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ORIGINAL ARTICLE

# Influence of Dab$^2$ and Pro$^3$ configuration of [Leu]-enkephalins on the interactions with $\beta$-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). Herein we describe the influence of Dab and Pro chirality of cyclic [Leu]enkephalins (X$^1$-c[Dab$^2$-Pro$^3$-$\beta$Nal(2)$^4$-Leu$^5$], where X = Tyr or Phe) on the binding constant with $\beta$-cyclodextrin and spatial and mutual orientation of guest and host molecules. The formation of complexes is enthalpy driven for all cyclic [Leu]enkephalins studied as well as for Nal and AcNalNH$_2$. Moreover, change of Dab residue configuration has a greater influence on changes of the binding constant of cyclic enkephalin with $\beta$-CD than change of Pro chirality has. Also, the replacement of Tyr$^1$ residue by Phe$^1$ substantially changes the peptide chain conformation. An analysis of 2D NMR spectra reveals that, apart from inclusion complex formed by penetration of cyclodextrin cavity from wider and narrow rims by Nal, Tyr or Phe or Leu residue, a side and/or bottom association complexes are formed.

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

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## Introduction

The main elements of opioid system are endogenous peptides which are natural ligands for opioid receptors ($\mu$, $\delta$, $\kappa$). Enkephalins are a part of $\beta$-endorphin and interact mainly with $\delta$-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. Apart from the protection of peptide bond, cyclodextrins are used as drug delivery systems [11, 12]. The $\alpha$-, $\beta$-, and $\gamma$-cyclodextrins (CD) are polysaccharides consisting of six to eight D-glucopyranose residues, respectively, linked by $\alpha$-1,4 glycosidic bonds into a macromolecule. Each CD 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 [13, 14]. Moreover, CDs can recognize not only the size and shape but also the chirality of amino acids and their derivatives [15]. 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 [16–20]. 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]-enkephalins with $\beta$-CD in water solution. The guest molecules contain aromatic amino acids residues (Nal, Tyr or Phe) as well as Leu residue which are able to form stable complexes with $\beta$-CD. The literature data concerning the formation of the inclusion complexes with amino acid derivatives are not abundant. From aforementioned amino acid residues the lowest binding constant of the complex formation with $\beta$-CD possesses Leu residue ($K = 9.7\ M^{-1}$) [21] whereas a little higher values are observed for Phe ($K = 11\ M^{-1}$ [22], $K = 18\ M^{-1}$ [23]) and $PheNH_2$ ($K = 22\ M^{-1}$ [24]). More data exists for Tyr residue and its derivatives. Binding constant of Tyr zwitterion with $\beta$-CD in water solution is about $50\ M^{-1}$ [22, 25–27] and increases to about $K = 130–150\ M^{-1}$ for AcTyr [22, 25] and $K = 120\ M^{-1}$ for $AcTyrNH_2$ [28]. There is a lack of data concerning the interactions of 2-Nal and its derivatives with $\beta$-CD. However, the binding of naphthalene and its derivatives has been extensively studied. In the most of these studies a 1:1 complexation stoichiometry was assumed and the binding constants of 2-alkylnaphthalene derivatives with $\beta$-CD are about $700\ M^{-1}$ [29, 30], however, much higher ($K = 1190\ M^{-1}$ [31]) and much lower ($K = 200\ M^{-1}$ [32]) values were also presented in the literature.

Herein we studied the influence of Dab and Pro chirality of cyclic [Leu]enkephalins ($X^1$-c[$Dab^2$-$Pro^3$-$\beta Nal(2)^4$-$Leu^5$], where X = Tyr or Phe) (Fig. 1) on the binding constant with $\beta$-CD in water as well as spatial and mutual orientation of guest and host molecules. In our studies we applied fluorescence spectroscopy, microcalorimetry and high resolution $^1H$ NMR spectroscopy (two-dimensional $^1H$–$^1H$ 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 [17, 22–26, 33–42].

## Experimental

### Materials and methods

$\beta$-CD was purchased from Roth, whereas cyclic analogues of [Leu]enkephalins ($X^1$-c[$Dab^2$-$Pro^3$-$\beta Nal(2)^4$-$Leu^5$], where X = Tyr or Phe) (see Table 1 for sequence and abbreviations) were synthesized according to the procedure published previously [5, 6]. The solutions were prepared by dissolving the appropriate amount of $\beta$-CD in water.

