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FEATURE ARTICLE

Photophysics and Biological Applications of 7-Azaindole and Its Analogs

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7-Azaindole is the chromophoric moiety of 7-azatryptophan, which is an alternative to tryptophan as an optical probe of protein structure and dynamics. The great power of the 7-azaindole chromophore is that it is red shifted both in absorption and emission from tryptophan, that its fluorescence decay is single exponential in water under appropriate conditions, and that its emission is sensitive to solvent. In addition, 7-azatryptophan can be incorporated into synthetic peptides and bacterial protein. In this article, the interactions of 7-azaindole with its environment are discussed. Special attention is directed to the difference in its fluorescence properties in water as opposed to nonaqueous solvents. The sensitivity of 7-azaindole to its environment is demonstrated and then exploited by studying it and its analogs in peptides and in complexes with larger proteins containing many tryptophan residues.

Introduction

A. Background. The standard optical probe of protein structure and dynamics has been tryptophan (Figure 1). The use of tryptophan, however, presents a number of problems. Most significant are the difficulties in the interpretation of the data posed by the intrinsic nonexponential fluorescence decay of tryptophan[1−11] and the occurrence of proteins containing more than one tryptophan residue (which consequently renders the origin of the signal derived from the multiple probes ambiguous). Consequently, we have devoted considerable effort to the development and characterization of the nonnatural amino acid 7-azatryptophan as an alternative probe;[12−19] and other groups have subsequently begun to exploit its properties.[20,21,74]

7-Azatryptophan is a promising alternative to tryptophan as a photophysical probe. In addition to having a single-exponential lifetime decay in water over most of the pH range, (e.g., 780 ps at pH 7 and 20 °C), 7-azatryptophan has optical spectra that differ significantly from those of tryptophan.[12−19] The absorption and emission spectra of 7-azatryptophan are red shifted 10 and 46 nm, respectively, from those of tryptophan.[82] We have proposed that the "well-behaved" fluorescence properties of 7-azatryptophan are a result of the interactions of the 1-nitrogen with the solvent[13] and the relatively low energy of its fluorescent state with respect to that of tryptophan.[17]

7-Azatryptophan thus has great potential as a probe of protein structure and dynamics, especially for investigations of protein−protein interactions, in which one of the proteins involved may contain several tryptophans. A dramatic illustration of the optical selectivity afforded by the lifetime and the spectroscopic distinguishability of 7-azatryptophan is that, in a solution 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;[12,17] and even when the ratio is 40:1 the 7-azatryptophan is easily detected in the mixture. 5-Hydroxytryptophan, which has been proposed as a useful biological

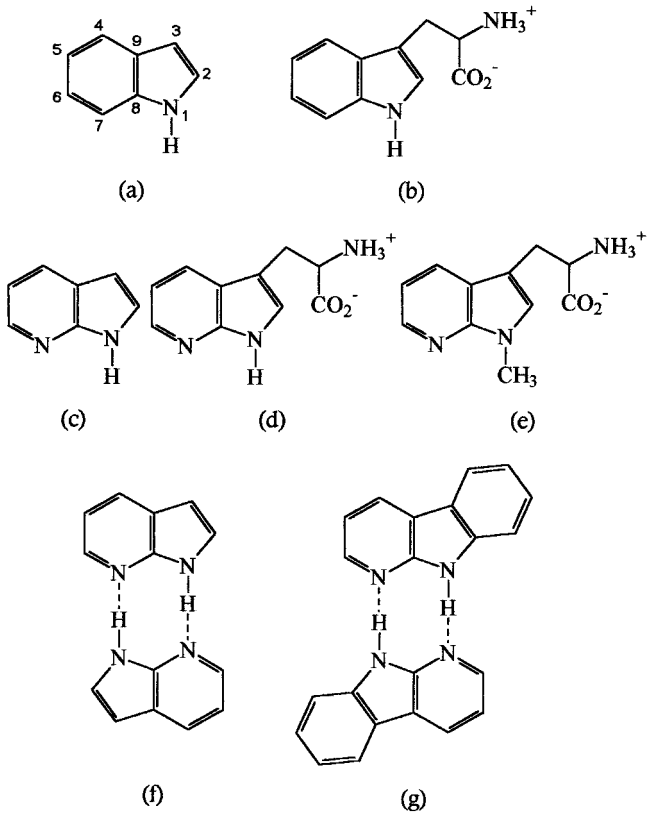

Figure 1. Structures of (a) indole, (b) tryptophan, (c) 7-azaindole, (d) 7-azatryptophan, (e) 1-methyl-7-azatryptophan, (f) 7-azaindole dimer, and (g) 1-azacarbazole dimer.

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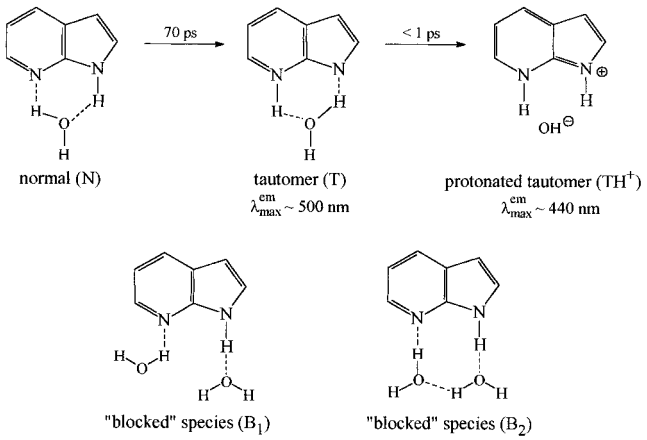

Figure 2. *Idealized* depictions of 7-azaindole/H_2O interactions. Our interpretation of the photophysics of 7-azaindole in H_2O is that most of the solute population is inappropriately solvated for proton transfer. The evidence for this conclusion is that time-resolved measurements indicate that not more than 20% of the solute population tautomerizes in ~70 ps. If more of the solute tautomerized, a characteristic tautomer emission at 500 nm should be observed. This is not the case. What is observed is a small population emitting at 440 nm, characteristic of a protonated tautomer.[13] In previous work we have schematically denoted the blocked species by the structure, B_1. Recent calculations[63,64] and experiments[65] indicate that, at least in the gas phase, a more realistic blocked structure is given by B_2.

probe,[22] has a fluorescence lifetime comparable to that of tryptophan (3.8 ns) and consequently cannot provide the same degree of optical selectivity.[17]

Kasha and co-workers were the first to realize that dimers of 7-azaindole (Figure 1f) can undergo excited-state tautomerization.[23] It was subsequently observed[24−26] that alcohols can provide a state of solvation (presumably a cyclic one, of the type indicated in the top of Figure 2) at room temperature in which such a double-proton transfer is possible with the monomer. In water, on the other hand, we[13] and Kasha and co-workers[27] have provided evidence that such a state of solvation is largely prohibited (Figure 2, bottom). Nevertheless, in water at room temperature, the fluorescence lifetimes of 7-azaindole and 7-azatryptophan are relatively short-lived (~900 and 780 ps, respectively), owing most likely to internal conversion[13,27−29] facilitated by the N_1-H stretch or, possibly, to dissociation of the bond itself. The 7-azaindole chromophore is thus remarkably sensitive to its state of solvation. The N_1-H interacts strongly with the solvent through hydrogen bonding, and the chromophore is poised to undergo an excited-state double-proton transfer. The nonradiative pathways permitted by the N_1-H group are very effectively shut down by methylation of the 1-nitrogen.[13] 1-Methyl-7-azaindole has a fluorescence lifetime of 21 ns and a fluorescence quantum yield of 0.55 in water at 20 °C.[13] This suggests, as has been borne out in our investigations, that 1-methyl-7-azatryptophan (Figure 1e) will have a similarly long-lived fluorescence decay and hence be an excellent probe of events transpiring on a more extended time scale.[19] The discussion of the 7-azaindole photophysics presented here centers on the excited-singlet states. Investigations of the triplet have been performed by Waluk et al.,[28] Bulska et al.,[29] and most recently by Maki and co-workers.[30]

In order for 7-azatryptophan to be useful as a biological probe and not merely a photophysical curiosity, it is required that 7-azatryptophan be amenable to incorporation into proteins and peptides and that these modified systems remain functional. Pardee, Prestige, and co-workers[31] and Brawerman and Yčas[32] were the first to demonstrate that bacteria can be grown in media containing 7-azatryptophan instead of tryptophan and that 7-azatryptophan can be incorporated into bacterial protein. Schlesinger[33] has shown that, in the tryptophan auxotroph of *Escherichia coli*, W3747, active alkaline phosphatase is formed when tryptophan is replaced by 7-azatryptophan in the growth medium. Other groups have investigated various aspects of 7-azatryptophan uptake in bacteria.[34−36] More recently, we have demonstrated that the *E. coli* tryptophan auxotroph (ATCC 23803) incorporates 7-azatryptophan and that the β-galactosidase expressed retains ~20% of its activity.[12] A powerful technique that can be used to incorporate nonnatural amino acids into wild-type or mutant proteins site specifically has been developed independently by the groups of Chamberlin and Schultz.[37,38] In this technique, a cell-free extract capable of protein synthesis is supplied with an mRNA containing a stop codon at the site of interest. An aminoacyl suppressor tRNA capable of recognizing the stop codon, which is not a substrate for the endogenous aminoacyl−tRNA synthetases, is chemically modified with a nonnatural amino acid and is then added to the extract. This results in suppression of the stop codon, insertion of a nonnatural amino acid, and continuation of protein synthesis. Finally, we have begun to explore the use of 7-azatryptophan and 1-methyl-7-azatryptophan for tagging small peptides or cofactors in order to study them in complex with larger proteins.[18,19]

B. Organization of this Article. The Results and Discussion section of this article is divided into three sections. First, we address the current understanding of the photophysics of the 7-azaindole chromophore, in particular how the photophysics are affected by the surrounding environment, for example, the solvent (section IIIA). Two topics have been at the forefront of the discussion here: the mechanism by which 7-azaindole executes a double-proton transfer in alcohols; and the extent to which the nonradiative processes differ in alcohols and water. *The sensitivity of 7-azaindole to its environment depends on both specific and bulk interactions with the solvent.* The distinction between these interactions, which is illustrated by the examples presented in this article, is elaborated upon in section IV. Specific interactions with the solvent are dominated by interaction with the N_1-H. These interactions can be exploited in the study of protein structure and dynamics either by using the onset of excited-state tautomerization as a probe of local environment or by inhibiting any nonradiative processes involving the N_1-H by substituting this hydrogen with a methyl group. The bulk interactions of 7-azaindole with the solvent are those that are typical of most indoles and find their origin in the large dipole change of one of the two closely lying excited states, the ^1L_a state, with respect to its value in the ground state. This dipole change causes the solvent to relax about the new dipole and consequently produces changes in the fluorescence maximum and lifetime of the chromophore. Meech et al.[69] have provided an initial, qualitative description of the bulk effects of solvent on the excited states of indoles. More detailed investigations of these states, in particular, the charge transfer character of the ^1L_a state and the interaction of the ^1L_a state with solvent, have been provided by Callis and co-workers and are discussed in a recent review article.[86]

Second, in order to incorporate the 7-azaindole chromophore into a peptide or a protein, it must be attached as an amino acid side chain. The differences in the photophysics of this nonnatural amino acid, 7-azatryptophan, with respect to its naturally occurring analog, tryptophan, are discussed (sections IIIBi). We report the synthesis of a 7-azatryptophan-containing tripeptide and octapeptide that mimic the active site of the potato chymotrypsin inhibitor II:[43] NAc-Pro-7-azatryptophan-Asn-NH_2 and NAc-Lys-Ala-Cys-Pro-7-azatryptophan-Asn-Cys-Asp-NH_2. It is of considerable significance that the fluorescence decay of

the tripeptide is single exponential but that of the octapeptide is nonexponential. This is discussed in detail (section IIIBii).

