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The Tyrosyl Fluorescence of Angiotensin II in Alcoholic Solvents[1]

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Angiotensin II is an octapeptide hormone and contains a single tyrosyl residue and no tryptophyl residues. Intramolecular interactions of the tyrosyl residue with, for example, ionizable side chains or hydrogen bond acceptors can potentially perturb its fluorescence properties. The intrinsic fluorescence of angiotensin II was used to determine if the interactions of the tyrosyl residue were altered, as a consequence of conformational changes induced by certain alcoholic solvents. Steady-state and time-resolved fluorescence data for angiotensin II in neutral aqueous buffer, isopropanol and 1,2-propanediol, provided no evidence for specific conformations, which facilitated intramolecular association of the tyrosyl residue with other moieties. Multiexponential decay kinetics in which the decay times were <5 ns were observed in all cases. No fluorescence which could be attributed to tyrosinate anion was detected in the solvent systems studied.

KEY WORDS: Angiotensin II; tyrosine; tyrosinate; fluorescence lifetime; time-resolved fluorescence.

INTRODUCTION

The spectral properties of a tyrosyl residue in peptides or proteins are usually found to be similar to those observed for the free amino acid, which has absorption and fluorescence spectral maxima at 275 and 303 nm. Ionization of the hydroxy group of aqueous tyrosine, to form tyrosinate, results in red shifts of the spectral maxima to 295 and 340 nm, respectively [1]. The observation of an emission band with a maximum near 340 nm from peptides or proteins at neutral pH, containing tyrosyl but not tryptophyl residues, has frequently been attributed to tyrosinate fluorescence (for a review, see Ref. 2).

In the ground state the phenolic hydroxyl pK_a of tyrosine is near 10 but is estimated to be only 4–5 in the first excited singlet state [3]. The rate of deprotonation of the phenolic hydroxyl is slow relative to the rate of deactivation of the excited state [4], and in aqueous solution the fluorescence quantum yield is found to be constant from pH 4 to pH 8. If, however, a strong proton acceptor is present at sufficiently high local concentrations, the rate of excited-state proton transfer may be enhanced, potentially leading to tyrosinate fluorescence at pH's significantly below the ground-state pK_a [3,5–8]. However, model studies of aqueous tyrosine in the presence of proton acceptor species [7] have recently suggested that excited-state hydroxyl proton transfer does not occur. The fluorescence decay kinetics provided evidence for the formation of ground-state hydrogen-bonded complexes, between tyrosine and the proton acceptor, which persisted in the excited state. Similar results were observed for aromatic alcohols in nonaqueous solvents [8].

It has been reported that the hormone angiotensin II (Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]) displays

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tyrosinate fluorescence in alcoholic organic solvents, such as isopropanol and 1,2-propanediol, but not in water solution [9,10]. The organic solvents were proposed to simulate the environment of the angiotensin II (ANG II) receptor and to induce hormone conformations in which interactions between Tyr^4 and potential proton acceptor moieties were favored, facilitating tyrosinate fluorescence. The proton acceptors were suggested to be the imidazole ring of His^6 and, to a lesser extent, the C-terminal carboxylate.

Decay times ascribed to tyrosinate fluorescence were very long, for example, 21 ns in 1,2-propanediol [9,10]. The values were surprising since they are almost three orders of magnitude greater than the lifetime of tyrosinate in water, which is 26 or 71 ps depending on the pH [7,11]. Such dramatic differences in the lifetime of the excited singlet state of tyrosinate clearly merit further investigation and here we report the results of steady-state and picosecond time-resolved fluorescence measurements on ANG II in alcoholic and aqueous solvents.

MATERIALS AND METHODS

ANG II was obtained from three sources, Sigma Chemical Co., Bachem Inc., and The Peptide Institute Inc. The samples were, according to the suppliers, "pure" by the criterion of reverse-phase high-performance liquid chromatography. Fluorescence-grade isopropanol was obtained from A & C American Chemicals Ltd. 1,2-Propanediol from Hartman-Leddon Co., also fluorescence grade, required further treatment with activated charcoal to remove trace fluorescent impurities. *N*-Acetyltyrosinamide (NAYA) was recrystallized three times from ethanol/methanol. Solutions of ANG II were prepared for fluorescence experiments according to one of two protocols. The hormone was dissolved in water and then added to a large volume of solvent or buffer. Alternatively, following Ref 10, hormone samples suspended in the organic solvent were warmed to 50°C, cooled to ambient temperature, and then filtered to remove insoluble material. The filters used were obtained from Millipore, Millex-GV_{13} (0.22 μm), or from PALL Trinity, PALL-Ultipor NR (0.2 μm).

