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Photophysics of phenylalanine analogues Part 2. Linear analogues of phenylalanine

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Abstract

The photophysical properties (fluorescence quantum yields and lifetimes) of linear analogues of phenylalanine (Phe) [β-homophenylalanine (β-Hph), β-phenylalanine (β-Phe), phenyglycine (Phg)] in water at pH = 6 and 1 have been measured. The obtained results indicate that relative space location of the amino, carboxyl groups and phenyl chromophore influence on photophysical properties at both pH. In acidic solution (pH = 1) lower fluorescence quantum yields and fluorescence lifetimes than observed at pH = 6 for all studied compounds suggest that the protonated carboxylic group efficiently quenches phenyl fluorescence. It was found that the proximity of the protonated amino group and the phenyl ring modify the photophysical properties of phenylalanine analogues β-Phe and Phg.

Keywords: Phenylalanine analogues; Fluorescence; Time-resolved fluorescence; Fluorescence quenching

1. Introduction

The fluorescence and ultraviolet absorption of peptides and proteins is mostly due to the presence of three aromatic amino acids: tryptophan, tyrosine and phenylalanine (Phe) [1,2]. Therefore, their photophysical properties have been studied extensively. Most of the effort, however, have been focussed on tryptophan and tyrosine because of low absorption and fluorescence quantum yield of Phe and because of the fact that many native proteins contain large numbers of Phe residues which make usage of Phe fluorescence in conformational studies rather difficult. The photophysical properties of the aromatic amino acids depend on the status of the amino and carboxyl groups (neutral, protonated, acylated, amidated) although they are separated form chromophore by two methylene groups [3–5]. In the case of tyrosine or tryptophan containing compounds multi-exponential kinetics of the fluorescence decay were observed [3–6]. Complex fluorescence decays were usually observed for tryptophan or tyrosine incorporated into proteins [3,4,7–24], peptides [25–32], and the free amino acids, and their derivatives or analogues [5,33–55]. An explanation of this behavior has been offered by Rayner and Szabo [34,35] based on the Gauduchon and Whal rotamer model [55]. This model assumes the existence of well-defined rotamers about the C^{α}–C^{β} bond, whose interconversion times are considerably longer than the excited-state lifetimes. The different lifetimes of the rotamers arise from quenching interactions between the fluorophore and quenching groups. The quenching efficiency is controlled by the orientation of the quenching groups and the process is distance dependent. The rotamer model of fluorescence decays of aromatic amino acids has been supported by the latest investigations [23,24,26,36] that included also studies of constrained tryptophan [43–45,56–58], tryptophan in protein crystals [12,13,59] and tryptophan in peptides with defined secondary structure [29,30].

In this work we present photophysical properties of linear analogues of Phe [β-homophenylalanine (β-Hph), β-phenylalanine (β-Phe), phenyglycine (Phg)] in solution at pH = 6 and 1 in order to explain influence of status of quenching groups (amino and carboxyl group) and influence of distances between the quenching groups and phenyl ring on quenching efficiency of the phenyl fluorescence by protonated carboxyl group.

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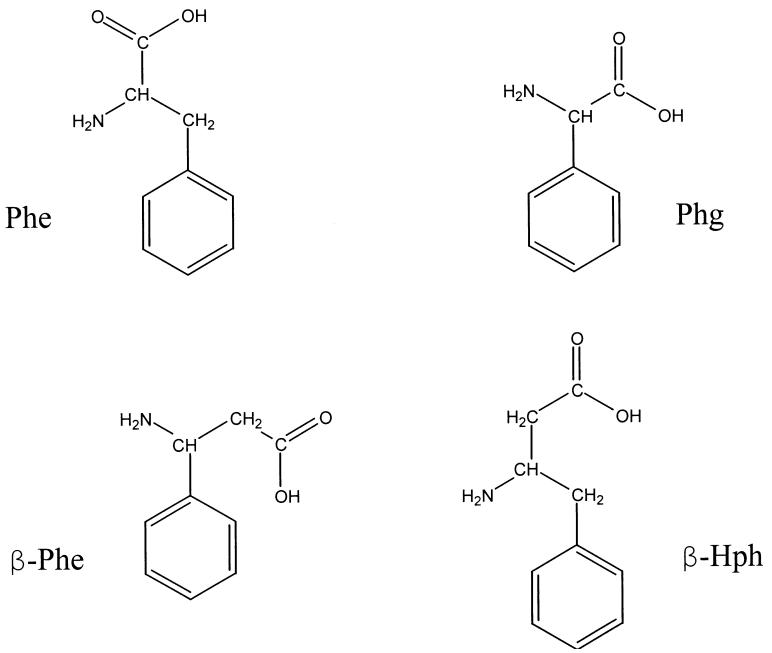

Fig. 1. Structure of phenylalanine and its linear analogues.