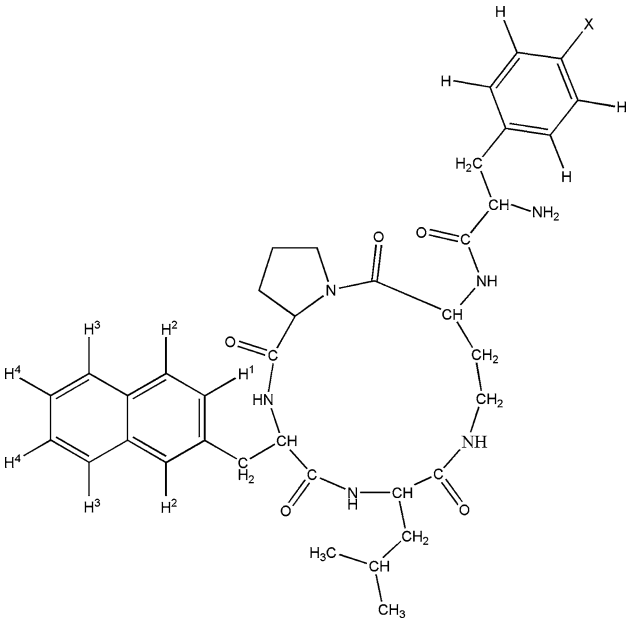

**Fig. 1** Structure of cyclic [Leu]enkephalin

**Table 1** Abbreviations and sequences of studied [Leu]-enkephalins

| Abbreviation | Sequence |
|---|---|
| F-[L,L]-EN | $Phe^1$-c[$Dab^2$ -$Pro^3$ -$\beta Nal(2)^4$ -$Leu^5$] |
| F-[L,D]-EN | $Phe^1$-c[$Dab^2$ -D-$Pro^3$ -$\beta Nal(2)^4$ -$Leu^5$] |
| F-D,L]-EN | $Phe^1$-c[D- $Dab^2$ -$Pro^3$ -$\beta Nal(2)^4$ -$Leu^5$] |
| F-[D,D]-EN | $Phe^1$-c[D- $Dab^2$ -D- $Pro^3$ -$\beta Nal(2)^4$ -$Leu^5$] |
| Y-[L,L]-EN | $Tyr^1$-c[$Dab^2$ -$Pro^3$ -$\beta Nal(2)^4$ -$Leu^5$] |
| Y-[L,D]-EN | $Tyr^1$-c[$Dab^2$ -D- $Pro^3$ -$\beta Nal(2)^4$ -$Leu^5$] |
| Y-[D,L]-EN | $Tyr^1$-c[D- $Dab^2$ -$Pro^3$ -$\beta Nal(2)^4$ -$Leu^5$] |
| Y-[D,D]-EN | $Tyr^1$-c[D- $Dab^2$ -D- $Pro^3$ -$\beta Nal(2)^4$ -$Leu^5$] |

### Spectroscopy measurements

Absorption spectra of all enkephalins studied 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 bandwidth for excitation and emission. The steady-state emission spectra were measured at 15, 20, 30, 40 and 50 °C. Temperature was maintained using Julabo F26-MP refrigerated circulator. To the aqueous solution of $\beta$-CD 200 µl of stock solution of appropriate [Leu]enkephalin was added ($c = 3*10^{-5}$ M). The optical density of the sample at the excitation wavelength ($\lambda = 280$ nm) did not exceed 0.1. All measurements were made triplicate.

### Determination of equilibrium constants

In the fluorimetric titration method the equilibrium constants of complex with 1:1 stoichiometry are usually

calculated using the non-linear least-square methods applying following equation [22, 26, 33, 41, 42]:

$$I_f = \frac{I^0_{EN} + I^0_{EN\bullet\beta CD} K[CD]_0}{1 + K[CD]_0} \quad (1)$$

or in linear form:

$$\frac{1}{I_f - I^0_{EN}} = \frac{1}{K\left(I^0_{EN\bullet\beta CD} - I^0_{EN}\right)[CD]_0} + \frac{1}{I^0_{EN\bullet\beta CD} - I^0_{EN}} \quad (2)$$

For successive 1:2 complex formation one obtained:

$$I_f = \frac{I^0_{EN} + I^0_{EN\bullet\beta CD} K_1[CD]_0 + I^0_{EN\bullet\beta CD^2} K_1K_2[CD]^2_0}{1 + K_1[CD]_0 + K_1K_2[CD]^2_0} \quad (3)$$

where $I_f$ is the fluorescence intensity of the chromophore in the presence of various [CD] concentration; $I^0_{EN}$ the fluorescence intensity of the chromophore in water; $I^0_{EN\bullet\beta CD}$ and $I^0_{EN\bullet\beta CD^2}$ the fluorescence intensity of 'pure' 1:1 (EN:$\beta$CD) and 1:2 (EN:$\beta CD^2$) complexes, whereas $K_1$ and $K_2$ denote the stepwise association constants for 1:1 and 1:2 complexes, respectively.

Equations 1, 2 and 3 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 [43–46].

