Journal of Fluorescence, Vol. 7, No. 4, 1997

The Photophysics of β-Tyrosine and Its Simple Derivatives

Wieslaw Wiczk,[1,2] Leszek Łankiewicz,[1] Cezary Czaplewski,[1] Stanislaw Oldziej,[1] Krystyna Stachowiak,[1] Alicja Michniewicz,[1] Beata Micewicz,[1] and Adam Liwo[1]

Received July 1, 1997; revised July 17, 1997; accepted October 30, 1997

Synthesis and photophysical studies of (*O*-methyl)-β-tyrosine (β-tyrosine; an analogue of tyrosine, in which the amino group is moved from the α- to the β-carbon, closer to the phenol ring) and its derivatives with a blocked amino and/or carboxyl group were performed to explain the nature of the fluorescence of tyrosine derived analogues. All β-tyrosine derivatives, except Ac-βTyr(Me), displayed the monoexponential fluorescence decay. The biexponential fluorescence decay observed for Ac-βTyr(Me) is assumed to be the result of the presence of two low-energy conformations (extended and with an intramolecular hydrogen bond). Higher quenching of the fluorescence of β-tyrosine derivatives by the *N*-acetyl group than by the *N*-methylamide group moved farther was found, contrary to the data found for the respective derivatives of natural tyrosine. The obtained photophysical data are discussed with theoretical calculations (AMBER, AM1) on the basis of the rotamer model.

KEY WORDS: Tyrosine; tyrosine analogue; conformer; fluorescence spectroscopy; time-domain fluorescence.

INTRODUCTION

The fluorescence of tyrosine, tyrosine derivatives, and the tyrosine residue in peptides and proteins is the subject of wide investigations. The tyrosine zwitterion and derivatives with an ionized α-carboxyl group exhibit monoexponential decay kinetics. Amidation or protonation of the α-carboxyl group results in a complex fluorescence decay.[1-4] Several explanations for the complex fluorescence kinetics of tryptophan and tyrosine have been forwarded, including the involvement of the ^1L_a and ^1L_b states,[5-7] an excited-state reaction,[8-10] and different lifetimes for the side-chain rotamers about the C^α–C^β bond.[3,11-22] In the rotamer model, multiexponential decay kinetics is proposed to be the result of the presence of a number of ground-state rotamers, some of which do not interconvert within the fluorescence time scale (typically 3–5 ns). Individual rotamers are assumed to exhibit monoexponential decay kinetics. This model, introduced by Gauduchon and Wahl,[3] suggests a charge transfer quenching between the excited aromatic chromophore (indole or phenol ring, respectively) as the donor and electrophilic units in the amino acid backbone (carbonyl or protonated amino group[15-17,20,21]) as the acceptor. As shown by Laws *et al.*[1,21] a shorter fluorescence decay lifetime was associated with the protonated carboxyl group, while a longer lifetime was associated with the ionized carboxylate. By the use of global and linked-function analysis, Laws *et al.*[1,20,21] were able to show that the rotamer model can explain the complex fluorescence decay of tyrosine analogues,[1,20] tyrosine,[20,21] and tryptophan residues[17] in small peptides. The rotamer model in the case of tyrosine analogues and peptides containing tyrosine or tryptophan residues showed that the rotamer in which the phenol or indole ring can come in the closest contact with the carbonyl group has the shortest fluorescence lifetime. The analysis of these results make clear that the rotamer interconver-

[1] Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland.
[2] To whom correspondence should be addressed.

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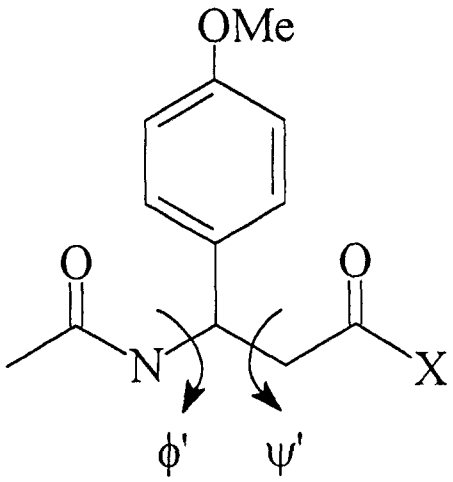

Fig. 1. Structure of β-tyrosine with marked rotation angles.

sion around the C^{α}–C^{β} bond in tyrosine is slower than the lifetime of the excited state. Unfortunately, for tyrosine components exhibiting a single-exponential decay, Laws *et al.*[1] were unable to establish whether (i) the slow-exchange rotamer model is the accurate description, but the three rotamers have similar unresolved fluorescence lifetimes, or (ii) the rotamer interconversion is fast enough, thereby averaging the emission. A rotamer model has also been used to explain acrylamide quenching of tyrosine amide.[22] The experimental basis of the rotamer theory was the observations by Cowgill[23] that the peptide carbonyl or the amide group is responsible for the quenching of tyrosine fluorescence in proteins and by Tourman *et al.*[24] that the carbonyl groups can quench the fluorescence of aromatic rings efficiently by a charge-transfer mechanism. In tyrosine different rotamers have different distances between the phenolic ring and quenching groups (amide and/or carboxyl), which may explain changes in the photophysical behavior of different rotamers. The charge-transfer reaction between the electrophilic unit in the amino acid backbone and the excited aromatic phenol subunit leads to a biexponential fluorescence decay of tyrosine in an acidic aqueous solution. This phenomenon was investigated by Kungl.[25] Based on the dynamics of tyrosine and the peptide Gly–Tyr–Gly *in vacuo* and water, with classical molecular dynamics and with stochastic computer simulations, he concluded that, since the rotamers frequently interconvert within the fluorescence lifetime of tyrosine, their contribution to the nonexponential fluorescence decay should be negligible.

