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Influence of the separation of the charged groups and aromatic ring on interaction of tyrosine and phenylalanine analogues and derivatives with β-cyclodextrin

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Abstract

Interactions of tyrosine and phenylalanine analogues with β-cyclodextrin have been examined in terms of structural features of the ligand such as the separation of the charged amino group and aromatic ring, the presence of additional functional group attached to the amino or phenyl ring, and the presence of a charge on amino or carboxyl group, and steric effects using steady-state and time-resolved fluorescence spectroscopy and microcalorimetry. The studied aromatic amino acids possess low binding constant to β-cyclodextrin, diversified with respect to the presence or absence of a substituent in para position of the phenyl ring. However, calculated, based on the global analysis of the fluorescence intensity decays, binding constants do not allow to estimate unequivocally the influence of the distance between the charged groups and phenol/phenyl ring on the inclusion complex stability because of their low diversification.

Keywords: Tyrosine analogues; Phenylalanine analogues; Cyclodextrin; Inclusion complexes; Fluorescence; Time-resolved fluorescence; Global analysis

1. Introduction

Naturally occurring α-, β-, γ-cyclodextrins are molecules shaped like a truncated cone. They possess a tapered cavity of about 8 Å depth [1] which enables them to act as a host for several classes of compounds which have been subjects of systematic studies [2], applying UV–Vis, circular dichroism, steady-state fluorescence, FT-IR spectroscopy [3–12], time-resolved fluorescence [13], NMR [14–18], molecular mechanics and molecular dynamics [19–22] as well as thermodynamic methods [2,23–27]. Aromatic amino acids [13,28–38] and peptides [39–42] are one group of compounds which interactions with cyclodextrins have been intensively studied because of their feature similar to enzyme–substrate complexes. Complexation reactions involving cyclodextrins are also highly important to drug delivery system technology [43]. Naturally occurring aromatic amino acids, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), possess fluorophore so the changes of their photophysical properties, such as fluorescence intensity or fluorescence lifetime, upon addition of the cyclodextrin solution can be utilized to determine stoichiometry and equilibrium constant of the inclusion complexes (amino acid–cyclodextrin) [13]. In this paper we have examined the interactions of phenylalanine and tyrosine derivatives with β-cyclodextrin in terms of structural features of the ligand using steady-state and time-resolved fluorescence spectroscopy and microcalorimetry. The structures of amino acids and their analogues studied in this paper are presented in Fig. 1. All studied compounds possess mono-exponential fluorescence intensity decays (fluorescence lifetimes collected in Table 1). However, the newest measurements and global analysis of the fluorescence intensity decays measured at 10 different wavelengths reveal for AcTyr two fluorescence lifetimes

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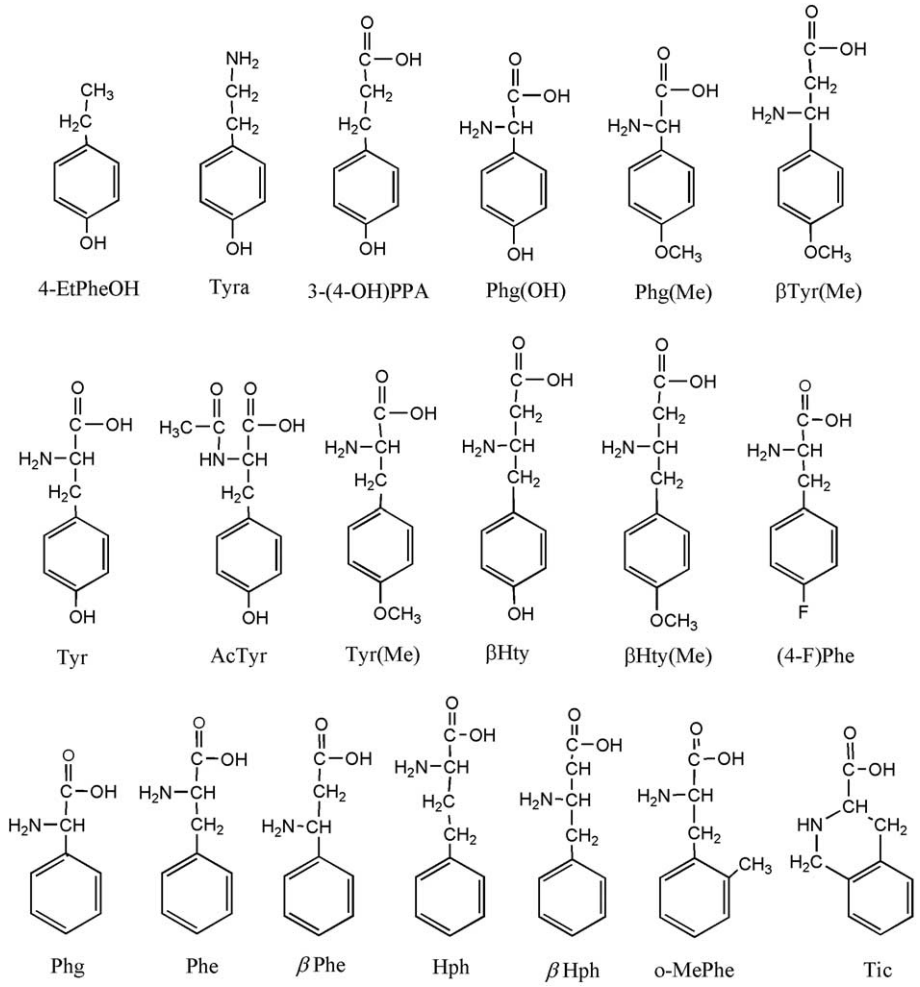

Fig. 1. Structures of tyrosine and phenylalanine derivatives and analogues studied.

(τ_1=3.27 ns with the contribution α_1=0.881 and τ_2=0.60 ns with the contribution α_2=0.119 [44]). The mono-exponential fluorescence intensity decay of guest molecule allows to apply time-resolved fluorescence spectroscopy and global analysis to obtain the fluorescence lifetimes of the free and encapsulated fluorophore as well as to calculate their relative contributions proportional to the pre-exponential factors and accordingly binding constant, as it has been done for tyrosine [13]. Time-resolved fluorescence spectroscopy and global analysis have this advantage that for diluted solution the fluorescence lifetime measured does not depend on the concentration of fluorophore, so small deviation in its concentration in prepared solution of cyclodextrin does not introduce additional error into equilibrium constant calculation as it is in the steady-state method.

2. Materials and methods

β-CD was purchased from Roth, whereas tyrosine (Tyr), tyramine hydrochloride (Tyra), *N*-acetyltyrosine (AcTyr), *O*-methyl tyrosine (Tyr(Me)), β-homotyrosine (βHty), 4-fluorophenylalanine ((4-F)Phe) were from Fluka, homo-phenylalanine (Hph) from Bachem, phenylalanine (Phe) from Roanal, 4-ethyl-phenol (4-EtPhOH) from Loba Chemie, phenylglycine (Phg) and (4-hydroxy)phenylglycine (Phg(OH)) from Aldrich. All these compounds were used as received after checking their purity using RP-HPLC. *O*-methyl-β-tyrosine (βTyr(Me)) was obtained as described in [49], *O*-methyl-β-homotyrosine (βHty(Me)) was obtained as described in [50], 4-methoxyphenylglycine was obtained as described in [51], 2′-methylphenylalanine (*o*-MePhe) and tetrahydroisoquinoline-3-carboxylic acid (Tic) were obtained as described in [52], whereas β-phenylalanine (βPhe) and β-homo-phenylalanine (βHph) were obtained as described in [53].

