Peptide Sequence and Conformation Strongly Influence Tryptophan Fluorescence

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ABSTRACT This article probes the denatured state ensemble of ribonuclease Sa (RNase Sa) using fluorescence. To interpret the results obtained with RNase Sa, it is essential that we gain a better understanding of the fluorescence properties of tryptophan (Trp) in peptides. We describe studies of *N*-acetyl-L-tryptophanamide (NATA), a tripeptide: AWA, and six pentapeptides: AAWAA, WVSGT, GYWHE, HEWTV, EAWQE, and DYWTG. The latter five peptides have the same sequence as those surrounding the Trp residues studied in RNase Sa. The fluorescence emission spectra, the fluorescence lifetimes, and the fluorescence quenching by acrylamide and iodide were measured in concentrated solutions of urea and guanidine hydrochloride. Excited-state electron transfer from the indole ring of Trp to the carbonyl groups of peptide bonds is thought to be the most important mechanism for intramolecular quenching of Trp fluorescence. We find the maximum fluorescence intensities vary from 49,000 for NATA with two carbonyls, to 24,400 for AWA with four carbonyls, to 28,500 for AAWAA with six carbonyls. This suggests that the four carbonyls of AWA are better able to quench Trp fluorescence than the six carbonyls of AAWAA, and this must reflect a difference in the conformations of the peptides. For the pentapeptides, EAWQE has a fluorescence intensity that is more than 50% greater than DYWTG, showing that the amino acid sequence influences the fluorescence intensity either directly through side-chain quenching and/or indirectly through an influence on the conformational ensemble of the peptides. Our results show that peptides are generally better models for the Trp residues in proteins than NATA. Finally, our results emphasize that we have much to learn about Trp fluorescence even in simple compounds.

INTRODUCTION

The fluorescence properties of the tryptophan residues in proteins provide a sensitive and informative probe for many different types of studies of proteins (1,2). In our studies of protein folding, we are able to do the same studies with much less protein when a Trp residue is present whose properties differ in the folded and unfolded conformations of the protein (3,4). When the protein does not contain a Trp, it is often useful to add one. In a previous study of the fluorescence properties of Trp residues in folded ribonuclease Sa (RNase Sa), we provided guidelines for how to do so (5).

The fluorescence properties of Trp residues in folded proteins vary widely (2,3,6,7). The quantum yields vary from near zero to 0.35, and the wavelength where the quantum yield is maximal, λ_{max}, varies from as low as 308 nm for buried Trp residues to near 350 nm for Trp residues that are largely exposed to solvent (5,8–10). In our studies of peptides described here, the differences in λ_{max} are small, but some of the differences in fluorescence intensity are large. Consequently, we will focus our attention on the factors that determine the fluorescence intensity of Trp residues in peptides and proteins.

In 1963, Cowgill began experimental studies of the fluorescence properties of tyrosine and tryptophan residues in proteins, peptides, and model compounds (11–13). He was able to show convincingly that ''Fluorescence was internally quenched by carbonyl groups.. . .'' (12). This has been confirmed by more recent studies (2,14,15). Especially convincing are studies of peptides in constrained conformations by the Barkley group that led to their conclusion: ''The peptide bond quenches tryptophan fluorescence by excited-state electron transfer, which probably accounts for most of the variation in fluorescence intensity of peptides and proteins.'' (16). The Callis group has provided theoretical support for this idea (9,17,18). Chen and Barkley (19) have also shown which groups in the side chains of proteins are capable of quenching Trp fluorescence. More recently, Nanda and Brand (20) have shown that Trp fluorescence can be quenched by a transient, excited state NH $\cdots$ π hydrogen bond to another aromatic residue.

We compare the fluorescence properties of Trp residues in unfolded RNase Sa with the fluorescence properties of Trp residues in pentapeptides with the same sequence as those in the proteins (21). Our goal was to see if fluorescence can show properties of the unfolded states of proteins that give us a better understanding of the denatured state ensemble of proteins. In this study, we compare the fluorescence properties of these peptides with the simpler models: *N*-acetyl-L-tryptophanamide (NATA), a tripeptide; AWA; and the pentapeptide, AAWAA. The results show a number

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of interesting findings that give us a better understanding of the fluorescence properties of peptides and proteins.