Third, the use of the methylated analog of 7-azatryptophan, 1-methyl-7-azatryptophan, to study interactions of cofactors and of small peptides with larger proteins containing many tryptophan residues. Two examples are investigated: the interaction of tagged biotin (*N*-α-biotinoyl-D,L-1-methyl-7-azatryptophan methyl ester) with avidin (section IIICi) and the interaction of a tagged antigen [SIIN(1-methyl-7-azatryptophan)EKL] with the class I major histocompatibility complex molecule (MHC) (section IIICii). Whereas the fluorescence lifetime of the biotinylated 1-methyl-7-azatryptophan is single exponential in the complex with avidin, complexation of the tagged antigen to MHC induces nonexponential fluorescence decay in 1-methyl-7-azatryptophan. This result points to the sensitivity of the chromophore to its environment, despite its lack of labile protons.

II. Experimental Section

A. Peptide Synthesis Involving 7-Azatryptophan and 1-Methyl-7-azatryptophan. D,L-7-Azatryptophan is obtained from Sigma. 1-Methyl-7-azatryptophan is prepared as described elsewhere.[19] The synthesis of peptides containing 7-azatryptophan and 1-methyl-7-azatryptophan is similar to that of the tripeptide, NAc-Pro-7-azatryptophan-Asn-NH_2.[15] Synthesis is performed with an Applied Biosystems 430A peptide synthesizer starting with benzhydrylamine resin. The purity of the peptides was verified by HPLC. Diasteriomeric peptides were separated on a Beckman HPLC system with a Vydac reverse-phase C-18 semipreparative column (10 × 250 mm). An important feature of the octapeptide NAc-Lys-Ala-Cys-Pro-7-azatryptophan-Asn-Cys-Asp-NH_2 is that it contains two cysteine residues, which provide the potential for disulfide formation. This eventuality is discussed and considered in the following experimental procedures. Since this octapeptide yields a double-exponential fluorescence lifetime (see below), its preparations (i.e., reduced, oxidized, and alkylated forms) were frequently checked for impurities or degradation by TLC using Sigma T-6770 precoated plates and several solvent systems: EtOH/H_2O, EtOAc/H_2O, and BuOH/H_2O/HOAc in various proportions. All results gave a single spot when viewed under UV light or after development in an I_2 chamber.

B. Derivatization of the 7-Azatryptophan Octapeptide. Several routes were employed to ensure complete reduction or oxidation of the cysteine thiols. Identical results were obtained for both the D- and the L-octapeptides.

i. Reduction of Cysteine Residues. Two methods for reducing the disulfide linkages within the octapeptide were employed. Dithiothreitol (DTT) was added to solutions of the octapeptide to a final concentration of 0.05 M DTT and allowed to react for 4 h under inert atmosphere before analysis to ensure significant reduction of the cysteine thiols. Alternatively, a solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl)[39] was prepared by dissolving 11.5 μmol in 100 μL of water. This TCEP·HCl solution was added to a solution of octapeptide (0.1 μmol) in 100 μL of water. The reduction of disulfide bonds appears to be immediate since there is no change in the fluorescence lifetime data when the reaction mixture is monitored over several hours.

ii. Alkylation of Cysteine Residues. Another strategy employed to remove disulfide linkages was to alkylate all the cysteine residues. Disulfide linkages in the octapeptide were reduced using TCEP·HCl as described above. 2-Methylaziridine (Aldrich) was added undiluted in a quantity representing a greater than 40-fold excess with respect to the number of sulfhydryl groups.[40] The solution was evaporated to dryness under argon to remove all excess 2-methylaziridine. The alkylated octapeptide was reconstituted in water. Solutions of the reduced and alkylated octapeptides were deoxygenated with argon; the concentration of dissolved oxygen was less than 6.25×10^{-6} M as measured by a Hach OX-2P kit. A positive pressure of argon was maintained throughout the measurements.

iii. Oxidation of Cysteine Residues. Pure oxygen was bubbled gently through a $\sim 10^{-5}$ M aqueous solution of D-octapeptide to induce disulfide bond formation. No noticeable solution evaporation occurred. The oxidation state of the thiols in the octapeptides was verified by a spot test.[41] The spot test depends on the ability of the thiol groups to catalyze the reaction $2NaN_3 + I_2 \rightarrow 2NaI + 3N_2$ (g). The presence of thiol groups is indicated by bubbling due to the evolution of N_2 and the loss of yellow color due to the consumption of I_2. This test is somewhat subjective and may not be sensitive enough for concentrations below 10^{-5} M. The results, however, indicate clearly the presence of −SH groups for the octapeptide under reducing conditions and are negative for the octapeptide under oxidizing conditions. Throughout all measurements, a positive pressure of oxygen was maintained in the sample cuvette.

C. Synthesis and Purification of Biotinylated 1-Methyl-7-azatryptophan. Biotinylated 1-methyl-7-azatryptophan (*N*-α-biotinoyl-D,L-1-methyl-7-azatryptophan methyl ester) was prepared from 1-methyl-7-azatryptophan following the procedure described for the 7-azatryptophan analog.[18] The raw product was purified twice by column chromatography on silica gel (mesh 40-140). All solvents used were HPLC grade (Fisher). Yellowish sample was dissolved in a minimum volume of chloroform and loaded on a 26-cm × 1.8-cm-diameter column. A chloroform/methanol mixture (63%:37%) was used as eluent to collect approximately 20 fractions of 3−5 mL each. The absorbance in the 260−400 nm wavelength range of each fraction was measured using a Perkin-Elmer Lambda 18 UV−Vis spectrometer. Only fractions having an absorbance >0.6 at 288 nm were pooled. The solvent was removed at 10 mmHg at ∼35 °C. The clear, amorphous residue was dried overnight at 1 mmHg and then recrystallized from hot $CHCl_3$ by addition of CH_3CN. Colorless crystals were washed with 3−5 mL of cold CH_3CN and dried overnight at 1 mmHg. Composition $C_{22}H_{29}N_5O_3S$ (MW 459.12 g/mol) was verified by ESI mass spectroscopy in 1:1 methanol/water mixture. Only three major peaks were observed with *m*/*z*: 460.1 (MH^+), 482.1 (MNa^+), 498.2 (MK^+).

D. Preparation of Antigen−MHC Samples. Murine class I MHC (H-2K^b) was provided by Dr. Luc Teyton of R. W. Johnson as a concentrated stock solution (0.32 mM) in PBS buffer, 0.02% NaN_3. The same buffer containing 1−5% reduced Triton X-100 as a surfactant (Aldrich Chemical Co.) was used to prepare peptide stock solutions. Amounts of MHC and antigen used were calculated based upon the K_D[44] for the peptide−MHC pair and the requirement for ≥88−90% of bound peptide given a total 1-methyl-7-azatryptophan chromophore concentration of 2−4 μM. The presence of surfactant was necessary to eliminate protein aggregation, to inhibit nonspecific binding, and to obtain reproducible results for the lifetime of the antigen−MHC complex. Concentrations of the MHC and peptide stock solutions were determined from absorption spectra using the extinction coefficients: $\epsilon(\text{H-2K}^b)_{280\ nm} = 69\ 200\ cm^{-1}\ M^{-1}$;[45] $\epsilon(\text{peptide})_{289\ nm} \simeq 8300\ cm^{-1}\ M^{-1}$.[13] For the time-resolved experiments (vide infra Figure 11), the total sample volume was maintained at ∼50 μL. 2.5-mm cuvettes were used for the sample and for obtaining the instrument response function.

E. Time-Resolved Experiments. Fluorescence lifetimes and fluorescence anisotropy decays were obtained by means of

TABLE 1: Photophysical Parameters of 7-Azaindole and its Analogs[a]

species	τ_1(ps)	τ_2(ps)	A_1	τ_r(ps)[b]	λ_{max}^{abs} (λ_{max}^{em}) (nm)[j]	λ_{ex}, λ_{em} (nm)[k]
tryptophan[1]	620 ± 50	3210 ± 120	0.22 ± 0.01	33.2 ± 5.5[87]	278 (351)	295, ≥320
7-azaindole[13]	886 ± 15		1.00	20 ± 2[c]	289 (385)	305, ≥320
7-azaindole/MeOH[12,46]	147[d]	730	0.90	34 ± 8[14 g]	288 (374, 505)	285, ≥320
7-azatryptophan[13,17]	780 ± 10		1.00	50 ± 3	288 (397)	305, ≥320
7-azatryptophan/DMSO	(15 400) ± 863[h]		1.00		(385)	288, ≥320
7-azatryptophan/CH_3CN	953[h]	6978	0.25		290 (364)	288, ≥335
1-methyl-7-azatryptophan	(21 700) ± 400		1.00		289 (409)	288, ≥335
NAc-KACP(7-AT)NCD-NH_2[e] (oxidized)	216 ± 30	874 ± 11	0.18 ± 0.02	297 ± 48	(389)	290, ≥335
NAc-KACP(7-AT)NCD-NH_2 (reduced)	189 ± 31	845 ± 9	0.20 ± 0.04	312 ± 17	(396)	290, ≥335
NAc-KACP(7-AT)NCD-NH_2 (alkylated)	232	931	0.28	465	(465)	285, ≥335
NAc-P(7-AT)N-NH_2	833 ± 20		1.00	103 ± 13	(397)	310, ≥345
biotinylated-7-azatryptophan[18]	646 ± 9	2690 ± 970	0.98 ± 0.02	108 ± 2	288 (390)	285, ≥335
biotinylated-7-AT/avidin[18]	417 ± 14	2300 ± 40	0.48 ± 0.01	80 ± 18/∞	282 (390)[i]	310, ≥400
biotinylated-1-methyl-7-AT	22 000		1.0	130	289 (410)	288, ≥335
biotinylated-1-methyl-7-AT/avidin	25 700		1.0	403/∞	283 (409)[i]	310, ≥435
SIINFE(1M7-AT)L[f]	(10 300) ± 300[h]		1.0	230 ± 10	289 (407)	310, ≥435
SIINFE(1M7-AT)L[f]/MHC	137[h]	1050	0.57	1820	∼280 (409)[i]	310, ≥435

[a] Unless otherwise specified, the measurement is carried out in water at 20 °C. Fluorescence lifetimes are usually described by two exponentially decaying components: $K(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$. [b] In most cases the fluorescence anisotropy decay is adequately described by a single exponentially decaying component. for the complexes, the anisotropy decay is best described by two exponentially decaying components: $r(t) = r_1(0) \exp(-t/\tau_{r1}) + r_2(0) \exp(-t/\tau_{r2})$. In this case, the longer-lived component does not decay on the time scale of the measurement and is reported in this table as ∞. See the text for further details. [c] 23.5 °C.[14] [d] The rise time for the formation of the tautomer band of 7-azaindole in methanol is 150 ps at room temperature.[13,14] [e] Model proteinase inhibitor in which leucine has been replaced by 7-azatryptophan (7-AT). [f] Model OVA-8 antigen in which lysine has been replaced with 1-methyl-7-azatryptophan (1M7-AT). [g] −9 °C.[14] [h] Measured on a 3-ns time scale. [i] Excited at 310 nm. [j] Steady-state absorption and emission maxima (excited at absorption maximum, unless otherwise specified). [k] Excitation and detection wavelengths for time-resolved measurements.

time-correlated single-photon counting using the apparatus described elsewhere.[13,14,18] Standard sample concentrations were $\sim 10^{-5}$ M. In the case of the antigen−MHC experiments, the concentration of MHC was typically 5 μM and that of the taggend antigen, 2−4 μM. In most cases the emission wavelength was selected by cutoff filters. When more spectral resolution was required, the samples were concentrated to $\sim 10^{-4}$ M (OD ≃ 0.7 at 290 nm for the 7-azatryptophan octapeptide) and emission was collected with an ISA H-10 monochromator with 2-mm slits. This provided a 16-nm band pass. Fluorescence lifetime data $K(t)$ were fit to one or to a sum of decaying exponentials (whose amplitudes are reported as normalized to unity) by an iterative convolution procedure using a nonlinear least squares algorithm. A simultaneous fitting procedure was employed for extracting the anisotropy decay $r(t)$ from parallel and perpendicular fluorescence emission profiles. The quality of the fit was determined by visual inspection of the residuals and the χ^2 criterion.[13,14,18] The results of time-resolved experiments are summarized in Table 1.

