole vapor as a standard. Crystals of indole in a 1-cm cuvette filled with argon were heated to 65 °C. Comparison of the absorption and the excitation spectra of the vapor indicated that the position of the sharp ^1L_b transition varied by 1 nm. A correction for this discrepancy was applied throughout our calculations. Corrections were also applied for the wavelength-dependent response of the fluorimeter.

The relative excitation-wavelength dependent fluoresence quantum yields were determined by two methods. The first method requires collecting an emission spectrum for each excitation wavelength and employs eq 1:

$$\frac{\phi_F(\lambda_1)}{\phi_F(\lambda_2)} = \frac{1 - 10^{-OD(\lambda_2)}}{1 - 10^{-OD(\lambda_1)}} \frac{\int_0^\infty I_{em}(\lambda_1, \bar{\nu})\, d\bar{\nu}}{\int_0^\infty I_{em}(\lambda_2, \bar{\nu})\, d\bar{\nu}} \frac{c(\lambda_2)}{c(\lambda_1)} \quad (1)$$

$OD(\lambda_i)$ is the optical density at the excitation wavelength, λ_i, $I_{em}(\lambda_i, \bar{\nu})$ is the emission intensity at the excitation wavelength λ_i, and $c(\lambda_i)$ is a correction factor taking into account fluctuations of the intensity of the xenon lamp of the fluorimeter as well as other factors discussed above. (Alternatively, the peak height may be used instead of the integrated spectrum since in the systems studied here the emission profile does not change shape or position over the range of excitation wavelengths investigated.)

The second method is based upon a comparison of the fluoresence excitation spectrum and the optical density as a function of the excitation wavelength, eq 2:

$$\frac{\phi_F(\lambda_1)}{\phi_F(\lambda_2)} = \frac{1 - 10^{-OD(\lambda_2)}}{1 - 10^{-OD(\lambda_1)}} \frac{I_{ex}(\lambda_1)}{I_{ex}(\lambda_2)} \quad (2)$$

$I_{ex}(\lambda_i)$ is the intensity of the excitation spectrum (monitored, in our case, at the emission maximum) at the excitation wavelength, λ_i.

As a check of our procedures, we determined the excitation-wavelength dependence of the fluorescence quantum yield for rhodamine B and pyrene. The fluorescence quantum yield across the range of excitation wavelengths scanned remained constant within an experimental error of ±15% for all the systems studied here except for that of the complex of biotinylated 7-azatryptophan and avidin. The excitation-wavelength dependence of the fluorescence quantum yield reported elsewhere is in error.[36]

Results

Figure 1 displays the fluorescence emission and excitation spectra of avidin, the biotin–7-azatryptophan adduct, and the complex of the biotin–7-azatryptophan adduct with avidin. Figure 2 demonstrates that, with an excitation wavelength of 310 nm, 7-azatryptophan is detected predominantly in the complex.

Figure 3 presents the fluoresence anisotropy decay of the biotinylated 7-azatryptophan; Figure 4, of avidin alone. Figure 5 presents the fluorescence anisotropy decay of the complex of biotinylated 7-azatryptophan with avidin. Even in the presence of 16 tryptophan residues, the 7-azatryptophan (1 per subunit) is detected unambiguously. The fluorescence anisotropy decay of the avidin–biotin complex that is detected by means of the 7-azatryptophan chromophore is clearly different from that observed from avidin itself (detected by means of the tryptophyl residues). Fluorescence lifetime and anisotropy decay data are summarized in Table 1.

The fluorescence lifetime of biotinylated 7-azatryptophan is dominated by emission from 7-azatryptophan and is well described by the function: $K(t) = 0.98 \pm 0.02 \exp(-t/646 \pm 9$ ps$) + 0.02 \pm 0.02 \exp(-t/2690 \pm 970$ ps). The residual 2% of this fluorescence decay is attributed to biotin itself, which is characterized by a fluorescence lifetime with a long component of about 3 ns.