MATERIALS AND METHODS

Materials

NATA and 3-[*N*-morpholino] propanesulfonic acid (MOPS) were obtained from Sigma-Aldrich (St. Louis, MO). The seven peptides used in this study were synthesized by AnaSpec (San Jose, CA) with >99% purity. Ultra-pure urea was obtained from Nacalai Tesque (Kyoto, Japan), and ultra-pure GdnHCl was obtained from ICN Biomedicals (Aurora, OH). Urea and GdnHCl stock solutions were prepared by weight, and the concentrations determined as described previously (22,23). All other reagents were of analytical grade.

Peptide solution concentrations

Concentrations were determined using a molar absorption coefficient at 280 nm, $\varepsilon_{280} = 5630\ M^{-1}\ cm^{-1}$ for NATA and the peptides containing a single Trp residue and no Tyr residues. For the two peptides containing both a Trp and Tyr residue, $\varepsilon_{280} = 6850\ M^{-1}\ cm^{-1}$ was used. These ε_{280} values are based on previous results (24). The error in determining peptide concentrations using these extinction coefficients is <3%.

Fluorescence emission spectra

The fluorescence spectra were determined on peptide samples with 10 μM concentrations in 30 mM MOPS buffer, pH 7.0, 25°C. They were studied in two denaturing solutions: 8.5 M urea and 6 M GdnHCl. All fluorescence measurements used an SLM 8100 spectrofluorometer. The samples were excited at 300 nm and the emission was recorded from 310 to 500 nm using 4 nm emission and excitation bandwidths and a 1-s integration time. Temperature was controlled using a Brinkman Lauda RM refrigerated water bath. The fluorescence contribution from the blank denaturant solutions was subtracted from that of the samples. Emission spectra were also corrected for the wavelength dependent instrument response. The emission λ_{max} is the wavelength where the greatest fluorescence intensity, I_F, was observed. The spectral moment was calculate using the following formula:

$$\text{spectral moment} = \sum_{i=310}^{500} \lambda_i I_{F,i} / \sum_{i=310}^{500} I_{F,I}. \quad (1)$$

NATA and five of the peptides contain only Trp residues and no Tyr residues, but two of the peptides contained both a Trp and a Tyr residue. More experiments were done to see if the additional Tyr residues or small shifts in the absorption spectrum would affect absorption at 300 nm and, also, to see if the use of a 4-nm excitation bandwidth would influence the absorption. We measured molar absorption coefficients at 300 nm (ε_{300}), in units of $M^{-1}\ cm^{-1}$, and the average of the molar absorption coefficients at 1-nm intervals between 298 and 302 nm ($\varepsilon_{298-302}$) for NATA and four of the peptides, AAWAA, EAWQE, HEWTV, and GYWHW. The ε_{300} values were: 958 (NATA), 977 (AAWAA), 912 (EAWQE), 976 (HEWTV), and 1028 (GYWHE); and the $\varepsilon_{298-302}$ values were: 985 (NATA), 1009 (AAWAA), 943 (EAWQE), 1007 (HEWTV), and 1060 (GYWHE). For the four molecules with no Tyr residue, the average $\varepsilon_{300} = 956 \pm 30$, and the average $\varepsilon_{298-302} = 986 \pm 31$. For the molecule containing a Tyr residue: $\varepsilon_{300} = 1028$ and $\varepsilon_{298-302} = 1060$. First, because the ε-values for the molecules containing a single Trp are identical within experimental error, we conclude that their absorption spectra are not shifted significantly. Second, the Tyr appear to absorb a small amount of the light at 300 nm, but it will not contribute to Trp fluorescence unless energy is transferred to the Trp and this should be small in peptides and unfolded proteins. Finally, the average value of $\varepsilon_{298-302}$ is just 3.2% higher than the ε_{300} value and this will have no influence on the interpretation of our results.

Acrylamide and iodide quenching

The quenching experiments were done at a peptide concentration of 10 μM at pH 7.0, 25°C in 30 mM MOPS buffer in the presence of 7.6 M urea or 3.8 M GdnHCl. It was necessary to use lower concentrations of urea and GdnHCl than those used for the fluorescence emission experiments because iodide was not soluble at higher urea and GdnHCl concentration. However, the denaturant concentrations used were well above those needed to unfold RNase Sa completely (25), which will be important in the companion study. The samples were excited at 300 nm and the emission measured at 350 nm. Four nanometer excitation and emission wavelength slits were used. The fluorescence intensity in the absence of quencher, F_0, was measured after the sample temperature had equilibrated. The quencher was then added and the fluorescence intensity in the presence of quencher, F, was measured after a 3-min equilibration that was found to be long enough to reach equilibrium. Using these results, F_0/F was calculated and plotted as a function of quencher concentration, [Q], to determine the Stern-Volmer quenching constant, K_{SV}, according to the following equation (1,26):

$$F_0/F = 1 + K_{SV}[Q]. \quad (2)$$

Each experiment was carried at least twice and the results were averaged. The results always differed by <1.7%, and generally by <1%.

