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ORIGINAL ARTICLE

Influence of Leu^5 configuration on the equilibrium constants of complexes of [Leu]-enkephalin with β-cyclodextrin studied by fluorescence spectroscopy, microcalorimetry and ^{1}H NMR spectroscopy

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Abstract Natural enkephalins and their analogues are very important as potential therapeutic agents (analgetics). In this paper we describe the influence of Leu chirality of cyclic [Leu]enkephalins on the binding constant with β-cyclodextrin and spatial and mutual orientation of guest and host molecules. The formation of complexes is enthalpy driven for both cyclic [Leu]enkephalins. Moreover, D-configuration of Leu residue causes an increase of the binding constant of cyclic enkephalin compared to L-analogue. An analysis of 2D NMR spectra reveals that, apart from inclusion complex formed by penetration of cyclodextrin cavity from wider and narrow rims by Trp or Leu residue, a side and/or bottom association complexes are formed.

Keywords Enkephalin · Cyclodextrin · Fluorescence spectroscopy · Microcalorimetry · NMR spectroscopy · Inclusion complex · Association complex

Introduction

The main elements of opioid system are endogenous peptides which are natural ligands for opioid receptors (μ, δ, κ). Enkephalins are a part of β-endorphin and interact mainly with δ-opioid receptors. Among their neurophysiological functions the most important is perception of pain sensation [1–3]. Natural enkephalins are very flexible molecules with many low energy conformations in solution. One of the methods to decrease the mobility of the peptide chain is cyclization [4–6]. It has been shown that the biological activity of cyclic opioid–peptide analogues depends on mutual orientation and conformational freedom of aromatic pharmacophore group [7–10], which in the case of enkephalins occurs at position 1 and 4. To protect peptide bonds from enzymatic degradation and modify conformational freedom both the peptide chain and side chains of aromatic residues, complexation with cyclodextrin is frequently used. The α-, β-, and γ-cyclodextrins (CD) are polysaccharides consisting of six to eight D-glucopyranose residues, respectively, linked by α-1,4 glycosidic bonds into a macromolecule. Each cyclodextrin has its own ability to form inclusion complexes with various guest molecules with suitable polarity and dimension because of their special molecular structure—hydrophobic internal cavity and hydrophilic external surface [11, 12]. Moreover, CDs can recognize not only the size and shape but also the chirality of amino acids and their derivatives [13]. However, molecules of many peptides and proteins are too hydrophilic and bulky to penetrate entirely into the CD cavity and the topological constrains of the peptide backbone may influence on the formation of inclusion complexes. Thus, their interaction with CDs could only be local meaning that accessible hydrophobic side chains may form inclusion complexes with CDs [14–18]. To further explain the influence of the side chain on interactions of CD with peptides, in this work we present the results of our studies on the binding process of the cyclic analoques of [Leu]enkephalin with β-cyclodextrin in water solution. Because of the Leu

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configuration influence not only on the conformation of the enkephalin studied but also on Trp photophysical properties [5], we decided to use β-CD which forms more stable inclusion complex with Trp than α-CD [12]. In our studies we applied fluorescence spectroscopy, microcalorimetry and high resolution ^{1}H NMR spectroscopy (two-dimensional ^{1}H-^{1}H nuclear Overhauser enhanced spectroscopy (NOESY) methods. It has been proven that these methods give reliable information about the stoichiometry, association constants, thermodynamic parameters and structure of inclusion complexes of CDs [15, 19–32].

Materials and methods

β-CD was purchased from Roth, whereas cyclic analogues of [Leu]enkephalin: H-c[D-Dab^2-Gly^3-Trp^4-(D or L)Leu^5] (D or L-EN) (Fig. 1), were synthesized according to the procedure published previously [4]. The solutions were prepared by dissolving the appropriate amount of β-CD in water. To the aqueous solution of CD 200 μL of stock solution of D- or L-EN was added ($C_{D\text{ or }L\text{-EN}} = 3 \times 10^{-5}$ M). The optical density of the sample at the excitation wavelength ($\lambda = 280$ nm) did not exceed 0.1.

Spectroscopy measurements

Absorption spectra of D- and L-EN in water were recorded using a Perkin–Elmer Lambda 40P spectrophotometer.

Fluorescence spectra were recorded using a Perkin–Elmer LS-50B spectrofluorimeter with 3.5 nm band-width for excitation and emission. The steady-state emission spectra were measured at 20 °C. 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 [33]. The emission wavelength ($\lambda = 350$ nm) was selected by means of monochromator (about 7.5 nm bandwidth). The fluorescence intensity decays were recorded at 20 °C with a polarizer set up at a magic-angle. The Ludox solution was used as a reference. The fluorescence lifetimes and global analysis were calculated using software delivered by Edinburgh Analytical Instruments, Level 1 and 2, respectively. Fluorescence intensity decay data were fitted by the iterative convolution to the sum of exponents:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \tag{1}$$

where α_i and τ_i are the pre-exponential factor and fluorescence lifetime, respectively.The pre-exponential factors were used to calculate so-called lifetime weighted quantum yield [34]:

$$<\tau>_I = \sum_i \alpha_i \tau_i \tag{2}$$

In the global analysis [19, 34] the measured fluorescence intensity decays for each concentration of CD were analyzed simultaneously assuming different kinetic model in which pre-exponential factors were non-global parameters, whereas the fluorescence lifetime was global parameter. The adequacy of the exponential decay fitting was judged by visual inspection of the plots of weighted residuals and the statistical parameter χ_R^2 and shape of the autocorrelation function of the weighted residuals, and serial variance ratio (SVR).