The complex of avidin and biotinylated 7-azatryptophan is fit well to a double-exponential fluorescence decay. The dominant component is again attributed to 7-azatryptophan and

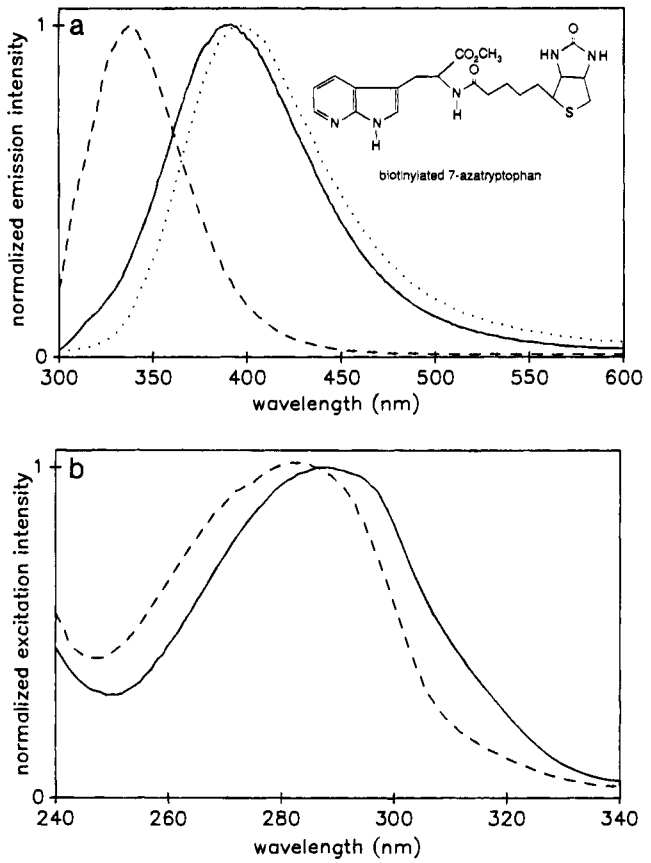

Figure 1. (a) Fluorescence spectra of avidin (λ_{em}^{max} = 339 nm) (- - -); biotinylated-7-azatryptophan (λ_{em}^{max} = 390 nm) (• • •), whose structure is displayed in the inset; and the complex of avidin and the biotinylated 7-azatryptophan (—). λ_{ex} = 310 nm. The excitation peak has been subtracted out of each spectrum. (b) Excitation spectra of biotinylated 7-azatryptophan (—) and of biotinylated 7-azatryptophan complexed with avidin (- - -). Samples were dissolved in a 95/5 water/methanol mixture. λ_{em} = 390 nm.

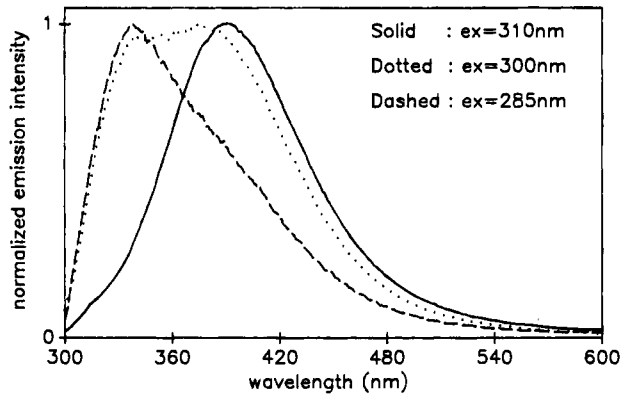

Figure 2. Fluorescence spectra of the complex of avidin and biotinylated 7-azatryptophan (1 per binding site) as a function of excitation wavelength. Note that at λ_{ex} = 310 nm essentially only emission from 7-azatryptophan is observed, whereas at bluer excitation wavelengths the contribution from tryptophan in avidin is more pronounced. For λ_{ex} = 310 nm, the excitation peak has been subtracted out of the spectrum.

has an apparent time constant of 420 ps. *Because we have chosen a 3-ns full-scale time base to investigate the rapid dynamics, we consequently have not made a very precise determination of either the magnitude or the duration of the longer-lived component, which arises mostly from tryptophan itself but contains a small contribution from biotin.* Fitting the data collected on a full scale of 3 ns indicates that about 50% of the emission collected arises from 7-azatryptophan.

