Synthesis and Photophysics of the Optical Probe N_1-Methyl-7-azatryptophan

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Abstract: The development of a new intrinsic optical probe of protein structure and dynamics, N_1-methyl-7-azatryptophan (1M7AT), is reported. The utility of this nonnatural amino acid derivative lies in its single-exponential, long-lived fluorescence decay (21.7 ± 0.4 ns) and in its high fluorescence quantum yield (0.53 ± 0.07). Its absorption and emission maxima are red-shifted 10 and 65 nm, respectively, from those of tryptophan. These characteristics permit its unambiguous detection with unprecedented discrimination against emission from multiply occurring native tryptophan residues. In a mixture of these two amino acids, no tryptophan signal is detected until the tryptophan:N_1-methyl-7-azatryptophan ratio exceeds 75:1. Consequently, N_1-methyl-7-azatryptophan is ideal for studying the interactions of small peptides containing it with large proteins.

Introduction

The difficulties in using tryptophan as an optical probe of protein structure and dynamics are well known. Tryptophan has an intrinsic nonexponential fluorescence decay, and it occurs multiply in most proteins of interest.[1−7] Consequently, we have devoted considerable effort to the development and characterization of the nonnatural amino acid 7-azatryptophan as an alternative optical probe,[8−19] and other groups have subsequently begun to exploit its properties.[20,21] 7-Azatryptophan has a single-exponential fluorescence decay (780 ps in water, pH 7 and 20 °C), and the locations of its absorption and emission maxima permit it to be detected unambiguously in the presence of up to 10 tryptophan residues.[8,11,16] Furthermore, it is amenable to peptide synthesis and can be incorporated into bacterial protein.[8,11] These qualities are extremely useful, especially when short-time dynamics are of interest and when there are a limited number of tryptophans present. We have, however, demonstrated in a recent study of biotinylated 7-azatryptophan in complex with avidin that the relatively low fluorescence quantum yield of 7-azatryptophan (0.03 in water, pH 7) can diminish its utility when long-time dynamics are of interest.[19]

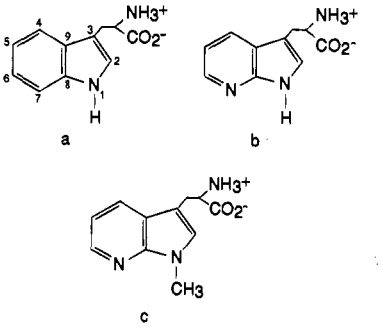

Figure 1. Structures of (a) tryptophan, (b) 7-azatryptophan, and (c) N_1-methyl-7-azatryptophan.

In order to address problems where long-time dynamics are of interest and many tryptophan residues are present, it is necessary that the optical probe have both a long-lived excited state and a high fluorescence quantum yield. We have already suggested that N_1-methyl-7-azatryptophan (Figure 1) conforms to these requirements[19] on the basis of our understanding of the photophysics of the 7-azaindole chromophore.[9−19] The most significant nonradiative properties of 7-azaindole (in particular, those that distinguish it from indole) are determined by the N_1 proton and its interactions with the solvent. In alcohols[9,15,17,22,23] (and to a small degree in water[13,24−26] this proton participates in an excited-state double-proton transfer. Internal conversion promoted by the interaction of this proton with the solvent has also been suggested.[27] The importance of the N_1 proton in the nonradiative process of 7-azaindole is demonstrated most vividly by the methylation of N_1: in water, the fluorescence lifetime and quantum yield increase from 910 ps and 0.03 to 21.0 ns

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Table 1. Summary of Photophysical Data

compound[a]	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)	ϵ[b] (M^{-1} cm^{-1})	ϕ_f	A_1[c]	τ_1 (ns)	τ_2[d] (ns)
tryptophan	280[35]	351[16]	5400[35]	0.18[22]	0.22 ± 0.01	0.620 ± 0.050	3.2 ± 0.1[1]
7-azatryptophan (7AT)	288[11]	397[16]	6200[11]	0.03 ± 0.01	1.00	0.780 ± 0.010[e,16]	
NAc-P(7AT)N-NH_2	289	397	6200[g]	0.02 ± 0.01	1.00	0.830 ± 0.010	
NAc-KACP(7AT)NCD-NH_2[f]	289	396	6200[g]	0.03 ± 0.01	0.84 ± 0.04	0.850 ± 0.010	0.190 ± 0.030
N-*t*-Boc-N_1-methyl-7-azatryptophan	289	414	8300[h]	0.47 ± 0.02	1.00	16.1 ± 1.1	
N_1-methyl-7-azatryptophan (1M7AT)	289	409	8300[h]	0.53 ± 0.07	1.00	21.7 ± 0.4	
SIIN(1M7AT)EKL[i]	289	407	8300[h]	0.52 ± 0.06	1.00	16.4 ± 0.3	