Time-resolved fluorescence measurements

Time-resolved fluorescence experiments were carried out in a K2 multifrequency cross-correlation phase and modulation fluorometer from ISS (Champaign, IL). Data were collected using the multifrequency phase and modulation method (27,28). The exciting light was from a Spectra-Physics Argon Ion laser tuned at 300 nm. The beam was passed through a 300-nm Melles Griot FIU004 interference filter to remove the 275-nm line present when the laser is operated in the deep ultraviolet region. The modulated beam was passed through a polarizer oriented parallel to the vertical laboratory axis and the emission was viewed through a polarizer oriented at the magic angle (54.7°) to eliminate the polarization effects on the lifetime measurements. Emission was collected through a Schott WG-345 cut-on filter to separate the fluorescence signal from the scattered light. Experiments were carried out between 2 and 250 MHz selecting 10–14 frequencies. A solution of *p*-terphenyl in ethanol was used as a reference lifetime with a value of 1.05 ns. Data analysis was carried out using Global Analysis (Global Unlimited, Urbana, IL) obtained from the Laboratory for Fluorescence Dynamics currently located at the University of California, Irvine, CA. The lifetime data were analyzed assuming different models including monoexponential, multiexponential or continuous lifetime distribution (28) decay models. The goodness of fit was determined by using the reduced χ^2 values.

RESULTS

To gain a better understanding of the results obtained with the proteins described in the companion study, a Trp model, *N*-acetyl-L- tryptophanamide (NATA), and seven peptides were studied: a tripeptide, AWA, and six pentapeptides, AAWAA, WVSGT (D1W), GYWHE (Y52W), HEWTV (Y55W), EAWQE (T76W), DYWTG (Y81W). The latter five peptides have the same sequence as those surrounding the tryptophans in the proteins studied in the companion study (21). (The corresponding protein variants are shown in parentheses after the peptide sequence.) For WVSGT, the peptide model for D1W,

the amino terminal amino group was not acetylated. For all of the other peptides, the α-amino group was acetylated and the α-carboxyl group was amidated.

The parameters measured were the spectral moment, SM, the wavelength where fluorescence intensity is maximal, λ_{max}, the fluorescence intensity at λ_{max}, I_F, the Stern-Volmer quenching constant, K_{SV}, the average excited state lifetime, τ_{ave}, and the width of the lifetime distribution. For reasons explained in Materials and Methods, the emission spectral measurements were done in 8.5 M urea and 6 M GdnHCl, and the quenching experiments were done in 7.6 M urea and 3.8 M GdnHCl. The latter denaturant concentrations are high enough to completely unfold the proteins described in our previous study (5).

Fluorescence emission spectra

Typical fluorescence emission spectra for the peptides are shown in Fig. 1. The parameters characterizing these spectra in 8.5 M urea and in 6 M GdnHCl are given in Table 1. The λ_{max} values range from 346 to 352. NATA always has the greatest λ_{max}, but the differences from the peptides are small. The λ_{max} values for the peptides are all 348 $\pm$ 2 nm and do not differ significantly. The I_F value for NATA is substantially larger than for the peptides (28–56% larger). Also, the I_F values are always greater in 8.5 M urea than they are in 6 M GdnHCl (6–28% larger).

Fluorescence quenching by acrylamide and iodide

Typical Stern-Volmer plots for the quenching of the Trp fluorescence of the peptides by iodide in 3.8 M GdnHCl are shown in Fig. 2. The Stern-Volmer quenching constants characterizing the results in 7.6 M urea and 3.8 M GdnHCl solutions are given in Table 2. For both iodide and acrylamide, the K_{SV} values are always substantially greater for NATA than for any of the peptides. In addition, the K_{SV} value is always substantially greater for WVSGT where the Trp is the N-terminal residue in the peptide than for any of the other peptides.