### 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 $\beta$-CD. Typically, 27 injection of 10 µl of $\beta$-CD solution (about 15 mM) were injected into a calorimetric cell containing ligand (concentrations of $\beta$-CD and ligands are shown in Table 3). Control experiments were performed to determine the heat of dilution for $\beta$-CD. Additionally, in the case where the binding constant was low, in a separate experiment 5 injections of the ligand into high concentration of $\beta$-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 ($t_{mix}$) was 0.50 s. In all cases enkephalin concentration was 6 mM whereas $\beta$-CD concentration was 12 mM. The NMR spectra were processed using VNMR 4.3 Varian Unity 500 Plus software.

### Mass spectra measurement

Water solution of the mixture of Y-[D,D-EN] (3 mM) and $\beta$-CD (12 mM) was diluted with methanol in the ratio of 1:1 and 0.1% of HCOOH was added. The analysis was performed using an Applied Biosystem mass spectrometer type Qstar XL MS–MS with nano-spray ionization using nitrogen as a carrier gas at Pomeranian Science and Technology Park, Gdynia, Poland.

## Results and discussion

### Steady-state fluorescence spectra

Addition of the $\beta$-CD to the aqueous solution of [Leu]-enkephalin caused an increase of naphthyl fluorescence intensity, as it was observed for 2-methylnaphthalene [29–32], without changing the shape and position of its structured emission band (Fig. 2). Because of the excitation wavelength used ($\lambda = 280$ nm) as well as low absorption coefficient and low fluorescence quantum yield of Phe its

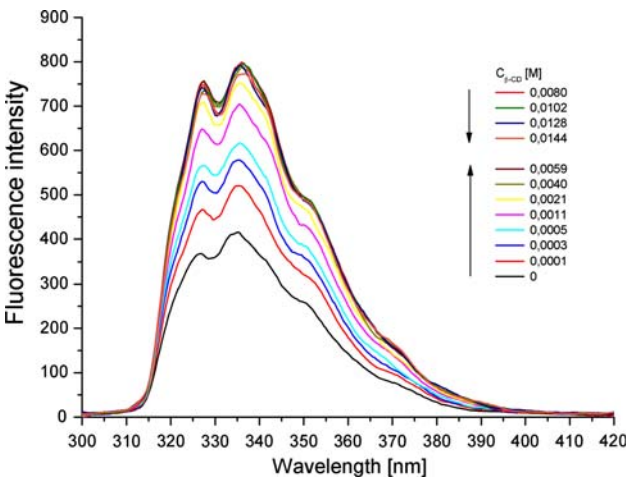


**Fig. 2** The changes of fluorescence spectra of Nal residue upon addition of $\beta$-CD to F-[D,D-EN] solution

emission was not observed for all derivatives of [Leu]-enkephalins containing Phe residue. Moreover, for enkephalin derivatives containing Tyr residue in position 1, which also absorbs excitation light, only in the case of Y-[D,D]-EN, apart from strong naphthyl emission, residual fluorescence of phenol chromophore was observed (Fig. 3). This indicates that the chirality of Dab and Pro residues substantially changes the mutual orientation of the phenol group and the peptide bond which efficiently quenched Tyr fluorescence [47–49]. As can be seen from Figs. 2 and 3 the increase of $\beta$-CD concentration above 0.004 M causes the decrease of naphthyl fluorescence intensity for all studied compounds except Nal. Application of Eq. 2 (Fig. 4) reveals deviation from a straight line for higher CD concentration. Thus, not only a simple complex with 1:1 stoichiometry but also mixed complexes (1:2 or 2:2) could be formed. The formation of inclusion complex with 2:2