[a] Zwitterionic forms of all amino acids, measured at 20 °C. Naturally occurring amino acids are abbreviated using the standard nomenclature.[37] [b] The extinction coefficients are measured at the absorption maxima. [c] Fluorescence lifetimes are fit to a double exponential of the form $F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$, where $A_1 + A_2 = 1.00$. [d] The absence of an entry indicates that the fluorescence decay was best fit to a single exponential. [e] Nonexponential fluorescence decay can be detected in 7-azatryptophan in water if emission is collected with a sufficiently narrow bandwidth on the blue or red edge of the spectrum.[13] [f] Experiments were performed under aqueous conditions in which the cysteine residues were assumed to be reduced thiols. [g] The extinction coefficient was assumed to be equal to that of 7-azatryptophan.[11] [h] The extinction coefficient was assumed to be equal to that of N_1-methyl-7-azaindole.[13] [i] Peptide dissolved in phosphate-buffered saline containing 0.5% gelatin and 0.02% NaN_3.

and 0.55.[13] We anticipated that the methylated analog of 7-azatryptophan would possess the same characteristics.[13,19] In this paper we demonstrate that it does and comment on the utility of this result.

Materials and Methods

Synthesis of N_1-Methyl-7-azatryptophan. The preparation of N_1-methyl-7-azatryptophan was performed in three steps: blocking the alkyl nitrogen of D,L-7-azatryptophan with a protecting group to form *N*-*t*-Boc-D,L-7-azatryptophan, deprotonation using lithium diisopropylamide and subsequent methylation of the pyrrolic nitrogen with methyl mesylate to form *N*-*t*-Boc-N_1-methyl-D,L-7-azatryptophan, and deprotection of the alkyl nitrogen to yield the final product N_1-methyl-D,L-7-azatryptophan.

Preparation of *N*-*t*-Boc-D,L-7-azatryptophan. The method used was slightly modified from that of Bodansky and Bodansky[28] and has been briefly outlined in our previous work.[11] Triethylamine (515 μL, 3.67 mmol; Kodak) was added to D,L-7-azatryptophan (500 mg, 2.44 mmol; Sigma) with stirring in 1.5 mL of water/1.5 mL of dioxane. As 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile (665 mg, 2.70 mmol; BOC-ON; Aldrich) was added, the mixture became yellow. After stirring for 1 h, all material had dissolved. The solution was stirred for 3 more h. Water (4 mL) and ethyl acetate (5 mL) were added to the solution, and the aqueous fraction was extracted twice with 5 mL portions of ethyl acetate. The aqueous layer was acidified to pH 4 with crystalline citric acid and was filtered. The precipitate was washed with dilute citric acid and ethyl acetate, and dried *in vacuo* to yield *N*-*t*-Boc-D,L-7-azatryptophan (0.70 g, 94%). Mp: 232−236 °C. ^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ 12.60 9s, 1H), 11.41 (s, 1H), 8.21 (d, 1H, J = 3.9 Hz), 7.98 (d, 1H, J = 7.8 Hz), 7.30 (s, 1H), 7.08 (dd, 1H, J_1 = 7.5 Hz, J_2 = 3.3 Hz), 4.18 (br, 1H), 3.19−2.94 (m, 3H), 1.34 (s, 9H). Anal. Calcd for $C_{15}H_{19}N_3O_4$: C, 59.01; H, 6.27; N, 13.76. Found: C, 58.77; H, 6.29; N, 13.52. MS (EISP): m/z 305.1 $[M]^+$.