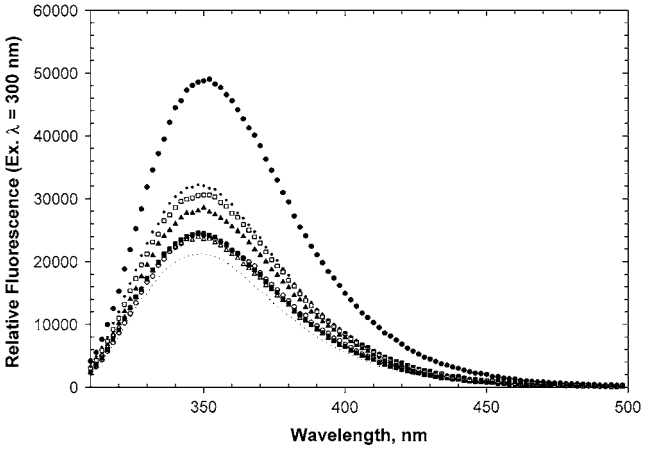

FIGURE 1 Fluorescence emission spectra after 300 nm excitation for NATA and seven peptides at 10 μM concentration in 8.5 M urea, pH 7.0, and 25°C. NATA (●), AWA (○), AAWAA (▲), WVSGT (D1W) (Δ), GYWHE (Y52W) (■), HEWTV (Y55W) (□), EAWQE (T76W) (●●●●), and DYWTG (Y81W) (▪▪▪▪).

Fluorescence excited-state lifetimes

Time-resolved fluorescence measurements were carried out as described above. The results for the peptides are given in Table 3. The fluorescence of NATA decayed monoexponentially, and a single value of the excited-state lifetime is given. For all of the peptides studied, the decay was more complex, and the excited-state lifetimes showed a Gaussian distribution. The τ_{ave} value, the center of the Gaussian distribution, and the width of the distribution are given. The τ_{ave} value for WVSGT is substantially larger than the τ-value for NATA and the τ_{ave} values for all of the other peptides.

DISCUSSION

Fluorescence emission spectra λ_{max}

In a previous study, we showed that λ_{max} for AWA increases as the solvent becomes more polar: 320 nm (hexane), 337 nm (ethanol), 348 nm (9 M urea), and 351 nm (water) (5). In a vacuum, it is estimated that $\lambda_{max} \approx 300$ nm for an indole group (1). This shows that λ_{max} for Trp red shifts as the environment becomes more polar. The Trp side chain in NATA is more exposed to solvent than the Trp side chains in the peptides and this is reflected in the λ_{max} values. For NATA, $\lambda_{max} = 352$ nm in urea and $\lambda_{max} = 351$ nm in GdnHCl and this is slightly higher than the $\lambda_{max} = 348$ nm $\pm$ 2 observed for the peptides (Table 1). It appears that the indole ring in the Trp side chain in pentapeptides is partially buried compared with the indole ring in NATA, but the difference is small. We will see in the companion study that the λ_{max} values for the denatured proteins do not differ significantly from those for the pentapeptides (21), and conclude that λ_{max} values are not sensitive to the long-range interactions in proteins that effect the I_F values and the k_q values characterizing acrylamide and iodide quenching.

I_F

Experimental (16) and theoretical (9) evidence suggests that electron transfer from Trp to the carbonyl groups of neighboring peptide bonds is the most important mechanism for intramolecular quenching of Trp fluorescence. Our results support this idea and show clearly the importance of conformational effects in the peptides in determining the extent of quenching. In this section, we will first discuss the results

TABLE 1 Comparison of the fluorescence properties of the peptides in 8.5 M urea and 6 M guanidine hydrochloride, pH 7.0, and 25°C

Peptide	SM* (nm) Urea	SM (nm) GdnHCl	$\lambda^{\dagger}_{max}$ (nm) Urea	λ_{max} (nm) GdnHCl	I_F at $\lambda^{\ddagger}_{max}$ Urea	I_F at λ_{max} GdnHCl	% Δ Urea to GdnHCl¶	%Δ I_F from NATA Urea§	%Δ I_F from NATA GdnHCl‖
NATA	363	364	352	351	49,000	35,500	28	0	0
AWA	362	362	349	346	24,400	22,100	9	50	38
AAWAA	362	362	350	350	28,500	23,100	19	42	35
WVSGT	361	363	347	350	23,900	22,100	8	51	38
GYWHE	361	361	347	347	24,600	23,100	6	50	35
HEWTV	361	362	349	349	30,800	25,700	17	37	28
EAWQE	361	362	347	348	32,300	24,900	23	34	30
DYWTG	361	362	347	347	21,500	18,400	14	56	48

*Spectral moment (SM) is the wavelength at which the total area under the emission spectrum is divided into two equal areas.
†λ_{max} is the wavelength of maximum fluorescence intensity. We estimate the error to be ±2 nm.
‡I_F at λ_{max} is the fluorescence intensity at λ_{max}. We estimate the error to be ±3%.
¶$\%\Delta = (I_{F,Urea} - I_{F,GdnHCl}/I_{F,Urea})\times 100$.
§$\%\Delta I_{F,Urea} = (I_{F,NATA} - I_{F,Peptide}/I_{F,NATA})\times 100$.
‖$\%\Delta I_{F,GdnHCl} = (I_{F,NATA} - I_{F,Peptide}/I_{F,NATA})\times 100$.

in urea given in Table 4 and then compare these results to those in GdnHCl given in Table 5 in a separate section.