Determination of equilibrium constants

In the fluorimetric titration method the equilibrium constants are usually calculated using the non-linear least-square methods applying following equation [19, 20, 30–32], for 1:1 stoichiometry:

$$I_f = \frac{I^0_{EN} + I^0_{EN\cdot\beta CD} K [CD]_0}{1 + K[CD]_0} \tag{3}$$

and for successive 1:2 complex formation:

$$I_f = \frac{I^0_{EN} + I^0_{EN\cdot\beta CD} K_1 [CD]_0 + I^0_{EN\cdot\beta CD^2} K_1 K_2 [CD]_0^2}{1 + K_1[CD]_0 + K_1 K_2 [CD]_0^2} \tag{4}$$

where: I_f—the fluorescence intensity of the chromophore in the presence of various [CD] concentration; I^0_{EN}—the fluorescence intensity of the chromophore in water; $I^0{}_{EN\beta CD}$ and $I^0_{EN\beta CD}{}^2$—the fluorescence intensity of 'pure' 1:1 (EN:βCD) and 1:2 (EN:βCD^2) complexes, whereas K_1

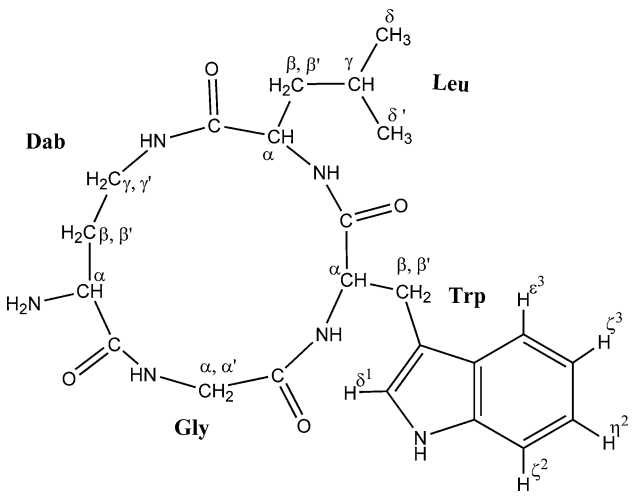

Fig. 1 Structure of cyclic [Leu]enkephalin

and K_2—denote the stepwise association constants for 1:1 and 1:2 complexes, respectively.

Equations 3 and 4 are valid for a large excess of CD over dyes and with the assumption that during the excited state lifetime the conversion of the uncomplexed dye to the complexed one and vice versa can be excluded since the corresponding guest exchange rate constants are small [35–37].

Calorimetric titration

The calorimetric titrations were performed with a Microcal Omega Titration Calorimeter (Northampton, MA). All experiments were performed at 25 °C in water. Because of low solubility of the ligands studied we titrated low concentration of the ligand with better soluble β-CD. Typically, 27 injection of 10 μL of β-CD solution (about 15 mM) were injected into a calorimetric cell containing ligand (concentrations of β-CD and ligands are shown in Table 3). Control experiments were performed to determine the heat of dilution for β-CD. Additionally, in the case where the binding constant was low, 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 constants.

NMR measurements

Proton NMR spectra were recorded on a 499.89 MHz Varian spectrometer at the Nuclear Magnetic Resonance Laboratory at the Technical University of Gdańsk. The experiments were carried out in D_2O. Two-dimensional ^1H-^1H nuclear Overhauser enhanced spectroscopy (NOESY) spectra were recorded at 30 °C, the mixing time (τ_{mix}) was 0.50 s. The NMR spectra were processed using VNMR 4.3 Varian Unity 500 Plus software.

Results and discussion

Steady-state fluorescence spectra

Addition of the β-CD to the aqueous solution of D- or L-EN caused not only a decrease of tryptophan fluorescence intensity but also changed the shape and position of its emission band (Fig. 2). The fluorescence band position of indole [38] as well as tryptophan [39] depends on polarity of its surrounding. The polarity of β-CD cavity is estimated to be close to that of 2-propanol [21] or ethanol [32]. Thus, the shift of Trp residue emission spectra of enkephalins to shorter wavelengths upon addition of cyclodextrin proves that the complex between Trp and CD forms. The emission spectra changes for both analogues are not identical, although they apply to the same fluorophore. In the case of D-EN, the emission band is blue-shifted without the significant change of the fluorescence intensity, whereas for L-EN the band is less shifted but the change of the fluorescence intensity is greater than for D-EN. Those results indicate that mutual orientation of Trp residue and peptide chain in each enkephalin must be different and probably is a result of Leu residue chirality as the difference between D-EN and L-EN relates only to the Leu configuration. The fluorescence quantum yields of both enkephalins studied are different and equal 0.063 and 0.187 for D-EN and L-EN, respectively [4]. Quenching of the indole fluorescence by peptide bond [40] in the case of D-EN suggests that the chromophore is closer to the peptide chain than in the case