Molecular dynamics simulations of the conformational dynamics of tryptophan were performed by Gordon *et al.*[26] They have obtained different results depending on the model used for hydrogen representation in the simulation. The rotamer model for the tryptophan zwitterion can be supported using the CHARMM intramolecular potential under the condition that hydrogen atoms are explicitly included in the model of tryptophan. They have also shown that the predicted relative populations of tryptophan rotamers are not consistent with the experimental data. On the other hand, the rotamer model is not supported by the results obtained by James and Ware for homo-tryptophan.[27] In homo-tryptophan (an analogue of tryptophan with an additional methylene group separating the indole ring from the α-carbon) the α–β conformers play a less significant role by virtue of the β–γ and γ–δ conformers. Homo-tryptophan, with a much larger number of conformations, exhibits the same qualitative behavior as tryptophan, but with fluorescence lifetimes somewhat larger.

In the case of β-tyrosine (βTyr) the carboxylic group remains in the same position compared to tyrosine, separated from the phenolic ring by the -CH-CH_2- linkage, and the NH_2 group is shifted closer to the phenolic ring (the amino group is connected to the β-carbon) (Fig. 1). According to the Newman projection of tyrosine, there are three possible positions of the phenolic ring in relation to the carboxylate,[1,4] because of the rotation about the C^{α}–C^{β} bond. In β-tyrosine, as in tyrosine, there is a rotation about the C^{α}–C^{β} bond and the number of possible rotamers that possess different distances between the phenol ring and the carboxylate is the same as in tyrosine. On the contrary to Tyr, the distance between the amino and the phenol group in βTyr is fixed.

In this paper we report the results of our photophysical studies of β-tyrosine derivatives to obtain some new insights into the rotamer model.

EXPERIMENTAL

Synthesis

The following derivatives of β-tyrosine (βTyr) were obtained: H-βTyr(Me)-OH, Ac-βTyr(Me)-OH, H-βTyr(Me)-NHMe, and Ac-βTyr(Me)-NHMe. A derivative of β-tyrosine Boc-βTyr(Me)-OH, the starting precursor for the synthesis of the all other derivatives, was obtained as described previously.[28,29] Boc-protec-

tion removal, to prepare derivatives of βTyr, was accomplished by an action of 4 *N* HCl/dioxane at room temperature.[30] *N*-Methyl amides were prepared from Boc-βTyr(Me)-OH using PyBOP as a coupling reagent and H_2N–CH_3·HCl as the amine source in the presence of TEA.[31] Acetylation of an amino group was carried out by reaction with Ac_2O in a THF/H_2O solvent system. The homogeneity of the amino acid derivatives was assessed by TLC (*n*BuOH–AcOH–H_2O = 4:1:1, $CHCl_3$–MeOH–AcOH = 85:10:5, $CHCl_3$–MeOH = 9:1, AcOEt–*n*-hexane = 1:1), analytical reversed-phase HPLC (a linear gradient of 0–80% CH_3CN in 0.1% TFA in H_2O over 60 min at a flow rate of 1 ml/min; column, Kromasil C-18; 4.6 × 250 mm; 5 μm), and mass spectrometry (FDMS or FABMS).

Spectroscopic Measurements

Fluorescence decays were collected by a time-resolved single-photon counting technique on an Edinburgh Analytical Instrument Type CD-900 fluorimeter interfaced with an IBM PC AT. The excitation source was a flash lamp filled with 0.5-atm hydrogen, operated at 40 kHz with about 6.5 kV across a 1-mm electrode gap. The half-width of the instrument response was 1.2 ns. The excitation (270-nm) and emission (310-nm) wavelengths were selected by means of monochromators (about 10-nm band width).

Fluorescence decays from the sample and the reference (Ludox; observation wavelength, 310 nm) were measured to $1 \cdot 10^4$ counts in the peak. The counting rate did not exceed 2% of the lamp repetition rate. The decay curves were stored in 1024 channels of 0.054 ns/channel. Fluorescence decay data were fitted by the iterative convolution to the sum of exponents:

$$I(\lambda,t) = \sum_i \alpha_i(\lambda)\exp(-t/\tau_i) \qquad (1)$$

where T_i is the decay time of the *i*th component and $\alpha_i(\lambda)$ is its preexponential factor at emission wavelength λ.

Since this model assumes that τ_i is independent of λ, the decay curves taken at different emission wavelengths can be analyzed simultaneously.[32,33] The data were analyzed by a global least-squares iterative convolution program supplied by the manufacturer (FLA-900 Level 2 software; Edinburgh Analytical Instruments). The adequacy of the exponential decay fitting was judged by inspection of the plots of weighted residuals and by the statistical parameters χ^2 and the shape of the autocorrelation function of the weighted residuals and serial variance ratio (SVR). Lifetimes distributions were obtained using FLA-900 Level 2 software, which does not assume any functional shape of the calculated lifetime distribution.