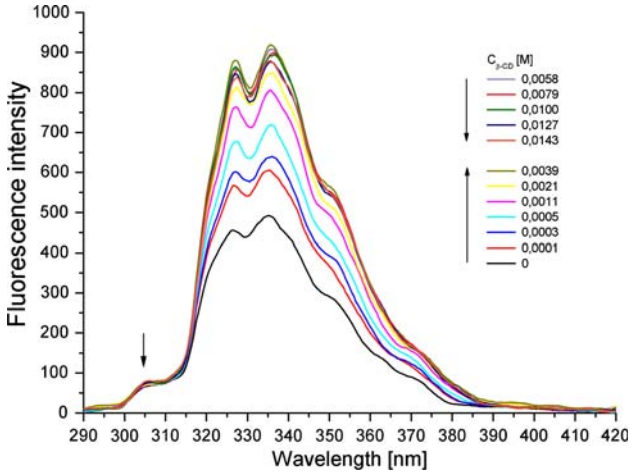


**Fig. 3** The changes of fluorescence spectra of Nal residue upon addition of $\beta$-CD to Y-[D,D-EN] solution

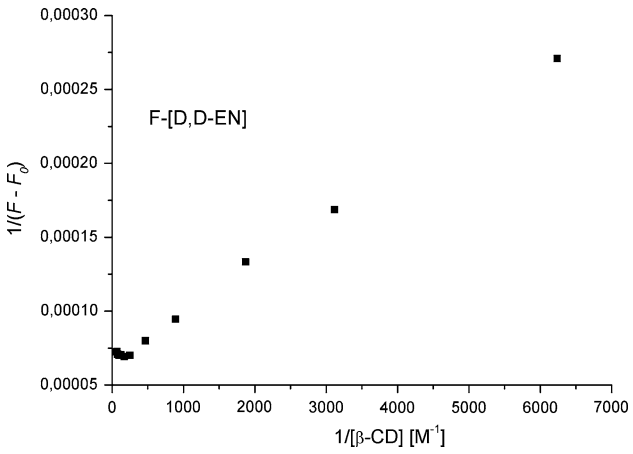


**Fig. 4** The dependence of reciprocal fluorescence intensity of naphthyl fluorescence *versus* reciprocal $\beta$-CD concentration

stoichiometry for naphthalene [50] and 2-methylnaphthalene [31] with $\beta$-CD in water solution was proposed by Hami basing on excimer fluorescence observed for naphthyl fluorophore. The structure of such type of complex for 2-naphthylmethanol and $\beta$-CD in the solid state was presented by Harada et al. [32]. Two $\beta$-CD molecules form a dimer in which the secondary hydroxyl group sides face to each other and two naphthylmethanol molecules are accommodated in the dimer cavity with two naphthyl ring nearly parallel. A parallel arrangement of naphthalene molecules is required for an excimer fluorescence. In our case, we did not observe any excimer fluorescence neither for Nal and $AcNalNH_2$ nor for enkephalins studied. Thus, this type of complex was excluded. Mass spectra recorded for the mixture of Y-[D,D-EN] and $\beta$-CD (1:4) using electro-spray ionization method reveal that apart from a peak of m/z = 1805.66 assigned for the inclusion complex [Leu]-EN:$\beta$-CD with 1:1 stoichiometry another weak peaks on MS spectrum are present: (m/z = 2270.66) which can be assigned to the complex of two $\beta$-CD molecules, (m/z = 2940.99) which can be assigned to the complex of enkephalin:$\beta$-CD with 1:2 stoichiometry, and another very small peaks which origin is unknown (Fig. 5). Thus, our fluorescence data, except for Nal, were analyzed according to the Eq. 3 assuming successive formation of two complexes with 1:1 and 1:2 stoichiometry. An example of nonlinear fitting of integral fluorescence intensity changes versus $\beta$-CD concentration for Nal, $AcNalNH_2$ and F-[D,D-EN] are presented in Fig. 6 whereas obtained binding constants at different temperatures are collected in Table 2. Naphthylalanine with free, unblocked amino acid moiety forms 1:1 inclusion complex with $\beta$-CD because of strong hydration sphere formed around charged amino and carboxyl groups what do not allow for deep penetration of $\beta$-CD cavity [22, 25, 51]. In the case of $AcNalNH_2$, a compound with only one chromophore, mixed complexes are also formed as in the case of enkephalins. The fluorescence data for $AcNalNH_2$ suggest that naphthyl chromophore can penetrate a $\beta$-CD cavity deeper than in the case of Nal, probably in such a way that a part of chromophore protrudes from the cavity enabling incorporation of the second $\beta$-CD molecule. However, in the case of the complex [Leu]-EN:$\beta$-CD 1:2, one $\beta$-CD molecule is bound to the naphthyl moiety whereas the other one may interact with Phe or Tyr or Leu residues changing the position of the naphthyl fluorophore in $\beta$-CD plausibly in such a way that its micro-environment is a little more hydrophilic causing a small decrease of fluorescence intensity. The direct interaction of the naphthyl chromophore of [Leu]-EN with the dimer of $\beta$-CD could be excluded because of the lack of 1:2 Nal:($\beta$-CD)$_2$ complex. Unfortunately, basing only on the fluorescence data unequivocal determination of the structure of the complex with 1:2 stoichiometry is impossible.