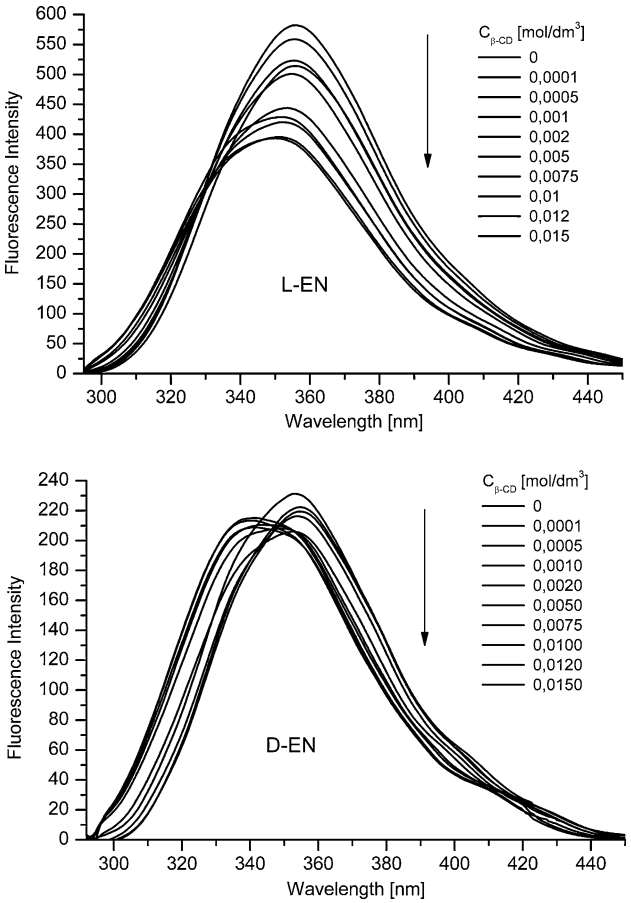

Fig. 2 The changes of fluorescence spectra of Trp residue upon addition of β-CD of L-EN (top) and D-EN (bottom) solution

of L-EN. Lowering the fluorescence intensity and shortening the fluorescence lifetime (see next paragraph) of both enkephalins observed upon β-CD addition, more obvious for L-EN than for D-EN, suggest conformational changes which lead to spatial reorientation of Trp resulting in its stronger interaction with peptide chain or/and forming stronger hydrogen bond between β-CD and nitrogen atom of Trp residue. However, a complicated pattern of fluorescence, especially lack of isosbestic point in fluorescence titration of both enkephalins indicates on the formation of various complexes rather than simple inclusion complex with 1:1 stoichiometry. Moreover, fitting of the fluorescence data to Eq. 3 or 4 failed (data not shown) confirming to some extent the presence of different type (inclusion and association) of complexes between enkephalin and cyclodextrin in solution.

Time-resolved measurements

Apart from the fluorescence intensity (fluorescence quantum yield) other photophysical properties proportional to the concentration of the free and bound guest molecule can be used for stoichiometry and binding constant calculation [19, 35, 41]. For a two component system normalized pre-exponential factors (α) of the fluorescence lifetime for all forms present in the solution obtained from the global analysis of the fluorescence intensity decays as well as the average fluorescence lifetime defined by Eq. 2 can be used for such analysis [19, 41, 42]. The fluorescence intensity decays of Trp residue of enkephalins studied with and without cyclodextrin are highly heterogenous (three exponents are needed for correct describing the fluorescence intensity decay for high CD concentration, Tables 1 and 2). Thus, it is impossible to assign particular lifetime to a particular form of enkephalin present in the solution in spite of observed regular changes of pre-exponential factors with increasing cyclodextrin concentration (Fig. 3). Moreover, application of average lifetime for estimation of binding constant give unreasonable results [42]. Nevertheless, different shape of the dependence of pre-exponential factor on β-CD concentration for each enkephalin studied as well as shorter average fluorescence lifetime $<\tau>_{av}$ (Tables 1 and 2) of D-EN than that of L-EN confirm the influence of Leu chirality on the conformation of enkephalin and its binding with β-CD.

Microcalorimetric titration

Reliable values of equilibrium constant and thermodynamic parameters of the complexation process can be obtained from microcalorimetric titration. In spite of formation of different type of complexes, in the case of microcalorimetric titration formation of only one complex with 1:1 stoichiometry was applied because of low molar ratio of enkephalin to cyclodextrin (about 2.5, much lower than in the fluorescence titration). The fit was not perfect but only this method give reasonable results and acceptable errors of fitted parameters and such data are presented in Table 3. A binding constant of D-EN is higher than that of L-EN. Interactions of enkephalins with β-CD are enthalpy driven in both cases. However, enthalpy of complexation obtained for both enkephalins are, in the range of experimental error, the same. Thus, the enthalpy of complexation obtained for D-EN and L-EN do not indicate that the conformation of D-EN facilitates the entrance of Trp residue to cyclodextrin cavity and strengthens their interaction in comparison with L-EN. For both enkephalins, regardless of Leu configuration, the decrease of entropy is observed, and the biggest changes are observed for L-EN, however, the differences are not so high. The decrease of entropy is probably a result of desolvation of Trp residue and peptide chain during complexation process [43]. Thus, weaker binding of L-EN to cyclodextrin is a result of entropy changes. The enkephalin molecule has two amino acid side chains which are able to form inclusion complex, Trp and Leu. The literature data concerning the formation of the inclusion complex with amino acid derivatives are not

Table 1 Fluorescence intensity decay parameters and average fluorescence lifetime of L-EN in water and for different β-cyclodextrin concentrations