Methylation of *N*-*t*-Boc-D,L-azatryptophan. Diisopropylamine (595 μL, 4.23 mmol; Sigma) was added to 7 mL of anhydrous THF and stirred under argon at −78 °C for 15 min. *n*-Butyllithium (2.35 mL, 2.1 M, 4.94 mmol; Johnson Matthey Catalog Co.) in hexanes was added dropwise to the amine solution. This mixture was stirred at −78 °C for 1 h and then warmed to room temperature. At this time, the solution was added dropwise to a slurry of *N*-*t*-Boc-D,L-7-azatryptophan (500 mg, 1.64 mmol) with stirring in 7 mL of anhydrous THF/1.5 mL of anhydrous DMSO chilled to −78 °C under an argon atmosphere. The slurry became yellow upon addition of the amide. This mixture was kept at −78 °C for 1 h and then allowed to warm to room temperature. The solution was again chilled to −78 °C, and methyl methanesulfonate (220 μL, 2.60 mmol; Aldrich)[29] was added. The acetone/dry ice bath was removed after 3 h, and the solution continued to stir for 15 h. The reaction was quenched with 15 mL of water and extracted three times with ethyl acetate. Isolation of this product was as for *N*-*t*-Boc-D,L-7-azatryptophan to yield *N*-*t*-Boc-D,L-N_1-methyl-7-azatryptophan. Mp: 217−218 °C. ^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ 12.67 (s, 1H), 8.27 (d, 1H, J = 4.2 Hz), 8.01 (d, 1H, J = 7.2 Hz), 7.34 (s, 1H), 7.10 (dd, 1H, J_1 = 6.9 Hz, J_2 = 4.2 Hz), 4.17 (br, 1H), 3.80 (s, 3H), 3.18−3.04 (m, 3H), 1.35 (s, 9H). Anal. Calcd for $C_{16}H_{21}N_3O_4 \cdot H_2O$: C, 56.97; H, 6.82; N, 12.46. Found: C, 57.91; H, 6.82; N, 12.65. MS (EISP): m/z 319.2 $[M]^+$.

Deprotection of *N*-*t*-Boc-D,L-N_1-methyl-7-azatryptophan. *N*-*t*-Boc-N_1-methyl-7-azatryptophan (100 mg, 0.32 mmol) was dissolved in 1 mL of concentrated hydrochloric acid and allowed to stir for 1 h. The solution was then concentrated to near dryness by rotary evaporation. Water (1 mL) was added, and the solution was again evaporated to near dryness. This last step was repeated, then 1 mL of water was added to the solution, and solid lithium hydroxide was added to neutralize the solution. The solution was then evaporated to dryness. After addition of 2 mL of 2-propanol to dissolve the lithium chloride, the solution was filtered and the remaining solids were washed repeatedly with 2-propanol to yield N_1-methyl-7-azatryptophan. The NMR confirmed the absence of the *N*-*t*-Boc protecting group, but otherwise agreed with that obtained for *N*-*t*-Boc-D,L-N_1-methyl-7-azatryptophan. Mp: decomposition began at ~235 °C, with sharp melting at 292 °C. MS (ESI+): m/z 220.1 $[MH]^+$.

Synthesis of Peptides. Synthesis of peptides containing 7-azatryptophan (7AT) or N_1-methyl-7-azatryptophan (1M7AT) was performed as described elsewhere,[11] and the purity of the peptides was verified by HPLC. The peptide sequences listed in Table 1 employ the standard one-letter abbreviations of the natural amino acid residues and our chosen abbreviations for the optical probes.

Spectroscopic Measurements. Fluorescence lifetimes were obtained by means of time-correlated single-photon counting.[13,14] Owing to the long fluorescence lifetime (Table 1) of N_1-methyl-7-azatryptophan, a 50-ns full-scale time window was required to characterize it properly. The fluorescence decays of mixtures of one N_1-methyl-7-azatryptophan to varying amounts of tryptophan were measured in order to determine what level of tryptophan produces an appreciable background signal. These measurements were performed on a 3-ns time scale in order to be more sensitive to onset of the tryptophyl fluorescence lifetime, especially its subnanosecond component (Table 2). The fluorescence decays of the mixtures were adequately fit to one or a sum of two exponentially decaying components: $F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$, where $A_1 + A_2 = 1.00$. Two exponentials only became necessary at probe:tryptophan ratios of less than 1:75.

(25) Charge transfer to the side chain is generally considered to be the major nonradiative process in tryptophan,[1,16,32] and different charge transfer rates owing to a distribution of ground-state conformers is attributed to the origin of the nonexponential fluorescence decay in tryptophan. Although 7-azaindole is capable of photoionization,[12,33] we have argued that charge transfer to the side chain is not operative in 7-azatryptophan because it is not thermodynamically favorable.[16] This argument is confirmed by the absence of nonexponential decay in the 7-azatryptophan tripeptide (Table 1) and its presence in all tryptophan-containing tripeptides.[1,16]

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Table 2. Average Fluorescence Lifetime of Mixtures of N_1-Methyl-7-azatryptophan (1M7AT) and Tryptophan

1M7AT:Trp	$\langle\tau\rangle^a$ (ns)	1M7AT:Trp	$\langle\tau\rangle^a$ (ns)
1:0	21.7 ± 0.4	1:150	9.1 ± 2.3
1:50	23.8 ± 0.3	1:175	6.6 ± 1.5
1:75	22.3 ± 0.4	1:200	5.2 ± 0.9
1:100	9.4 ± 1.5		

[a] Data are collected at pH 7 and 20 °C. $\langle\tau\rangle = A_1\tau_1 + A_2\tau_2$; see Materials and Methods. λ_{ex} = 310 nm; $\lambda_{em} \geq$ 455 nm.