The solutions were prepared by dissolving the appropriate amount of β-CD in water. To the aqueous solution of β-CD, 100 μl of stock solution of amino acid was added. The pH of solutions of all studied amino acids and their derivatives was in the range of 5.6–6.0, except for 3-(4-OH)PPA which was measured at pH=8 and 2. Because of better solubility of Hph was measured at pH=2, its fluorescence was measured at this value.

Table 1
The fluorescence lifetime of studied compounds in water at different pH

Compound	τ [ns]	pH	Reference
Tyr	3.25	5.6	[13]
	3.37	5.5	[44]
	3.76	6.14	[45]
	3.38	5.2	[46]
	3.34	7.0	[47]
	3.27	6.0	[54]
	0.90	1.0	[47]
Tyr(Me)	4.82	5.5	[44]
	5.06	6.14	[45]
Tyra	3.16	6.0	[48]
	3.2	4.4–6.3	[46]
	3.30	7.0	[47]
βTyr(Me)	5.03	7.0	[49]
βHty	3.36	7.0	[50]
βHty(Me)	4.77	7.0	[50]
Phg(OH)	2.89	7.0	[51]
Phg(Me)	4.07	7.0	[51]
(*o*-Me)Phe	6.17	7.0	[52]
3-(4-OH)PPA	3.46	6.27	[45]
	0.60	2.58	[45]
Phe	7.12	7.0	[52]
	4.54	1.0	[52]
(4-F)Phe	7.24	7.0	[52]
	4.87	1.0	[52]
Tic	20.04	7.0	[52]
	16.75	1.0	[52]
βPhe	3.30	6.0	[53]
	2.82	1.0	[53]
βHph	5.18	6.0	[53]
	4.60	1.0	[53]
Phg	4.78	6.0	[53]
	2.47	1.0	[53]
AcTyr	3.60	5.24	[45]
	3.2	6.88	[46]
	3.32	7.0	[47]

In the fluorescence studies the optical density of the sample at the excitation wavelength (λ=275 nm for tyrosine derivatives and its analogues and 250 nm for phenylalanine and its analogues) did not exceed 0.1.

3. Spectroscopic measurements

Absorption spectra of all studied compounds in water were recorded using a Perkin-Elmer Lambda 40P spectrophotometer, whereas fluorescence spectra were recorded using a Perkin-Elmer LS-50B spectrofluorimeter with 3.5-nm bandwidth for excitation and emission. The steady-state emission spectra were measured at 20 °C. Additionally, for 3-(4-OH)PPA and Hph the steady-state measurements were performed at 15, 20, 30, 40 and 50 °C to calculate thermodynamic parameters of the inclusion complex formation. Temperature was maintained using Julabo F26-MP refrigerated circulator.

Fluorescence intensity decays were collected using a time-correlated single-photon counting apparatus (the picosecond/femtosecond laser system, Ti/sapphire 'Tsunami' laser pumped with an argon ion laser 'BeamLok' and thermoelectrically cooled MCP-PTM R3809U-05) at the Laboratory of Ultrafast Laser Spectroscopy, Adam Mickiewicz University, Poznań, Poland [55]. The excitation wavelength was 275 nm for phenol chromophore and 260 nm for phenyl chromophore. The emission wavelength (λ=315 nm for tyrosine derivatives and its analogues and derivatives and 310 nm for phenylalanine and its analogues) was selected by means of monochromator (about 7.5-nm bandwidth). All fluorescence intensity decays were recorded at 20 °C with a polarizer set up at a magic-angle up to 10^4 counts at maximum, whereas the instrument response function was measured using the Ludox solution (up to 10^5 counts at maximum). The fluorescence lifetimes and global analysis were calculated using software (Level 1 and 2) delivered by Edinburgh Analytical Instruments.

Fluorescence intensity decay data were fitted by the iterative convolution to the sum of exponents:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where α_i and τ_i are the pre-exponential factor and fluorescence lifetime, respectively.

In the global analysis [56] the measured fluorescence intensity decays for each concentration of β-CD were analysed simultaneously assuming different kinetic model in which pre-exponential factors were non-global parameters, whereas the fluorescence lifetimes were global parameters. The adequacy of the exponential decay fitting was judged by visual inspection of the plots of weighted residuals and by the statistical parameter χ^2_R and shape of the autocorrelation function of the weighted residuals, and serial variance ratio (SVR).

3.1. The equilibrium constants determination

Equilibrium constants were calculated applying the non-linear least-squares methods using the following equation [13]:

$$I = \frac{I_0 + I_1 \cdot K \cdot [\mathrm{CD}]}{1 + K \cdot [\mathrm{CD}]} \quad (2)$$

where: I—the fluorescence intensity of a chromophore in the presence of various [CD] concentration; I_0—the fluorescence intensity of a chromophore in water; I_1—the fluorescence intensity of 'pure' complex. The same equation was used for calculation of equilibrium constants from the time-resolved measurements using pre-exponential factors instead of the fluorescence intensity. Equilibrium constant calculations using the non-linear least-squares methods were performed using Origin 6.1 software from OriginLab, Northampton, MA.

3.2. Calorimetric titration

The calorimetric titrations were performed with a Microcal (Northampton, MA) Omega Titration Calorimeter. All experiments were performed at 25 °C in water.

Typically, 27 injections of 10 μl of β-CD solution (14 mM) were injected into a calorimetric cell containing ligand (concentrations shown in Table 5). Control experiments were performed to determine the heat of dilution for β-CD. Additionally, in the case of low binding constant, in a separate experiment 5 injections of the ligand into high concentration of β-CD (14 mM) were made, enabling independent determination of the reaction enthalpy. This value was then used as a starting guess in a fitting of the enthalpy of binding. The obtained raw data were fitted using software delivered by manufacturer. We chose the best fitted "one set for sites" binding model. Based on information obtained from other experiments, we assumed binding ratio as 1:1 and fixed it upon fitting process. Such procedure significantly improved the fitted results, especially in the cases with low binding constant. Because of low solubility of most ligands we titrated low concentration of the ligand with better soluble β-CD.

4. Results

4.1. Steady-state fluorescence

The fluorescence intensity and lifetime of tyrosine does not depend on hydronium ion concentration in the pH range from about 3 to 8 [13,47]. Thus, at pH close to neutral both amino and carboxyl functional groups are ionized and one form is present in the solution. Moreover, at pH=2 the carboxyl group of 3-(4-OH)PPA and Hph (pK_a=4.6 and $pK_a \approx 3.3$ for β- or β-homo amino acids [57]) is almost totally protonated because of the difference between pK_a and pH.