C_{CD} [mol/dm^3]	τ_1 [ns]	τ_2 [ns]	τ_3 [ns]	α_1	α_2	α_3	χ^2_R	τ_{av} [ns]
0	4.26			1.000			1.71	3.98
	4.34	1.00		0.891	0.109		1.30	
0	4.04			1.000			1.63	3.68
	4.11	0.71		0.873	0.127		1.17	
0	4.00			1.000			1.98	3.62
	4.12	0.99		0.840	0.160		1.13	
0	3.98			1.000			2.21	3.57
	4.13	1.04		0.818	0.182		1.09	
0.002	3.93			1.000			3.54	3.38
	4.18	1.10		0.742	0.258		1.13	
0.005	3.82			1.000			6.68	3.1
	4.26	1.18		0.622	0.378		1.19	
0.007	3.93			1.000			9.39	3.09
	4.54	1.26		0.557	0.443		1.13	
0.01	3.71			1.000			9.78	2.86
	4.31	1.18		0.537	0.463		1.15	
0.0119	3.68			1.000			10.33	
	4.35	1.18		0.519	0.481		1.26	2.85
	4.62	2.44	0.85	0.390	0.271	0.339	1.18	2.75
0.0151	3.64			1.000			10.77	
	4.29	1.15		0.516	0.484		1.26	2.77
	4.45	1.93	0.7	0.429	0.273	0.298	1.28	2.64

Table 2 Fluorescence intensity decay parameters and average fluorescence lifetime of D-EN in water and for different β-cyclodextrin concentrations

C_{CD} [mol/dm^3]	τ_1 [ns]	τ_2 [ns]	τ_3 [ns]	α_1	α_2	α_3	χ^2_R	τ_{av} [ns]
0	1.58			1.000			1.87	1.48
	1.77	1.03		0.611	0.389		1.08	
0	1.45			1.000			3.07	1.39
	1.70	1.03		0.543	0.457		1.08	
0	1.45			1.000			3.53	1.38
	1.69	0.95		0.577	0.423		1.04	
0.001	1.44			1.000			3.99	1.37
	1.80	1.07		0.422	0.578		1.12	
0.002	1.44			1.000			4.68	1.37
	1.87	1.07		0.371	0.629		1.15	
0.005	1.43			1.000			6.47	1.34
	2.09	1.11		0.239	0.761		1.18	
0.007	1.40			1.000			7.56	
	1.97	0.94		0.313	0.687		1.35	1.28
	1.33	0.46	2.86	0.731	0.210	0.059	1.12	1.24
0.01	1.44			1.000			7.48	
	2.00	0.98		0.673	0.327		1.38	1.32
	1.45	0.57	3.7	0.727	0.245	0.025	1.07	1.29
0.0119	1.40			1.000			8.27	
	2.28	1.10		0.182	0.818		1.25	1.32
	1.38	0.51	3.47	0.718	0.248	0.034	1.11	1.24
0.0151	1.38			1.000			8.91	
	2.27	1.06		0.187	0.813		1.25	1.29
	1.62	0.89	4.17	0.448	0.536	0.016	1.19	1.27

abundant. For Ac-Trp the binding constants of the complex formation with β-CD is higher (17.1 [44]) than that of Leu residue (K = 9.7) [45]. The obtained binding constants for enkephalins studied are higher than literature data for aforementioned amino acid confirming participation of the whole enkephalin molecule in guest-host interactions. However, because of assumption made in fitting procedure in microcalorimetric titration the obtained results are only apparent values of the complexation process.

^{1}H NMR studies

2D NOESY spectra of L-EN and D-EN with β-CD in D_2O presenting a cross-peaks indicating on dipole–dipole interactions between the protons of β-CD and Trp residue are presented in Fig 4a for L-EN and 4b for D-EN, respectively. Moreover, in Fig 5a and b cross-peaks indicating on dipole–dipole interactions between the protons of Leu residue and CD for L-EN and D-EN, respectively, are also presented. Additionally, in Fig. 6a and b cross-peaks indicating on dipole–dipole interactions between Trp and Leu residues for both enkephalins are shown. As can be

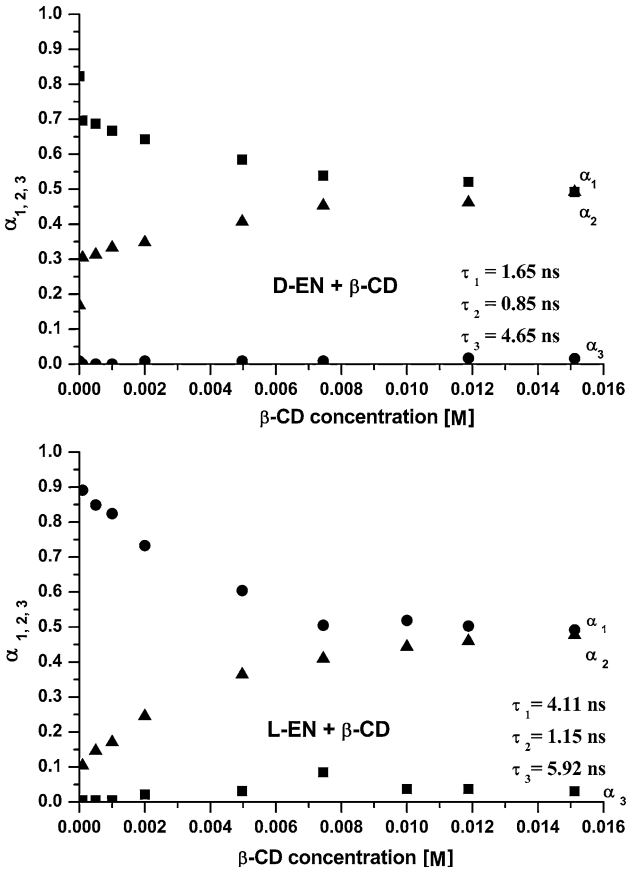

Fig. 3 The dependence of normalized ($\Sigma\alpha_i = 1$) pre-exponential factors obtained from global analysis of Trp fluorescence intensity decays of enkephalins on β-CD concentration. The global parameters were fluorescence lifetimes equal $\tau_1 = 1.65$ ns, $\tau_2 = 0.85$ ns and $\tau_3 = 4.65$ ns for D-EN (top) and $\tau_1 = 4.11$ ns, $\tau_2 = 1.15$ ns and $\tau_3 = 5.92$ ns for L-EN (bottom), respectively