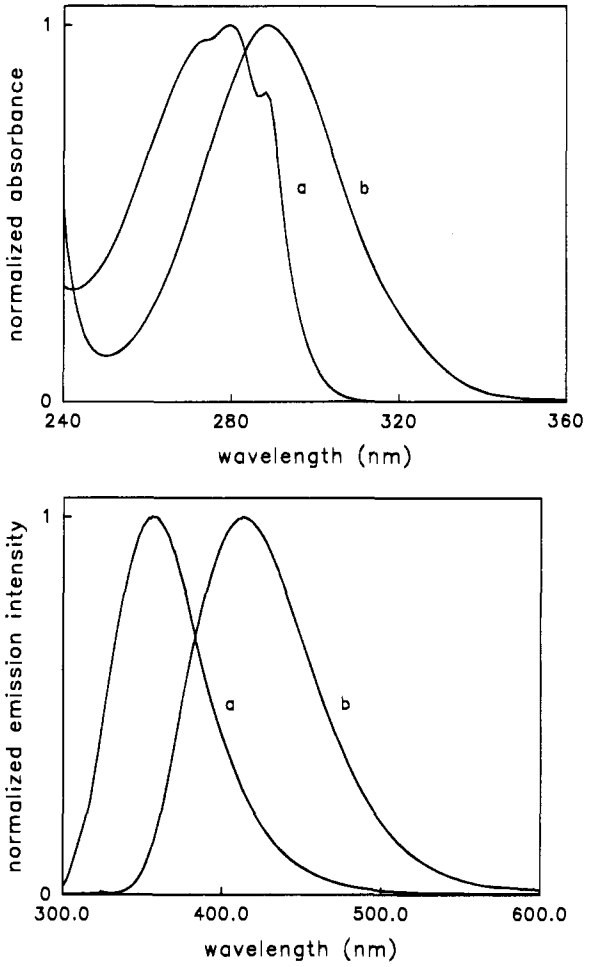

Figure 2. Comparison of the normalized absorption (top) and emission (bottom) of (a) tryptophan and (b) N_1-methyl-7-azatryptophan.

Consequently, we made no attempt to attach physical significance to the lifetimes or weights, but concentrated on the visual deviation of the fluorescence decays of the mixtures with respect to the probe alone and on the average fluorescence lifetime computed from the decay parameters: $\langle\tau\rangle = A_1\tau_1 + A_2\tau_2$ (Figure 4 and Table 2). The N_1-methyl-7-azatryptophan concentration was 2−20 μM for these experiments, and the tryptophan concentration was adjusted accordingly.

Fluorescence quantum yield determinations were performed using tryptophan ($\phi_f = 0.20$[36]) as a standard. Absorbance measurements were made using a Perkin-Elmer Lambda 18 double-beam UV/vis spectrophotometer. Fluorescence measurements were obtained with a Spex Fluoromax fluorimeter. All measurements were conducted at room temperature (~21 °C).

Results and Discussion

The absorption and emission spectra of N_1-methyl-7-azatryptophan and tryptophan are shown in Figure 2. Table 1 summarizes the steady-state and the time-resolved data. Because of the shift in the absorption and emission spectra of N_1-methyl-7-azatryptophan with respect to tryptophan, and because of the very large fluorescence quantum yield of N_1-methyl-7-azatryptophan (0.53 ± 0.07), it is expected that its fluorescence decay can be uniquely detected in the presence of many background tryptophan residues. A comparison of the fluorescence decays of N_1-methyl-7-azatryptophan, tryptophan, and 7-azatryptophan is given in Figure 3. To illustrate the usefulness of N_1-methyl-7-azatryptophan as an optical probe in an environment containing multiple tryptophans, we measured the fluorescence decay lifetime of the mixture of these two amino acids. The fluorescence decay profile of the mixture exactly overlaid that of N_1-methyl-7-azatryptophan up to a 75-fold excess of tryptophan, where the average fluorescence lifetime begins to become perceptibly shorter. The tryptophyl contribution to the fluorescence decay becomes much more apparent as the probe: tryptophan ratio approaches 1:200 (Table 2, Figure 4). Few, if any, naturally occurring biological systems contain this many tryptophans; clearly, emission from our optical probe would be unambigously observed when incorporated into proteins. *Finally, the single-exponential fluorescence decay of N_1-methyl-7-azatryptophan permits a simplified interpretation of time-resolved data. Any change in its fluorescence decay can be directly attributed to its environment.*