III. Results and Discussion

A. Photophysics of 7-Azaindole. *i. Probing Solvent/7-Azaindole Interactions.* In alcohols, the fluorescence spectrum of 7-azaindole is bimodal. In methanol, for example, the maximum of the higher energy band is at 374 nm and that of the lower-energy band is at 505 nm. The former band arises from the so-called "normal" species that decays into the latter band by double-proton transfer.[24−26,46] In alcohols, the tautomerization or double-proton transfer reaction has been traditionally depicted (Figure 2) as being mediated by one solvent molecule, which forms a cyclic complex with the solute.

On the other hand, in water 7-azaindole has a smooth fluorescence spectrum with a single maximum.[83] 7-Azaindole exhibits a single-exponential fluorescence decay of 910 ps in water at neutral pH and 20 °C if emission from the entire band is collected.[12,13,46] The fluorescence decay, however, deviates from single exponential if emission is collected with a limited band pass.[13] At the blue edge of the emission, an acceptable fit is obtained using two exponentially decaying components and it indicates that about 20% of the fluorescent emission decays with a time constant between 40 and 100 ps (depending on the full-scale time base chosen for the experiment). A component with a 70-ps decay time is also detected in the transient absorbance of 7-azaindole in water.[47] There is no such rapid component in the fluorescence decay or the transient absorption of the 7-methyl and 1-methyl derivatives of 7-azaindole.[13,47] At the red edge of the emission, a rising component with a similar time constant is observed.[13] We have thus attributed this rapid component to a *small* population of 7-azaindole molecules that undergo excited-state tautomerization.

The 910-ps component that is resolved when emission is collected over the entire band is attributed to the majority of the 7-azaindole molecules that are not capable of excited-state tautomerization because they exist in a "blocked" state of solvation (Figure 2). Similar blocked states of solvation have been observed in argon matrices at 10 K for the much studied model of excited-state proton transfer, 3-hydroxyflavone.[48] The groups of Barbara,[49] Kasha,[50] and Harris[51] have discussed the importance of intermolecular hydrogen bonding, cyclic hydrogen-bonded complexes with one solvent molecule, and doubly solvated hydrogen-bonded complexes.

Finally, at the red edge of the emission band, the fluorescence decay is observed to lengthen from 910 to 980 ps (Figure 3a). This lengthening of the lifetime at long emission wavelengths can be attributed to *the protonated tautomer*. That is, 980 ps represents the weighted average of 910 ps and a longer lifetime of ∼1100 ps, which corresponds to that of protonated (pH < 3) 7-azaindole (Figure 3b).[13] Also, if the majority of 7-azaindole in water did tautomerize, significant emission should be observed at ∼500 nm. No emission is observed at 500 nm because so little tautomer is produced and because the tautomer that is produced is rapidly protonated (owing to the presence of the very basic N_1 nitrogen, $pK_a \simeq 13$[13]) and has an emission maximum at ∼440 nm.

Finally, Maroncelli and co-workers have proposed that 7-azaindole and 1-azacarbazole[52] can be used as model systems in which to investigate the coupling of solvent, especially, alcohols, to proton transfer reactions. Their observations are the following: (1) The proton transfer processes of 7-azaindole

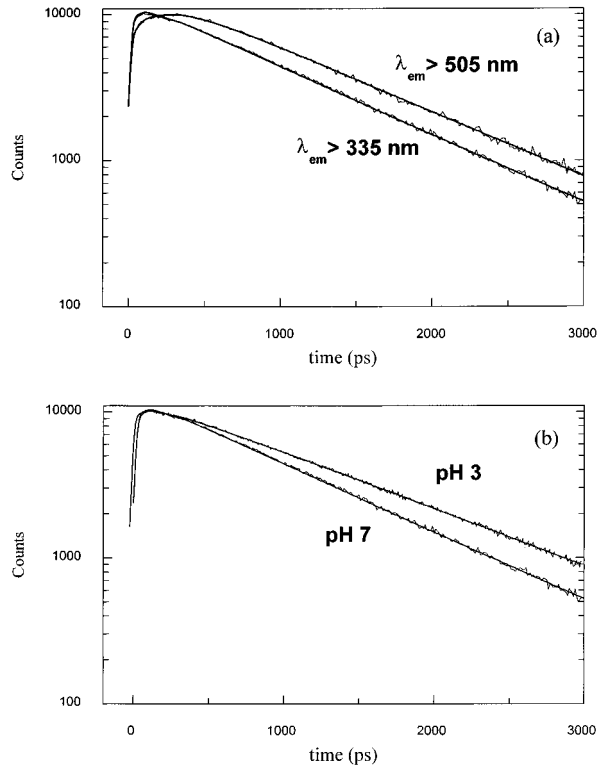

Figure 3. Fluorescence decay of 7-azaindole in water at 20 °C, $\lambda_{ex} = 288$ nm. (a) Decay of the entire emission band at pH 7, $\lambda_{em} \geq 335$ nm: $K(t) = 1.00 \exp(-t/909\ \text{ps})$, $\chi^2 = 1.34$. Decay of the red edge of the emission band, $\lambda_{em} \geq 505$ nm: $K(t) = -0.85 \exp(-t/201\ \text{ps}) + 1.85 \exp(-t/964\ \text{ps})$, $\chi^2 = 1.05$. (b) The decay of the entire emission band at pH 7, as in panel a, is compared with the decay of the entire emission band at pH 3: $K(t) = -0.19 \exp(-t/549) + 1.19 \exp(-t/1060\ \text{ps})$, $\chi^2 = 1.13$.

and 1-azacarbazole are nearly identical except for the 1-azacarbazole reaction being about 5 times slower. (2) The isotope effect on the reaction rate is (very nearly) temperature and solvent independent: ~3 in 7-azaindole and ~5 in 1-azacarbazole. (3) The activation energies for the 7-azaindole and 1-azacarbazole reactions are often, but not always, close to the viscosity activation energy. (4) The rates of both of these reactions show a logarithmic dependence on the polarity as determined by the $E_T(30)$ value[88] of the solvent in the case of mono-alcohols. (5) The rates in water and other poly-OH compounds are much smaller than expected based on the correlation with $E_T(30)$ noted above.

Maroncelli and co-workers interpret these results as follows: (1) The small rate of reaction of 7-azaindole and 1-azacarbazole in the solvents studied compared to their dimers and isolated complexes reflects the difficulty of achieving the proper form for proton transfer. (2) They propose that the proper form is a 1:1 cyclic complex with a single alcohol solvent molecule; such complexes are very rare in bulk alcohol solvents. (3) They interpret the proton transfer reaction in terms of the transition-state-theory expression $k = k_{PT} \exp(-G/kT)$, where k_{PT} is the rate of proton transfer once the system is in the proper configuration for reaction and G is the free energy of activation required to rearrange the solvent to the proper form. Their current interpretation is in spirit similar to that proposed in their initial article,[24] but it differs in one important respect. They no longer propose that the rate of solvent equilibration is relevant to the overall rate. Rather, it is merely the equilibrium solvation free energy needed to achieve the cyclic form G that controls the rate.

ii. Assessing the Roles of Water and Alcohols: Application of the Proton Inventory to the Nonradiative Process in 7-Azaindole. The isotope effect on proton transfer reactions is rarely a linear function of solvent deuterium content. Gross and Butler explained this phenomenon by noting that either the H/D composition in the proton site may be different with respect to the solvent or more than one proton is in flight during the rate-limiting step.[53−57] The Gross−Butler equation relates the rate of the process in the protiated solvent k_o to the rate in a solution of mole fraction n, k_n, of the deuteriated solvent and *to all the protons in the reactant and transition states involved*.

The data for 7-azaindole in methanol and in ethanol[46] are such that a plot of $(k_n/k_o)^{1/2}$ vs n yields a straight line. This result suggests that only two protons are involved in the excited-state tautomerization of 7-azaindole in alcohols and is also consistent with the "cyclic complex" of 7-azaindole and alcohol (Figure 2) that has been traditionally assumed to be required for the tautomerization to proceed.

On the other hand, the data for 7-azaindole in H_2O/D_2O mixtures suggests that more than two protons are involved in the transition state of the nonradiative deactivation process and that the major nonradiative process in water cannot be the same as that in alcohols. As noted above, we have argued that not more than 20% of the 7-azaindole population in water is capable of executing double-proton transfer and that this process can be observed only under conditions of sufficient wavelength and time resolution.[13,17,46]

Two related studies of 7-azaindole in water have been performed. Chou et al.[27] investigated 7-azaindole in mixtures of water and aprotic solvents. Small additions of water to polar aprotic solvents produced tautomer-like emission. They proposed that excited-state tautomerization is possible only when there are significant concentrations of 1:1 complexes of 7-azaindole and water. They further proposed that in pure water the formation of higher-order aggregates inhibits tautomerization during the excited-state lifetime. Chapman and Maroncelli performed similar experiments.[58] They propose, however, that the longer, ~900 ps, decay time of the entire emission band is a measure of the tautomerization rate. Their scheme requires that the nonradiative decay rate of the tautomer is greater than the rate of tautomerization. They estimate that the rate of tautomerization is $1.2 \times 10^9\ \text{s}^{-1}$. Our observations and conclusions more nearly approach those of Chou et al.