Decay-associated spectra (DAS), the emission spectra associated with each individual decay component,[34,35] were calculated from

$$I_i(\lambda) = I_{SS}(\lambda)[\alpha_i(\lambda)\tau_i I \sum_i \alpha_i(\lambda)\tau_i] \qquad (2)$$

where $I_i(\lambda)$ is the emission spectrum associated with the *i*th component, I_{ss} is the total steady-state spectrum, and $\alpha_i(\lambda)$ is the fractional intensity of fluorescence of the *i*th component at wavelength λ.

The steady-state spectra were obtained on a Perkin–Elmer LS-50 B spectrofluorimeter with a 2.5-nm band width for excitation and emission. The excitation wavelength was 270 nm. The quantum yields were measured relative to a value of 0.14 for tyrosine in water at room temperature.[36] The sample concentration was about $5 \cdot 10^{-5}$ in steady-state measurements and $1 \cdot 10^{-4}$ *M* in time-resolved experiments. All measurements were made in doubly deionized water, pH 7.0, at room temperature.

Theoretical Calculations

Rule-based conformational searches were performed on model compounds: (1) Ac–βTyr and (2) Ac–βTyr–NHMe using the Chem-X program with standard parameters[37] (see Fig. 1 for designation of variable angles). The generated conformations were then subjected to energy minimization using the AMBER 4.1 force field.[38] Then a minimal-tree cluster analysis of the energy-minimized conformations was carried out, using the RMS deviation between all backbone atoms as a distance measure. The RMS cutoff to separate families was 0.5 Å. For each family, the conformation with the lowest energy was taken as the representative of the entire family. Families with an energy more than 5 kcal/mol above the lowest-energy conformation were discarded. For the lowest-energy representatives of the remaining families, we calculated the electrostatic-solvation energy, using the boundary-element approach in which the Poisson–Boltzmann equation is solved for the solute molecule immersed in a continuous dielectric hereafter referred to as the IBEM method.[39–41] The dielectric constant of the solvent was taken as $\varepsilon = 80$, which corresponds to water, while the dielectric constant of the solute was assumed to be $\varepsilon = 2$. To make a better assessment of the relative thermodynamic stability of the obtained conformations in solution, we also computed energies for the selected conformations using the semiempirical AM1

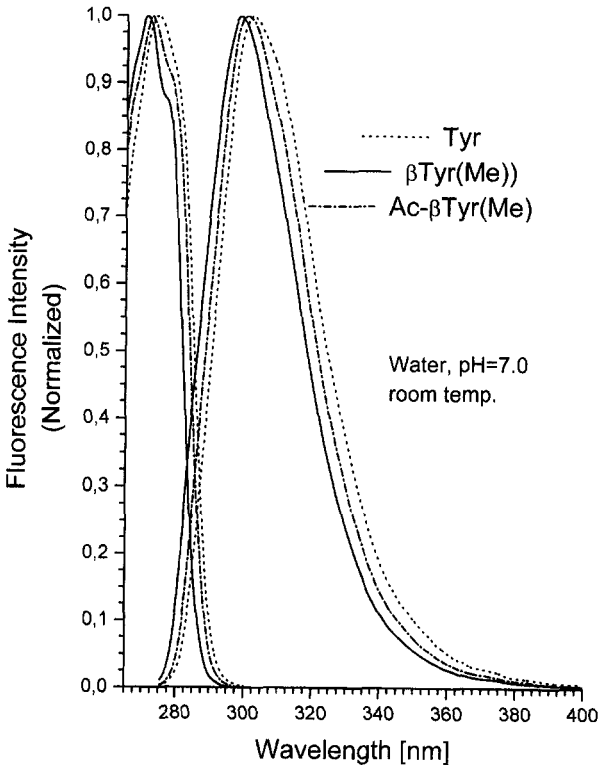

Fig. 2. Fluorescence and fluorescence excitation spectra of tyrosine and βTyr(Me) derivatives in water at pH 7.0 at room temperature.

methods[42] with the Conductor Like Screening Model approach to calculate electrostatic solvation, with the assuming dielectric constant $\varepsilon = 80$ for the solvent.

RESULTS AND DISCUSSION

Steady-State Fluorescence

The fluorescence spectra of derivatives of (*O*-methyl)-*β*-tyrosine are shown in Fig. 2. In our studies we used *O*-methylated derivatives of *β*Tyr just to exclude possible additional effects connected with an interaction of the free phenolic hydroxyl with the solvent.[4,43] For comparison the fluorescence spectrum of tyrosine measured under the same conditions is also presented. Generally, the fluorescence spectra of *β*Tyr(Me) derivatives are slightly shifted toward short wavelengths, but only the form of the N-terminal group (*N*-acetylamino or amino) is important for these shifts. The form of the carboxylic group (ionized, amidated) plays a minor role. The derivatives of *β*Tyr(Me) with a protonated amino group have emission spectra shifted more toward shorter wavelengths than the *N*-acetylated derivatives. On the other hand, the shape of the emission spectra of *β*Tyr(Me) derivatives is almost-identical to that of tyrosine.