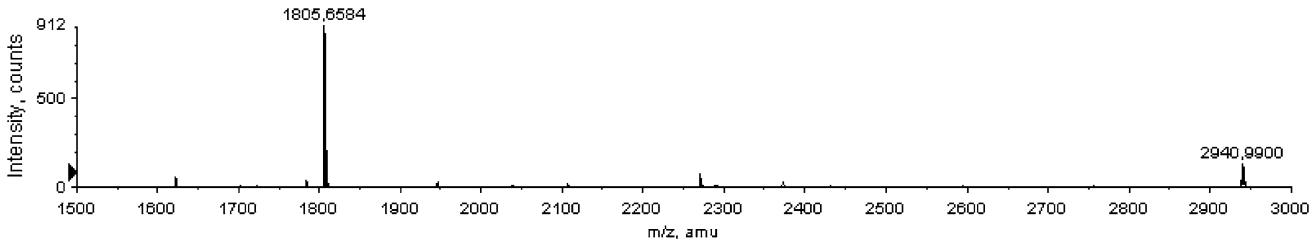


**Fig. 5** MS spectrum of the solution of Y-[D,D-EN] and $\beta$-CD (1:4)

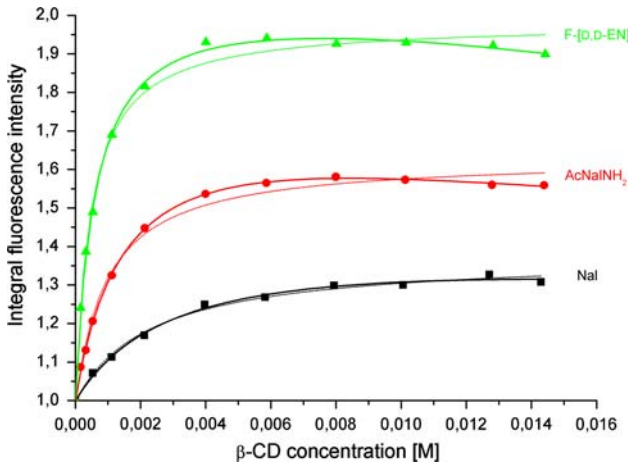


**Fig. 6** Nonlinear fit of fluorescence intensity changes of naphthyl fluorescence versus $\beta$-CD concentration according to Eq. 3

As can be seen from the data presented in Table 2 the values of the binding constants for complexes with 1:2 stoichiometry are much lower than those for 1:1 complexes and also unreliable as determined errors are higher than binding constant at all temperatures studied. The measurements were repeatable, so the high errors of the second equilibrium constant are the results of a small decrease of fluorescence intensity at higher $\beta$-CD concentration and correlation between fitted parameters ($K_1$ and $K_2$) [52]. Thus, only binding constant for 1:1 complex will be discussed. The lower binding constant recorded for Nal compared to that observed for $AcNalNH_2$ is consistent with literature data showing that the ionized guest molecules form weaker inclusion complexes with cyclodextrins than neutral ones [14, 23–25]. Data collected in Table 2 show a substantial influence of the peptide chain on the binding constant of inclusion complexes with $\beta$-CD. Such increase of the binding constants of enkephalins compared to $AcNalNH_2$ is probably a result of stabilization of the inclusion complex by hydrogen bond network between hydroxyl groups of $\beta$-CD and amide bonds of the peptide. The different chirality of Dab and Pro residues as well as the presence of hydroxyl group on phenyl ring of tyrosine caused the conformational changes of the peptide chain of enkephalins studied [5, 6]. Thus, the differences between binding constants among enkephalins studied seems to be connected with different chirality of Dab and Pro residues. Generally, the binding constants of enkephalin analogues containing L-Dab residue are higher than those of D-Dab analogues. Comparatively lower changes of the binding constants are observed for the analogues in which the chirality of Dab residue is conserved but chirality of Pro residue which is next to Nal residue changes (F-[L,L-EN] K = 2470 and F-[L,D-EN] K = 2700; F-[D,L-EN] K = 1850 and F-[D,D-EN] K = 1700). A bigger difference is observed in the case when Dab chirality changes but Pro chirality is the same. This suggests that Dab chirality has greater influence on the peptide chain conformation and accessibility of the naphthyl chromophore to $\beta$-CD in spite of being situated further from Nal residue than Pro residue in the amino acid sequence. The same is also observed for analogues containing Tyr residue. The influence of the hydroxyl group attached to the phenyl ring of amino acid residue in position 1 can be estimated comparing the equilibrium constants for the complexes of the analogues with the same chirality of Dab and Pro residues. The OH group provides a significant enthalpy stabilization for the ligand-$\beta$-CD complex [53]. For the enkephalins studied, tyrosine analogues with D-Dab residue have higher binding constants than those of Phe analogues, e.g.: Y-[L,L-EN] K = 3591 and F-[L,L-EN] K = 2470 as well as Y-[L,D-EN] K = 3565 and F-[L,D-EN] K = 2700. However, for D-Dab analogues there is no difference in the binding constants of Tyr or Phe analogues with $\beta$-CD indicating on the different mutual orientation of Tyr and Phe residues in both D-Dab and L-Dab analogues. Moreover, it indicates that the interactions of Tyr and Phe residues with $\beta$-CD molecule in L-Dab analogues are similar.