In large part because of the rapid (1.4 ps) tautomerization observed in dimers of 7-azaindole (figure 1f),[59] the tautomerization of dilute solutions of 7-azaindole in alcohols has been discussed in terms of a two-step process.[60] The first step, which is rate limiting, involves obtaining the correct solvation of the solute by the alcohol, the second faster step, double-proton transfer. The interpretation of our isotopic substitution experiments depends on whether the two-step model is appropriate and if it is, whether the solvation step is slow, fast, or comparable to tautomerization. If the rate-limiting step in the double-proton transfer reaction is the formation of the cyclic complex, then the isotope effects we discuss above require reinterpretation. Dimers of 7-azaindole may not, however, be an appropriate paradigm for the tautomerization of the 7-azaindole−alcohol complex. For example, Fuke and Kaya[61,62] observe that in supersonic jets the rate of excited-state double-proton transfer of 7-azaindole dimers is $10^{12}\ \text{s}^{-1}$, while in dimers of 1-azacarbazole (Figure 1g) and in complexes of 7-azaindole with 1-azacarbazole the rate is $10^9\ \text{s}^{-1}$. The reduction in rate by a factor of 10^3 is surprising given the very similar hydrogen bonding in the three types of complexes. It is therefore most likely premature to assume that tautomerization in a 7-azaindole complex occurs as rapidly as in a 7-azaindole dimer. Fuke and Kaya suggest that detailed considerations of the coupling of

proton motion with intermolecular vibrational motion are required in order to predict the rate of such tautomerization reactions.[61]

Calculations on complexes of 7-azaindole with increasing numbers of solvent molecules will enhance our understanding of the role played by the cyclic complex and how easily it is formed. Gordon[63] has performed ab initio calculations on 7-azaindole and its 1:1 complexes with water and methanol. Geometry optimizations using restricted Hartree−Fock wave functions and a double-ζ plus polarization basis set were performed on the minima of the normal and the tautomer species and the transition states connecting these minima. Energetics[84] were predicted using second-order perturbation theory. The *ground-state* activation energy for tautomerization is predicted to decrease from nearly 60 kcal/mol in 7-azaindole to about 26 kcal/mol for the 1:1 complexes. Vertical excitation of the 1:1 complex with water, qualitatively estimated using singly excited configuration interaction, is predicted to reverse the order of stability of the two tautomers.

Gordon has also performed calculations involving more than one solvent molecule.[64] In these cases, all the energies were obtained by first optimizing the geometries at the SCF/6-31G-(d) level of theory and then doing MP2/DZP energies at the SCF/6-31G(d) geometries. For the two water molecules, the tautomer is 9.6 kcal/mol above the normal form. The barrier for the proton transfer is 22 kcal/mol, compared with 26 kcal/mol with one water. For three water molecules, two differently solvated normal species are found with slightly different energies. The energy difference between these two normal forms is about 1.5 kcal/mol. With respect to the lower energy structure, the tautomer is 10.9 kcal/mol higher, and the barrier for proton transfer is 22.9 kcal/mol. None of these results includes zero-point vibrational energy (ZPE) corrections. The ZPE corrections will have only a small effect on the tautomer energy, but will reduce the barrier by about 4 kcal/mol. An interesting result, based on calculations of animated reaction paths, is that, during the double-proton transfer, the participating waters must move in much closer before the water-assisted proton trnasfer occurs. When three waters are present, the third water basically gets in the way and must first remove itself before the other two waters carry out the transfer. For the two methanol molecules, the tautomer is 9.4 kcal/mol above the normal isomer. The barrier for proton trnasfer is 21.1 kcal/mol. Thus, there is not much difference between one or two methanols and one or two waters. The structures determined in the calculations of Gordon have been observed experimentally by Miller, Kaya, and co-workers.[65]

Recently, on the basis of the observation of double-exponential decay of the signal of 7-azaindole dimers in a molecular beam, Zewail and co-workers have proposed that the excited-state double-proton transfer occurs in a two-step process via a zwitterionic intermediate.[66] Results of an ab initio calculation by Douhal et al. have been cited in support of this mechanism.[67] These calculations (CIS geometry optimizations for the excited state) reveal two transition states and suggest zwitterionic character, in agreement with the proposal of Zewail and co-workers. At the end of their discussion, however, Douhal et al. note that, when they take their CIS points on the excited-state surface and add correlation (MP2), they find a completely downhill path from the vertically excited normal species to the excited tautomer. Thus, the results of Douhal et al. indicate that at a higher level of theory both transition states disappear. Given the experimental evidence in support of concerted proton transfer reactions,[46,53−57] some of which is cited above for 7-azaindole, verification of the sequential mechanism proposed by Zewail and co-workers would be an important and surprising result. The proposal of a zwitterionic intermediate in the gas phase is also intriguing. Gordon and co-workers have shown that for glycine gas phase zwitterions are highly unstable, and that, in order to obtain a minimum on the potential energy surface, at least two water molecules must be present.[68]

iii. 1-Methyl-7-azaindole and 1-Methyl-7-azatryptophan: Toward More Powerful Optical Probes. The most significant nonradiative properties of 7-azaindole (in particular, those that distinguish it from indole[13,18,19]) are determined by the N_1 proton and its interactions with the solvent. Internal conversion promoted by the interaction of this proton with the solvent has also been suggested.[28] 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 and 0.55.[13,19] We anticipated that the methylated analog of 7-azatryptophan would possess the same characteristics.[13,18,19] Consequently, we synthesized N_1-methyl-7-azatryptophan (Figure 1) in order to address problems where long-time dynamics are of interest and many tryptophan residues are present. Because of the shift in the absorption and emission spectra of 1-methyl-7-azatryptophan with respect to tryptophan, and because of its very high fluorescence quantum yield (0.53 ± 0.07) and its long fluorescence lifetime (21.7 ± 0.4 ns), a 1:75 mixture of 1-methyl-7-azatryptophan and tryptophan is such that the 1-methyl-7-azatryptophan can be detected uniquely. A comparison of the absorption and emission spectra of 1-methyl-7-azatryptophan and tryptophan is made in Figure 4. The immediate and most powerful use of 1-methyl-7-azatryptophan is to incorporate it into small peptides of known biological interest and to study the interactions of these tagged peptides with larger proteins. Such applications are discussed below in section C. As will be seen in the second example presented in this section, although methylation of N_1 eliminates excited-state proton transfer as a nonradiative process, it should not be assumed that the fluorescence properties of 1-methyl-7-azaindole or of 1-methyl-7-azatryptophan are insensitive to the environment. For example, the fluorescence lifetime of 1-methyl-7-azaindole is approximately six times longer in D_2O than that of 1-methyl-7-azaindole in cyclohexane.[58] This sensitivity to environment can most likely be rationalized in terms of the scheme presented by Meech et al.,[69] who have discussed the different fluorescence properties of the ^1L_a and ^1L_b excited singlet states of indoles and the ability of solvent to determine the energetics of the ^1L_a state.

B. 7-Azatryptophan, Isolated and in Peptides. *i. Single-Exponential Fluorescence Decay of 7-Azatryptophan in Water: Sensitivity to Environment.* The nonexponential fluorescence decay of tryptophan renders its use as an optical probe at best cumbersome. We have argued that this decay arises from efficient charge transfer processes of different stable conformations of the indole ring to the side chain.[1] It seems likely that 7-azatryptophan would exist in similar conformations. It is then natural to ask why the fluorescence lifetime of 7-azatryptophan in water is single exponential (when emission is collected over most of the band).[12,15,17] We have addressed this problem by considering the thermodynamics of excited-state charge transfer processes in 7-azatryptophan.[17] It is important to recall that in water the emission maximum of 7-azatryptophan lies at lower energy than that of tryptophan: 397 as opposed to 351 nm. Consequently, it can be argued that the excited state of 7-azatryptophan does not possess sufficient driving force to effect a charge transfer reaction during the lifetime of the excited state. Consistent with this interpretation is the observation that 7-azatryptophan in CH_3CN has an emission maximum that is blue shifted with respect to that in water (374 nm) and that

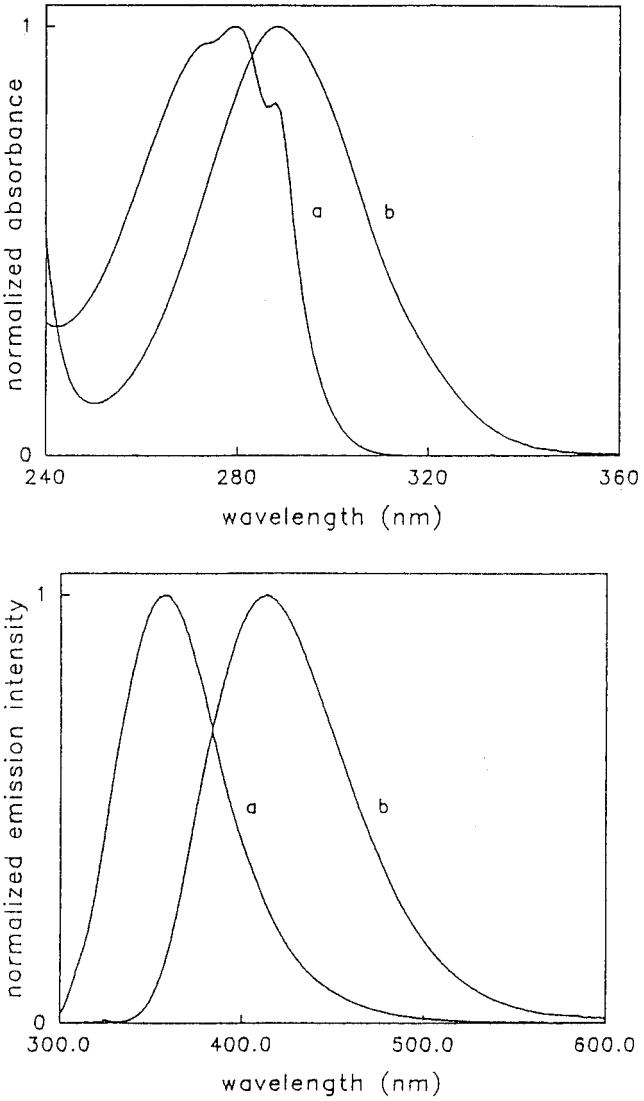

Figure 4. Comparison of the normalized absorption (top) and emission (bottom) spectra of (a) tryptophan and (b) 1-methyl-7-azatryptophan.

CH_3CN induces nonexponential fluorescence decay in 7-azatryptophan (Figure 5).

This result is of extreme importance, not only for a comparative understanding of the photophysics of tryptophan and of 7-azatryptophan, but also for the use of 7-azatryptophan as an optical probe. It indicates that, aside from the ability of its 7-azaindole chromophore to execute double-proton transfer under conditions of propitious solvation, 7-azatryptophan exhibits other sensitivity to solvent environment. Consequently, in some cases, as we shall demonstrate below, 7-azatryptophan may have a nonexponential fluorescence decay when it is incorporated into a peptide or a protein matrix. It is important to appreciate, however, that, in using 7-azatryptophan as the optical probe, our explanation of its fluorescence decay is not hobbled from the outset by complicated photophysics of the probe itself.

ii. Fluorescence Properties of 7-Azatryptophan in Peptides. a. Fluorescence Lifetime and Anisotropy Decay. The fluorescence maximum of the model tripeptide containing D- or L-7-azatryptophan, NAc-Pro-7-azatryptophan-Asn-NH_2, is 397 nm at 20 °C and pH 7. The fluorescence lifetime of the 7-azatryptophan tripeptide is single-exponential of ~830-ps duration (when emission is collected over the entire band).[12,15] Thus, not only is the fluorescence decay of 7-azatryptophan in water single-exponential, but so is that of the tripeptide containing 7-azatryptophan. In contrast, most derivatives of tryptophan