The excitation spectra of *β*Tyr(Me) derivatives observed at wavelength $\lambda = 310$ nm and the excitation spectrum of tyrosine are also presented in Fig. 2. Excitation spectra of *β*Tyr(Me) derivatives are, like the above-mentioned emission spectra, shifted toward shorter wavelengths compared with tyrosine, and the shifts of the spectra depend on the character of the amino group. The highest shifts were observed for the derivatives with a free amino group. *N*-Acetylation resulted in a lower shift. The status of the carboxylic group (free carboxyl or amide) does not influence the position of the spectra. The same lack of influence was observed for the emission spectra. The *β*Tyr(Me) derivatives with a free amino group exhibited a more pronounced vibrational structure of the excitation spectra than tyrosine. On the other hand, the *N*-acetyl derivatives of *β*Tyr(Me), by means of the vibrational structure, are similar to tyrosine.

The direction of the changes in the emission and excitation spectra (shape, position of bands) for *β*Tyr(Me) derivatives and for (*p*-hydroxy)phenylglycine [Phg(OH)] derivatives[44] is the same compared with the spectra of tyrosine, although the carboxylate in *β*-tyrosine is farther from the phenolic chromophore than in Phg(OH), by one additional -CH_2- group. But in both [*β*Tyr and Phg(OH)] the amino groups are located in the same, very close position with respect to the phenolic ring [the amino and phenolic groups in Phg(OH) and *β*Tyr are connected to the same carbon]. The proximity and interaction of the amino and phenolic groups are observed as the changes of the emission and excitation spectra. The changes in the character of the amino group because of acylation (lack of charge and increased steric hindrance) also influence the shape of the spectra (shift of the maximum).

The quantitative characteristics of the studied *β*Tyr(Me) derivatives are presented in Table 1. The quantum yield for *β*Tyr(Me) is about 2.5 times larger than that for Tyr. This is in good agreement with the literature data on increased decay times and quantum yields for (*O*-methylated)tyrosine derivatives.[1,4,21,44]

The conversion of the carboxylic group into the *N*-methylamide practically does not change (within the measurement error) the fluorescence quantum yield. This is very interesting, especially in terms of the quantum yields obtained for tyrosine amide[1,2,4,20–23] and peptides

Table I. Quantum Yields and Fluorescence Lifetimes for β-Tyrosine Derivatives in Water, pH = 7.0, at Room Temperature

Compound	Quantum yield	Lifetime (ns)		Preexponential factors		χ^2_R	
		τ_1	τ_2	α_1	α_2	1 exp	2 exp
H–Tyr–OH	0.14	3.35	—	1.000	—	0.98	—
HCl·βTyr(Me)–OH	0.34	5.03	—	1.000	—	1.11	—
Ac–βTyr(Me)–OH	0.194	3.49	—	1.000	—	6.22	—
		1.38	3.94	0.432	0.568	—	0.93
Ac–βTyr(Me)–NHMe	0.268	3.93	—	1.000	—	1.09	—
HCl·βTyr(Me)–NHMe	0.33	4.88	—	1.000	—	1.05	—

with tyrosine at the N-terminus.[4,20,21,45] In these derivatives the amide group and an amino acid residue coupled to the carboxylate of tyrosine significantly decreased the fluorescence and changed the decay times.

The acylation of the N-terminal amino group has a quite different effect on the fluorescence spectra. The quantum yield for Ac–βTyr(Me)–OH is almost two times lower than that obtained for nonacylated βTyr(Me)–OH. The quantum yield for the other derivatives of Ac–βTyr(Me) also depends on the kind of the C-terminal group. Generally the low quantum yield obtained for acetylated β-tyrosine is even lower for the derivative with a free (ionized) carboxylic group, although the carboxylate anion was not thought to be a quencher of tyrosine fluorescence, because of its low electron-acceptor properties (due to its negative charge).[4,45]

Decay of Fluorescence Intensity

The decay times of all (*O*-methyl)β-tyrosine derivatives are presented in Table I. The fluorescence decay of the parent compound, H–βTyr(Me)–OH, is monoexponential, with a decay time of τ=5.03 ns. Almost the same decay time was observed for (*O*-methyl)tyrosine.[1,4] The weak interaction between the charged amino group and the phenolic chromophore observed in the emission and fluorescence excitation spectra did not change the decay times. The derivative of βTyr(Me) with only the carboxylate protected (*N*-methylamide) exhibited monoexponential fluorescence decay. The decay time for βTyr(Me)–NHMe, τ=4.88 ns, is only slightly shorter than that for the parent βTyr(Me). The lack of effect of amidation observed for β-tyrosine is opposite to the data obtained for Tyr and Tyr–NH_2. A literature survey of decay-time data for tyrosine amide (Tyr–NH_2) revealed that the intensity-averaged lifetime calculated from the equation

$$\langle\tau\rangle_I = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \tag{3}$$