In the presence of β-cyclodextrin, the absorption spectra of the studied compound possessing free hydroxyl group shift to the longer wavelengths for a few nanometers, comparing to that in pure water, while the compounds which have blocked phenol hydroxyl group (methoxy group) or devoid of hydroxyl substituent in para position in the phenyl ring do not show any changes of the shape and position of the absorption band. Such behavior was also observed by Ross and Rekharsky [58]. Moreover, a small red shift of the emission spectra (about 1 nm) as in the case of tyrosine [13,58] and an increase of the fluorescence intensity of the emission band was observed as a result of addition of β-CD to water solution of tyrosine analogues with free hydroxyl group. However, the shape and position of the emission spectra of the compounds devoid of free hydroxyl substituent do not change, whereas the fluorescence intensity increases (data not shown). The fluorescence spectra of tyrosine analogues with free hydroxyl group do not contain the emission of tyrosinate. In spite of the fact that phenol hydroxyl group is more acidic in the excited state than in the ground state, the deprotonation of hydroxyl group in the excited state can be excluded because the proton transfer to water is too slow to compete effectively with other deactivation pathways in the absence of a strong proton acceptor [45,59].

The double-reciprocal plot, according to Eq. (3), was used to check the stoichiometry and estimate the binding constant of the inclusion β-CD-amino acid complexes:

$$\frac{1}{I - I_0} = \frac{1}{I_1 - I_0} \cdot \frac{1}{K[\mathrm{CD}]_0} + \frac{1}{I_1 - I_0} \tag{3}$$

where $[CD]_0$ represents the initial concentration of β-CD, which is much larger than that of the fluorescent substrate in this study, I stands for the total fluorescence intensity of the substrate in β-CD solution while I_0 and I_1 stand for the fluorescence intensities of the substrate in pure water and the 1:1 complex, respectively, and K is the equilibrium constant. In all cases studied for which the fluorescence intensity changes were enough for calculation, the plot $1/(I-I_0)$ versus $1/[CD]_0$ is a straight line with correlation coefficient not lower than 0.98. However, for Phg(OH), Phg(Me), Tic, βPhe, *o*-MePhe and (4-F)Phe the fluorescence intensity changes were too low to calculate binding constants. Calculated from Eq. (2) equilibrium constants (K) of the formation of the complex of tyrosine or phenylalanine and their analogues with β-CD are presented in Tables 2 and 3. The values of equilibrium constants are diversified and depend on the separation of the charged groups and aromatic ring as well as the presence and state (free or blocked) of the hydroxyl group.

4.2. Time-resolved fluorescence

The fluorescence intensity decays of tyrosine and phenylalanine analogues in water and in water solution of β-CD (concentration of cyclodextrin in the range from 0 up to 0.0145 M) were recorded at 25 °C. The increase of CD concentration causes an increase in the fluorescence lifetime of compounds studied as shown for 4-ethyl-phenol in Fig. 2. The representative results of the analysis of each fluorescence intensity decay curve of Phg(Me), βTyr(Me) and 4-EtPhOH in the solution containing different β-CD concentrations with an assumption of mono-exponential fluorescence decay of the guest are presented in Table 4. The fluorescence lifetime of Phg(Me), βTyr(Me) and 4-EtPhOH, calculated according to Eq. (1) as well as the χ^2_R values increase when β-CD concentration rises. For all β-CD concentrations, the χ^2_R for a mono-exponential fit is within or slightly higher than that corresponding to the 95% confidence limit as in the case of tyrosine [13]. The same tendency was also observed for the remaining tyrosine analogues studied (data not shown). A systematic increase

Table 2
The equilibrium constants of complex formation between β-CD and tyrosine or phenylalanine analogues calculated using pre-exponential factors obtained from the global analysis of fluorescence intensity decay at 20 °C with two lifetimes variable and linked for all fitted files (column 2), with two lifetimes linked and the one lifetime (determining the fluorescence lifetime of amino acid in water) fixed (column 3) and steady-state fluorescence measurements (column 4)

Compounds	K_g (α_1^L, α_2^L) (global 10 files)	K_g (α_1^L, α_2^L, τ_g^F) (global 10 files)	$K_{s.s}$ (surface)	K lit value
Phg(OH)	18±10[a]	21.7±9.1[a]	–[b]	–
	$-\infty \leq <K \leq 43$[c]	$13.2 \leq <K \leq 33.6$[c]		
Phg(Me)	43±15[a]	54±13[a]	–[b]	–
	$18.3 \leq <K \leq 91.7$[c]	$19.2 \leq <K \leq 110.7$[c]		
Tyr[d]	40±5[a]	50.2±5.4[a]	50±4[a]	33 (at pH=7) [32]
		$38.6 \leq <K \leq 66.3$[c]		147 (at pH=11) [37]
AcTyr	164.4±16.3[a]	–	157.4±17.9[a]	130 [23]
	$146.9 \leq <K \leq 186.2$[c]			
Tyr(Me)	26.1±4.8[a]	22.0±5.8[a]	20±14[a]	–
	$18.6 \leq <K \leq 32.7$[c]	$17.6 \leq <K \leq 27.1$[c]		
βTyr(Me)	15.9±5.2[a]	21.0±5.7[a]	19±6[a]	–
	$9.4 \leq <K \leq 20.9$[c]	$9.1 \leq <K \leq 35.9$[c]		
βHty	118±4[a]	121.0±4.4[a]	137±58[a]	–
	$109.2 \leq <K \leq 126.3$[c]	$117.8 \leq <K \leq 130.4$[c]		
βHty(Me)	115.2±4.8	117.8±3.3[a]	116±40[a]	–
	$105.8 \leq <K \leq 122.6$[c]	$117.2 \leq <K \leq 123.3$[c]		
Tyra	26.2±5.6[a]	43.1±6.0[a]	45±11[a]	70 [56], 63 [60], 79 [2]
	$24.2 \leq <K \leq 28.6$[c]	$38.0 \leq <K \leq 47.4$[c]		
3-(4-OH)PPA	588.4±19.8[a]	589.4±50.3[a]	687±22[a]	
(pH=2)	$574.5 \leq <K \leq 617.2$[c]	$561.0 \leq <K \leq 609.1$[c]		
(pH=8.0)	–	–	285±28[a]	297 (at pH=7) [56],
4-EtPhOH	582.8±11.1[a]	581.9±15.3[a]	653±58[a]	251 (at pH=4.2) [2]
	$573.5 \leq <K \leq 605.6$[c]	$560.8 \leq <K \leq 609.1$[c]		
Hph (pH=2)	98.7±8.7[a]	78.2±4.9[a]	81.4±8.9[a]	–
	$87.3 \leq <K \leq 110.2$[c]	$79.2 \leq <K \leq 83.0$[c]		

[a] Asymptotic standard error.
[b] Too small fluorescence intensity changes for equilibrium constant determination.
[c] Error from plane error analysis.
[d] Data from [13].