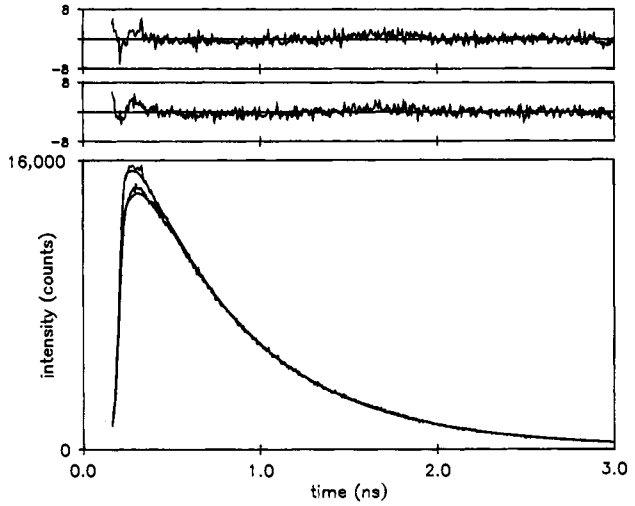

Figure 3. Parallel and perpendicular fluorescence intensity profiles from which the anisotropy decay is calculated[6] for biotinylated 7-azatryptophan: λ_{ex} = 285 nm, λ_{em} > 335 nm, 20 °C. The fluorescence anisotropy decay was fit to a single exponential: $r(t)$ = 0.06 exp(−t/110 ps), χ^2 = 1.62. Displayed above the polarized fluorescence profiles are the residuals for the parallel and the perpendicular emission, respectively. The fluorescence lifetime of this compound was fit to the function: $K(t)$ = 0.98 exp(−t/642 ps) + 0.02 exp(−t/2484 ps), χ^2 = 1.50. The residual contribution of long-lived component is due to biotin itself, which is weakly fluorescent. Biotin has an average fluorescence lifetime of 1.5 ns (and a long-lived component of about 3 ns) in a 95/5 water/methanol mixture. The values of the limiting anisotropies, $r(0)$, reported here and in Figures 4 and 5 are consistent with steady-state measurements obtained in glasses.[6,34,35]

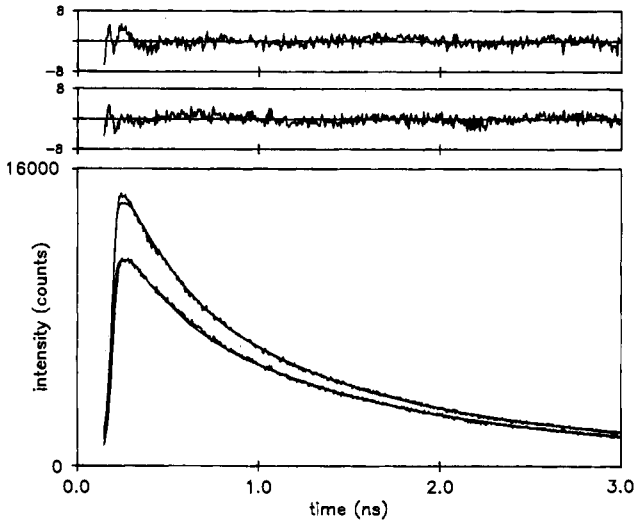

Figure 4. Parallel and perpendicular fluorescence intensity profiles from which the anisotropy decay is calculated for avidin: λ_{ex} = 285 nm, λ_{em} > 335 nm, 20 °C. The fluorescence anisotropy decay was fit to a single exponential: $r(t)$ = 0.08 exp(−t/1586 ps), χ^2 = 1.56. The fluorescence lifetime of avidin was fit to the function: $K(t)$ = 0.41 exp(−t/272 ps) + 0.59 exp(−t/1757 ps), χ^2 = 1.36. The upper set of residual corresponds to emission polarized parallel to the excitation source; the lower, perpendicular to the excitation source.

It is important to note that detection of such a relatively small contribution of 7-azatryptophan emission in the complex is unexpected given the respective optical properties of 7-azatryptophn and tryptophan. The contribution of fluorescence detected from a particular chromophore as a function of time, $C(t)$, depends on several factors: the optical density at the excitation wavelength, the radiative rate, the fraction of the emission spectrum over which the fluorescence lifetime is measured, and the fluorescence lifetime of the chromophore itself. The