The instrumentation and procedures for time-resolved and steady-state fluorescence measurements have been described previously [11,12]. Time-resolved fluorescence measurements were performed using the technique of time-correlated single-photon counting with laser/microchannel plate-based instrumentation. In this study the data were collected in 2048 channels at 10 ps/channel for samples in organic solvents and at 5 ps/channel for samples in aqueous buffer. The instrument response function was determined from the Raman scattering of the excitation by water (at 316 nm for the 287-nm excitation) [11] and had a fwhm of 60 ps. Each decay curve typically contained 0.5–1 $\times$ 10^6 total counts. For all samples corrections were made for the signal (less than 6%) from the appropriate sample-free blank. All measurements were performed at 20°C with a spectral resolution of 4 or 8 nm as indicated. Procedures for the simultaneous or "global" analysis [13] of multiple time-resolved data sets have been described elsewhere [12].

RESULTS

The fluorescence decay parameters for ANG II in isopropanol are summarized in Table I. Data were collected to at least 1.3 $\times$ 10^6 total counts at 10 ps/channel in 2048 channels, for eight emission wavelengths (300, 305, and 325 nm and every 5 nm to 350 nm; 8-nm band pass). Wavelengths to the red of the tyrosine emission maximum were included to enhance the contribution from tyrosinate and hydrogen-bonded tyrosine species, if present. Acquisition times typically ranged from 180 to 600 s. At each emission wavelength the fluorescence decay was well described by double-exponential kinetics, with decay times of approximately 1 and 4 ns. Global analysis, fitting for two decay times which were emission wavelength independent, gave an excellent fit (χ^2 = 1.03, SVR = 1.95). The quality of the global fit indicated that photodamage was negligible. Decay times recovered from the global analysis were 4.092 ± 0.003 and 0.966 ± 0.010 ns. Contributions from this 1-ns component were small; the normalized preexponentials ranged from 0.10 at 300 nm to 0.25 at 350 nm and the corresponding fractional fluorescences ranged from 0.03 to 0.07.

To prepare the sample, ANG II was heated in isopropanol to 50°C, cooled, and then filtered. This sample was filtered through a Millipore filter and control experiments showed that no detectable fluorescence was extracted from this filter by isopropanol. The isopropanol solutions of ANG II, after filtration, had an optical density of 0.03 at 275 nm. With anhydrous isopropanol we were unable to reproduce the ANG II concentrations of 0.25 to 1 mg/ml, corresponding to an optical density of 0.34 to 1.34 (1-cm pathlength), reported earlier [10].

Our results for the fluorescence decay kinetics of ANG II in isopropanol may be compared with the literature values. Double-exponential decay kinetics with decay times of 3.88 ± 0.18 and 15.49 ± 0.25 ns, and with fractional fluorescences of 0.21 and 0.79 at 350

Table I. Fluorescence Parameters for ANG II and NAYA[a]

Sample	No.[b]	τ_1[c]	τ_2	τ_3	α_1[d]	α_2	α_3	f_1[e]	f_2	f_3	χ^2[f]	SVR[g]
ANG II, isopropanol	8	4.09	0.97		0.75	0.25		0.93	0.07		1.03	1.95
ANG II, 96% isopropanol/water	4	3.78	0.81		0.76	0.24		0.94	0.06		1.02	1.88
ANG II, 99% 1,2-propanediol/water	4	4.35	1.16		0.78	0.22		0.93	0.07		1.04	1.92
ANG II, buffer[h]	4	1.86	0.62	0.04	0.31	0.18	0.51	0.82	0.15	0.03	1.03	1.98
NAYA, buffer	4	1.64	0.16		0.77	0.23		0.97	0.03		1.03	1.92

[a] The excitation wavelength was 287 nm and optical densities at the excitation wavelength were <0.05 (1-cm pathlength). Parameters are given for a global fit to a series of emission wavelengths.

[b] Number of data sets in the global analysis. For eight data sets emission wavelengths were 300, 305, and 325 to 350 nm in 5-nm steps (8-nm band pass). For four data sets emission wavelengths were 320 to 350 nm in 10-nm steps (4-nm band pass).

[c] Fluorescence decay times (ns) recovered from global analysis. The standard errors, derived from the diagonal elements in the nonlinear least-squares analysis, were typically <0.02.

[d] Normalized preexponential at 350 nm.

[e] Fractional fluorescence at 350 nm.

[f] Reduced χ^2 for the global fit ($\chi^2 = 1$ for an ideal fit).

[g] The serial variance ratio of the residuals for the global fit (SVR = 2 for a random distribution of residuals).