## Microcalorimetric titration

Reliable values of equilibrium constant and thermodynamic parameters of the complexation process can be obtained from microcalorimetric titration. In the case of microcalorimetric titration formation of only one complex with 1:1 stoichiometry was applied because of much lower molar ratio of enkephalin to $\beta$-CD than in the fluorescence titration. The fit was not perfect (Fig. 7) probably because

**Table 2** Calculated bindings constants of [Leu]enkephalins as well as Nal and $AcNalNH_2$ with $\beta$-CD studied at different temperature

| Binding constant of inclusion complex with 1:1 stoichiometry $K_1$ ($M^{-1}$)<br>Binding constant of inclusion complex with 2:1 stoichiometry $K_2$ ($M^{-2}$)<br>Correlation coefficient $r^2$ | | | | | | ΔH (kJ/mol) | ΔS (J/mol*K) |
|---|---|---|---|---|---|---|---|
| Tenperature(°C) | 15 | 20 | 30 | 40 | 50 | | |
| F-[L,L-EN] | 2470 ± 590 | 1900 ± 460 | 1170 ± 340 | 760 ± 154 | 224 ± 241 | −39.2 ± 4.4 | −70.9 ± 14.2 |
| | 108 ± 160 | 160 ± 190 | 137 ± 208 | 16 ± 150 | 1226 ± 2090 | $r^2 = 0.982$ | |
| | 0.997 | 0.997 | 0.995 | 0.998 | 0.996 | | |
| F-[L,D-EN] | 2700 ± 175 | 1760 ± 180 | 1330 ± 190 | 370 ± 180 | 98 ± 164 | −48.8 ± 5.4 | −104.0 ± 12.7 |
| | 38 ± 30 | 65 ± 46 | 0 ± 98 | 206 ± 311 | 1020 ± 2610 | $r^2 = 0.953$ | |
| | 0.999 | 0.999 | 0.997 | 0.994 | 0.990 | | |
| F-[D,L-EN] | 1850 ± 260 | 1310 ± 290 | 970 ± 240 | 685 ± 186 | 443 ± 193 | −03.4 ± 3.0 | −43.5 ± 9.7 |
| | 20 ± 61 | 47 ± 103 | 80 ± 190 | 85 ± 207 | 581 ± 640 | $r^2 = 0.983$ | |
| | 0.997 | 0.994 | 0.994 | 0.995 | 0.996 | | |
| F-[D,D-EN] | 1690 ± 200 | 1580 ± 140 | 1050 ± 120 | 787 ± 114 | 421 ± 90 | −26.4 ± 3.5 | −29.5 ± 11.3 |
| | 0 ± 53 | 0 ± 50 | 0 ± 97 | 0 ± 186 | 0 ± 191 | $r^2 = 0.969$ | |
| | 0.998 | 0.999 | 0.999 | 0.997 | 0.995 | | |
| Y-[L,L-EN] | 3590 ± 420 | 3530 ± 390 | 2250 ± 270 | 1880 ± 340 | 1820 ± 480 | −18.4 ± 3.3 | 4.5 ± 11.0 |
| | 0 ± 47 | 0 ± 110 | 0 ± 74 | 231 ± 590 | 0 ± 658 | $r^2 = 0.926$ | |
| | 0.996 | 0.984 | 0.997 | 0.995 | 0.987 | | |
| Y-[L,D-EN] | 3565 ± 360 | 2860 ± 260 | 2090 ± 190 | 1240 ± 240 | 1000 ± 390 | −28.7 ± 1.8 | −31.7 ± 5.9 |
| | 69 ± 86 | 60 ± 50 | 0 ± 65 | 0 ± 107 | 0 ± 610 | $r^2 = 0.993$ | |
| | 0.998 | 0.998 | 0.998 | 0.996 | 0.993 | | |
| Y-[D,L-EN] | 1920 ± 330 | 1500 ± 440 | 1020 ± 360 | 730 ± 250 | 635 ± 220 | −27.2 ± 2.1 | −31.9 ± 7.0 |
| | 150 ± 160 | 180 ± 385 | 148 ± 243 | 122 ± 194 | 35 ± 130 | $r^2 = 0.988$ | |
| | 0.997 | 0.990 | 0.990 | 0.993 | 0.992 | | |
| Y-[D,D-EN] | 1730 ± 190 | 1330 ± 270 | 1050 ± 170 | 900 ± 170 | 709 ± 480 | −19.5 ± 2.4 | −6.2 ± 7.8 |
| | 48 ± 103 | 40 ± 104 | 0 ± 88 | 0 ± 142 | 0 ± 750 | $r^2 = 0.967$ | |
| | 0.998 | 0.995 | 0.997 | 0.996 | 0.995 | | |
| $AcNalNH_2$ | 600 ± 64 | 445 ± 80 | 373 ± 60 | 350 ± 150 | 329 ± 68 | −13.3 ± 4.1 | −5.6 ± 6.4 |
| | 42 ± 63 | 48 ± 97 | 8 ± 83 | 221 ± 384 | 46 ± 176 | $r^2 = 0.839$ | |
| | 0.991 | 0.988 | 0.998 | 0.997 | 0.998 | | |
| Nal | 460 ± 74 | 428 ± 57 | 305 ± 60 | 278 ± 40 | 206 ± 38 | −16.4 ± 1.9 | −5.6 ± 6.4 |
| | 0.991 | 0.994 | 0.989 | 0.995 | 0.992 | $r^2 = 0.958$ | |