in both fluorescence lifetime and χ_R^2 values with growing β-CD concentration suggests the presence of both "free" fluorophore and its complex with cyclodextrin. The global analysis of the fluorescence decay curves with an assumption of the mono-exponential decay function and the same fluorescence lifetimes for all data sets (global parameter) gives an unacceptably high value of χ_R^2 (Table 5). The global fit to a double-exponential function with two fluorescence lifetimes, common for all analysed data sets (global parameter) and independent (non-global) pre-exponential factors, gives acceptable χ_R^2 values which only slightly decreased when the fit to the double-exponential function with two non-global fluorescence lifetimes was applied. The addition of a third component to the fitted function does not improve the χ_R^2 values. The fluorescence lifetimes, pre-exponential factors and the χ_R^2 values obtained from the global analysis applying mono- or bi-exponential function together with the local χ_R^2 values obtained for the particular β-CD concentration are also presented in Table 5. Two fluorescence lifetimes obtained from the global analysis of the fluorescence intensity decay measured for 10 different β-CD concentrations indicate the presence of two individuals in the solution. The fluorescence lifetimes of tyrosine and its derivatives in organic solvent are longer than in water [43,60]. Therefore, the longer fluorescence lifetime can be connected with the fluorescence lifetime of encapsulated phenol fluorophore of tyrosine analogues because of hydrophobic interior of the β-cyclodextrin cavity. However, the shorter fluorescence lifetime corresponds to the fluorescence lifetime of the free fluorophore, though, it differs slightly from the values obtained from a single file analysis (mono-exponential fit) of the fluorescence intensity decay of compounds studied measured in water. The fluorescence lifetimes obtained from the global analysis using a bi-exponential function are

Table 3
The equilibrium constants of complex formation between β-CD and phenylalanine analogues calculated using pre-exponential factors obtained from the global analysis of two files of fluorescence intensity decay at 20 °C with two lifetimes linked and the one lifetime (determining the fluorescence lifetime of amino acid in water) fixed

Compound	K_{2f} (τ_g^F) (global 2 files)	$K_{s.s.}$ (surface)	K lit values
Phe	15.3	10.5±6.2	18 [37], 106 (at pH=11) [37]; 3 (at pH=5) [32]
βPhe	25.1	–	–
o-MePhe	51.8	–	–
βHph	23	32.6±7.6	–
Tic	11.5	–	–

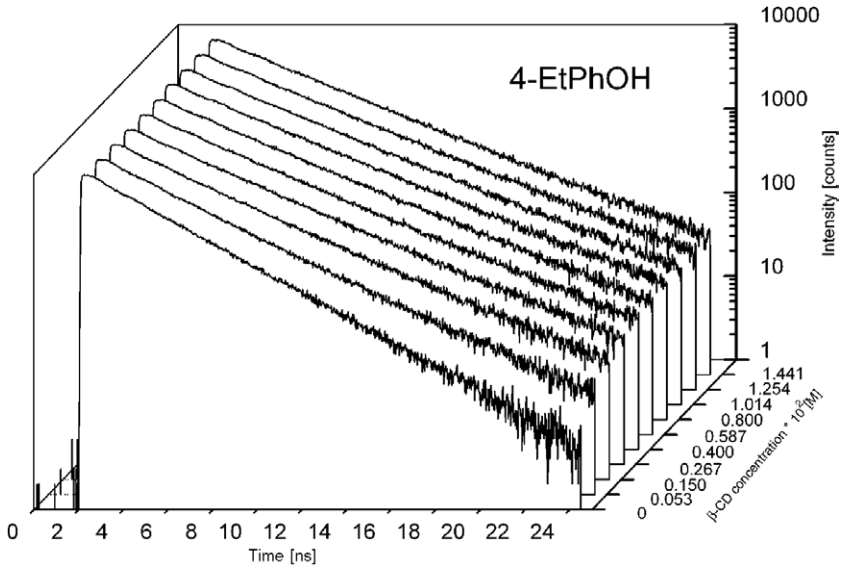

Fig. 2. Fluorescence intensity decays of 4-ethyl-phenol at different β-cyclodextrin concentrations.

generally shorter because of a small contribution (about a few percent) of a component with a longer lifetime corresponding to the complexed fluorophore. The exception is Tyr(Me). For this amino acid, the fluorescence lifetime in water solution ($c_{CD}=0$), obtained from the global analysis, contains large (about 20%) contribution of the component with long fluorescence lifetime. This is a result of a small difference between the fluorescence lifetimes of free and complexed fluorophore and cross-correlation between fluorescence lifetimes and pre-exponential factors [56,61]. The global analysis of the fluorescence intensity decays with the assumption of a bi-exponential function with two fluorescence lifetimes common for all analysed data sets (global parameter) and the one corresponding to the fluorescence lifetime of free fluorophore in water solution fixed, and independent (non-global) pre-exponential factors gives acceptable χ^2_R values which are only slightly higher when the fit to the double-exponential function with two global but varied fluorescence lifetimes was applied. The values of the pre-exponential factors and χ^2_R are presented in Table 5. The fluorescence lifetimes for the inclusion complex of amino acid with β-CD obtained in this case seem to be more accurate because of the lower cross-correlation between parameters [56,61].

Table 4
The fluorescence lifetime and the values of quality of fit (χ^2_R) obtained from the fit of mono-exponential function to the fluorescence intensity decay of Phg(Me), βTyr(Me) and 4-EtPhOH in solution of different cyclodextrin concentration

Phg(Me)			βTyr(Me)			4-EtPhOH		
CD [M]	τ [ns]	χ^2_R	CD [M]	τ [ns]	χ^2_R	CD [M]	τ [ns]	χ^2_R
0.0	3.91	1.13	0	4.67	1.11	0	2.98	1.18
0.0001	3.9	1.1	0.0001	4.07	1.13	0.005	3.28	1.41
0.0005	3.9	1.07	0.0005	4.74	1.17	0.0013	3.56	1.46
0.001	3.91	1.17	0.001	4.81	1.19	0.0027	3.73	1.33
0.002	3.98	1.21	0.002	4.92	1.26	0.004	3.82	1.33
0.005	3.97	1.17	0.005	5.12	1.26	0.006	3.9	1.41
0.0075	3.96	1.15	0.0075	5.24	1.21	0.008	3.96	1.35
0.01	4	1.18	0.01	5.33	1.3	0.010	3.99	1.2
0.012	4	1.12	0.012	5.36	1.23	0.0125	4	1.31
0.0145	4.07	1.4	0.0145	5.44	1.26	0.0144	4.04	1.3

The pre-exponential factors (α_1 and α_2) determining the contribution of the particular chemical individual in the fluorescence intensity decay, presented in the Table 5, were used to calculate the equilibrium constant according to Eq. (2) in which the fluorescence intensity was replaced by the pre-exponential factor. The correctness of such a method of equilibrium constant calculation was demonstrated in the calculation of the equilibrium constants of tyrosine with cyclodextrins [13] and pK calculation using Henderson–Hasselbalch equation [44,47]. The graphical examples of the dependence of pre-exponential factors on the CD concentration for a weak (Phg(Me)), medium (βHty(Me)) and relatively strong (3-(4-OH)PPA)) complexes are presented in Fig. 3. The differences between the equilibrium constant calculated using pre-exponential factors obtained from the global analysis with two linked but varied lifetimes and two linked but one fixed lifetime are in the range of experimental error the same. However, the global analysis with the fixed guest lifetime gives more accurate fluorescence lifetime of the inclusion complex and in our opinion this method should be used. Most software for non-linear least-squares analysis report uncertainties which are based on the assumptions of no correlation between parameters. These are called the asymptotic errors. Because lifetimes and pre-exponential factors are cross-correlated, the best way to determine the range of parameters consisted with the data is to examine the χ^2_R surface, which is also called a support plane analysis [56]. To determine the confidence interval, the values of χ^2_R with a fixed parameter value, χ^2_R (par), is compared with the minimal values of χ^2_R with all