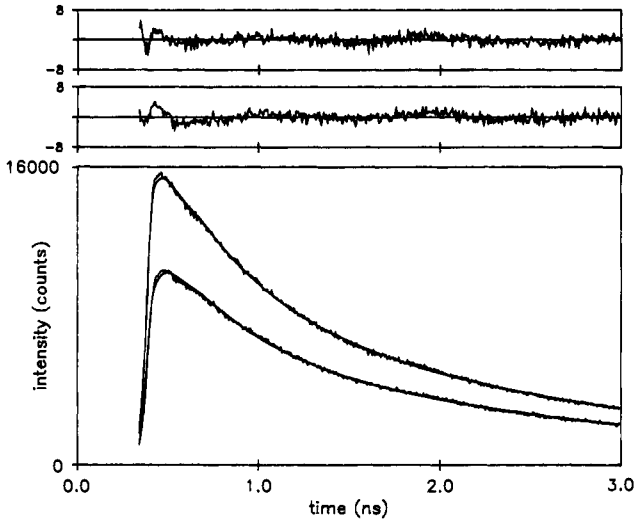

Figure 5. Parallel and perpendicular fluorescence intensity profiles from which the anisotropy decay is calculated for the complex of avidin and biotinylated 7-azatryptophan (1 per binding site), λ_{ex} = 310 nm, λ_{em} > 400 nm. As indicated in the Results, about 50% of the emission comprising the anisotropy decay is due to the 7-azatryptophan chromophore. The anisotropy decay is well described by two components: $r(t)$ = 0.06 exp(−t/64 ps) + 0.13; χ^2 = 1.51. The second component of the anisotropy decay reflects the overall tumbling of the protein itself and is too long-lived to be accurately determined on a 3-ns time scale, on which it appears to be infinite. The fluorescence lifetime of the biotinylated 7-azatryptophan in the complex was fit the function: $K(t)$ = 0.48 exp(−t/423 ps) + 0.52 exp(−t/2265 ps), χ^2 = 1.17. The upper set of residuals corresponds to emission polarized parallel to the excitation source; the lower, perpendicular to the excitation source.

expression used for determining the contribution of fluorescence observed from 7-azatryptophan in the presence of four tryptophyl residues (a model system for avidin) when the excitation wavelength is 310 nm and when fluoresence is collected at wavelengths longer than 400 nm is shown in eq 3, where $F_{em} = \int_{\tilde{\nu}}^{\infty} I_{em}(\tilde{\nu})\, d\tilde{\nu} / \int_{0}^{\infty} I_{em}(\tilde{\nu})\, d\tilde{\nu}$. ϵ_{Trp} (310 nm) = 100 cm^{-1} M^{-1}

$$C^{7AT}(t) = [\epsilon_{310}^{7AT} k_R^{7AT} F_{em}^{7AT} \exp(-t/780\text{ ps})]/ \{\epsilon_{310}^{7AT} k_R^{7AT} F_{em}^{7AT} \exp(-t/780\text{ ps}) + 4\epsilon_{310}^{Trp} k_R^{Trp} F_{em}^{Trp}[0.22 \exp(-t/620\text{ ps}) + 0.78 \exp(-t/3210\text{ ps})]\} \quad (3)$$

and ϵ_{7AT} (310 nm) = 1100 cm^{-1} M^{-1}.[3] The radiative rates used for 7-azatryptophan and for tryptophan are 3.8 × 10^7 s^{-1} and 5.0 × 10^7 s^{-1}, respectively. The quantity $C(t)$ is plotted for 7-azatryptophan in the presence of four tryptophan residues and for 1-methyl-7-azaindole in the presence of four tryptophan residues for different excitation wavelengths and on different time scales to demonstrate the selectivity of the 7-azatryptophan chromophore as an optical probe (Figure 6). 1-Methyl-7-azaindole is presented to illustrate the enormous optical selectivity that persists at longer times as a result of blocking the nonradiative processes afforded by the interactions of the N_1 proton with the solvent[5,12] (see Conclusions).

Detection of emission wavelengths longer than 400 nm was chosen to accelerate the data collection time and to ensure discrimination against emission from tryptophan itself. In principle, the "homogeneity" of the signal can be improved by selecting a combination of a lower energy emission wavelength cutoff and a lower energy excitation wavelength, as is demonstrated elsewhere and in Figure 6.

Our data reveal an interesting complication in the photophysics of the complex of avidin and biotinylated 7-azatryptophan. Namely, despite the high 7-azatryptophan to tryptophan ratio in the complex, the contribution of 7-azatryptophan emission detected is essentially constant (50−60%) regardless of whether the excitation wavelength is 285, 290, or 310 nm. The explanation of this phenomenon is revealed by Figure 1b, which compares the fluorescence excitation spectra of biotinylated 7-azatryptophan free and complexed with avidin. Complexation induces a blue shift and a slight change in shape of the absorption spectrum that renders preferential optical excitation of 7-azatryptophan less efficient.