seen, the chemical shifts of protons of particular amino acid residue are different for each enkephalin, however, the biggest differences are observed for Leu residue. For L-EN the chemical shifts of protons of methyl groups are at 0.218 and 0.542 ppm ($\Delta\delta = 0.324$ ppm, Figs. 5a and 6a), whereas for D-EN at 0.125 and 0.645 ppm ($\Delta\delta = 0.520$ ppm, Figs. 5b and 6b). Smaller but distinct differences between position of the particular proton signals in both L- and D-EN are observed also for $C^{\alpha}H$ proton of Dab residue as well as for one of $C^{\alpha}H$ glycine protons (not shown). Indole protons for both enkephalins are in the same order, however, for each ekephalin they are positioned at different δ value. In the presence of the peptide, oligosaccharide protons are also shifted in comparison with the free β-CD (for β-CD proton description, see Fig. 7). The biggest shift is observed for internal H^3 proton of β-CD equal $\Delta\delta = 0.036$ ppm and $\Delta\delta = 0.055$ ppm for D-EN and L-EN, respectively, smaller one for H^5 whereas the shift of external ones (H^6 and H^2) is negligible. On presented NOESY spectra dipole–dipole interactions between

Table 3 Equilibrium constants for complex formation of D- and L-EN with β-CD in aqueous solution obtained based on microcalorimetric titration at T = 298 K, concentration of β-CD = 13.77 mM

Compound	Concentration [mM]	K [M^{-1}]	ΔG [kJ/mol]	ΔH [kJ/mol]	ΔS [J/mol*K]	$T\Delta S$ [kJ/mol]
D-EN	0.803	269 ± 27	−14.1	−20.8 ± 1.2	−22.5	−6.7
L-EN	0.745	162 ± 4	−12.6	−20.3 ± 0.4	−26.0	−7.7

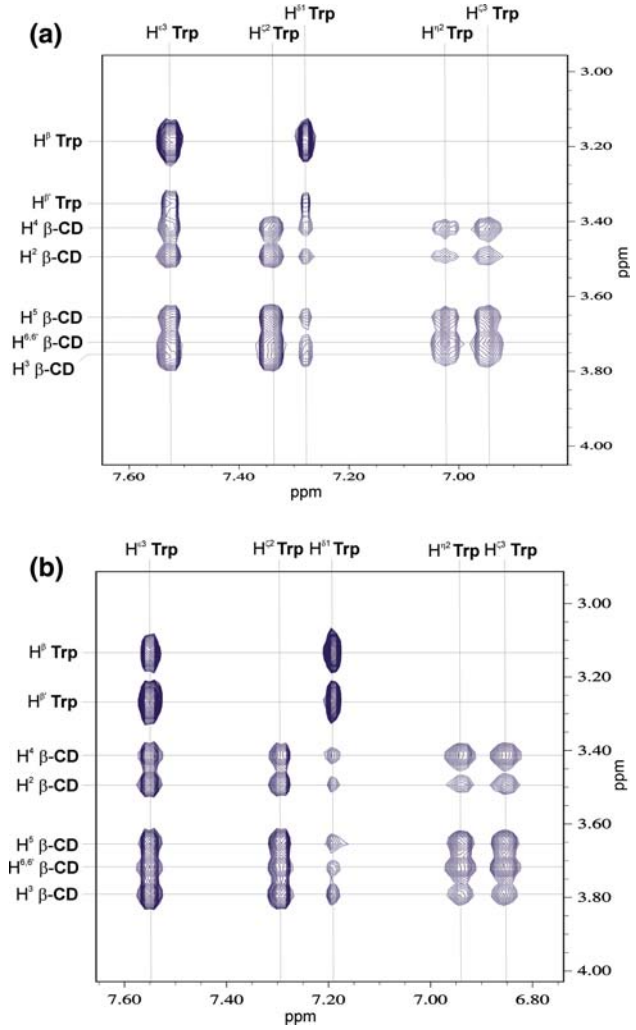

Fig. 4 NOESY NMR spectrum of (**a**) L-EN and (**b**) D-EN complexes with β-CD. The crosspeaks indicating the interaction of β-CD and Trp are presented