is 1.75 ns[1] or 1.11 ns[3] (depending on the data used in the calculations), and it is almost three times shorter than for the parent tyrosine. Although the distance between the chromophore (phenolic group) and the carboxyamide in Tyr–NH_2 and in βTyr(Me)–NHMe is the same, the efficiency of the fluorescence quenching in the tyrosine derivative is higher. This phenomenon can be explained partially as a result of the difference in acceptor characteristics (electron affinity) of -CO-NH_2 and -CO-$NHCH_3$ groups. The donor properties of the substituents at the nitrogen atom of the carboxyamide group can influence the acceptor capacity of the carbonyl group due to a modification of the charge distribution on its oxygen and carbon. The donor properties of N-substituents for the discussed carboxyamides increase in the order H < -CH_3 < -$CH_2COO^{\ominus}$. The acceptor properties of the resulting amides, modified by different substituents, should influence the fluorescence quenching ability of these amides. The most efficient quencher of the fluorescence of the phenolic chromophore is the -CO-NH_2 group, followed by primary and secondary amides. Confirmation of these quenching properties of the amides came from the studies of average fluorescence lifetimes for the dipeptide Tyr–Gly and Tyr–NH_2 performed by Laws[1] and Gauduchon and Wahl.[3] They found that the average fluorescence lifetime of Tyr–Gly is longer by 20% than that of Tyr–NH_2. This particular influence of N-substituents (exhibiting different donor properties) on the acceptor properties of the carboxyamide carbonyl proved that the quenching of the fluorescence of the phe-

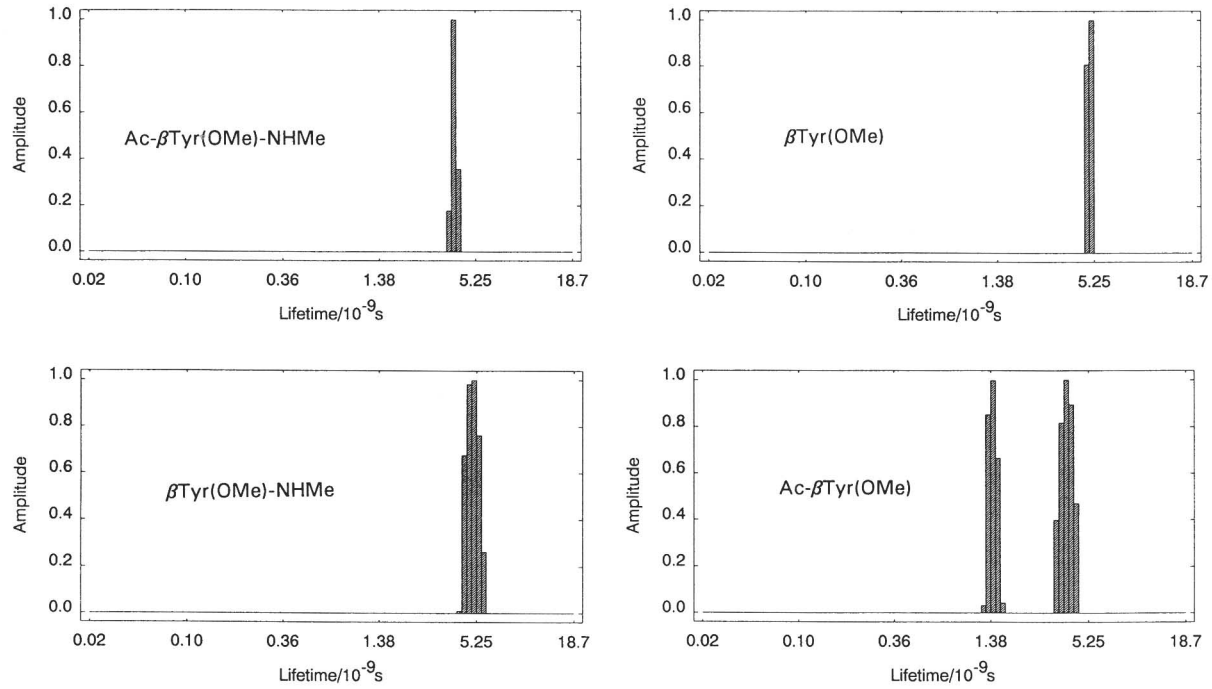

Fig. 3. Lifetime distributions of βTyr(Me) derivatives in water at pH 7.0 at room temperature.

nolic chromophore is governed by a charge-transfer mechanism.

The quenching efficiency of the ammonium group in tyrosine has been discussed in terms of the electrostatic effect on the quenching efficiency of the carbonyl group and not attributed directly to a direct quenching process.[3,23,45] According to Cowgill,[45] the intramolecular quenching process of tyrosine fluorescence requires a hydrated carbonyl group and has strict spatial requirements. The presence of the charged amino group ($NH_3^{\oplus}$) close to carboxyamide should alter the dipole moment of the CO group by a field effect and cause enhanced hydration, resulting in facilitating the fluorescence quenching of the phenol fluorescence of a tyrosine derivative by a charge-transfer mechanism. An increased fluorescence decay time caused by the *N*-acetylation of the amino group was observed for Tyr–NH_2.[1,3] According to Laws,[1] the fluorescence decay time for Ac–Tyr–NH_2 is $\langle\tau\rangle_I$ = 2.26 ns, and according to Gauduchon and Wahl,[3] it is $\langle\tau\rangle I$ = 1.61 ns, which is longer than the average decay time observed for Tyr–NH_2, 1.75 ns[1] and 1.11 ns.[3] The increased fluorescence decay time due to the incorporated protection of the amino group (acetylation) has proved an influence of the charged $NH_3^{\oplus}$ group on the quenching abilities of the carboxyamide. Such a mechanism of tyrosine fluorescence quenching by amides and the involvement of the $NH_3^{\oplus}$ group in this process are also supported by our result of the small fluorescence quenching by the amide group in βTyr(Me)–NHMe. Separation of the $NH_3^{\oplus}$ group from the amide quenching moiety by the additional carbon, as observed in βTyr derivatives, significantly diminished its quenching capacity. The small distance between the phenolic chromophore and the $NH_3^{\oplus}$ group in βTyr derivatives and the independence of the fluorescence decay time from this distance support the fact that the ammonium group is not involved directly to a quenching process.