The number of backbone carbonyl groups in NATA and the peptides varies as follows: NATA = 2, AWA = 4, WYSGT = 5, and all of the other pentapeptides = 6. With less carbonyl groups, NATA would be expected to have the highest I_F, and this is observed. However, AWA has an I_F value 14% lower than AAWAA, despite having two less carbonyl groups. This suggests that the four carbonyl groups of AWA are better able to quench the fluorescence than the six carbonyl groups in AAWAA, presumably because of differences in the average conformations of the peptides in solution. It will be interesting to see if these differences can be accounted for by studies of the two peptides through computational approaches, such as those used by Callis et al. (9).

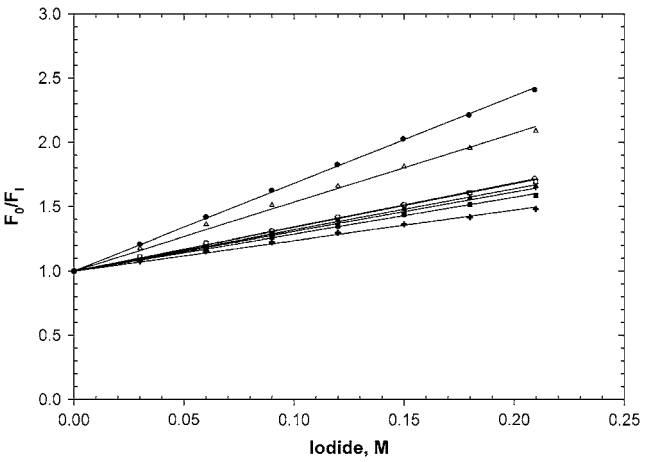

FIGURE 2 Iodide fluorescence quenching (300 nm excitation, 350 nm emission) of NATA and seven peptides at 10 μM concentration in 3.8 M GdnHCl, pH 7.0, and 25°C. NATA (●), AWA (○), AAWAA (▲), WVSGT (D1W) (Δ), GYWHE (Y52W) (■), HEWTV (Y55W) (□), EAWQE (T76W) (●●●●), and DYWTG (Y81W) (▪▪▪▪).

For AAWAA and the other four pentapeptides with Trp in the central position, the I_F values range from 21,500 for DYWTG to 32,300 for EAWQE with an average value of 27,540 (Table 4). These pentapeptides all contain six backbone carbonyl groups and have Trp at the same position so the large differences between the I_F values are interesting. Chen and Barkley (19) have shown that some of the side chains in proteins are capable of collisional quenching of Trp fluorescence. Considering just the side chains found in our peptides, the best quenchers are the protonated His side chain and the Tyr side chain, but neither quenches as well as acrylamide. The α-amino group, and the side chains of Asn, Gln, and the unprotonated form of His are less efficient quenchers. It is not clear, of course, whether these collisional quenching results can be used to predict the effect of side-chain quenching in our peptides. EAWQE is the only peptide with a carbonyl group in the side chain adjacent to the Trp, and it has the greatest I_F value. This suggests that this side-chain carbonyl does not make a large contribution to the

TABLE 2 Stern-Volmer constants for acrylamide and iodide quenching of the peptide fluorescence in 7.6 M urea and 3.8 M guanidine hydrochloride, pH 7.0, and 25°C

Peptide	Urea Acrylamide K_{SV} (M^{-1})	Urea Iodide K_{SV} (M^{-1})	Urea % Δ*	GdnHCl Acrylamide K_{SV} (M^{-1})	GdnHCl Iodide K_{SV} (M^{-1})	GdnHCl % Δ†
NATA	19.86	8.85	55	16.89	6.81	60
AWA	9.84	3.95	60	9.80	3.42	65
AAWAA	9.86	4.03	59	8.66	3.19	63
WVSGT	12.81	7.73	40	10.52	5.35	49
GYWHE	7.90	2.63	67	7.60	2.86	62
HEWTV	10.06	3.72	63	8.85	3.39	62
EAWQE	9.59	2.92	70	8.08	3.07	62
DYWTG	8.39	2.49	70	7.20	2.36	67