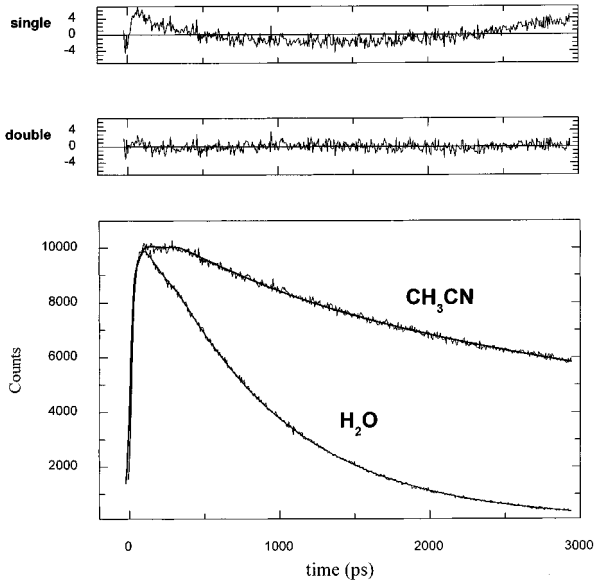

Figure 5. Fluorescence decay of 7-azatryptophan at 20 °C in (a) water, $\lambda_{ex} = 288$ nm, $\lambda_{em} \geq 320$ nm and (b) acetonitrile, $\lambda_{ex} = 288$ nm, $\lambda_{em} \geq 335$ nm. The residuals displayed are for single (upper) and double (lower) exponential fits of the acetonitrile data. (a) Water, $K(t) = 1.00 \exp(-t/824\text{ ps})$, $\chi^2 = 1.13$. (b) Acetonitrile, single-exponential fit: $K(t) = 1.00 \exp(-t/4340\text{ ps})$, $\chi^2 = 4.31$; double-exponential fit: $K(t) = 0.25 \exp(-t/953\text{ ps}) + 0.75 \exp(-t/6980\text{ ps})$, $\chi^2 = 1.14$. Care was taken to ensure that the acetonitrile used was very dry. Acetonitrile (Fischer HPLC grade) was distilled three times: first over CaH_2, then over P_2O_5, and again over CaH_2. No molecular sieves were added to the solution. The presence of molecular sieves in DMSO in an earlier investigation induced artifactual nonexponential fluorescence decay in 7-azatryptophan[17] (see Table 1).

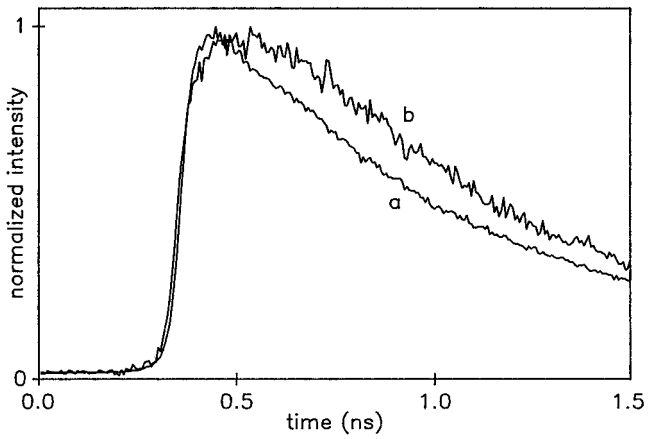

Figure 6. Fluorescence lifetime decays of the reduced D-7-azatryptophan octapeptide at 20 °C. (a) $\lambda_{em} = 375$ nm, $K(t) = 0.19 \exp(-t/100\text{ ps}) + 0.68 \exp(-t/706\text{ ps}) + 0.13 \exp(-t/1658\text{ ps})$; $\chi^2 = 1.155$. (b) $\lambda_{em} \geq 505$ nm, $K(t) = -0.24 \exp(-t/151\text{ ps}) + 0.92 \exp(-t/656\text{ ps}) + 0.32 \exp(-t/1504\text{ ps})$; $\chi^2 = 1.12$. The latter measurement was collected to a maximum of 2000 counts instead of 10 000 owing to the low fluorescence intensity in this region.

except for the anomalous *N*-acetyl-tryptophanamide exhibit fluorescence decays that can be fit only to a sum of exponentials.[1,2]

The fluorescence lifetime of the 7-azatryptophan octapeptide is nonexponential under all conditions (Figure 6 provides a representative example), i.e., whether the octapeptide contains D- and L-7-azatryptophan or whether the octapeptide is reduced, oxidized, or alkylated. The fluorescence spectra of the reduced and the oxidized 7-azatryptophan octapeptides are quite similar. It is unlikely that an impurity is the cause of the nonexponential fluorescence decay of the octapeptide. One would expect a species contributing 20% to the fluorescence lifetime decay to be apparent in the steady-state emission spectrum. This is not

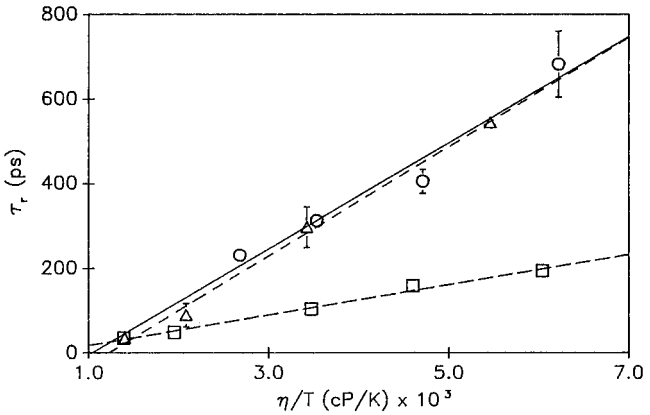

Figure 7. Plots of the rotational diffusion (reorientation) time τ_r against η/T for the D-7-azatryptophan tripeptide (□) and the reduced (○) and the oxidized (△) D-7-azatryptophan octapeptides. Identical results were obtained for the L forms. The τ_r data presented are averages from at least three measurements at a given viscosity. $\lambda_{ex} = 310$ nm and $\lambda_{em} \geq 345$ nm. The samples were dissolved in tris buffer (see Experimental Section). The viscosity values used were those of water.[81] The data are fit to the relation, $\tau_r = c\eta + \tau_0$, where $c = V/kT$ if the diffusing species is a sphere. The tripeptide data yield a volume of 470 ± 10 Å^3. The reduced and oxidized octapeptide data both yield a volume of 1700 ± 200 Å^3.

the case, since the reduced and alkylated octapeptide emission spectra (measured at pH 7.8) are nearly identical to that of 7-azatryptophan in water (data not shown).

The fluorescence anisotropy decay of the 7-azatryptophan tripeptide and the 7-azatryptophan octapeptide in the reduced and oxidized states were measured as a function of temperature. Identical results were obtained for both the D and the L forms. The fluorescence anisotropy decays of the 7-azatryptophan tripeptide and reduced octapeptide are both very well described by a single exponential ($\chi^2 \simeq 1.1-1.3$) (Figure 7). On the other hand, the fit of the anisotropy decay of the 7-azatryptophan oxidized octapeptide to a single exponential is typically not very good ($\chi^2 \simeq 2$); but a double-exponential fit does not yield a significantly better result. We have observed that the limiting value of the anisotropy decay of 7-azaindole and its analogs is less than the theoretical maximum of 0.40. We have attributed this result to the presence of closely lying excited singlet states whose transition dipoles lie at large angles to each other.[14] These states are traditionally referred to as ^1L_a and ^1L_b.[14,70,71]

Assuming the peptide solvent complex is spherical, plots of the anisotropy decay against temperature can be used to estimate a hypothetical volume[14] (Figure 7 and caption). The volume obtained for the tripeptide is 470 ± 10 Å^3. The volume obtained for both the reduced and oxidized octapeptides is 1700 ± 200 Å^3. Given that the reduced and oxidized forms are likely to have extended and hairpin geometries, respectively, we conclude that the *7-azatryptophan is in both cases probing similar forms of local depolarizing motion* that is independent of the gross conformation of the octapeptide. (Similar results have been observed for a series of ACTH fragments of varying lengths.[6])

b. Possible Role of Proline Isomerization in Octapeptide. A factor that may contribute to the nonexponential fluorescence decay of the 7-azatryptophan octapeptide is the isomerization of the proline residue. Proline is the only amino acid that occurs significantly in the *cis* conformation in peptides and proteins. 5.7% of the Pro residues occurring in proteins have *cis* peptide bonds.[72] On the other hand, all the other residues combined occur in the *cis* conformation only 0.5% of the time.[72] In the octapeptide, it is possible that a *cis* conformation of the Cys-Pro peptide bond contributes to a different state of solvation of the 7-azatryptophan residue, which in turn gives rise to a population of chromophores capable of undergoing excited-state tautomerization. Attempts to perturb the *cis/trans* population of the reduced octapeptide by addition of the enzyme peptidyl-prolyl-*cis/trans*-isomerase (PPIase)[73] produced no measurable changes in the weights or the lifetimes of the fluorescence decay. This was also verified by NMR. A sample of tripeptide with a 70:1 ratio of peptide to enzyme was monitored over a 24 h period. There was no observable change in the *cis* population over this time. These results can be rationalized by noting that time constants of 10−100 s are typical for *cis/trans* isomerization of X-Pro bonds in denatured proteins.[72] The PPIase will not perturb the relative populations if equilibrium between them is reached rapidly with respect to the time required to perform the optical or the NMR experiment.

c. Nonexponential Fluorescence Decay of 7-Azatryptophan in Peptides. In the context of the categories of solvent and peptide heterogeneity discussed here, it is important to bear in mind that, given the above description of solvation of 7-azaindole, even a homogeneous state of solvation can yield a nonexponential fluorescence decay, if it affords a "cyclic" solute−solvent complex (Figure 2). For example, the fluorescence decay of 7-azaindole (and 7-azatryptophan) in methanol at room temperature that is obtained by collecting all emission wavelengths is nonexponential owing to the presence of tautomer that is formed by the normal species.[13] On the other hand, in water at room temperature,[13] the blocked configuration is predominant in the fluorescence decay of 7-azaindole and of 7-azatryptophan.

In interpreting the results described above, our organizing assumption is that the double-exponential decay observed for the 7-azatryptophan octapeptide is a consequence of excited-state tautomerization that is induced by the peptide itself. The outstanding question is then to what extent this production of chromophores, which are susceptible to excited-state tautomerization, is a result of direct interaction with the peptide, the ability of the peptide to reorganize solvent about the chromophore, or a distribution of solvation environments.

That the octapeptide does undergo excited-state tautomerization is suggested by the fluorescence decay profiles obtained as a function of emission wavelength (Figure 6). Emission collected on the red edge of the spectrum yields a rise time commensurate to the shorter-lived component observed when emission is collected either over the entire spectrum or at individual bluer wavelengths: 150 ps. *What distinguishes the octapeptide from 7-azatryptophan, 7-azaindole, and the tripeptide in water is the prominence of the shorter-lived decay component and the observation of nonexponential fluorescence decay even when emission is collected over the entire band.* This implies that for the octapeptide in water the tautomer band and the normal band are essentially centered upon one another.

There are two results in whose context the remainder of the data must be considered. (1) *The 7-azatryptophan octapeptide is the only compound containing 7-azaindole considered up to now whose fluorescence lifetime is nonexponential in pure water when emission is collected over the entire band.* (Szabo and co-workers have reported nonexponential fluorescence decay for the tripeptide Lys-7-azatrp-Lys at an emission wavelength of 400 nm.[74] Lifetime measurements at other wavelengths were not cited. It is not clear whether these measurements report on intrinsic tautomerization of the chromophore or on effects that are induced by the lysine residues.) (2) That reducing and oxidizing conditions significantly influence the secondary structure of the octapeptide is demonstrated by the spot tests and by the different rotation times. We cannot rule out additional heterogeneity resulting from *cis/trans* isomerization of the proline bond.