The lack of multiexponential fluorescence decay observed for βTyr(Me)–NHMe, despite the same possibility of rotamer formation as for Tyr–NH_2,[1,2] could be the result of a too small difference in decay times observed for individual rotamers—due to a small propensity toward quenching. Using our equipment we observed biexponential decay for Tyr–NH_2, with lifetimes τ_1 = 0.45 ns and τ_2 = 1.15 ns as in work by Gauduchon and Wahl (τ_1 = 0.4 ns and τ_2 = 1.2 ns),[3] despite the possibility of the existence of three rotamers. The relatively wide distribution of decay times for βTyr(Me)–NHMe in comparison with βTyr(Me) (Fig. 3) proved the previous statement about the small differences in decay times related to individual rotamers. A similar effect is observed for Ac–Tyr: the acetyl group slightly quenching the fluorescence of the phenolic chromophore di-

minished the decay times but not significantly enough to be detected. Despite the same possibility for Ac–Tyr and Tyr–NH_2 of the existence of three rotamers in ground state in a solution, stable during the lifetime of the excited state of the chromophore, simple monoexponential fluorescence decay was observed for Ac–Tyr, even using state-of-the-art equipment.[1]

For Ac–*β*Tyr(Me)–NHMe, as for *β*Tyr(Me)–NHMe, the fluorescence decay also is monoexponential, but the decay time is shorter by about 20%. Comparing the decay times of *β*Tyr(Me) derivatives with and without an *N*-acetyl group, one can conclude that the quenching effect of the carboxyamide moiety is negligible and the 20% decrease in the decay time is due only to the presence of the *N*-acetyl group. The higher efficiency of the fluorescence quenching of the derivatives of β-tyrosine by the acetyl group than that of tyrosine could be explained as a result of the shorter distance between the chromophore (phenol ring) and the quenching group (acetyl) in β-tyrosine (the amino group in tyrosine is farther from the chromophore by one $-CH_2-$ moiety).

Moreover, assuming that the quenching of the fluorescence of tyrosine and analogues, and their derivatives, caused by the *N*-acetyl group or by the carboxyamide occurs according to a charge-transfer mechanism,[4,24,45] the *N*-acetyl group in βTyr derivatives, being closer to the phenolic chromophore than the carboxyamide group (by one $-CH_2-$ moiety), should display a more pronounced, higher quenching effect. Because there is only one space orientation of the acetyl and the chromophore, the fluorescence decay is monoexponential and the decay time distribution is narrow (Fig. 3), despite the fact that Ac–βTyr(Me)–NHMe exists in a solution as a mixture of conformers (see Conformational Analysis, below).

The shortest, average fluorescence decay time was observed for Ac–βTyr(Me), the derivative with an ionized carboxylate. The monoexponential decay function did not fit well to the experimental data obtained for this derivative, giving a high value of χ^2_R (χ^2_R = 6.22; Table 1). The measured fluorescence decay can be, on the other hand, relatively well described by a superposition of two exponents (χ^2_R = 0.93) with the decay times τ_1 = 3.94 ns and τ_2 = 1.38 ns and normalized to one preexponential factor equal, respectively, to α_1 = 0.568 and α_2 = 0.432. The longer decay time observed for Ac–βTyr(Me) (τ_1 = 3.94 ns) is the same as the one for the disubstituted derivative, Ac-βTyr(Me)–NHMe. Thorough analysis of the fluorescence decay time distribution obtained for Ac–βTyr(Me) revealed two well-separated and narrow distribution functions, with average decay times τ_1 = 3.9 ± 0.32 ns (amplitude, 58%) and τ_2 = 1.42 ± 0.08 ns (amplitude, 42%) and with χ^2_R almost-ideal (χ^2_R = 0.99) (Fig. 3). These data, obtained for Ac–βTyr(Me), are essentially different from the data for Ac–Tyr, for which monoexponential fluorescence decay was observed. The decay times obtained by Laws *et al.*[1] for Ac–Tyr and Tyr are 3.6 and 3.76 ns, respectively, whereas Gauduchon and Wahl[3] obtained shorter decay times, 3.2 ns for Ac–Tyr and 3.38 ns for Tyr, but they used different conditions for measurements.