[h] 10 m*M* Tris, pH 7.5.

nm, have been reported [10]. We find no evidence for the long decay time component. Reanalysis of our data assuming triple-exponential decay kinetics and constraining one decay time to a value of 15.49 ns did not significantly improve the quality of the fit ($\chi^2 = 1.02$, SVR = 1.99). Furthermore, in this analysis the contribution of the 15.49-ns decay time was zero, within error, at all emission wavelengths. Similar results were obtained for ANG II in 96% isopropanol/water and in 99% 1,2-propanediol/water (Table I). We did not detect any long (>5-ns) decay time components in those solvents.

In aqueous buffer (10 m*M* Tris, pH 7.5) the fluorescence decay kinetics of ANG II were more complex and a double-exponential model gave a poor fit ($\chi^2 = 1.27$, SVR = 13). The triple-exponential decay analysis (Table I) included a short decay time component of 43 ps. In contrast, the model compound NAYA displayed biexponential decay kinetics in buffer. The recovered decay times of 1.64 and 0.16 ns for NAYA are the same as the literature values of 1.66 and 0.11 ns [14] and contrast with other values of 3.01 and 1.19 ns [10].

At wavelengths greater than 275 nm, where the contribution of Phe[8] is negligible, the absorption spectra of ANG II, in each of the solutions listed in Table I, were the same shape as those of the model compound NAYA under comparable conditions. The absorption maxima were 276 nm in buffer and 279 nm in the alcoholic solvents. Steady-state fluorescence emission spectra of ANG II and NAYA were also identical, with maxima at 302 nm in buffer and 303 nm in the alcoholic solvents. Finally, we note that ANG II samples from three sources showed essentially identical properties.

DISCUSSION

In water the zwitterion form of tyrosinate has a fluorescence lifetime of 69 ps and an emission maximum of 340 nm [7]. As we noted in the Introduction, in order to observe tyrosinate emission at neutral pH the rate of phenolic hydroxyl proton transfer must be increased in some way, so that it can compete with the rate of deactivation of the excited state. A strong proton acceptor, present at a high local concentration, could enhance proton transfer [3,5–8] and facilitate tyrosinate emission. However, recent fluorescence kinetic studies in water have indicated that the phenolic hydroxyl of tyrosine does not undergo excited-state proton transfer in the presence of proton acceptors which have pK_a's typical of those found in proteins and peptides [7].

Potential proton acceptor species formed ground-state, hydrogen-bonded complexes with the phenolic hydroxyl of tyrosine, which persisted in the excited state. The hydrogen-bonded complexes displayed fluorescence properties that were correlated with the pK_a of the "proton acceptor" species. As the pK_a of the proton acceptor was increased, the charge transfer component of the hydrogen-bonded complex's excited-state increased, re-

sulting in a progressive red shift in the emission maximum and decrease in the associated decay time component. The "tyrosinate-like" fluorescence properties (λ_{max}, 340 nm; τ, ~70 ps) of the ion pair were generally observed only when the proton acceptor pK_a approached that of the ground-state phenolic hydroxyl pK_a (10.3). Imidazole however, behaved anomalously, since the complex it formed with tyrosine showed a higher degree of charge transfer character than would be predicted from its pK_a of 7, approaching that of the imidazolium–tyrosinate ion pair.

The charge transfer character of the complex is also influenced by the polarity and polarizability of the solvent [7,8]. Low-polarity solvents do not favor ion-pair formation, and consequently the fluorescence properties of the hydrogen-bonded complex will become more "tyrosine-like" as the solvent polarity is reduced [8].

On the basis of the above discussion, an interaction between Tyr^4 and the imidazole ring of His^6 in ANG II may be expected to result in the formation of a ground-state hydrogen-bonded complex, which upon excitation would have a fluorescence decay time of the order of 70 ps and an emission maximum of 340 nm, similar to that of tyrosinate. Alcoholic solvents such as isopropanol are of intermediate polarity and this may affect the fluorescence properties to some extent. However, they are polarizable and are capable of hydrogen-bonding. It is unlikely therefore, that the charge transfer component of the structure of the hydrogen-bonded complex should be considerably reduced in isopropanol, compared to water.

Turner *et al.* [9,10] proposed that ANG II exhibited tyrosinate fluorescence in 1,2-propanediol, isopropanol, and trifluoroethanol on the basis of time-resolved fluorescence measurements. A long decay time component (13–21 ns) was observed for ANG II, but not for the model compound NAYA, in the alcoholic solvents at an emission wavelength of 350 nm. The long decay time was ascribed to tyrosinate formed as a result of excited-state proton transfer from Tyr^4 to the imidazole ring of His^6. Long decay time components were not detected in neutral aqueous solutions of ANG II or NAYA. It was further observed that the contribution of the long decay time increased with increasing wavelength over the range 320 to 350 nm. The contributions were significant; for example, the fractional fluorescence of the 15-ns component observed for ANG II in isopropanol was 0.8[10].