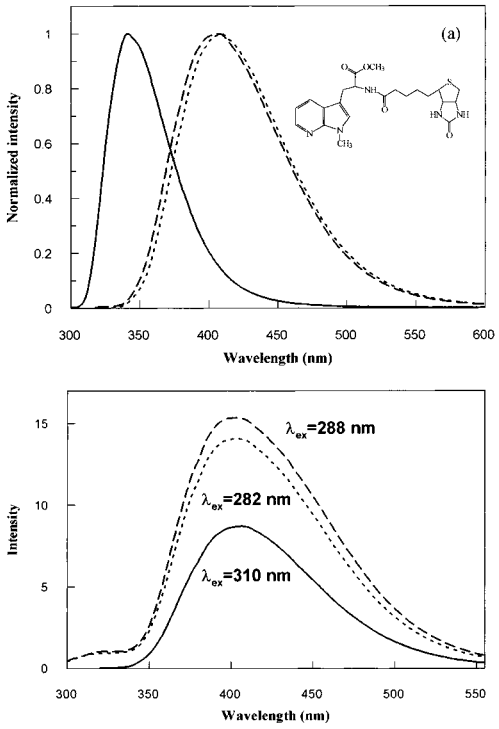

Figure 8. (a) The emission spectrum of avidin ($\lambda_{ex} = 288$ nm, solid line) and the emission spectra of biotinylated 1-methyl-7-azatryptophan ($\lambda_{ex} = 310$, short dashed line) and of the complex of biotinylated 1-methyl-7-azatryptophan with avidin ($\lambda_{ex} = 310$, long dashed line). The structure of biotinylated 1-methyl-7-azatryptophan is incorporated in the inset. (b) Emission spectra of the complex of biotinylated 1-methyl-7-azatryptophan and avidin at three different excitation wavelengths. The contribution from tryptophan is the small feature that is noticeable at ~325 nm.

C. Probing Interactions with Larger Proteins. *i. The Complex of Biotinylated 1-Methyl-7-azatryptophan and Avidin.* In a previous work, we prepared and studied biotinylated 7-azatryptophan by itself and in complex with avidin.[18] 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. The complex of avidin with biotinylated 7-azatryptophan was originally investigated in order to determine the *limits of the spectroscopic distinguishability of 7-azatryptophan* from tryptophan and to demonstrate its *usefulness as an optical probe*. In this initial study, although the emission of biotinylated 7-azatryptophan was clearly evident, the ability of the protein matrix (and the biotin) to influence the fluorescence properties of 7-azatryptophan were also readily apparent. In the complex with avidin, the fluorescence lifetime of biotinylated 7-azatryptophan represented only 50% of the amplitude of the total fluorescence intensity and the 7-azatryptophan lifetime was reduced from its value of 780 ps in water to 420 ps.

In our earlier work, we commented on these potential limitations and suggested the utility of employing 1-methyl-7-azatryptophan in instances where 7-azatryptophan might be significantly quenched. Figure 8a presents the *normalized* emission spectrum of avidin excited at 289 nm and the emission spectrum of the complex of avidin and biotinylated 1-methyl-7-azatryptophan excited at 310 nm. The spectral distinguishability is clearly evident. A more telling comparison, however, is made in Figure 8b.

Figure 9 presents the decay of the parallel and perpendicular emission intensity for this complex, from which the fluorescence anisotropy decay is obtained: $r(t) = r_1(0)\exp(-t/\tau_1) + r_2(0)\exp(-t/\tau_2) = (0.017 \pm 0.006)\exp(-t/513 \pm 166\text{ ps}) + (0.034 \pm 0.004)\exp(-t/(16\,900) \pm 2700\text{ ps})$, for the average of six experiments collected on a *25-ns time scale*; $r(t) = (0.020 \pm 0.004)\exp(-t/410 \pm 171\text{ ps}) + (0.032 \pm 0.010)$, for the average of four experiments collected on a *3-ns time scale*. The time constant for the longer lived anisotropy component is taken as infinity for the 3-ns data because of the limitations of obtaining an accurate value on this time scale.

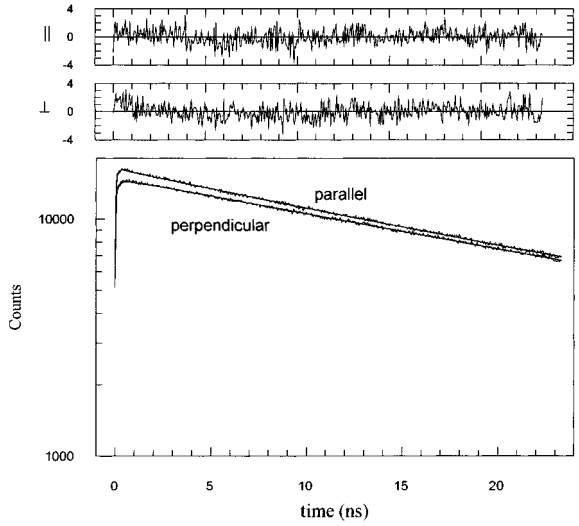

Figure 9. Decay of fluorescence intensity polarized parallel and perpendicular to the laser excitation for the complex of biotinylated 1-methyl-7-azatryptophan and avidin. $\lambda_{ex} = 310$ nm and $\lambda_{em} > 435$ nm. The fluorescence anisotropy decay obtained from the curves is given by $r(t) = 0.023\exp(-t/403\text{ ps}) + 0.031\exp(-t/14\,400\text{ ps})$, $\chi^2 = 1.178$. The fluorescence decay of the complex is single exponential and is given by $K(t) = 1.00\exp(-t/25\,700\text{ ps})$. As suggested from Figure 8, contamination from tryptophan emission is completely negligible under the conditions of detection and excitation used in our investigations.

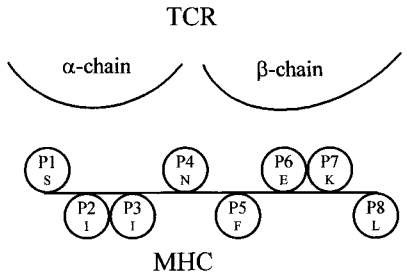

Figure 10. Schematic diagram (based on reference 85) of the contacts between the T-cell receptor (TCR) and the complex of the antigen, OVA-8 (SIINFEKL), and class I MHC (H-2K^b). The antigen residues that are believed to be "buried" in the MHC are shown below the horizontal line. Those that are believed to be exposed to solvent lie above the line. (The structure of reference 85 does not contain OVA-8 but dEV8.)

That the fluorescence anisotropy decay is fit to two exponentials indicates that we are probing the rapid librational motion of the 1-methyl-7-azatryptophan probe with respect to avidin as well as the overall tumbling motion of 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. $S^2 = [r(t)/r(0)]\exp(t/\tau_r) = r(0^+)/r_{eff}(0)$. 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 are equivalent to τ_2 and r_2, respectively. $r_{eff}(0)$ is the initial value of the anisotropy less those nonmotional factors contributing to the anisotropy decay.[75] 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,

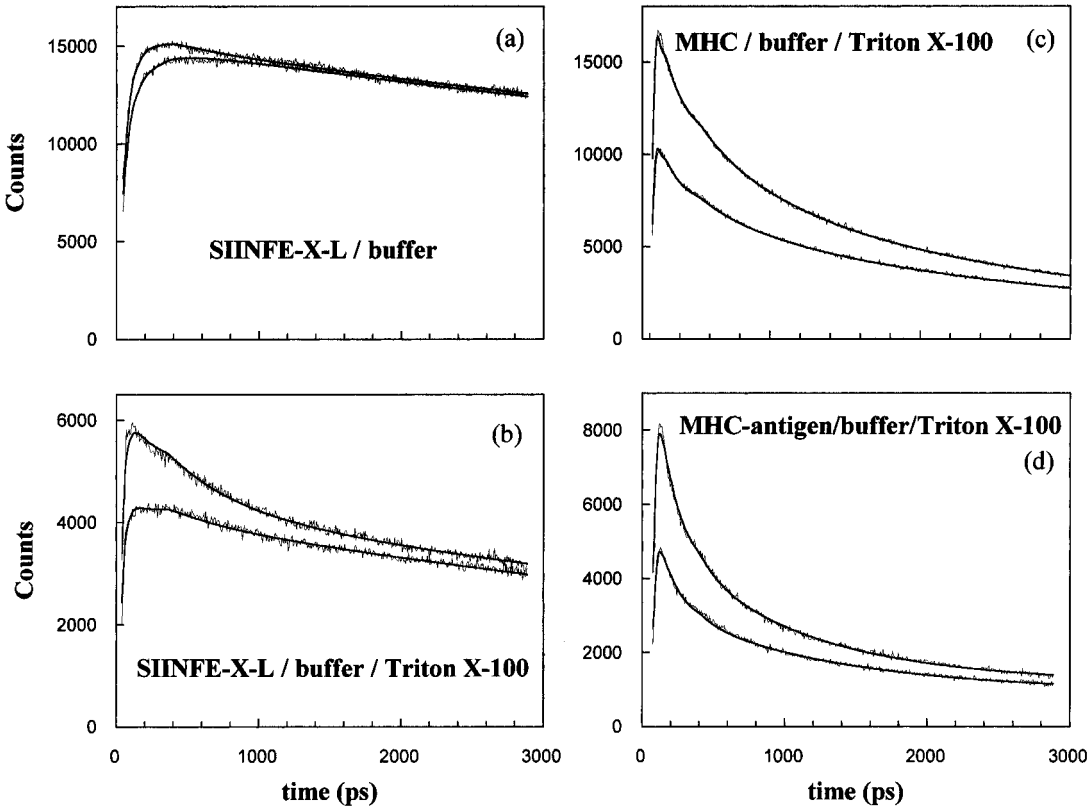

Figure 11. Fluorescence decay of the modified OVA-8 peptide, SIINFE(1-methyl-7-azatryptophan)L at room temperature (20 °C). Data are obtained on a full scale of 3 ns in order to resolve the more rapid time-dependent features. In each panel, the upper curve represents the emission polarized parallel to the excitation pulse, and the lower curve, emission polarized perpendicular. (a) Peptide in PBS buffer, $\lambda_{ex} = 310$ nm, $\lambda_{em} \geq 375$ nm: $r(t) = 0.062 \exp(-t/257 \text{ ps})$ and $K(t) = 1.00 \exp(-t/10\,300 \text{ ps})$; $\chi^2 = 1.33$. (b) Peptide in PBS buffer and 1% reduced Triton X-100, $\lambda_{ex} = 310$ nm, $\lambda_{em} \geq 375$ nm: $r(t) = 0.118 \exp(-t/608 \text{ ps})$; $K(t) = 0.25 \exp(-t/381 \text{ ps}) + 0.75 \exp(-t/6800 \text{ ps})$; $\chi^2 = 1.34$. (c) MHC (H-2K^b) in PBS buffer, 0.02% azide, and 1% reduced Triton X-100, $\lambda_{ex} = 288$ nm, $\lambda_{em} \geq 335$ nm: $r(t) = 0.175 \exp(-t/2710 \text{ ps})$; $K(t) = 0.36 \exp(-t/55.4 \text{ ps}) + 0.26 \exp(-t/338 \text{ ps}) + 0.38 \exp(-t/1850 \text{ ps})$; $\chi^2 = 1.30$. The 11 tryptophan residues in the MHC molecule give rise to a fluorescence decay that is satisfactorily fit to the triple exponential. Of course, no particular physical significance is accorded to this result. (d) Peptide bound to class I MHC (H-2K^b) in PBS buffer, 0.02% azide, and 1% reduced Triton X-100, $\lambda_{em} \geq 435$ nm: $r(t) = 0.213 \exp(-t/1820 \text{ ps})$; $K(t) = 0.57 \exp(-t/137 \text{ ps}) + 0.43 \exp(-t/1050 \text{ ps})$; $\chi^2 = 1.41$. We note that fitting the fluorescence lifetime to three exponentials yields $K(t) = 0.35 \exp(-t/68 \text{ ps}) + 0.35 \exp(-t/292 \text{ ps}) + 0.30 \exp(-t/1680 \text{ ps})$; $r(t) = 0.204 \exp(-t/1500 \text{ ps})$; $\chi^2 = 1.18$. The important point here is not the exact functional form to which the lifetime is fit, but the observation that interaction with the protein induces nonexponential fluorescence decay.