Steady-state and time-resolved data were combined to generate decay-associated spectra (DAS), which represent the relative contribution of individual lifetime components, as a function of wavelength, to the total fluorescence. The information from DAS can be used to assign the lifetime components to individual emitting species. The decay-associated spectra obtained for Ac–βTyr(Me) (not shown), for shorter and longer decay time components, are the same; they are just the emission of the phenolic chromophore quenched at different levels by the *N*-acetyl group. Thus, it cannot be concluded, on the basis of DAS, that two emitting species exist in different surroundings.

Two well-separated fluorescence decay times, on the other hand, testify to the existence of two stable [during the lifetime of the excited state of Ac–βTyr(Me)] species different in the efficiency of their fluorescence quenching of the phenolic chromophore by the acetyl group. As mentioned previously, since there is only one space orientation of the phenol ring and the acetyl group, the heterogeneity of fluorescence decay is due to a different interaction of the carboxylic group (nonquencher) with the quencher (acetyl). Theoretical calculation revealed that there is a possibility of six-membered ring formation in Ac–βTyr(Me) as a result of the presence of a hydrogen bond between the hydrogen from the acetylated NH (from the α-amino group) and an oxygen from carboxylate. A similar, stable hydrogen bond was detected previously by ^1H NMR[47] in peptides containing an aspartic acid residue (the interaction of the ionized aspartyl β-carboxylate with amide protons). Therefore a longer fluorescence decay time for βTyr(Me), equal to the decay time for Ac–βTyr(Me)–NHMe, is associated with the conformer in which the ionized carboxylate is relatively far from the acetyl group (an extended conformation), whereas a shorter fluorescence decay time is associated with the conformer with a hydrogen bond. The strong fluorescence quenching observed for the second conformer is the result of a modification of the acceptor properties of the acetyl group (hydrogen bond formed between the carboxylate and the acetylated NH), but it is difficult to explain whether the charge field of the carboxylate or a change

Table II. Energies (kcal/mol) of Representative Conformations of Ac–βTyr(Me)–$COO^{\ominus}$ and Ac–βTyr(Me)–CONHMe Calculated Using the AMBER Force Field and AM1 Semiempirical Methods, Respectively, Without (AMBER and AM1) and With (AMBER + IBEM and AM1 COSMO) Solvation in the Continuum-Dielectric Models

Comformation	AMBER	AMBER + IBEM	AM1	AM1 COSMO
		Ac–βTyrCOO$^{\ominus}$		
E	−18.40	−350.69	−177.96	−282.04
H6	−22.86	−350.36	−186.58	−280.80
		Ac–βTyr–CONHMe		
E	−9.75	−230.38	−102.36	−132.31
H6	−8.93	−227.94	−104.46	−131.53
H8	−10.73	−229.93	−104.21	−131.74

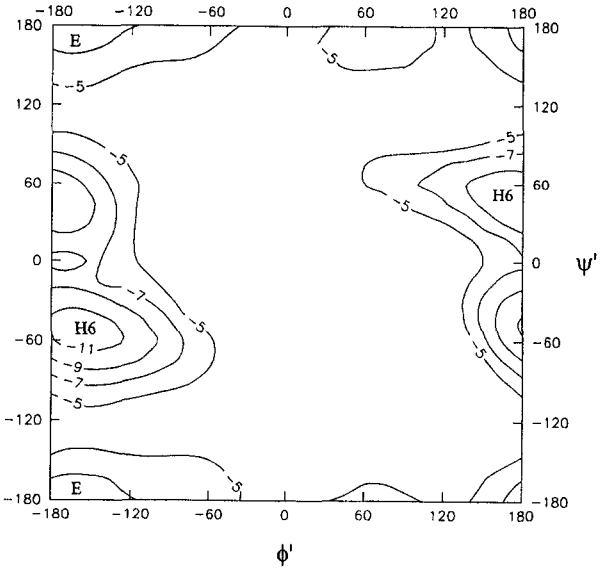

Fig. 4. AMBER energy-countour map of Ac–βTyr with the ionized carboxylate. The regions of the E and H6 conformations are labelled.

in a hydration level of the acetyl due to the ionized carboxyl in the neighborhood, or a favorable and stable space orientation of the chromophore (phenol group) and quencher (acetyl group) because of the intramolecular hydrogen bond, is responsible for that process. The weak tyrosine fluorescence quenching by the acetyl in Ac–Tyr, a compound containing an ionized carboxylate, suggests that the charge field of $COO^{\ominus}$ does not change the efficiency of the quenching abilities of acetyl. On the other hand, in the β-tyrosine derivative [Ac–βTyr(Me)] the acetyl is definitely closer to the chromophore, therefore the effect of the charge field of $COO^{\ominus}$ can be more significant. It is possible that all of the reasons mentioned above could play an important role in the quenching process. The presence of two major, low-energy conformers in Ac–βTyr(Me) (one with an extended main chain and the second with an intramolecular hydrogen bond between $COO^{\ominus}$ and acetylated NH) was also supported by theoretical calculations.