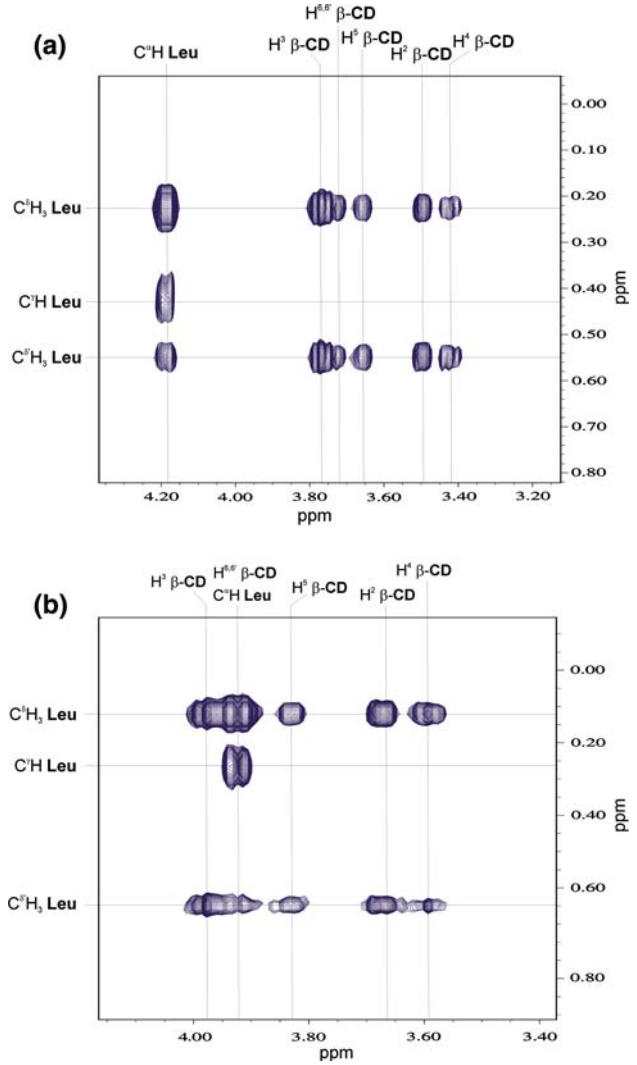

Fig. 5 NOESY NMR spectrum of (**a**) L-EN and (**b**) D-EN complexes with β-CD. The crosspeaks indicating the interaction of β-CD and Leu are presented

indole and both methyl groups of leucine protons and internal (H^3 and H^5) and external (H^2 and H^6) β-CD protons are observed (Fig. 4a, b; Fig. 5a and b, respectively). Additionally, for both enkephalin analogues dipole–dipole interactions between indole and both methyl groups of leucine protons are observed too (Fig. 6a and b).

The comparison of the strength of dipole–dipole interactions (distances) between particular protons between two studied complexes of EN with βCD can be done taking into the mind that the concentration of both complexes are different and equal D-EN · β-CD/D-EN = 0.78 and L-EN · β-CD/L-EN = 0.66, respectively (calculated based on equilibrium constant obtained from microcalorimetric titration assumed that the stoichiometry used in the microcalorimetric studies holds also in NMR experiments). The strength of dipole–dipole interactions between protons of Trp or Leu residue and cyclodextrin protons as well as between Leu and Trp residues protons are presented in Figs. 8a, b and 9a, b and 10a, b as a thickness of line (determined based on a volume of the cross-peak). More

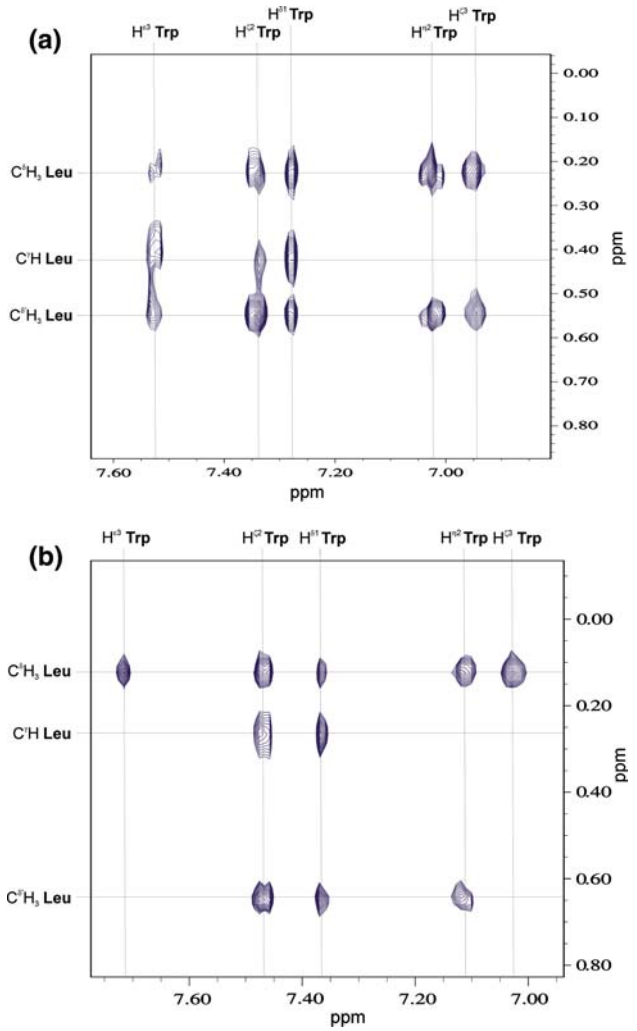

Fig. 6 NOESY NMR spectrum of (**a**) L-EN and (**b**) D-EN complexes with β-CD. The crosspeaks indicating the interaction of Trp and Leu are presented