Despite the shift of the absorption spectrum induced upon complexation, there is significant spectral overlap between the absorption spectrum of biotinylated 7-azatryptophan and the emission spectrum of tryptophan. The possibility of thus exploiting tryptophan to 7-azatryptophan energy transfer as an additional tool is confirmed by Figures 7 and 8. Figure 7e demonstrates that the *fluorescence quantum yield of 7-azatryptophan increases when the excitation wavelength is scanned through the region corresponding to tryptophyl adsorption.* Parts a−d in Figure 7 indicate no such variation of fluorescence quantum yield and are presented as control experiments. Figure 8 presents time-resolved data that verify the presence of energy transfer: at 330 nm where the emission of tryptophan is predominant, the average lifetime is significantly shorter than that of uncomplexed avidin; at emission wavelengths greater than 505 nm where the emission of 7-azatryptophan is predominant, a rise time in the emission is evident and is fit to a time constant of ~800 ps. Determination of the energy transfer time employed the spectral overlap integral determined from the absorption spectrum of biotinylated 7-azatryptophan bound to avidin (Figure 1b) and the emission spectrum of avidin: 2.84 × 10^{-15} cm^6/mol. This corresponds to a critical distance, R_0, of 12.3 Å. Distances between the tryptophan donor and the 7-azatryptophan acceptor were estimated as the distance from the midpoint between the 8 and 9 carbons of the indole ring of tryptophan and the carbonyl carbon of the biotin alkyl chain.[25] Three potential donors were investigated: tryptophans 70 and 97 of one monomer and tryptophan 110 of the other monomer. These tryptophans yielded the following donor−acceptor distances and energy transfer times, respectively: 4.9 Å, 6.7 ps; 11.2 Å, 960 ps; 29.1 Å, 290 ns. (An orientation factor of 2/3 was employed.) Our measured energy transfer time is in excellent agreement with Trp-97 being the donor.

Discussion

The steady-state absorption and fluorescence properties of 7-azatryptophan are sufficiently different from those of tryptophan that selective excitation and detection may be effected. The absorption maximum of 7-azatryptophan is red shifted by 10 nm with respect to that of tryptophan. There is also a significant red shift of about 50 nm of the maximum of the fluorescence spectrum of 7-azatryptophan with respect to that of tryptophan. We have measured the fluorescence decays of mixtures of tryptophan and 7-azatryptophan. Only when the ratio of tryptophan to 7-azatryptophan is as great as 10:1 does the tryptophyl emission become detectable. Furthermore, as opposed to aqueous tryptophan at pH 7, the fluorescence decay of 7-azatryptophan is single exponential. This result holds across the emission band and over the pH range we have studied, from 4 to 13. The monoexponential fluorescence decay in itself indicates the enormous preference for using 7-azatryptophan instead of tryptophan as a fluorescent probe.

Recently, there have been reports suggesting that 5-hydroxytryptophan is a useful biological probe as well.[28] While in some cases 5-hydroxytryptophan may prove useful (if relatively long

Table 1. Fluorescence Lifetime and Anisotropy Decay Parameters[a]

species	τ_{F_1} (ps)	τ_{F_2} (ps)	A_1	τ_{r_1} (ps)	τ_{r_2} (ps)	$r_1(0)$	$r_2(0)$
7ATB	646 ± 9	2690 ± 970	0.98 ± 0.02	108 ± 2		0.08 ± 0.01	
avidin	268 ± 9	1730 ± 70	0.42 ± 0.03	1300 ± 260		0.09 ± 0.01	
complex[b]	417 ± 14[c]	2300 ± 40	0.48 ± 0.01	80 ± 18	∞	0.06 ± 0.02	0.14 ± 0.01

[a] Fluorescence decays are fit to the function $K(t) = A_1 \exp(-t/\tau_{F_1}) + A_2 \exp(-t/\tau_{F_2})$. Anisotropy decays are fit to the function $r(t) = r_1(0) \exp(-t/\tau_{r_1}) + r_2(0) \exp(-t/\tau_{r_2})$. All measurements were performed at 20 °C and with $\lambda_{ex} = 285$ nm and $\lambda_{em} \geq 335$ nm, unless otherwise specified. [b] $\lambda_{ex} = 310$ nm, $\lambda_{em} \geq 400$ nm. [c] The apparent shortening of the lifetime of 7-azatryptophan in the complex may be attributed in part to a weighted average of emission from itself and tryptophan.