Table 5
The pre-exponential factors and lifetimes and χ^2_R values for mono- (1exp) and bi-exponential (2exp) fits with lifetimes as global parameters

β-CD [M]	χ^2 1exp	Guess lifetime non-fixed			Guess lifetime fixed		
		α_1	α_2	χ^2 2exp	α_1	α_2	χ^2 2exp
Phg(OH)	1.59	τ_1^L=2.73±0.05 ns τ_2^L=3.98±0.41 ns		1.117	τ_1^{LF}=2.67 ns τ_2^L=3.62±0.28 ns		1.123
0	1.432	0.979	0.021	1.054	0.938	0.062	1.061
0.0001	1.309	0.979	0.021	0.953	0.937	0.063	0.963
0.0005	1.381	0.979	0.021	1.103	0.931	0.069	1.112
0.001	1.528	0.979	0.021	1.210	0.931	0.069	1.211
0.002	1.396	0.972	0.028	1.167	0.924	0.076	1.164
0.005	1.171	0.945	0.055	1.098	0.883	0.117	1.094
0.00745	1.493	0.918	0.082	1.244	0.848	0.152	1.243
0.01	1.928	0.897	0.103	1.240	0.814	0.186	1.237
0.012	1.788	0.896	0.104	1.052	0.812	0.188	1.058
0.0145	2.433	0.873	0.127	1.084	0.782	0.218	1.090
Phg(Me)	1.275	τ_1^L=4.00±0.06 ns τ_2^L=5.45±0.86 ns		1.136	τ_1^{LF}=3.91 ns τ_2^L=4.68±0.31 ns		1.139
0	1.253	1	0	1.124	0.887	0.113	1.136
0.0001	1.242	1	0	1.098	0.892	0.108	1.109
0.0005	1.228	1	0	1.065	0.893	0.107	1.069
0.001	1.278	0.993	0.007	1.162	0.88	0.12	1.168
0.002	1.199	0.992	0.008	1.183	0.823	0.177	1.182
0.005	1.174	0.958	0.042	1.134	0.81	0.19	1.13
0.00745	1.095	0.957	0.043	1.054	0.807	0.193	1.05
0.01	1.263	0.943	0.057	1.143	0.773	0.227	1.14
0.012	1.263	0.936	0.064	1.078	0.759	0.241	1.079
0.0145	1.755	0.921	0.079	1.323	0.721	0.279	1.323
Tyr(Me)	1.515	τ_1^L=4.71±0.25 ns τ_2^L=0.41±0.25 ns		1.152	τ_1^{LF}=4.73 ns τ_2^L=5.53±0.28 ns		1.151
0	1.547	0.814	0.186	1.209	0.856	0.144	1.209
0.0001	0.1582	0.818	0.182	1.115	0.875	0.125	1.149
0.0005	0.1464	0.806	0.194	1.108	0.863	0.137	1.08
0.001	1.358	0.768	0.232	1.193	0.826	0.174	1.193
0.002	1.276	0.755	0.245	1.132	0.819	0.181	1.132
0.005	1.174	0.651	0.341	1.161	0.737	0.263	1.161
0.00745	1.242	0.597	0.403	1.117	0.683	0.317	1.116
0.01	1.582	0.544	0.456	1.227	0.63	0.370	1.226
0.012	1.722	0.500	0.500	1.117	0.594	0.406	1.115
0.0145	2.203	0.435	0.565	1.134	0.563	0.464	1.13
βTyr(Me)	1.731	τ_1^L=4.84±0.13 ns τ_2^L=6.31±0.56 ns		1.198	τ_1^{LF}=4.89 ns τ_2^L=6.67±0.76 ns		1.201
0	1.72	0.967	0.033	1.136	1	0	1.143
0.0001	1.601	0.961	0.039	1.143	0.994	0.006	1.146
0.0005	1.755	0.965	0.035	1.233	0.994	0.006	1.232
0.001	1.652	0.955	0.045	1.309	0.983	0.017	1.311
0.002	1.338	0.938	0.062	1.165	0.972	0.028	1.162
0.005	1.26	0.887	0.113	1.228	0.932	0.068	1.228
0.00745	1.34	0.864	0.136	1.176	0.915	0.085	1.176
0.01	1.61	0.833	0.167	1.092	0.889	0.111	1.092
0.012	2.083	0.816	0.184	1.309	0.877	0.123	1.309
0.0145	2.948	0.77	0.230	1.209	0.838	0.162	1.208
βHty	6.23	τ_1^L=3.33±0.17 ns τ_2^L=4.52±0.22 ns		1.119	τ_1^{LF}=3.28 ns τ_2^L=4.45±0.211 ns		1.122
0	8.788	1	0	1.075	0.937	0.063	1.089
0.0001	8.419	0.965	0.035	1.104	0.937	0.063	1.122
0.0005	5.478	0.923	0.077	1.019	0.887	0.113	1.016
0.001	3.952	0.887	0.113	1.219	0.851	0.149	1.212
0.002	1.693	0.81	0.19	1.081	0.768	0.232	1.083
0.005	2.468	0.638	0.362	1.131	0.596	0.404	1.132
0.00745	4.922	0.545	0.455	1.163	0.500	0.500	1.164
0.01	6.818	0.489	0.511	1.055	0.440	0.560	1.054
0.012	8.753	0.447	0.553	1.156	0.397	0.603	1.156
0.0145	11.006	0.397	0.603	1.192	0.348	0.652	1.192

(continued on next page)

Table 5 (*continued*)