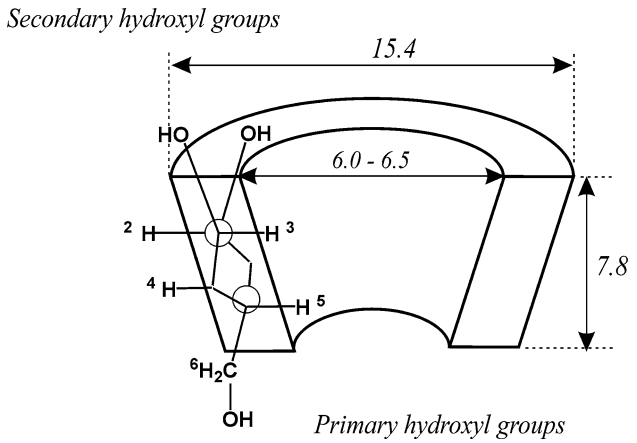

Fig. 7 Structure and protons description of β-CD

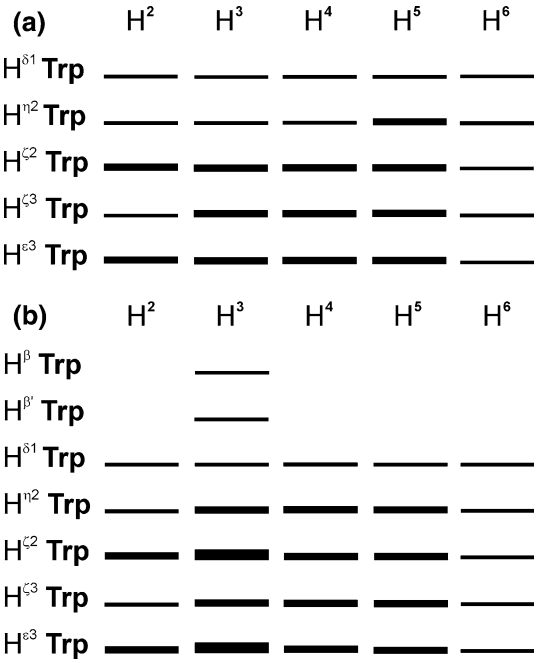

Fig. 8 Weighted volumes of observed signals of pairs of proton belonging to βCD and Trp of (**a**) L-EN·CD and (**b**) D-EN·CD complexes in 2D NOESY spectra

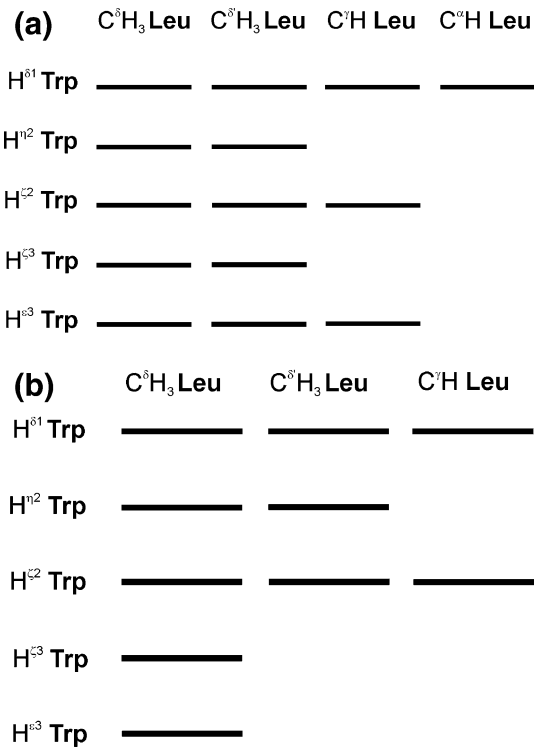

Fig. 9 Weighted volumes of observed signals of pairs of proton belonging to Leu and Trp of (**a**) L-EN·CD and (**b**) D-EN·CD complexes in 2D NOESY spectra

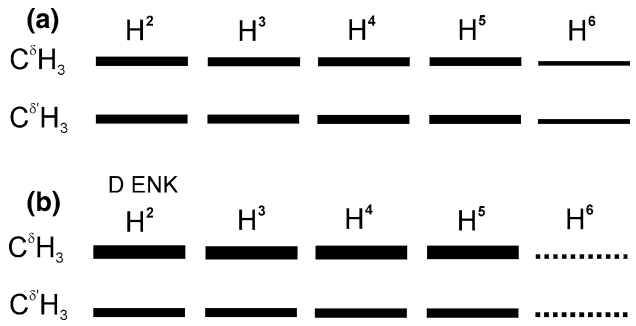

Fig. 10 Weighted volumes of observed signals of pairs of proton belonging to βCD and Leu of (**a**) L-EN·CD and (**b**) D-EN·CD complexs in 2D NOESY spectra

distinct interaction between $C^{\beta}H_2$ protons of Trp and H^3 of glucose (Figs. 11, 8a and b) suggests a deeper penetration of CD cavity by indole ring of D-EN than L-EN for which such interactions are not observed. Additionally, for D-EN distances between protons of both Leu methyl groups and CD protons are more diversified (Fig. 10a and b), whereas for L-EN analogue they are nearly equal. Such differences have to be a result of different spatial orientation of methyl groups of Leu in both analogues in relation to the indole ring as well as cyclodextrin molecule. For D-EN, one of Leu methyl group is much closer to the indole ring than the other, while for L-EN these distances are less diversified (Fig. 9a and b). Moreover, only for L-EN both methyl groups interact with four indole protons (Fig. 9a). Furthermore, only for L-EN, interaction between $C^{\alpha}H$ of Leu and $H^{\delta 1}$ of indole proton is observed (Fig. 12) what suggests that the plane made by $^{\delta}CH_3$-$C^{\gamma}H$-$^{\delta'}CH_3$ carbon atoms is almost parallel to the plane of indole ring whereas for D-EN it is turned round some angle.