2. Experimental

2.1. Materials

Phe and Phg were purchased from Fluka, were p.a. quality and were used without further purification. β-Hph was prepared as described previously [51] whereas β-Phe was obtained according to the literature [52] and were purified by semi-preparative RP-HPLC. Structures of studied compounds are presented in Fig. 1. The pH adjustments were performed by adding HCl or NaOH solution (Merck, Suprapur).

2.2. Spectroscopic measurements

Photophysical parameters were determined as it was described in our previous similar studies of constrained analogues of Phe modified at phenyl ring [60]. The absorption spectra were measured using a Perkin-Elmer Lambda 18 spectrophotometer. The steady-state fluorescence spectra were recorded using a Perkin-Elmer LS-50B spectrofluorimeter with 4 nm band-width for excitation and emission. The excitation wavelength was 258 nm. The optical density of the sample at the excitation wavelength did not exceed 0.1. The fluorescence quantum yields of Phe analogues were calculated by comparing the integral intensity of the steady-state emission spectra excited at 258 nm (corrected for refractive index of solvent and absorbance) with that of tyrosine in water, using a value of 0.14 [61] for the later. Fluorescence decays were collected by the time-correlated single photon counting technique on an Edinburgh Analytical Instruments type CD-900 fluorometer interfaced with an IBM PC. 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 1.1 ns. The excitation (258 nm) and emission (280 nm) wavelengths were selected by means of monochromators (about 10 nm bandwidth). Fluorescence decays from sample and reference (Ludox, observation wavelength 280 nm) were measured to 10^4 counts at peak. The counting rate did not excced 2% of the repetition rate. The decay curves were stored in 1024 channels of the 0.054 ns per channel. Fluorescence decay data were fitted by the iterative convolution to the sum of exponents:

$$I(t) = \sum_i \alpha_i \exp\left(\frac{-t}{\tau_i}\right) \quad (1)$$

where τ_i is the decay time of the i-th components and α_i the pre-exponential factor. The adequacy of the exponential decay fitting was judged by visual inspection of the plots of weighted residuals, by the statistical parameter X_R^2, by shape of the autocorrelation function of the weighted residuals, and also by serial variance ratio (SVR). In steady-state measurements, the sample concentration was about 5×10^{-4}, whereas 1×10^{-3} M in time-resolved experiments. All measurements were performed in double deionized water (Millipore) at pH = 6 or 1, at room temperature.

3. Results and discussion

Absorption spectra of the linear analogues of Phe are presented in Fig. 2 (the absorption spectrum of Phe is added for comparison). Absorption spectra of Phe and its analogues studied in water solution at pH = 1 and 6 are characteristic

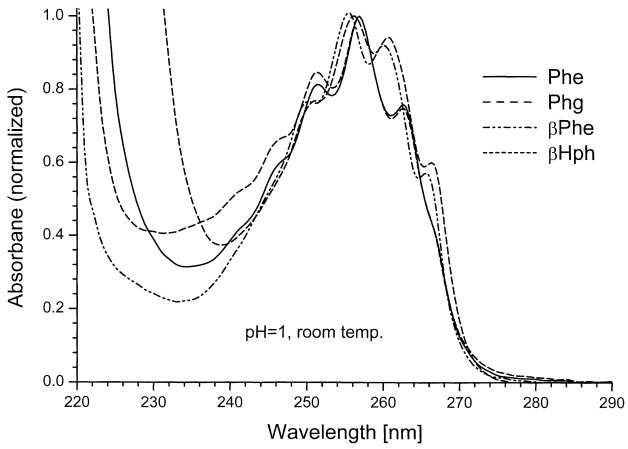

Fig. 2. Normalized absorption spectra of Phe, Phg, β-Phe and β-Hph.