β-CD [M]	χ^2 1exp	Guess lifetime non-fixed			Guess lifetime fixed		
		α_1	α_2	χ^2 2exp	α_1	α_2	χ^2 2exp
βHty(Me)	4.1	τ_1^L=4.65±0.21 ns	τ_2^L=6.00±0.31 ns	1.154	τ_1^{LF}=4.67 ns	τ_2^L=6.06±0.33 ns	1.163
0	5.789	1	0	1.088	1	0	1.098
0.0001	5.019	0.966	0.034	1.124	0.983	0.017	1.136
0.0005	4.083	0.944	0.056	1.165	0.955	0.045	1.15
0.001	2.705	0.9	0.1	1.154	0.911	0.089	1.186
0.002	0.156	0.862	0.174	1.203	0.844	0.156	1.217
0.005	0.171	0.685	0.315	1.164	0.708	0.292	1.212
0.00745	0.310	0.595	0.405	1.124	0.624	0.376	1.1
0.1	0.462	0.534	0.466	1.213	0.568	0.432	1.199
0.012	0.537	0.506	0.494	1.164	0.537	0.463	1.191
0.145	0.717	0.452	0.548	1.142	0.486	0.514	1.143
Tyra	2.804	τ_1^L=3.28±0.1 ns	τ_2^L=4.60±0.35 ns	1.037	τ_1^{LF}=3.24 ns	τ_2^L=4.47±0.32 ns	1.04
0	5.239	1	0	0.968	1	0	0.979
0.00113	2.71	0.983	0.02	0.984	0.955	0.045	0.989
0.00269	1.403	0.932	0.07	0.993	0.893	0.107	0.99
0.00404	1.223	0.891	0.109	1.045	0.866	0.134	1.045
0.00592	1.551	0.83	0.17	1.074	0.782	0.218	1.07
0.00808	2.066	0.793	0.207	1.047	0.736	0.264	1.034
0.01023	2.973	0.741	0.259	1.054	0.682	0.318	1.05
0.01265	3.661	0.701	0.299	1.087	0.644	0.356	1.085
0.0145	4.406	0.674	0.326	1.161	0.605	0.395	1.121
Tyr[a]	4.91	τ_1^L=3.35±0.05 ns	τ_2^L=4.32±0.34 ns	1.117	τ_1^{LF}=3.25 ns	τ_2^L=4.05±0.25 ns	1.176
0					0.902	0.098	1.115
0.000094					0.887	0.113	1.248
0.0005					0.874	0.126	1.194
0.001					0.859	0.141	1.195
0.0027					0.809	0.191	1.169
0.005					0.767	0.233	1.213
0.0075					0.717	0.283	1.142
0.01					0.679	0.321	1.082
0.0145					0.613	0.387	1.167
AcTyr	6.998	τ_1^L=3.08±0.12 ns	τ_2^L=4.70±0.19 ns	1.13	τ_1^{LF}=3.27 ns	τ_2^L=5.41±0.27 ns	1.188
0	18.01	0.933	0.07	1.08	1	0	1.294
0.00054	17.07	0.887	0.113	1.14	0.97	0.03	1.302
0.0015	3.824	0.791	0.209	1.11	0.896	0.104	1.124
0.0027	2.747	0.746	0.254	1.32	0.868	0.142	1.389
0.004	2.091	0.672	0.328	1.18	0.791	0.209	1.227
0.0059	3.109	0.588	0.412	1.1	0.731	0.269	1.111
0.0081	5.456	0.526	0.474	1.0	0.672	0.328	1.028
0.0102	7.091	0.496	0.504	1.17	0.647	0.353	1.175
0.0127	7.476	0.489	0.511	1.15	0.637	0.363	1.155
0.0145	9.105	0.456	0.544	1.08	0.612	0.388	1.073
3-(4-OH)PPA	1.973	τ_1^L=0.64±0.17 ns	τ_2^L=2.74±0.07 ns	1.05	τ_1^{LF}=0.48 ns	τ_2^L=2.70±0.06 ns	1.07
0	1.05	0.996	0.004	1.05	0.812	0.188	1.093
0.00113	4.248	0.635	0.365	1.03	0.587	0.413	1.042
0.00269	2.27	0.44	0.56	0.979	0.413	0.587	0.984
0.00404	1.668	0.349	0.65	0.909	0.355	0.645	0.913
0.00592	1.446	0.295	0.705	1.061	0.289	0.711	1.063
0.00808	1.566	0.25	0.75	1.092	0.244	0.756	1.116
0.01023	1.579	0.227	0.773	1.151	0.222	0.778	1.164
0.01265	1.463	0.205	0.795	1.066	0.186	0.814	1.081
0.01454	1.543	0.167	0.833	1.082	0.155	0.845	1.101
4-EtPhOH	9.78	τ_1=2.86±0.24 ns	τ_2=4.20±0.17 ns	1.199	τ_1^{LF}=2.98 ns	τ_2^L=4.32±0.20 ns	1.22
0	46.21	0.948	0.1	1.249	1	0	1.36
0.00053	13.09	0.737	0.263	1.164	0.806	0.194	1.196
0.00149	2.122	0.531	0.469	1.176	0.598	0.402	1.21
0.00267	1.561	0.402	0.598	1.138	0.479	0.521	1.166

Table 5 (*continued*)

β-CD [M]	χ^2 1exp	Guess lifetime non-fixed			Guess lifetime fixed		
		α_1	α_2	χ^2 2exp	α_1	α_2	χ^2 2exp
4-EtPhOH	9.78	τ_1=2.86±0.24 ns τ_2=4.20±0.17 ns		1.199	τ_1^{LF}=2.98 ns τ_2^{L}=4.32±0.20 ns		1.22
0.004	2.664	0.33	0.67	1.171	0.406	0.594	1.184
0.00587	4.388	0.26	0.74	1.215	0.344	0.656	1.243
0.008	5.911	0.216	0.784	1.292	0.289	0.711	1.269
0.0101	6.594	0.198	0.802	1.157	0.271	0.729	1.168
0.0125	7.136	0.177	0.82	1119	0.26	0.74	1.187
0.01441	8.111	0.156	0.844	1.243	0.229	0.771	1.243
Hph	3.128	τ_1=3.64±0.56 ns τ_2=9.18±0.17 ns		1.042	τ_1^{LF}=3.70 ns τ_2^{L}=9.13±0.89 ns		1.043
0	7.989	1	0	0.702	1.0	0	0.996
0.0015	3.078	0.889	0.111	0.983	0.889	0.111	1.071
0.004	1.592	0.731	0.27	0.1081	0.731	0.269	1.111
0.0059	1.749	0.64	0.36	1.106	0.64	0.360	1.041
0.008	2.14	0.577	0.42	1.151	0.577	0.423	1.093
0.0102	2.343	0.5	0.50	1.088	0.500	0.500	1.091
0.0125	2.762	0.458	0.54	1.083	0.458	0.542	0.975
0.0145	3.373	0.4	0.60	1.111	0.400	0.600	1.022
Phg	1.158	τ_1^{L}=5.22±0.35 ns τ_2^{L}=5.28±0.3 ns		1.132	τ_1^{LF}=5.32 ns τ_2^{L}=5.14 ns		1.096
0	1.132	–	–	1.064	–	–	0.955
0.0015	1.036	–	–	0.989	–	–	0.917
0.004	1.083	–	–	1.089	–	–	0.99
0.0059	1.081	–	–	1.103	–	–	1.083
0.008	1.093	–	–	1.093	–	–	1.091
0.0102	1.17	–	–	1.186	–	–	1.178
0.0125	1.19	–	–	1.152	–	–	1.159
0.0145	1.459	–	–	1.39	–	–	1.396
(4-F)Phe	1.094	τ_1^{L}=6.84±0.38 ns τ_1^{L}=8.97±1.45 ns		1.066	τ_1^{L}=7.13 ns τ_2^{L}=10.2±6.5 ns		1.074
0	1.025	–	–	0.985	–	–	1.002
0.0015	1.118	–	–	1.082	–	–	1.095
0.004	1.082	–	–	1.072	–	–	1.071
0.0059	1.138	–	–	1.139	–	–	1.136
0.008	1.039	–	–	1.023	–	–	1.033
0.0102	1.126	–	–	1.018	–	–	1.113
0.0125	1.079	–	–	1.05	–	–	1.07
0.0145	1.145	–	–	1.068	–	–	1.073

[a] Data from [13].

parameters variable, χ^2_R (min). The range of parameters values is expanded until χ^2_R (par) exceeds the F_i value for the number of parameters, degree of freedom and chosen probability, typically P=0.32. In our case the F_i values at P=0.32 is equal to 1.28. Both types of errors of the binding constant determinations are presented in Table 2.