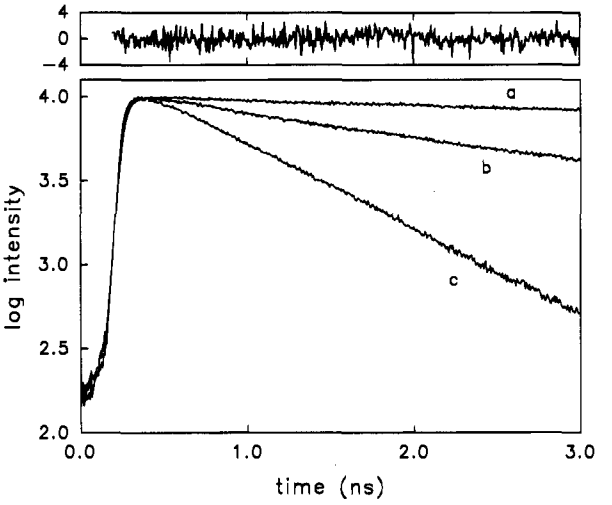

Figure 3. Fluorescence decay of (a) N_1-methyl-7-azatryptophan in water (pH 7.0, 20 °C, λ_{ex} = 310 nm, $\lambda_{em} \geq$ 455 nm, $F(t) = 1.00\exp(-t/22.8\text{ ns})$, $\chi^2 = 1.07$), (b) tryptophan (pH 7.0, 20 °C, λ_{ex} = 310 nm, $\lambda_{em} \geq$ 320 nm, $F(t) = 0.16\exp(-t/332\text{ ps}) + 0.84\exp(-t/3.08\text{ ns})$, $\chi^2 = 1.13$), and (c) 7-azatryptophan in water (pH 7.0, 20 °C, λ_{ex} = 310 nm, $\lambda_{em} \geq$ 375 nm, $F(t) = 1.00\exp(-t/812\text{ ps})$, $\chi^2 = 1.16$). Fluorescence decay parameters of each species are listed in Table 1. These experiments were performed on a 3-ns time scale to emphasize the monoexponential fluorescence decay lifetime of N_1-methyl-7-azatryptophan. Displayed above the fluorescence decay profiles are the residuals for N_1-methyl-7-azatryptophan in water.

Sensitivity to the Environment of the Fluorescence Properties of the Chromophores 7-Azaindole and N_1-Methyl-7-azaindole. Unlike the photophysics of tryptophan where blue shifts or red shifts of the fluorescence spectrum can only be crudely interpreted in terms of, respectively, nonpolar or polar *continuous* environments and where nonexponential decay can only be rationalized by a sweeping invocation of conformational heterogeneity, the delicate nature of the photophysics of 7-azaindole provides the possibility of gleaning much more detailed and specific information on the environment of the chromophore. *This is because the photophysics of 7-azaindole can only be satisfactorily explained by understanding in microscopic detail its interactions with its solvation environment: a continuum picture of the environment is not sufficient.*

It has been suggested[20] that if the 7-azaindole chromophore is located in a hydrophobic pocket, it will exhibit a long