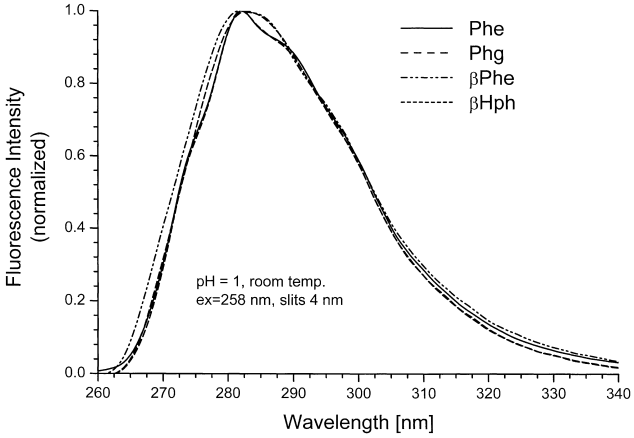

Fig. 3. Normalized emission spectra of Phe, Phg, β-Phe and β-Hph.

for well defined vibrational structure and they are consisted with literature data for Phe [62,63,66,70]. The absorption spectrum of β-Hph is nearly the same as the absorption spectrum of phenylalanine except the short-wavelength part (Fig. 2) The absorption spectra of β-Phe and Phg because of proximity of the phenyl ring and protonated amino group (both groups are attached to the same carbon atom) are different than the spectrum of Phe and β-Hph. The small blue shift of absorption maximum and modification of the relative intensity of vibrational bands were observed. Such changes in absorption spectra of β-Phe and Phg indicate that positively charged (protonated) amino group interacts with phenyl ring because of its electron-withdrawing character.

Fluorescence spectra of studied compounds in water solution at pH = 1 are shown in Fig. 3. Emission spectra of β-Hph and Phg differ from that of Phe and β-Phe, similarly like in the case of the absorption spectra. The shape and bands positions of the fluorescence spectra of these analogues are similar to the fluorescence spectrum of Phe but vibrational structure is less pronounced. The maximum of fluorescence intensity for all compounds studied is located at about 282 nm regardless of pH. Fluorescence quantum yields measured at pH = 1 and 6 are collected in Table 1.

Fluorescence quantum yields for all studied compounds are higher at pH = 6 than at pH = 1 indicating that protonated carboxylic group quenches fluorescence of Phe analogues. The fluorescence quantum yields for Phe and β-Hph in water at pH = 6 are the same (0.02), whereas for β-Phe and Phg are lower (0.014 and 0.018, respectively). Decrease of the fluorescence quantum yields of β-Phe and Phg in comparison to Phe can be explained by interaction of the protonated amino group with the phenyl ring. These phenomena were observed in absorption and fluorescence spectra of these compounds. The lowest fluorescence quantum yield in water solution at pH = 1 was observed for Phg in which the quenching group (COOH) and the fluorophore are in the closest proximity, higher were for Phe and β-Phe, whereas the highest was for β-Hph in which the distance between the quenching group and the fluorophore is the largest.

Fluorescence decays for all linear analogues of Phe at pH = 6, as well as at pH = 1, are mono-exponential. Moreover, shorter fluorescence lifetimes are obtained at pH = 1 rather than at pH = 6. As it was observed for derivatives of tyrosine and their analogues [5,53,64–69], Phe and in ring substituted Phe analogues [60,70,71] protonation of the carboxylic group caused changes of their photophysical properties. Protonation of carboxylic group of studied Phe

Table 1
Fluorescence quantum yields, fluorescence lifetimes and calculated radiative and non-radiative rate constants of β-Hph, β-Phe, Phg and Phe in water at pH = 1 and 6

Compound	pH	Quantum yield (QY)	τ (ns)	X_R^2	$k_f = QY/\tau^a$ ($\times 10^{-6}$ s^{-1})	$k_{nr} = (1-QY)/\tau^a$ ($\times 10^{-8}$ s^{-1})
Phe[a]	1	0.014	4.54	1.05	3.08	2.17
	6	0.020	7.28	1.15	2.81	1.38
β-Hph	1	0.018	4.60	0.95	3.91	2.13
	6	0.020	5.18	1.03	4.05	1.88
β-Phe	1	0.014	2.82	1.12	4.96	3.49
	6	0.016	3.30	1.14	4.92	2.98
Phg	1	0.009	2.47	1.07	3.85	4.01
	6	0.018	4.78	1.06	3.77	2.09

[a] Values from [60].