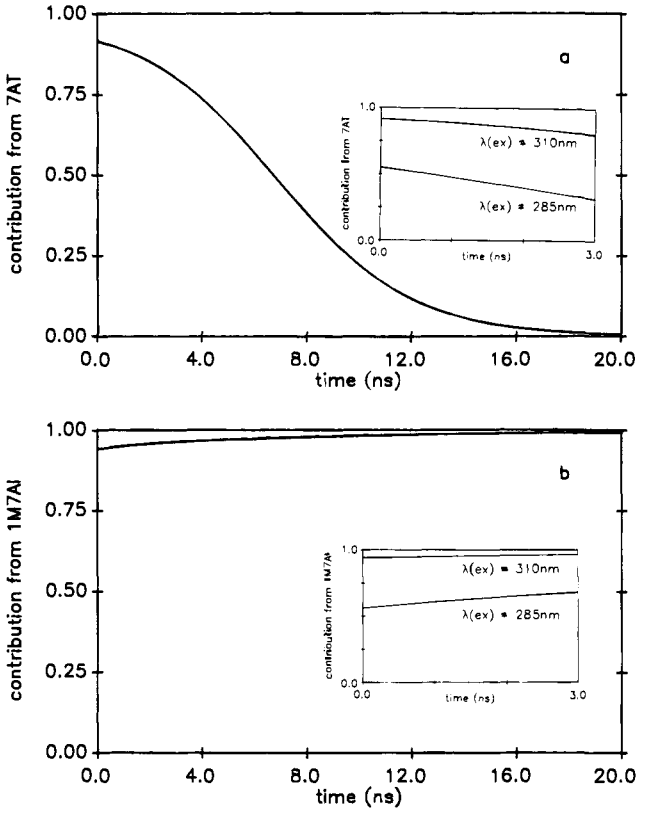

Figure 6. Percentage of emission observed from 7-azatryptophan and related compounds as a function of time for various experimental conditions as determined from eq 3. (a) 7-Azatryptophan/4 tryptophans, $\lambda_{ex} = 310$ nm, $\lambda_{em} \geq 400$, 20-ns full scale. The inset depicts the difference in emission (on a 3-ns scale) observed from 7-azatryptophan when the excitation wavelength is changed from 310 nm to 285 nm. (b) 1-Methyl-7-azaindole/4 tryptophans, $\lambda_{ex} = 310$ nm, $\lambda_{em} \geq 400$, 20-ns full scale. The inset depicts the difference in emission observed from 7-azaindole when the excitation wavelength is changed from 310 nm to 285 nm. The rise in percentage emission is a result of the 21-ns fluorescence lifetime of 1-methyl-7-azaindole as compared to the ~2.6-ns average fluorescence lifetime of tryptophan (eq 3).

excitation wavelengths, ~320 nm, are employed), we have demonstrated[10] that, because its fluorescence spectrum and lifetime are similar to those of tryptophyl chromophores, it can be more difficult to distinguish from tryptophan than is 7-azatryptophan. *This is due in large part to the 3.8-ns lifetime of 5-hydroxytryptophan, which is similar to that of the long component of tryptophan.* In mixtures of 5-hydroxytryptophan and the tryptophyl chromophore, NATA (*N*-acetyltryptophanamide), in a ratio as high as 1/10, the presence of 5-hydroxytryptophan cannot be discriminated from the mixture ($\lambda_{ex} = 305$ nm; $\lambda_{em} > 335$ nm). On the other hand, when the ratio of 7-azatryptophan to NATA is as low as 1/40, the 7-azatryptophan is easily detected.[10]

The spectroscopic distinguishability of 7-azatryptophan in the presence of avidin is demonstrated clearly in the figures. As is noted above and in the caption to Figure 5, about 50% of the emission of the complex of biotinylated 7-azatryptophan with avidin is attributable to 7-azatryptophan. *More importantly, the emission attributable to 7-azatryptophan decays according to a single exponential even though there are four biotin binding sites in avidin.* As indicated in the caption to Figure 4, however, the fluorescence decay of avidin itself requires two exponentials in order to be adequately fit. This is not surprising considering that there are 16 tryptophans present and that the fluorescence decay of tryptophan is intrinsically nonexponential.