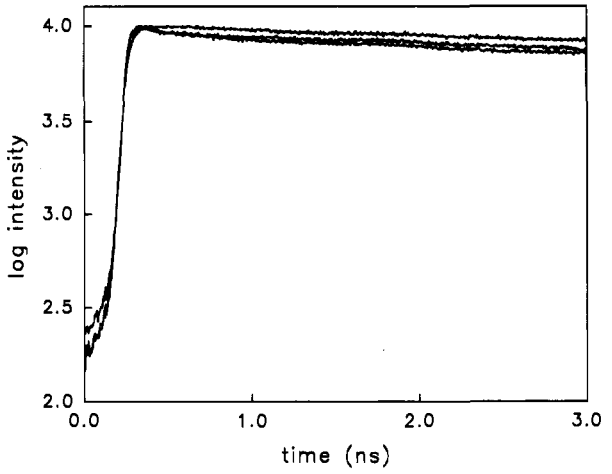

Figure 4. Fluorescence decay of (top trace) N_1-methyl-7-azatryptophan ($F(t)$ = 1.00 exp(−t/22.8 ns), χ^2 = 1.07) and mixtures of N_1-methyl-7-azatryptophan and tryptophan (middle trace, 1:100, $F(t)$ = 0.35 exp(−t/83 ps) + 0.65 exp(−t/12.3 ns), χ^2 = 1.28; bottom trace, 1:200, $F(t)$ = 0.41 exp(−t/96 ps) + 0.59 exp(−t/10.4 ns), χ^2 = 1.21) (pH 7.0, 20 °C, λ_{ex} = 310 nm, $\lambda_{em} \geq$ 455 nm). No contribution from tryptophan to the fluorescence decay lifetime is apparent up to a 75-fold excess of tryptophan. In our work with 7-azatryptophan, we performed similar measurements[11] with mixtures of *N*-acetyltryptophanamide (NATA) because it is known to have an anomalous single-exponential lifetime of 3-ns duration. This lifetime component consequently provides a telling contrast to the 780-ps lifetime of 7-azatryptophan. In the measurements displayed in this figure and reported in Table 2, tryptophan itself affords the more rigorous test because of the presence of its subnanosecond component, which is expected to stand out in starkest contrast against the 21-ns lifetime of the probe. The nonnatural amino acid 5-hydroxytryptophan has also been proposed as an optical probe.[34] Its fluorescence lifetime is not, however, sufficiently different from that of tryptophan to provide a significant contrast in mixtures.[16]

fluorescence lifetime. It must be kept in mind, however, that one of the primary nonradiative processes in 7-azaindole is excited-state proton transfer, as observed in dimers,[31] in alcohols,[9,15,17,22,23] and to a much lesser extent in water.[13,24-26] This excited-state tautomerization can occur if an appropriate hydrogen bonding interaction is established between the solvent molecule, the N_1 proton, and N_7. Consequently, the ability of 7-azaindole to tautomerize does not necessarily depend on the presence of either a hydrophobic or a hydrophilic environment but rather on the specific nature of the available molecular species that can provide suitable hydrogen bonding partners.

Table 1 provides a comparison of the behavior of 7-azaindole and N_1-methyl-7-azaindole as fluorescent probes in systems of varying complexity. While 7-azatryptophan and the tripeptide NAc-Pro-7-AzaTrp-Asn-NH_2 exhibit single-exponential fluorescence decay, the octapeptide NAc-Lys-Ala-Cys-Pro-7-Aza-Trp-Asn-Cys-Asp-NH_2 provides a nonexponential fluorescence decay. Clearly, the nonexponential fluorescence decay in the octapeptide must be induced directly by the amino acid side chain residues or indirectly by their ability to reorganize water about the chromophore.[25] On the other hand, both N_1-methyl-7-azatryptophan and the octapeptide Ser-Ile-Ile-Asn-(1M7AT)-Glu-Lys-Leu display single-exponential fluorescence decay because of the absence of the N_1 proton.

Although methylation of N_1 in 7-azaindole eliminates excited-state proton transfer as a nonradiative process, it should not be assumed that the fluorescence properties of N_1-methyl-7-azaindole or of 1M7AT are insensitive to the environment. For example, the fluorescence lifetime of N_1-methyl-7-azaindole increases by a factor of ~6 in going from cyclohexane to D_2O as a solvent.[26]

Conclusions

Previously we have shown that 7-azatryptophan can be uniquely detected in an environment of up to a 10-fold tryptophan excess. We have improved the optical selectivity of the probe by methylating N_1. N_1-Methyl-7-azatryptophan (1M7AT) has numerous advantages over other intrinsic fluorescent probes currently in use. It has red-shifted absorption and emission spectra with respect to those of tryptophan and a very high fluorescence quantum yield. This latter feature allows for a shorter data collection time and analysis of smaller or more dilute sample volumes. This probe is characterized by a long-lived monoexponential fluorescence decay that is also clearly distinguishable from that of tryptophan. In addition, 1M7AT is amenable to incorporation into peptide sequences. These combined factors allow for unambiguous detection of the probe signal in biological systems where site-specific analyses are expected to be difficult, if not impossible, owing to an overwhelming tryptophan content. The immediate and most powerful use of 1M7AT will be to incorporate it into small peptides of known biological interest and to study the interactions of these tagged peptides with larger proteins.

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