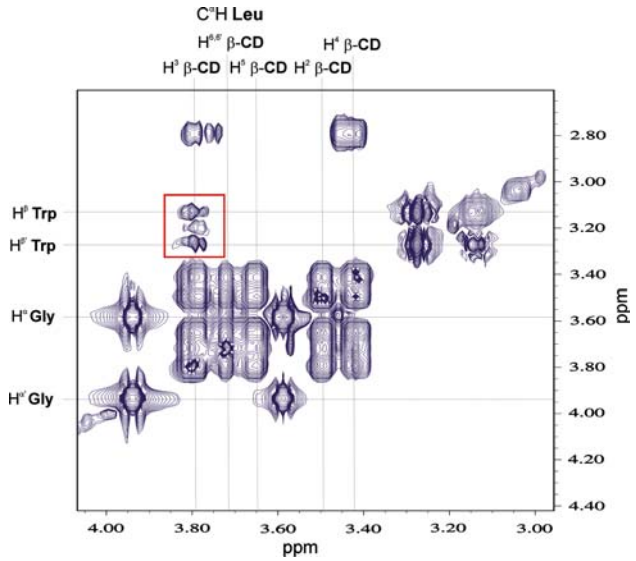

Fig. 11 NOESY NMR spectrum of L-EN complex with β-CD. The crosspeaks indicating the interactions of H^{β} protons of Trp with H^3 β-CD proton are presented

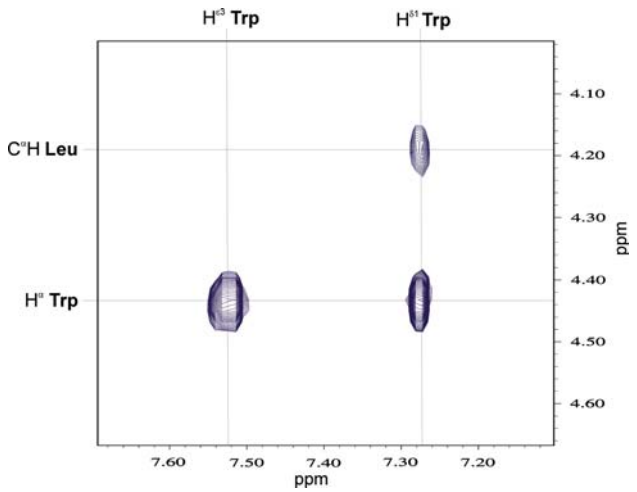

Fig. 12 NOESY NMR spectrum of L-EN complex with β-CD. The crosspeak indicating the interaction of $H^{\delta 1}$ proton of Trp with $C^{\alpha}H$ of Leu proton is presented

As can be seen from Fig. 4a, b and Fig. 8a, b for both enkephalins studied the distances between H^3 and H^5 cyclodextrin protons and indole protons are very similar indicating on the formation of two types of inclusion complexes in which indole ring penetrates cyclodextrin cavity from both sides—from primary and secondary hydroxyl groups. The presence of crosspeaks between leucine methyl groups and cyclodextrin protons (H^2, H^3, H^4, H^5 and H^6) demonstrates that also Leu residue penetrates CD cavity from both sides of cyclodextrin. Moreover, the Leu configuration influences the depth of penetration by particular methyl group (Fig. 10a, b). Because internal diameter of β-CD is about 6.5 Å (Fig. 7), it is rather impossible that both Trp and Leu residues penetrate CD cavity simultaneously. In our opinion, in spite of low binding constant of tryptophan and leucine itself, two different types of complexes are formed; one with Trp residue incorporated into CD cavity and the other one with leucine residue. A formation of these complexes is possible because the peptide chain and CD hydroxyl groups interact by means of hydrogen bonds [46]. Moreover, the presence of crosspeaks between external protons of CD (H^2, H^4 and H^6) and indole protons suggests that beside inclusion complexes an outside association and/or bottom association type of complexes are formed. The thickness of CD wall (about 4.5–5 Å) seems to be too big to observe the interactions of included indole or leucine protons with an external CD protons. The same type of interaction of methyl protons of leucine residue is observed as well, however, for D-EN contrary to L-EN, the interaction with H^6 CD protons is difficult to unequivocally describe. Different type of complexes (inclusion, outside and bottom association) were also suggested by Nau and Zhang for 2,3-diazabicyclo[2,2,2]oct-2-ene and β-CD [34].

However, as suggested by one of the referees, for the fraction of molecule showing the CD/Leu or CD/Trp inclusion the Trp or Leu residue may interacts with the nearby external wall of the CD ring, thus the formation of association complexes can be ruled out. The presence of multiple forms of complex with unknown binding constants in solution makes the analysis difficult and causes that the distances thus derived are rather less reliable. For these reasons we do not calculate the binding constant using the NMR spectroscopy method. However, general consistence of data obtained from different methods support such interpretation.

Conclusion

Incorporation of L-Leu or D-Leu residue into a peptide chain of cyclic enkephalin changes the spatial conformation of enkephalin itself as well as changes the mutual orientation of Trp and Leu side chains. Different chirality of Leu in L- and D-EN manifested by diversified interaction with cyclodextrin causes differences in the binding constant as well as mutual orientation of host–guest molecules. Beside a small binding constant of Ac-Trp and Leu amino acid alone, interaction of the peptide chain with CD molecule increases stability of a complex formed by enkephalin. The presence of two side chains which are able to penetrate CD cavity as well as possibility of hydrogen bonds formation between CD and a peptide chain and hydrophobic interaction of the peptide side chains causes a formation of mixed complexes. The analysis of 2D NMR spectra of complexes of enkephalins studied with β-CD allows to determine the spatial and mutual differences between host and guest molecules as well as the spatial orientation of Trp and Leu residues caused by different leucine chirality. However, the binding of enkephalins studied to β-cyclodextrin is too weak to consider it as a carrier or enantiomeric discriminator.

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