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 of the chromophore can diffuse:[76] $S = {}^1/_2 \cos(\theta_0(1 + \theta_0))$. Using biotinylated 1-methyl-7-azatryptophan, we obtain $\theta_0 = 29 \pm 4°$ for data obtained with a 25-ns full scale and $\theta_0 = 32 \pm 5°$ for data obtained with a 3-ns full scale. These values are in very good agreement with those obtained used the 7-azatryptophan probe in our earlier study ($\theta_0 = 29 \pm 5°$). This agreement is rather surprising given the extent to which the 7-azatryptophan emission is quenched by interactions with the biotin and the protein matrix.

This example of the complex of biotinylated 1-methyl-7-azatryptophan with the structurally well characterized protein, avidin,[77] demonstrates the utility and the enormous potential for using 1-methyl-7-azatryptophan for studying interactions in large proteins containing many tryptophans. A very rich example follows.

ii. Antigens Tagged with 1-Methyl-7-Azatryptophan and Their Interactions with MHC. The class I major histocompatibility complex molecule (MHC) binds antigenic peptides and presents these peptide sequences to cytotoxic T-cell receptors (TCR). The binding of foreign peptides to class I MHC molecules is thus the first step in the cascade of T-cell activation. Molecular recognition is consequently a fundamental process in the immune response.

Crystallographic structures[78] of the class I MHC molecule have been obtained revealing considerable detail on the peptide binding groove. It has also been determined that at least two anchor residues are required in order for the antigen to bind to MHC with high affinity.[44] Binding experiments were carried out with the OVA-8 (SIINFEKL) peptide and empty murine class I MHC (H-2K^b).[79] The binding constants obtained in these measurements were compared with those obtained from the corresponding peptides with each residue substituted by an alanine, or with multiple serine substitutions. It was observed that residues 3 and 5, isoleucine and phenylalanine, are essential for binding the antigen to MHC.[44] It has further been suggested that residues 4, 6, and 7 of OVA-8 (aspargine, lysine, and leucine) are crucial for identifying the TCR.[80] A schematic illustration of the TCR/antigen/MHC complex, based upon a recent x-ray structure,[85] is provided in Figure 10.

Studying the interactions of antigen with MHC and of the antigen MHC complex with TCR is a problem that can be addressed extremely well using 1-methyl-7-azatryptophan. The sensitivity of this chromophore to environment and its spectroscopic distinguishability from tryptophan (H-2K^b contains 11 tryptophan residues) can be exploited by synthesizing analogs of the SIINFEKL peptide that are substituted with 1-methyl-7-azatryptophan in the 1 through 8 positions. Such a procedure opens many potential avenues of research. For example, the interactions of the antigen with the binding pocket can be probed by placing 1-methyl-7-azatryptophan in positions 3 or 5. Furthermore, if TCR can recognize an analog with 1-methyl-

7-azatryptophan in position 7, this would have much to say about what it is recognizing in lysine, since 1-methyl-7-azatryptophan and lysine map a nitrogen off the peptide backbone by the same number of carbon atoms.

Figure 11a,b,d present the parallel and perpendicular emission profiles of the tagged antigen in water, detergent (reduced Triton X-100), and in detergent in complex with MHC, respectively. (Figure 11c presents the fluorescence of the tryptophans of MHC itself, which is a control experiment.) Because MHC is a membrane-bound protein, the presence of detergent is necessary to obviate aggregation of the protein. The differences in the emission profiles in the presence of detergent and protein is striking. Detergent, and protein, shorten the lifetime of the probe and induce nonexponential fluorescence decay. The *induction* of nonexponential fluorescence decay is significant because it demonstrates the sensitivity of 1-methyl-7-azatryptophan to its environment. Finally, unlike the example above for biotinylated 1-methyl-7-azatryptophan and avidin, the anisotropy decay of the complex of tagged antigen and MHC can be well described with only one exponential (which may nevertheless represent a sum of at least two exponentials that we cannot resolve). Insofar as the anisotropy decay can be fit to a single exponential, we interpret the data as indicating that the peptide is rather rigidly held in the binding pocket of MHC and that even the residue in position 7, which is believed to point out towards the solvent, is rigidly held by the protein matrix. Again, this latter conclusion is also borne out by the nonexponential character and magnitude of the time constants of the fluorescence decay of the 1-methyl-7-azatryptophan probe.

IV. Summary and Conclusions

7-Azaindole is capable of undergoing excited-state double-proton transfer in certain solvents under appropriate conditions. The details of this process have yet to be thoroughly understood. We have argued that in water this process is largely impeded.

7-Azatryptophan is clearly more sensitive than tryptophan to the onset of whatever secondary structure or partial secondary structure the peptide may have assumed. This is most vividly illustrated by comparing the *single exponential fluorescence decay* of NAc-Pro-7-azatryptophan-Asn-NH_2 with the *nonexponential fluorescence decay* of NAc-Lys-Ala-Cys-Pro-7-azatryptophan-Asn-Cys-Asp-NH_2. As we have noted in the Introduction, the fluorescence decay of tryptophan is not only nonexponential in peptides containing it, but in the amino acid itself.

The octapeptide containing the 7-azaindole chromophore discussed here is the first 7-azaindole derivative reported to display a nonexponential fluorescence decay in water when its emission is collected over the entire band. Clearly there is a structural change in going from the tripeptide to the octapeptide that induces the nonexponential fluorescence decay. The 7-azaindole chromophore is furthermore sensitive to structural changes induced by subjecting the octapeptide to reducing or oxidizing conditions: distinct differences are apparent in both the steady-state and time-resolved fluorescence data for the 7-azatryptophan octapeptide.

The ability of 7-azatryptophan to probe the secondary structure imposed by the octapeptide by manifesting a nonexponential fluorescence decay should be put into perspective. For example, it is likely that the majority of small peptides capable of assuming some degree of secondary structure will exhibit nonexponential fluorescence behavior similar to that of the octapeptide, presumably from the enhancement of the population of chromophores capable of excited-state tautomerization. Therefore, in situations where it is already known that the peptide in question possesses secondary structure, an alternative and possibly more useful optical probe is 1-methyl-7-azatryptophan:[19] it possesses a high fluorescence quantum yield and a long fluorescence lifetime, 0.55 and 21 ns in water, respectively, and these fluorescence properties are solvent dependent. Also, because its N_1 nitrogen is methylated, it cannot undergo excited-state tautomerization. *7-Azaindole is a chromophore of enormous potential utility because it can be modified to probe different environments and different phenomena*.

It is sometimes said that the 7-azaindole chromophore is a "water-quenched fluorescence probe." Such a sweeping statement is obviously contradicted by the octapeptide result above and, as we have noted previously,[19] more generally fails to take into account the different types of interactions of the solvent: namely, bulk interactions that will influence the relative spacings and positions of the ^1L_a and ^1L_b states, as discussed in the case of indoles by Meech et al.,[69] and specific interactions that promote excited-state proton transfer reactions and other nonradiative processes.[13] It is helpful to keep in mind the tabulation compiled by Chapman and Maroncelli:[58] a comparison of the lifetimes in cyclohexane and water, respectively, yields 1.72 and 0.82 ns for 7-azaindole, 5.27 and 26.1 ns for 1-methyl-7-azaindole, and 1.67 and 0.49 ns for 7-methyl-7-azaindole. The 1-methyl-7-azaindole result is especially significant. Given circumstances in which the ability of the N_1 proton to interact specifically with the solvent is "turned off", the bulk properties of the solvent take over. In fact, under these conditions, in nonpolar solvents, the fluorescence lifetime and quantum yield are reduced with respect to those measured in water. This is what is observed for the interaction of the tagged antigen with MHC (Figure 11). In a heterogeneous environment such as a protein, both the bulk and specific interactions will contribute to different extents depending on the protein.

A great value of 7-azatryptophan (or 1-methyl-7-azatryptophan) will be to probe the interactions of a smaller peptide or protein containing it with another protein that may contain several tryptophans. These intrinsically tagged molecules can then be studied individually or in complex with their target. This approach will be useful in studying complexes between cofactors or peptides containing 7-azatryptophan and large proteins that may contain many tryptophans.

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References and Notes

(1) Petrich, J. W.; Chang, M. C.; McDonald, D. B.; Fleming, G. R. *J. Am. Chem. Soc.* **1983**, *105*, 3824. Chang, M. C.; Petrich, J. W.; McDonald, D. B.; Fleming, G. R. *J. Am. Chem. Soc.* **1983**, *105*, 3819.

(2) Szabo, A. G.; Rayner, D. M. *J. Am. Chem. Soc.* **1980**, *102*, 554.

(3) Szabo, A. G.; Rayner, D. M. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 909.

(4) Werner, T. C.; Forster, L. S. *Photochem. Photobiol.* **1979**, *29*, 905.