The fluorescence anisotropy decay of biotinylated 7-azatryptophan in complex with avidin is fit well to two exponentially decaying components, the second of which is very long lived: $r(t) = r_1(0) \exp(-t/\tau_1) + r_2(0) \exp(-t/\tau_2) = (0.06 \pm 0.02) \exp(-t/80 \pm 8 \text{ ps}) + 0.14 \pm 0.01$ (Figure 5). That the fluorescence anisotropy is fit to two exponentials indicates that we are probing the rapid librational motion of the 7-azatryptophan probe with respect to avidin as well as the overall tumbling motion of the avidin itself. For probes attached to globular proteins, the order parameter, S^2, is a model independent measure of the extent to which restricted motion can occur.[29] $S^2 = [r(t)/r(0)] \exp(t/\tau_r) = r(0^+)/r_{eff}(0)$. τ_r and $r(0^+)$ are determined by the fit of the long-time behavior of the anisotropy decay (the overall protein reorientation or tumbling) to a single exponential and are equivalent to τ_2 and $r_2(0)$, respectively. $r_{eff}(0)$ is the initial value of the anisotropy less those nonmotional factors contributing to the anisotropy decay.[30] In the treatment of the data, $r_{eff}(0) = r_1(0) + r_2(0)$. S^2 gives an indication of the magnitude of the depolarizing motions that are present in addition to the overall protein reorientation. Thus a value of $S^2 < 1$ implies local motion of the chromophore with respect to the body of the protein, and $S^2 = 1$ implies a rigid chromophore that undergoes depolarization only by means of overall protein motion. The order parameter can be related to a *hypothetical* cone semiangle, θ_0, within which the transition dipole moment can diffuse:[29,31] $S = {}^1/_2 \cos\theta_0(1 + \cos\theta_0)$. In this example, $\theta_0 = 29 \pm 5°$. The relatively large value for the cone semiangle indicates that, while the biotin itself is firmly attached to the avidin, the 7-azatryptophan tag lies either in a mobile part of the protein or is partially exposed at the exterior of the protein. The latter of these possibilities is more likely given the large contribution of the rapid component of the anisotropy decay and the similarity of the fluorescence spectrum of 7-azatryptophan in the complex (Figures 1 and 2) to 7-azatryptophan in water.[5,10] If 7-azatryptophan were buried in the protein interior, not only would the rapid component be much less pronounced (or absent) but its fluoresence spectrum would be expected to resemble more closely what is observed

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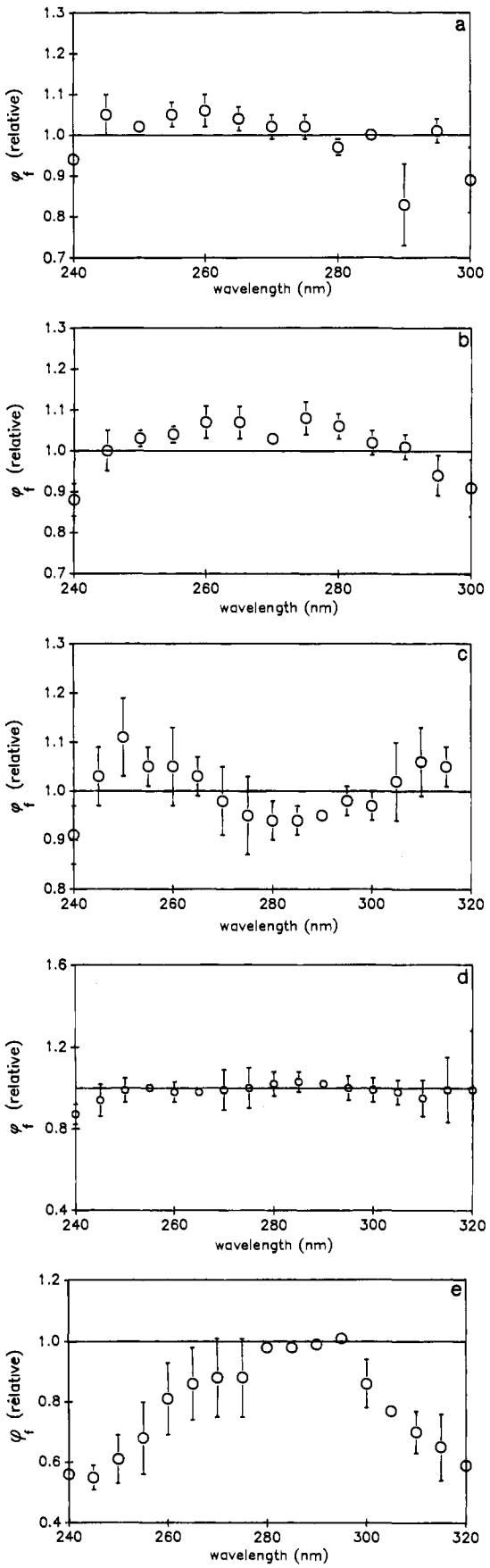

Figure 7. Relative fluorescence quantum yields. ϕ_F, as a function of excitation wavelength at neutral pH (unless otherwise indicated) for (a) indole, (b) 5-methoxyindole, (c) 7-azatryptophan, (d) rhodamine B in ethylene glycol, (e) complex of biotinylated 7-azatryptophan and avidin. The quantum yields presented here are relative to those obtained at the absorption maximum.