Decay times of 13–21 ns for tyrosinate fluorescence are far removed from the expected subnanosecond values. We note also that based on the quantum yield of tyrosinate (0.006 at pH 13 [1]) and the corresponding observed fluorescence lifetime (26 ps [7,11]), the *radiative* lifetime of tyrosinate in water is only 4.3 ns. For tyrosinate to have an observed lifetime of 21 ns in isopropanol, this solvent would have to increase the *radiative* lifetime at least fivefold. The long (13- to 21-ns) decay time components were not detected in our study. Given that significantly more counts in each decay curve were collected in this study and that the long decay time components were reported to account for up to 80% of the fluorescence signal in the wavelength range 320–350 nm, we would have expected to detect them, if present.

We found that the absorption spectra of ANG II and NAYA were the same shape (above 275 nm) under comparable solvent conditions. In particular, there was no evidence for an increased extinction coefficient at 295 nm, the absorption maximum for (ground-state) tyrosinate. Ground-state hydrogen-bonding between the phenolic hydroxyl proton and some other group in the ANG II molecule was also largely excluded, since this would be expected to result in a red shift of the ANG II absorption spectrum relative to that of the model compound NAYA [6,7,8,15]. The steady-state emission spectra of ANG II and NAYA were also the same shape under comparable conditions for all of the solvent systems studied. As the emission spectrum of tyrosinate is red-shifted by some 40 nm from that of tyrosine, this observation indicates that there is no significant excited-state tyrosinate formation in ANG II.

If the long decay time components reported earlier [9,10] were not due to tyrosinate, what was their origin? Why were they observed for ANG II in the alcoholic solvents but not in water and why, if they were artifacts, were they not observed for NAYA samples. One possibility is that the long decay time components were due to fluorescent contaminants extracted from the filters used to remove insoluble material from the samples [10]. We speculate that the alcoholic solvents may have been more efficient at extracting the contaminants than water. The neutral NAYA molecule with its aromatic side chain is more soluble in the alcoholic solvents than the octapeptide and therefore may not have required filtration. Unfortunately details of the filters used in Ref. 10 were not given. However, they are thought to have been PALL-Ultipor NR, 0.2 μm, from PALL Trinity (R. S. Roche, personal communication). We were able to extract material from these filters (with alcoholic solvents) absorbing at 275 nm and having fluorescence properties similar to those ascribed to tyrosinate in the study by Turner *et al.* [10]. For example, passing 3 ml of isopropanol at room temperature once through the filter increased the fluorescence signal at 350 nm (288 nm excitation) by an order of magnitude. The extracted material had fluorescence decay times of ca. 10 ns (46%), 3 ns (44%), and 1 ns (11%). Water and neutral buffer solutions extracted

much less fluorescence, which if analyzed as a double-exponential decay in a time-resolved experiment, displayed ca. 4- and 1-ns decay components. These values are similar to those reported for ANG II and NAYA in buffer [10].

Our high time resolution data for ANG II in buffer (pH 7.5) showed the presence of a 40-ps decay component which, as we have discussed above, is close to the value expected for a tyrosine–imidazole hydrogen-bonded complex [7]. The contribution of this component was small and persisted in the absence of buffer salts. However, a preliminary investigation indicated that the excitation and emission spectra associated with this component were not consistent with those expected for a hydrogen-bonded complex [7]. Additionally, the contribution of the 40-ps component was not significantly affected by pH over the range of the His^6 imidazole pK_a of 6.5 [16] (data not shown). This suggests that the 40-ps component does not result from an interaction between Tyr^4 and His^6.

In buffer the fluorescence decay of ANG II was triple exponential. Complex decay kinetics have been observed for the tyrosyl residues of other peptide hormones and have been explained in terms of rotamer populations about the C_α–C_β bond of the Tyr side chain (reviewed in Ref. 2). It is interesting to note that according to NMR, all three staggered C_α–C_β rotamers of Tyr^4 in ANG II (aq) are significantly populated [16]. Only two decay components were required to fit the fluorescence data in the alcoholic solvents. This suggests that the alcoholic solvents induce the hormone to adopt a conformation which limits the rotational freedom of the Tyr^4 side chain.

In summary, this study does not support earlier reports that ANG II displays "long-lifetime tyrosinate fluorescence" in alcoholic solvents [9,10]. We find no evidence for significant tyrosinate fluorescence from ANG II in neutral aqueous or alcoholic solvents. The results of this and other studies [7,11] question the assignment of long decay time components, emitting at 350 nm, to tyrosinate in other peptides and proteins containing tyrosyl but not tryptophyl residues [9,10,17]. Our data also indicate that conformations of ANG II in which the hydroxyl of Tyr^4 is involved in an intramolecular hydrogen bond are not significantly populated, in both neutral aqueous and alcoholic solvents.

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