Conformational Analysis

The energies of the selected conformations of Ac–βTyr with an ionized carboxylate and Ac–βTyr–CONHMe are summarized in Table II. For Ac–βTyr, there are effectively two types of conformation: with an extended backbone (E) and with a six-member hydrogen-bonded ring in the backbone (H6). The H6-type conformations are favored *in vacuo* for both the AMBER force field and the AM1 semiempirical method. However, after including solvation, the E-type conformations became equally or even more favored. These two types of conformations can easily be identified as broad regions of low energy in the energy-contour map (Fig. 4). For Ac–βTyr–CONHMe, in addition to the E and H6 families, an H8 family can be distinguished which comprises conformations with an Ac(CO)...HN(NMe) hydrogen bond. This conformation is the lowest in energy *in vacuo* and the extended conformation is favored after including solvation. It can also be noted that because of the increased number of variable dihedral angles, the conformational space of Ac–βTyr–NHMe is more diffuse than in the case of Ac–βTyr. Because energy depends significantly on three backbone torsional angles, in contrast to Ac–βTyr, the conformational space of Ac–βTyr–NHMe cannot be visualized as a two-dimensional energy-contour map.

The results of theoretical calculations obtained agree well with the fluorescence measurements. Two conformers found for Ac–βTyr(Me) (H6 and E), with the E conformer slightly more favored, are responsible for the heterogeneity of the fluorescence decay. The presence of a conformer of the H6 type existing at a lower concentration in a solution (higher energy) explains the short fluorescence decay time (42% of the whole fluorescence decay), whereas a conformer of the E type ought to be associated with a longer decay time (52% of whole fluorescence decay). The presence of three families of conformers for Ac–βTyr(Me)–NHMe, with a small difference in energy, existing in equilibrium, causes averaging of the fluorescence decay times and therefore the small quenching effect of the *N*-methylamide is practically not observable.

CONCLUSIONS

During our fluorescence measurements of βTyr(Me) derivatives, we did not observe any reaction in the excited state. HPLC profiles of the studied compounds before and after measurements showed the same pattern—no new compound was observed after excitation. In our studies we used the *O*-methylated derivatives of βTyr to prevent a possible reaction in excited states such as proton transfer and to exclude an interaction of the phenolic hydroxyl with the solvent.

In the rotamer model, three chemically distinct environments exist for the phenol ring about the C^{α}–C^{β} bond and each rotamer has its own associated decay constant, and the relative weighting of the amplitudes is set by the ground-state rotamer populations. Rotamers with different lifetimes have also been suggested as the cause of multiexponential decay of tryptophan systems.[11–16,46] The data described on the fluorescence decay measurements for βTyr(Me) derivatives have only partially confirmed the rotamer model of quenching. Theoretical calculations discovered that there is only one space orientation of the chromophore and the acetyl group for Ac–βTyr(Me)–NHMe [molecular modeling revealed the lack of a rotamer with a stable hydrogen bond as for Ac–βTyr(Me) and steric hindrance of the NHMe group allows only a rotamer with an extended conformation to exist] and therefore the fluorescence decay of this compound is monoexponential. The results of the fluorescence decays obtained for other βTyr(Me) derivatives are inconsistent with the rotamer model, but they provide new suggestions regarding the quenching mechanism of the fluorescence of tyrosine analogues. The fluorescence quenching by N-terminal or C-terminal protecting groups depends on the distance between the chromophore (phenolic ring) and a quenching moiety (acetyl, *N*-methylamide) and their direct surroundings. Thorough analysis of the dependence of the effectiveness of the quenching groups on the distance between the chromophore and the quencher led us to the conclusion that the previously suggested charge-transfer mechanism[3,24] is very probable [the weak quenching of tyrosine fluorescence by the acetyl in Ac–Tyr and the strong quenching by the acetyl in the case of Ac–βTyr(Me)]. We observed similar results (shorter decay time, higher quenching) in the case of Ac–Phg(OH) derivatives—in both βTyr and Phg(OH), the amino group is closer to the chromophore than in Tyr.[44] The results obtained in our investigations partially explained the influence of the $NH_3^{\oplus}$ group on the quenching of tyrosine fluorescence. This group is not directly involved in the quenching process, which is consistent with the previous suggestions,[3,45] but it influences the quenching ability of the amide group through its close space location. The separation of the $NH_3^{\oplus}$ group from the amide by the additional $-CH_2-$ moiety reduces considerably the quenching abilities of the amide. Unfortunately, on the basis of these data, no satisfactory explanation for the mechanism of the interaction between the $NH_3^{\oplus}$ group and the amide bond could be found. The effect of the charge field of the $NH_3^{\oplus}$ group and related to this better hydration of the amide bond suggested by Cowgill[45] seems to be a reasonably good explanation for the mechanism of the $NH_3^{\oplus}$ group action. On the other hand, the biexponential decay of fluorescence of Ac–βTyr(Me) with the ionized carboxylate, caused by the intramolecular hydrogen bond, makes the interpretation of the fluorescence decays of tyrosine and analogues incorporated within a peptide chain rather difficult. Therefore, during the fluorescence studies of peptides or proteins containing tyrosine or analogues, the possibility of the formation of relatively stable hydrogen bonds able to change the quenching properties of acylating groups has to be concerned. Full explanation of the fluorescence decay mechanism for tyrosine analogues requires further studies such as heterocorrelated NMR measurements to establish the ratio of rotamers and studies of model compounds with different conformational freedom of the chromophore and protecting groups at the N and C termini.

ACKNOWLEDGMENT

This work was supported by the Polish State Committee for Scientific Research (KBN) under Grant PB 0582/P3/03/04

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