Calculated as described in Materials and Methods. We estimate the error to be ±3%.
*$\%\Delta_{A\rightarrow I,Urea} = (K_{SV,Acrylamide} - K_{SV,Iodide}/K_{SV,Acrylamide})\times 100$.
†$\%\Delta_{A\rightarrow I,GdnHCl} = (K_{SV,Acrylamide} - K_{SV,Iodide}/K_{SV,Acrylamide})\times 100$.

TABLE 3 Average fluorescence lifetimes determined by analyzing a Gaussian distribution of the lifetimes for the peptides in 8.5 M urea and 6 M guanidine hydrochloride, pH 7.0, and 25°C

	τ^*_{ave} (ns)	τ^*_{ave} (ns)	Width (ns)	Width (ns)	χ^2	χ^2
Peptide	Urea	GdnHCl	Urea	GdnHCl	Urea	GdnHCl
NATA†	4.04 ± 0.02	2.80 ± 0.07	–	–	1.02	1.91
AWA (B/S)‡	2.40 ± 0.04	–	0.55 ± 0.05	–	2.32	–
AWA	2.46 ± 0.02	1.90 ± 0.05	0.60 ± 0.02	0.40 ± 0.05	0.57	1.40
AAWAA	2.80 ± 0.05	2.05 ± 0.08	0.92 ± 0.04	0.53 ± 0.07	1.68	1.98
WVSGT	4.59 ± 0.08	3.80 ± 0.04	3.37 ± 0.07	2.16 ± 0.05	2.51	1.69
GYWHE	2.25 ± 0.08	2.01 ± 0.02	1.05 ± 0.06	0.72 ± 0.03	1.28	0.65
HEWTV	2.95 ± 0.03	2.42 ± 0.04	1.07 ± 0.02	0.80 ± 0.05	0.83	1.17
EAWQE	3.10 ± 0.04	2.30 ± 0.03	1.07 ± 0.04	0.78 ± 0.04	1.05	0.78
DYWTG	2.48 ± 0.05	1.91 ± 0.06	1.17 ± 0.03	0.78 ± 0.03	1.83	1.04

*τ-value at the center of the Gaussian distribution.
†Monoexponential decay.
‡A blank was subtracted for this measurement.

quenching. GYWHE has a significantly higher I_F value than DYWTG despite the fact that there are two potential quenchers in the side chains adjacent to the Trp in the first compound, but only one in the second. This also suggest that quenching by adjacent side-chains does not make a large contribution to the I_F values. It will be difficult to distinguish side-chain quenching from the effects that side chains have on the average conformations of the peptides in solution. Any structural change that varies the orientation or distance between the indole ring and the backbone carbonyl groups or potential side-chain quenchers may be more important.

As expected, there is a correlation between the I_F values and the τ_{ave} values shown in Table 4. For NATA, AWA, and the five pentapeptides with Trp at the central position, the correlation coefficient is 0.97. The I_F for WVSGT is intermediate among the values for the other pentapeptides, but the τ_{ave} value is much larger than for any of the other compounds. Eftink et al. (29) have suggested that the α-ammonium group quenches Trp fluorescence by excited state proton transfer to carbon 4 of the indole ring. This quenching mechanism would not be possible in NATA or the other peptides. This quenching mechanism and the positive charge on the α-amino group may both contribute to the unique results observed for WVSGT.

It was clear from studies of simple indole-containing model compounds that fluorescence intensities are remarkably sensitive to the chemical structure (13,30). It is difficult experimentally to determine the conformational ensemble of a peptide and this makes it difficult to develop computational methods for predicting peptide conformations. Our results may prove useful in gaining a better understanding of the conformational ensembles of peptides and of the intramolecular quenching of Trp fluorescence in peptides and proteins.

k_q

Intermolecular, collisional quenching is described by the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q], \quad (3)$$

where F and F_0 are the fluorescence intensities in the presence and absence of the quencher, K_{SV} is the Stern-Volmer quenching constant, [Q] is the molar concentration of

TABLE 4 Fluorescence properties of the peptides in urea, pH 7.0, and 25°C

	I_F at λ^*_{max}	$\tau^\dagger_{ave}$	$k_q^\ddagger$ (M^{-1} ns^{-1})	
Peptide	(nm)	(ns)	Acrylamide	Iodide
NATA	49,000	4.04	4.92	2.19
AWA	24,400	2.44	4.00	1.61
AAWAA	28,500	2.80	3.52	1.44
WVSGT	23,900	4.59	2.79	1.68
GYWHE	24,600	2.25	3.51	1.17
HEWTV	30,800	2.95	3.41	1.26
EAWQE	32,300	3.10	3.09	0.94
DYWTG	21,500	2.48	3.38	1.00

*From Table 1.
†From Table 3.
‡$k_q = K_{SV}/\tau_{ave}$ using the K_{SV} values from Table 2.