The fluorescence lifetimes of (4-F)Phe (Fig. 4) and Phg (data not shown) do not depend on cyclodextrin concentration (Table 5) precluding binding constant calculation. For some phenylalanine derivatives the measurements of fluorescence intensity decays were done only in water and at highest cyclodextrin concentration (0.0145 M). The global analysis of the fluorescence intensity decays using only two files: amino acid in water and at the cyclodextrin concentration used gives the pre-exponential factors proportional to the concentrations of free and encapsulated fluorophore which allows to estimate the equilibrium constants which are presented in Table 3.

4.2.1. Calorimetry

The thermodynamic parameters of the formation of inclusion complex of selected amino acids with β-cyclodextrin are presented in Table 6. For βHty, βHty(Me) and 4-EtPhOH they were obtained from microcalorimetric titration, whereas for Hph and 3-(4-OH)PPA (at pH=2) they were calculated from the dependence of equilibrium constant determined from the fluorimetric measurements on temperature using Vant Hoff's equation.

4.2.2. Discussion

The modification of the photophysical properties of aromatic amino acid upon the addition of cyclodextrin can be utilized to determine the stoichiometry and binding constant of the inclusion complex. However, encapsulation of fluorophore into the hydrophobic interior of CD does not always change its properties because of lack of sensitivity to hydrophobic–hydrophilic environments or insufficient deep penetration of the cyclodextrin cavity as

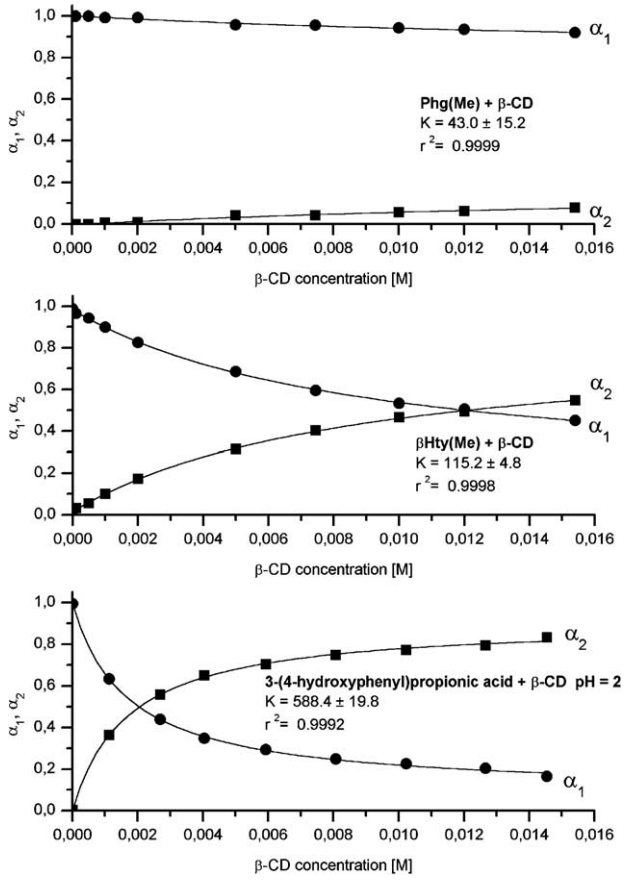

Fig. 3. The dependence of pre-exponential factors α_1 and α_2 defining a free guest and an inclusion complex concentration, respectively on β-cyclodextrin concentration for Phg(Me) (top), βHty(Me) (middle) and 3-(4-OH)PPA (bottom).

in the case of Phg and (4-F)Phe. The remaining studied amino acids show sufficiently distinct changes of the fluorescence intensity and/or fluorescence lifetime (Table 5) enabling to determine stoichiometry and binding constant. The binding constants obtained from the fluorescence data are generally reasonably consistent with published data, however, the difference between presented data and literature values (collected in Tables 2 and 3) are probably connected with a method applied for their determination. The literature values of equilibrium constants of Phe, Tyr, Tyra, 3-(4-OH)PPA and 4-EtPhOH were obtained applying the microcalorimetry because of a low binding of these amino acids with cyclodextrin. Much higher amino acid concentration, about two orders of magnitude, used in isothermal titration calorimetry than in fluorimetric method could lead to amino acid association resulting in different values of the equilibrium constants [62]. The example of such a behavior is 4-EtPhOH which equilibrium constant obtained from the microcalorimetric method ($K=457\pm9$, Table 6) is lower than that determined by fluorimetric method ($K=581.9\pm15.3$, Table 2).

Data presented in Table 2 indicate that among the studied substances higher binding constants possess neutral compounds in comparison to the corresponding charged species derived from the same guest molecule, for instance: 3-(4-OH)PPA at pH=2 and pH=8, Tyr and AcTyr (Table 2) which is in accordance with the literature data [2]. Another examples are Phe at different pH ($K=107$ at pH=11 [38] and $K=3$ at pH=5 [2]); AcPhe ($K=61$ [23]) and Phe as well as phenylalanine amide at different pH ($K=22$ and K=107 at pH=5 and 10, respectively) [32]. It has been shown that the phenolic group in a guest as tyramine or 3-(4-OH)PPA forms hydrogen bonds inside cyclodextrin cavity [23,58]. The same interactions were observed for tyrosine analogues studied causing that tyrosine and its analogues with free hydroxyl of the phenol group interact with β-cyclodextrin stronger than phenylalanine and its appropriate analogues (Tyr and Phe, βHty and βHph, Tables 2 and 3); phenylethylamine and tyramine [58,62]; 3-phenylpropionate and 3-(4-hydroxyphenyl)propionate [58].

As stated by Rekharsky et al. [64], for β-cyclodextrin values of the equilibrium constants of the formation of inclusion complexes increase in the order $OCH_3 > CH_3 > OH > H$. However, based on the presented data, it is difficult to estimate unequivocally the influence of methoxy group on binding constant. Methylation of the hydroxyl group of *p*-hydroxyphenylglycine increases the equilibrium constant of the inclusion complex with β-CD two times, whereas for tyrosine it decreases two times while for β-homo-tyrosine it remains unchanged (Table 2). A small increase of equilibrium constant was observed after methylation of the hydroxyl group of tyramine [62]. Also, comparing the methoxy-tyrosine analogues with appropriate phenylalanine analogues, it is not possible to estimate unequivocally the influence of the methoxy group on the binding constant. Only for βHty(Me) (Table 2) the binding constant is substantially higher than that for βHph (Table 3), whereas for remaining corresponding analogues the binding constants are, in the range of experimental error, the same. Van der Waals forces and hydrophobic interactions play the major role in β-CD complexation of guest molecule devoid of polar groups [2,34,37,58], however, for tyrosine derivatives and analogues the hydrogen bond formation is also important [37,58]. For the interaction of amino acid with cyclodextrins the hydration of amino acid moiety is also essential beside the presence of charged amino and carboxyl groups being outside the binding site. The hydration shell around the charged group (amino or carboxyl) includes about two C–C bond lengths [23]. Also, the cyclodextrin possesses its own strong and diversified sphere [23,25,63]. Thus, the displacement of the amino group from α- to β-carbon atom in the phenylalanine or tyrosine analogues (β-analogues) while the carboxyl group stands at α-carbon atom stretches the hydration sphere to the phenyl (phenol ring) causing shallow penetration of the cyclodextrin cavity resulting in lower constant binding. Moreover, the stand-off the carboxyl group from the phenyl group for more