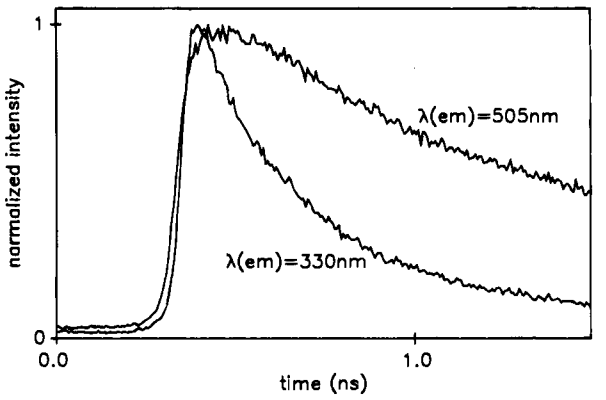

Figure 8. Fluorescence decay of biotinylated 7-azatryptophan bound to avidin at emission wavelengths of 330 ± 8 nm and ≥ 505 nm at 20 °C. λ_{ex} = 290 nm. (a) λ_{em} = 330 ± 8 nm, $K(t)$ = 0.60 exp(−t/78 ps) + 0.34 exp(−t/420 ps) + 0.06 exp(−t/1830 ps), χ^2 = 1.65. (b) λ_{em} ≥ 505 nm, $K(t)$ = −0.26 exp(−t/828 ps) + 1.03 exp(−t/822 ps) + 0.23 exp(−t/6580 ps), χ^2 = 1.40. In both measurements, data were collected only to a maximum of 3800 counts given the low fluorescence intensity in the emission wavelength range of interest.

in pure alcohols. In alcohols, a second maximum is observed at lower energies. This second band arises from excited-state tautomerization,[2,5,8,19,32] which does not occur to any significant extent either in pure water[5,8,9,33] or in the complex studied here. The conclusion concerning the degree of freedom afforded to the 7-azatryptophan moiety is also confirmed by the X-ray structure of the avidin−biotin complex,[25] which indicates that carboxylate groups of the valeryl side chain of biotin (used to form the linkage with 7-azatryptophan) lie at the surface of the protein.

Conclusions

1. Fluorescence anisotropy measurements of the complex of biotinylated 7-azatryptophan with avidin suggests the utility of 7-azatryptophan as probe of small molecule−protein interactions owing to its spectroscopic distinguishability with respect to tryptophan and to its intrinsic single-exponential fluorescence decay.

2. The spectroscopic distinguishability of 7-azatryptophan in the complex of biotinylated 7-azatryptophan and avidin is less than that expected from a comparison of the individual optical properties of 7-azatryptophan and tryptophan (50−60% as opposed to 85% at time zero, Figure 6). This result provides an example of the sensitivity of the 7-azaindole chromophore to its environment and indicates that not all such interactions may be favorable to its role as a probe molecule. As we have discussed elsewhere, much of the sensitivity of the fluorescence of 7-azaindole to its environment is a consequence of the proton bound to the 1-nitrogen, which can interact with the solvent and promote either internal conversion to the ground state or states of solvation that favor excited-state tautomerization.[4,5,8,9,12] In certain circumstances, it may be more convenient to use a chromophore where this interaction with the solvent is prohibited.[12] An excellent candidate is afforded by 1-methyl-7-azaindole, which has a fluorescence lifetime and quantum yield

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in water of 21 ns and 0.55, respectively.[5,32] The long fluorescence lifetime of 1-methyl-7-azaindole provides the additional advantage of permitting the measurement of rotational diffusion times on a time scale of tens of nanoseconds. The spectroscopic distinguishability of this chromophore is clearly demonstrated in Figure 6.

3. The occurrence of energy transfer from tryptophan to 7-azatryptophan is demonstrated and suggests another role for 7-azatryptophan as a probe of structure and environment.

4. Biotinylated 7-azatryptophan binds tightly to avidin (as demonstrated by chromatography). The 7-azatryptophan moiety can be modelled as diffusion in a cone with a half angle of 29 ± 5°.

5. The four avidin binding sites provide equivalent environments as indicated by the fluoresence lifetime and anistropy decay of the bound biotinylated 7-azatryptophan.

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