TABLE 5 Fluorescence properties of the peptides in guanidine hydrochloride, pH 7.0, and 25°C

	I_F at λ^*_{max}	$\tau^\dagger_{ave}$	$k_q^\ddagger$ (M^{-1} ns^{-1})	
Peptide	(nm)	(ns)	Acrylamide	Iodide
NATA	35,500	2.80	6.03	2.43
AWA	22,100	1.90	5.17	1.80
AAWAA	23,100	2.05	4.22	1.56
WVSGT	22,100	3.80	2.77	1.41
GYWHE	23,100	2.01	3.78	1.42
HEWTV	25,700	2.42	3.66	1.40
EAWQE	24,900	2.30	3.51	1.33
DYWTG	18,400	3.80	3.77	1.41

*From Table 1.
†From Table 3.
‡$k_q = K_{SV}/\tau_{ave}$ using the K_{SV} values from Table 2.

quencher, τ_0 is the is the lifetime of the fluorophore in the absence of quencher, and k_q is the bimolecular rate constant that limits the collision of the fluorophore and the quencher. The k_q values are given in Tables 4 and 5. They were calculated from the K_{SV} values in Table 2, and the τ_{ave} values in Table 3. In a given solvent, the k_q values should depend mainly on the accessibility of the Trp side chains to the quencher. Qualitatively, our peptide results support this. For both acrylamide and iodide quenching, the k_q values decrease in the order NATA > AWA > Pentapeptides with internal Trp in both urea and GdnHCl solutions (Tables 4 and 5). In addition, acrylamide is always a better quencher than iodide. If we exclude WVSGT, the k_q values for acrylamide are 2.32 ± 0.22 higher than the values for iodide in urea and 2.71 ± 0.76 higher in GdnHCl. The quenching of NATA fluorescence by acrylamide and iodide was studied in depth by Zelent et al. (26), and they also observed that acrylamide is a better quencher of indole fluorescence than iodide. They also show that based on molecular models the average radii of the two quenchers are similar.

A comparison of the k_q values for the Trp residue at the amino terminus with those at internal positions is interesting. The peptide with a Trp residue at the N-terminus, WVSGT, has a charge of $\sim +1.0$ at pH 7, whereas all of the other peptides will bear negative charges of ~ -0.75 for GYWHE and HEWTV, -1.0 for DYWTG, and -2.0 for EAWQE (31). For the quenchers, acrylamide is uncharged and iodide has a negative charge. For acrylamide, WVSGT has a lower k_q value than NATA or any of the other peptides (Table 4). This is a surprising result. The N-terminal Trp should be more accessible to acrylamide than the internal Trp residues in the peptides. Our guess is that the positive charge on the α-amino group somehow interferes with the ability of acrylamide to quench the fluorescence of the N-terminal Trp.

The results are quite different for quenching by iodide with its negative charge. The k_q value for the peptide with Trp at the N-terminus is higher than the k_q values for all of the other peptides. In this case there is a good correlation, 0.95, between the net charge on the molecules and the k_q values from Table 4: 0.94 (-2), 1.00 (-1), 1.26 and 1.17 (~ -0.75), 1.61 and 1.44 (0 charge), and 1.68 ($+1$). This suggests that charge–charge interactions between the quencher and Trp containing molecules influence the k_q values. The $k_q = 2.19$ value for NATA is greater than for any of the peptides, presumably because the indole ring is more accessible to the iodide ion than in the peptides and this is more important than the electrostatic interactions.

Comparison of urea and GdnHCl results

I_F values are always greater in urea solutions than in GdnHCl solutions (Tables 4 and 5). This indicates that guanidinium ions are better Trp fluorescence quenchers than urea molecules. In part, this may result because the guanidinium cation interacts more favorably with the indole ring than a urea molecule. Several lines of evidence suggest that cations can interact with aromatic rings (32).