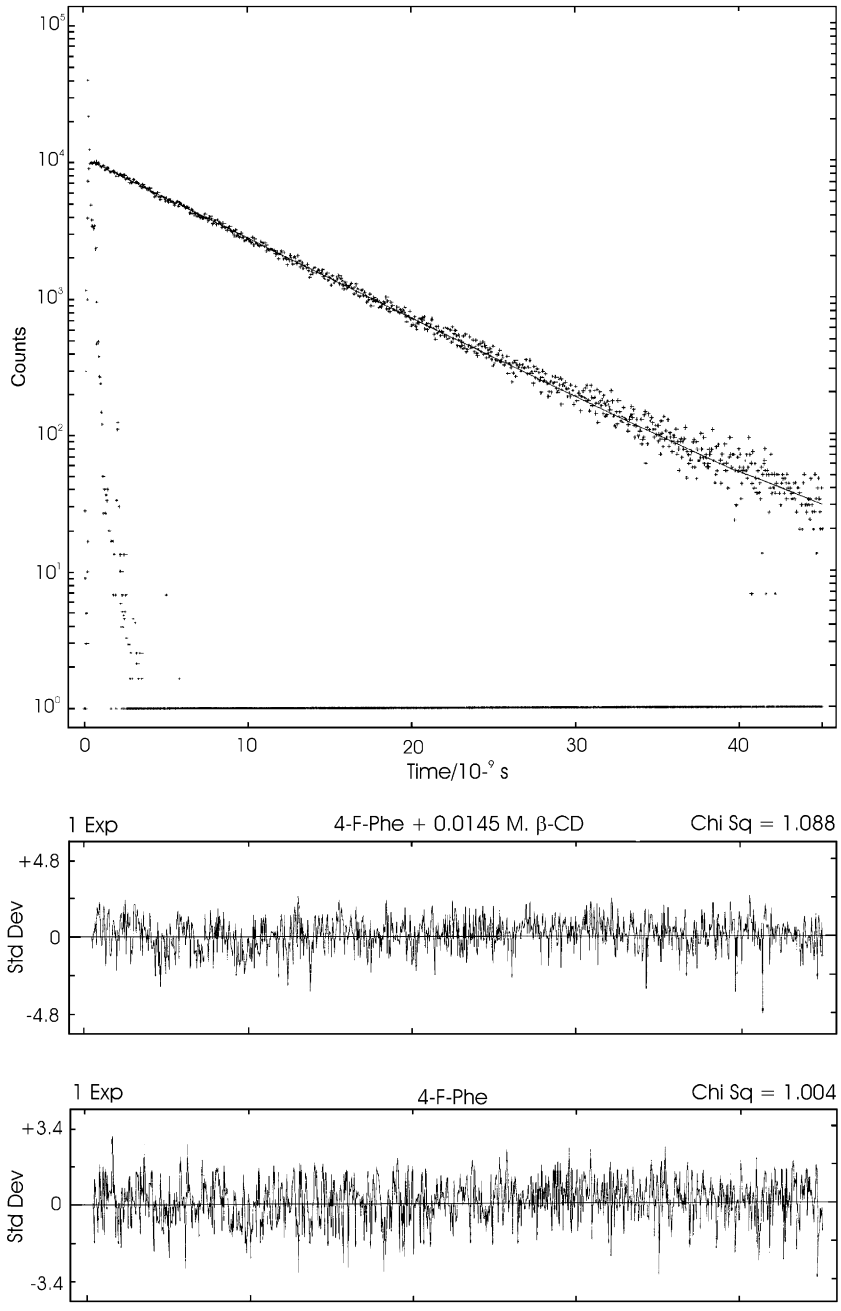

Fig. 4. Measured (dot) and fitted (solid line) fluorescence intensity decay versus time (left points represents a laser profile) of (4-F)Phe in β-CD (0.0145 M) solution. Weighed residuals for mono-exponential fit (in the presence and absence of β-CD) are plotted in the lower portion of the figure.

than one methylene group increases substantially the binding constant of benzoic acid analogues with β-CD [26,58]. This conclusion is also supported by the data presented in Table 2 ((Phg(OH), Tyr and βHty; Tyr(Me) and βHty(Me)). Such conclusion cannot be drawn from the binding constant data for phenylalanine analogues because of their low diversification.

The binding constants of phenylalanine derivatives (Table 3) depend on the substitution in the phenyl ring of phenylalanine. Small methyl group in position 2 of phenylalanine (*o*-MePhe) causes an increase of hydrophobic interaction rising the binding constant with β-CD, whereas bigger substituent as in the case of Tic decreases its value because of steric hindrance and unfavorable location of charged amino group.

The thermodynamic parameters collected in Table 6 indicate that the formation of inclusion complexes of amino acids studied with β-CD is enthalpy driven, except βHty(Me) for which entropy contribution is substantial. The release of water molecules that were originally residing within the cavity and the induced dehydration of peripheral hydroxyl groups of cyclodextrin and the guest molecule appear to be jointly responsible for an intrinsic entropy gain [2,23]. The positive entropy of association of βHty(Me) and

Table 6
Equilibrium constant and thermodynamic parameters obtained from the calorimetry measurements of β-CD (16 mM) and βHty, βHty(Me) and 4-EtPhOH at 298 °K in water and for 3-(4-OH)PPA at pH=2 and Hph from the dependence of equilibrium constant determined by fluorimetry on temperature

Compound (concentration [mM])	K [M^{-1}]	ΔG [kJ/mol]	ΔH [kJ/mol]	$T\Delta S$ [kJ/mol]	ΔS [J/mol K]
βHty (9.15 mM)	82.5±5.7	−10.9	−12.33±0.67	−1.43	− 4.61
βHty(Me) (1.96)	138±12	−12.15	−3.50±0.22	8.65	28.9
4-EtPhOH (23.43)	457±9	−15.15	−14.02±0.15	1.13	3.77
3-(4-OH)PPA pH=2			−19.5±0.4		−12.3±1.3
Hph			−17.3±5.7		−21.2±18.9

4-EtPhOH with β-CD could be a result of more richly dehydration of more weakly bonded water to the guest molecule.

Data presented in Table 6 reveals that the thermodynamic parameters obtained from microcalorimetric titration are more precise than those from spectroscopic measurements.

5. Conclusion

The global analysis of fluorescence intensity decays of guest molecule is a useful method allowing to determine not only the binding constant of the inclusion complex but also the fluorescence lifetime of guest–host complex. This method is applicable to the guest molecules possessing a mono-exponential fluorescence intensity decay because of a need to identify individuals present in a solution.

The studied aromatic amino acids, tyrosine or phenylalanine derivatives and analogues possess low binding constant to β-cyclodextrin, diversified with respect to the presence or absence of a substituent in para position of the phenyl ring. However, the specific hydrogen bond-network between the amino acid moiety possessing a strong hydration sphere and water molecules in the vicinity of host and the possibility to form the hydrogen bond between the hydrophilic group of amino acid and CD hydroxyl groups and water molecules remaining in the CD cavity [63] do not allow to estimate unequivocally the influence of the distance between the charged groups and phenol/phenyl ring on the inclusion complex stability and its binding constant.

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