(5) Ross, J. B. A.; Rousslang, K. W.; Brand, L. *Biochemistry* **1981**, *20*, 4361.
(6) Chen, L. X.-Q.; Petrich, J. W.; Fleming, G. R.; Perico, A. *Chem. Phys. Lett.* **1987**, *139*, 55.
(7) Cockle, S. A.; Szabo, A. G. *Photochem. Photobiol.* **1981**, *34*, 23.
(8) Donzel, B.; Gauduchon, P.; Wahl, Ph. *J. Am. Chem. Soc.* **1974**, *96*, 801.
(9) Engh, R. A.; Chen, L. X.-Q.; Fleming, G. R. *Chem. Phys. Lett.* **1986**, *126*, 365.
(10) Creed, D. *Photochem. Photobiol.* **1984**, *39*, 537.
(11) Tilstra, L.; Sattler, M. C.; Cherry, W. R.; Barkley, M. D. *J. Am. Chem. Soc.* **1990**, *112*, 9176.
(12) Négrerie, M.; Bellefeuille, S. M.; Whitham, S.; Petrich, J. W.; Thornburg, R. W. *J. Am. Chem. Soc.* **1990**, *112*, 7419.
(13) Chen, Y.; Rich, R. L.; Gai, F.; Petrich, J. W. *J. Phys. Chem.* **1993**, *97*, 1770.
(14) Rich, R. L.; Chen, Y.; Neven, D.; Négrerie, M.; Gai, F.; Petrich, J. W. *J. Phys. Chem.* **1993**, *97*, 1781.
(15) Rich, R. L.; Négrerie, M.; Li, J.; Elliott, S.; Thornburg, R. W.; Petrich, J. W. *Photochem. Photobiol.* **1993**, *58*, 28.
(16) Smirnov, A.; Rich, R. L.; Petrich, J. W. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 1007.
(17) Chen, Y.; Gai, F.; Petrich, J. W. *J. Phys. Chem.* **1994**, *98*, 2203.
(18) Rich, R. L.; Gai, F.; Lane, J. W.; Petrich, J. W.; Schwabacher, A. W. *J. Am. Chem. Soc.* **1995**, *117*, 733.
(19) Rich, R. L.; Smirnov, A. V.; Schwabacher, A. W.; Petrich, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 11850.
(20) Hogue, C. W. V.; Szabo, A. G. *Biophys. Chem.* **1993**, *48*, 159.
(21) Hasselbacher, C. A.; Rusinova, E.; Waxman, E.; Lam, W.; Guha, A.; Rusinova, R.; Nemerson, Y.; Ross, J. B. A. *Proc. SPIE—Int. Soc. Opt. Eng.* **1994**, *2137*, 312.
(22) Senear, D. F.; Laue, T. M.; Ross, J. B. A.; Waxman, E.; Eaton, S.; Rusinova, E. *Biochemistry* **1993**, *32*, 6179. Ross, J. B. A.; Senear, D. F.; Waxman, E.; Kombo, B. B.; Rusinova, E.; Huang, Y. T.; Laws, W. R.; Hasselbacher, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 12023. Hogue, C. W. V.; Rasquinha, I.; Szabo, A. G.; MacManus, J. P. *FEBS Lett.* **1992**, *310*, 269. Kishi, T.; Tanaka, M.; Tanaka, J. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 1267.
(23) Taylor, C. A.; El-Bayoumi, M. A.; Kasha, M. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 253.
(24) Moog, R. S.; Maroncelli, M. *J. Phys. Chem.* **1991**, *95*, 10359.
(25) McMorrow, D.; Aartsma, T. J. *Chem. Phys. Lett.* **1986**, *125*, 581.
(26) Konijnenberg, J.; Huizer, A. H.; Varma, C. A. G. O. *J. Chem. Soc., Faraday Trans. 2* **1988**, *84*, 1163.
(27) Chou, P.-T.; Martinez, M. L.; Cooper, W. C.; Collins, S. T.; McMorrow, D. P.; Kasha, M. *J. Phys. Chem.* **1992**, *96*, 5203.
(28) Waluk, J.; Pakula, B.; Komorowski, S. J. *J. Photochem.* **1987**, *39*, 49.
(29) Bulska, H.; Grabowska, A.; Pakula, B.; Sepiol, J.; Waluk, J.; Wild, U. P. *J. Lumin.* **1984**, *29*, 65.
(30) Wu, J. Q.; Oxarowski, A.; Davis, S. K.; Maki, A. H. *J. Phys. Chem.* **1996**, *100*, 11496.
(31) Pardee, A. B.; Shore, V. G.; Prestidge, L. S. *Biochim. Biophys. Acta* **1956**, *21*, 406. Pardee, A. B.; Prestidge, L. S. *Biochim. Biophys. Acta* **1958**, *27*, 330.
(32) Brawerman, G.; Yčas, M. *Arch. Biochem. Biophys.* **1957**, *68*, 112.
(33) Schlesinger, S. *J. Biol. Chem.* **1968**, *243*, 3877.
(34) Foote, J.; Ikeda, D. M.; Kantrowitz, E. R. *J. Biol. Chem.* **1980**, *255*, 5154.
(35) Rabinovitz, M.; Finkleman, A.; Reagan, R. L.; Breitman, T. R. *J. Bacteriol.* **1969**, *99*, 336.
(36) Goldberg, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 422.
(37) Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R. *J. Am. Chem. Soc.* **1989**, *111*, 8013.
(38) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182.
(39) Burns, J. A.; Butler, J. C.; Moran, J.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 2648.
(40) Cobb, K. A.; Novotny, M. V. *Anal. Chem.* **1992**, *64*, 879.
(41) Feigl, F. *Spot Tests in Organic Analysis*; Elsevier: Amsterdam, 1960.
(42) Schonbaum, G. R.; Zerner, B.; Bender, M. L. *J. Biol. Chem.* **1961**, *236*, 2930.
(43) Bryant, J.; Green, T. R.; Gurusaddaiah, T.; Ryan, C. A. *Biochemistry* **1976**, *15*, 3418.
(44) Baito, Y.; Peterson, P. A.; Matsumura, M. *J. Biol. Chem.* **1993**, *268*, 21309.
(45) Matsumura, M.; Saito, Y.; Jackson, M. R.; Song, E. S.; Peterson, P. A. *J. Biol. Chem.* **1992**, *267*, 23589.
(46) Chen, Y.; Gai, F.; Petrich, J. W. *J. Am. Chem. Soc.* **1993**, *115*, 10158.
(47) Gai, F.; Chen, Y.; Petrich, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 8343.
(48) Brucker, G. A.; Kelley, D. F. *J. Phys. Chem.* **1987**, *91*, 2856.
(49) Strandjord, A. J. G.; Barbara, P. F. *J. Phys. Chem.* **1985**, *89*, 2355.
(50) McMorrow, D.; Kasha, K. *J. Phys. Chem.* **1984**, *88*, 2235.
(51) Schwartz, B. J.; Peteanu, L. A.; Harris, C. B. *J. Phys. Chem.* **1992**, *96*, 3591.
(52) Presentation at Proton Transfer Symposium, 210th National Meeting of the American Chemical Society, Fall 1995, Chicago, IL. Private communication. Reynolds, L. E.; Boryschuk, S. J.; Chapman, C. F.; Maroncelli, M. Excited-state proton transfer of 1-azacarbazole and 7-azaindole in alcohol solvents: a revised model for solvent-mediated Proton Transfer. In preparation.
(53) Schowen, K. B. J. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; p 225.
(54) Limbach, H.-H.; Henning, J.; Gerritzen, D.; Rumpel, H. *Faraday Discuss.* **1982**, *74*, 229.
(55) Eliason, R.; Kreevoy, M. M. *J. Am. Chem. Soc.* **1978**, *100*, 7037.
(56) Kresge, A. J. *Pure Appl. Chem.* **1964**, *8*, 243.
(57) Albery, J. W. *J. Phys. Chem.* **1986**, *90*, 3774.
(58) Chapman, C. F.; Maroncelli, M. *J. Phys. Chem.* **1992**, *96*, 8430.
(59) Share, P. E.; Sarisky, M. J.; Pereira, M. A.; Repinec, S. T.; Hochstrasser, R. M. *J. Lumin.* **1991**, *48/49*, 204.
(60) McMorrow, D.; Aartsma, T. J. *Chem. Phys. Lett.* **1986**, *125*, 581.
(61) Fuke, K.; Kaya, K. *J. Phys. Chem.* **1989**, *93*, 614−621.
(62) Fuke, K.; Tsukamoto, K.; Kaya, K. *J. Chem. Phys.* **1991**, *95*, 4074−4080.
(63) Gordon, M. S. *J. Phys. Chem.* **1996**, *100*, 3974.
(64) Gordon, M. S. Private communication.
(65) Nakajima, A.; Hirano, M.; Hasuni, R.; Kaya, K.; Watanabe, H.; Carter, C. C.; Williamson, J. M.; Miller, T. A. Preprint.
(66) Douhal, A.; Kim, S. K.; Zewail, A. H. *Nature* **1995**, *378*, 260.
(67) Douhal, A.; Guallar, V.; Moreno, M.; Lluch, J. M. *Chem. Phys. Lett.* **1996**, *256*, 370.
(68) Jensen, J. H.; Gordon, M. S. *J. Am. Chem. Soc.* **1995**, *117*, 8159.
(69) Meech, S. R.; Phillips, D.; Lee, A. G. *Chem. Phys.* **1983**, *80*, 317.
(70) Eftink, M. R.; Selvidge, L. A.; Callis, P. R.; Rehms, A. A. *J. Phys. Chem.* **1990**, *94*, 3469−3479.
(71) Muino, P. L.; Callis, P. R. *Proc. SPIE—Int. Soc. Opt. Eng.* **1994**, *2137*, 362−371.
(72) Creighton, T. E., Ed. *Protein Folding*; W. H. Freeman and Co.: New York, 1992.
(73) Lang, K.; Schmid, F. X.; Fischer, G. *Nature* **1987**, *17*, 268.
(74) Brennan, J. D.; Clark, I. D.; Hogue, C. W. V.; Ito, A. S.; Juliano, L.; Paiva, A. C. M.; Rajendran, B.; Szabo, A. G. *Appl. Spectrosc.* **1995**, *49*, 51.
(75) Cross, A. J.; Waldeck, D. H.; Fleming, G. R. *J. Chem. Phys.* **1983**, *78*, 6455. Ruggiero, A. J.; Todd, D. C.; Fleming, G. R. *J. Am. Chem. Soc.* **1990**, *112*, 1003.
(76) Lipari, G.; Szabo, A. *J. Am. Chem. Soc.* **1982**, *104*, 4559. Petrich, J. W.; Longworth, J. W.; Fleming, G. R. *Biochemistry* **1987**, *26*, 2711.
(77) Livnah, O.; Bayer, E. A.; Wilchek, M.; Sussman, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5076.
(78) Fremont, D. H.; Matsumura, M.; Stura, E. A.; Peterson, P. A.; Wilson, I. A. *Science* **1992**, *257*, 919. Matsumura, M.; Fremont, D. H.; Peterson, P. A.; Wilson, I. A. *Science* **1992**, *257*, 927. van Bleek, G. M.; Nathenson, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11302.
(79) Jackson, M. R.; Song, E. S.; Yang, Y.; Peterson, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11302.
(80) Kelly, J. M.; Sterry, S. J.; Cose, S.; Turner, S. J.; Fecondo, J.; Rodda, S.; Fink, P. J.; Carbone, F. R. *Eur. J. Immunol.* **1993**, *23*, 3318.
(81) Hatschek, E. *The Viscosity of Liquids*; G. Bell and Sons, Ltd.: London, 1928; p 68.
(82) Some of our earlier reports cite 414 nm as the fluorescence maximum of 7-azatryptophan in water. This result is due to a systematic error of the fluorimeter employed. The correct result is 397 nm.[17]
(83) Avouris, P.; Yang, L. L.; El-Bayoumi, M. A. *Photochem. Photobiol.* **1976**, *24*, 211.
(84) In the course of this discussion in this section, "energy", unless otherwise indicated, refers to potential energy, namely, electronic energy obtained from ab initio calculations plus the zero-point vibrational energy correction. Hence, the energy may be thought of as ΔH at 0 K.
(85) Garcia, K. C.; Degano, M.; Stanfield, R. L.; Brunmark, A.; Jackson, M. R.; Peterson, P. A.; Teyton, L.; Wilson, I. A. *Science* **1996**, *274*, 209.
(86) Callis, P. R. The ^1L_a and ^1L_b transitions of tryptophan: applications of theory and experimental observations to the fluorescence of proteins. *Methods in Enzymol.* Submitted for publication.
(87) Chen, L. X.-Q.; Engh, R. A.; Fleming, G. R. *J. Phys. Chem.* **1988**, *92*, 4811.
(88) The $E_T(30)$ scale is an empirical measure of solvent polarity based on the position of the lowest energy absorption band of a betaine dye in that particular solvent. Dimroth, K.; Reichardt, C.; Siepman, T.; Bohlman, F. *Justus Liebig Ann. Chem.* **1963**, *661*, 1.