The last two columns collect thermodynamic parameters obtained from vant't Hoff equation

of the formation of different types of complexes, but only this method gave reasonable results and acceptable errors of fitted parameters and such data are presented in Table 3. The binding constants obtained from microcalorimetric measurements are lower than those from fluorescence titrations, however, the relation between corresponding enkephalins pairs is conserved. Figure 8 illustrates the correlation between binding constants obtained from microcalorimetric and fluorimetric titrations. In the microcalorimetric titrations the influence of the peptide chain presence as well as its conformation, dependent on chirality of Dab and Pro residues, on the binding constant value is more significant than in the case of fluorimetric titrations. The exceptions are Nal, $AcNalNH_2$ as well as Y-[L,D-EN] and Y-[L,L-EN]. For the first two compounds, devoid of a peptide chain, roughly linear dependence can be established. The introduction of the peptide chain to naphthyl fluorophore caused a jumping increase of the binding constant for about 500 which was not observed for data obtained from microcalorimetric titration indicating that the peptide chain interactions with $\beta$-CD molecule stronger keep naphthyl chromophore inside the $\beta$-CD cavity. Moreover, higher binding constants, determined from fluorescence data, for Y-[L,D-EN] and Y-[L,L-EN] compared to F-[L,D-EN] and F-[L,L-EN] suggest that Tyr residue interacts stronger with $\beta$-CD than Phe residue. For the remaining enkephalins studied, a linear correlation between binding constants obtained from two different

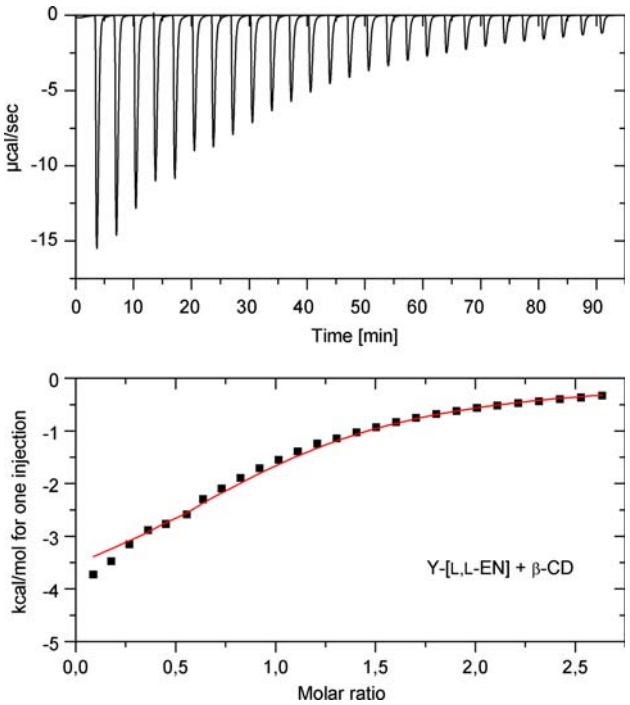


**Fig. 7** Microcalorimetric titration of Y-[D,D-EN] by $\beta$-CD (*top*) and fitting data assuming a formation of the complex with 1:1 stoichiometry (*bottom*)

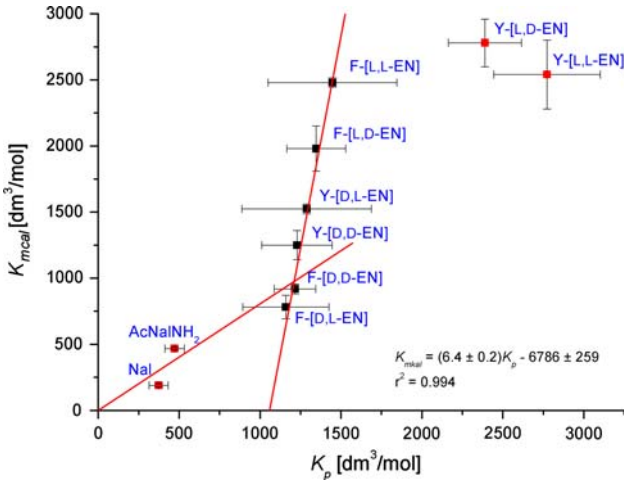


**Fig. 8** Plot of the binding constants values obtained from microcalorimetric titration *versus* the binding constants calculated basing on fluorescence titration