analogues also resulted in decrease of the fluorescence quantum yields and lifetimes (Table 1), without significant changes of the absorption and emission spectra. Decrease of the fluorescence quantum yields and the fluorescence lifetimes at acidic solution (pH = 1) testifies that the protonated carboxylic group works as an efficient quencher of fluorescence of the aromatic side chain of amino acid [5,45,53,60,64–71]. The fluorescence quenching proceeds according to the electron transfer mechanism from the excited phenyl chromophore (donor) to the protonated carboxylic group or amide group, as an electron (charge) acceptor, [5,25,33,55,68,72–74]. The quenching efficiency can be expressed as a ratio of fluorescence quantum yields or lifetimes measured at pH = 6 to that at pH = 1. Calculated, based on the fluorescence lifetimes, quenching efficiencies are 1.13, 1.17, 1.56 and 1.19 for β-Hph, β-Phe, Phe and Phg, respectively. The same values (with range of experimental error) were obtained from the fluorescence quantum yields. The consistence of the quenching efficiencies obtained from steady-state and time-resolved fluorescence measurements indicates that the fluorescence quenching of phenyl ring by protonated carboxylic group in studied Phe analogues is an intra-molecular process proceeding in the excited state without formation of a "dark complex" in the ground state. This interference is supported by the calculated fluorescence and non-radiative rate constants which are presented in Table 1. The fluorescence rate constant in solutions at pH = 6 and 1 for each of the Phe derivatives are the same, with range of experimental error, whereas these rate constants are not the same for all Phe analogues. It is known from the work of Fayet and Whal [75] that the amino acid side chain of tyrosine interacts with the excited phenol ring. Thus, the differences in fluorescence rate constant observed for Phe analogues can be caused by the interaction of amino acid side chain with the excited phenyl ring. The different distances between phenyl ring and the carboxyl and the amino groups as well as mutual orientation of the phenyl ring and amino-acid part modify the interactions of the fluorophore with other part of the molecule and, therefore, the photophysical properties of Phe analogues. The non-radiative rate constant increases with the decrease of pH of the solution (Table 1).

It is interesting to compare the fluorescence and non-radiative rate constants for Phe and its linear analogues as a function of the distance between carboxylic group to the phenyl ring and the position of the amino group in relation to the phenyl ring. It is well know that the protonated amino group, as well as the ionized carboxylic group, do not quench the fluorescence of tyrosine and Phe [5,51–53,55,60,65–71,76,77]. On the other hand, it is also known that the protonated amino group possessing electron-withdrawing character strongly enhances the quenching ability of the quenching groups (the protonated carboxylic group or amide group) which are close to the fluorophore [5,25]. Phe and all its analogues studied in water solution at pH = 6 are zwitterions, thus, they do not possess a protonated carboxylic group. The differences in the quantum yields and especially in the fluorescence lifetimes between the particular analogues of Phe arise from different distance between of the carboxyl group to the excited phenyl ring and arrangement of protonated amino group. For β-Phe, in which the distance between fluorophore and carboxylic group is the same as for Phe, the fluorescence lifetime should be equal to the fluorescence lifetime of Phe but it is shorter, because of proximity of the amino group and phenyl ring. The quenching efficiency of the protonated carboxylic group for the linear analogues of Phe in acidic solution depends on the distance between the phenyl ring and carboxylic group and is modify by mutual position of amino and carboxylic groups. Greater quenching efficiency observed for Phg than for Phe, as well as for β-Phe than for β-Hph confirms that the quenching process is distance dependent [5,33,55]. These results confirm also the Werner's and Forster's statement [25] that the protonated amino group enhances the quenching ability of the protonated or amidated carboxylic group.

Conformational studies of rotational isomerism of Phe and its derivatives by circular dichroism (CD) and nuclear magnetic resonance (NMR) have discovered possibilities of the presence of a stable rotamers in ground state [62,63,78–82] which population does not depend on the pH [79]. The presence of the stable rotamers should also be revealed by fluorescence spectroscopy (according to the rotamer model [5,31,34,35,53,55]) but because of either similar decay times of different rotamers or low energy of rotamers interconversion, the fluorescence decays of Phe and its analogues are monoexponential. Fast rotamers interconversion during the excited state lifetime was confirmed by molecular mechanic calculations for tyrosine [83], tryptophan [39,84], homotryptophan [85] and Phe in protein [86]. Because the results of the molecular mechanics calculation depend on the number of critical assumptions and moreover, there is substantial evidence for assigning the multiexponential decays of the fluorescence of tyrosine and tryptophan to rotamers [12,13,23,24,26,29,30,36,43–45,56–59], the most likely explanation of single exponential behavior of Phe and its analogues is that the decay times of each of the rotamers are comparable.

4. Conclusions

In our work we measured the fluorescence quantum yields and lifetimes of the linear analogues of Phe in water solution at different pH. The relative position of the protonated amino group, carboxylic group and phenyl fluorophore modifies the photophysical properties of the studied compounds. The protonated carboxylic group quenches the fluorescence of the phenyl fluorophore and the quenching efficiency depends on the distance between the quenching group and phenyl ring and the relative position of the amino and carboxylic groups. Mono-exponential fluorescence decays of all Phe analogues

studied in solution at pH = 6 and 1 can be explained based on the rotamer theory, making an assumption that the decay times of each of the rotamers are equal.

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