The differences between the I_F values in urea and GdnHCl range from 1500 for GYWHE to 13,500 for NATA. There is a good correlation (0.98) between the values in urea and GdnHCl, but the individual differences are interesting. The I_F values are greater in urea than GdnHCl by 38% for NATA, 10% for AWA, and 23% for AAWAA. These molecules are all uncharged, and the indole group accessibilities decrease in the order: NATA > AWA > AAWAA. This suggests that the differences are not due to charge effects or to accessibility. For protein denaturants like urea and GdnHCl, the polypeptide backbone is known to be preferentially solvated by the denaturants relative to water and that this difference is greater for GdnHCl than it is for urea (33,34). Perhaps the two extra peptide bonds in AAWAA increase the local concentration of guanidinium cations more than urea molecules and this accounts for the larger effect on AAWAA than on AWA.

There are also some surprisingly large differences between the I_F values in urea and GdnHCl solutions for the pentapeptides. For example, for GYWHE the urea value is only 1500 greater than the value in GdnHCl, but for EAWQE the urea value is 7400 greater. Two factors may contribute to these differences. First, the net charges on the pentapeptides vary from $+1$ to -2 and this may influence the local concentration of guanidinium cations. Second, the conformational ensembles of the peptides may differ in the two solvents, in part because the ionic strength will be much greater in the GdnHCl solutions than in the urea solutions. The two smallest differences are for GYWHE (1500) and WVSGT (1800). In both cases there is a partial positive charge near the Trp that might repel guanidinium cations and reduce the observed difference between urea and GdnHCl. The largest difference for EAWQE (7400) may result because the net charge of -2 on the peptide may increase the local concentration of guanidinium ions or it may result because the high ionic strength shields the electrostatic repulsion between the carboxyl groups of the terminal Glu residues in EAWQE and changes the conformational ensemble present relative to GYWHE.

For NATA, AWA, and the pentapeptides with Trp residues at internal positions, the k_q values are always smaller in urea solutions than in GdnHCl solutions, but the correlation coefficient between the k_q values is 0.98. This may reflect a difference in the viscosity of the solutions. For the 3.8 M GdnHCl solution, the relative viscosity is 1.25, but for the 7.6 M urea solution it is 1.60 (35). The solution viscosity has been shown to effect k_q values in other studies (36).

The results in urea and GdnHCl for the peptide with Trp at the N-terminus differ from those for the peptides with internal Trp residues. For acrylamide quenching, the k_q values do not differ significantly in urea and GdnHCl. However, for iodide ion quenching, the k_q value is greater in urea solutions than in GdnHCl solutions, probably because the higher ionic

strength in the GdnHCl solutions shields electrostatic interactions and reduces the k_q value.

N-acetyl-L-tryptophanamide (NATA) is not a good model for the tryptophan residues in proteins

NATA is often used as a model for the Trp residues in proteins and peptides. Our results suggest that it is not the best model. The λ_{max} value is only slightly higher than the λ_{max} values for the peptides, but the I_F value for NATA is from 34% to 56% higher in urea solutions and from 30% to 48% higher in GdnHCl solutions than in the peptides. In addition, the k_q values for NATA are substantially greater than the values for AWA, AAWAA, or any of the pentapeptides. For many studies, either AWA or AAWAA would be better models for the Trp residues at internal positions. Our results also show that the properties of Trp residues at the N-terminus differ substantially from those at internal positions. In part, this is due to the positive charge on the α-amino group, so it may also be true for Trp residues at the C-terminus. Consequently, NATA, AWA, and AAWAA are probably not good models for Trp residues at the N- or C-terminus.

CONCLUSIONS

For the pentapeptides, the I_F values range from 21,500 to 32,300 in 8.5 M urea and from 18,400 to 25,700 in 6 M GdnHCl (Tables 4 and 5). In both cases, the smallest I_F value is found for DYWTG and it is 50% lower than the largest I_F value for EAWQE in urea and 40% lower than the largest I_F for HEWTV in GdnHCl. These large differences between peptides show that the amino acid sequence influences the I_F values, either directly through quenching by the side chains or, more likely, indirectly through an effect on the conformational ensembles of the peptides. The fluorescence quenching studies show that the k_q values that govern quenching depend on electrostatic interactions between the peptides and the quenchers. Finally, the results show that the conformational ensembles of the peptides differ in urea and GdnHCl solutions. The fluorescence properties of pentapeptides are remarkably complex, and it will be difficult to account for them using the computational methods available currently.

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