methods holds, however, the slope of the correlation line is high and equal about six indicating on the peptide chain interactions with $\beta$-CD molecules which only partially could be observed by fluorimetric method. This fact can be rationalized taking into account that fluorimetric measurements give information about the changes which proceed in the neighbourhood of the fluorophore. Different chirality of Dab and Pro residues causes the conformational changes of the peptide chain thereby modifying in higher degree its interactions with $\beta$-CD molecules rather than photophysical properties of the naphthyl fluorophore by changing its position inside the $\beta$-CD cavity. Moreover, the microcalorimetric titration gives information about the total heat effects of the interaction of enkephalin and $\beta$-CD molecules. However, easy anchorage of the peptide chain to the $\beta$-CD hydroxyl groups does not have to be connected with strong heat effect, thus relatively small differences between binding constants of Nal, $AcNalNH_2$ and enkephalins are observed in microcalorimetric method opposite to fluorimetry. Also, thermodynamic parameters ($\Delta H$, $\Delta S$) derived by these two methods differ significantly (Tables 2 and 3). Values of enthalpy changes obtained from fluorimetry are in the range from $\Delta H = -13.3$ kJ/mol for $AcNalNH_2$ to $\Delta H = -48.8$ kJ/mol for F-[L,D-EN] whereas from microcalorimetry the enthalpy changes are lower and in the range from $\Delta H = -8.2$ kJ/mol for $AcNalNH_2$ to $\Delta H = -20.4$ kJ/mol for F-[L,D-EN].

**Table 3** Binding constants and thermodynamic parameters of inclusion complex between $\beta$-CD and enkphalin and $AcNalNH_2$ and Nal at 25 °C obtained from microcalorimetry measurements

| | Concentration (mM) | $\beta$-CD (mM) | K ($M^{-1}$) | $\Delta G$ (kJ/mol) | $\Delta H$ (kJ/mol) | $\Delta S$ (J/molK) | $T\Delta S$ (kJ/mol) |
|---|---|---|---|---|---|---|---|
| F-[L,L-EN] | 0.877 | 13.77 | 2480 ± 35 | −19.3 | −18.7 ± 0.1 | 2.1 | 0.6 |
| F-[L,D-EN] | 2.319 | 13.77 | 1980 ± 170 | −18.8 | −20.4 ± 0.4 | −5.6 | −1.7 |
| F-[D,L-EN] | 1.389 | 13.77 | 781 ± 89 | −16.5 | −18.0 ± 0.9 | −5.2 | −1.6 |
| F-[D,D-EN] | 1.020 | 13.77 | 918 ± 37 | −16.9 | −20.0 ± 0.4 | −10.5 | −3.1 |
| Y-[L,L-EN] | 1.141 | 13.77 | 2540 ± 260 | −19.4 | −19.5 ± 0.6 | −0.2 | −0.1 |
| Y-[L,D-EN] | 1.250 | 13.77 | 2780 ± 180 | −19.6 | −16.8 ± 0.3 | 9.4 | 2.8 |
| Y-[D,L-EN] | 0.383 | 13.77 | 1524 ± 37 | −18.1 | −14.4 ± 0.2 | 12.7 | 3.8 |
| Y-[D,D-EN] | 0.850 | 13.77 | 1250 ± 110 | −17.6 | 12.2 ± 0.4 | 16.2 | 5.5 |
| $AcNalNH_2$ | 0.298 | 13.77 | 486 ± 9 | −15.2 | −11.8 ± 0.1 | 11.3 | 3.4 |
| Nal | 0.525 | 13.77 | 190 ± 11 | −12.9 | −8.2 ± 0.3 | 15.9 | 4.8 |

However, some regularity is present. For all cases, the complexation process is enthalpy driven and less negative values of enthalpy changes are obtained for Nal and Ac-NalNH$_2$ than for enkephalins. More distinct differences are observed for entropy changes obtained from microcalorimetric and fluorimetric titrations. From the fluorescence experiment, except for AcNalNH$_2$ and Y-[L,L-EN] for which ΔS values are zero, in the range of experimental error, values of entropy changes are negative whereas microcalorimetric method gives a negative value of ΔS for enkephalins containing in position 1 Phe residue (except F-[L,L-EN]) and positive value for enkephalins containing in position 1 Tyr residue (except Y-[L,L-EN] for which ΔS is close to zero) as well as for Nal and AcNalNH$_2$. The hydrogen bonding between adjacent water molecules at hydrophobic cavity interfaces is weak. Furthermore, interactions between these water molecules and the organic phase result in substantial orientation of these weakly hydrogen-bonded water molecules in the interfacial region causing that the water molecules in the first solvation sphere of the hydrophobic molecule are more ordered compared to bulk water [54–56]. Thus, it seems that the presence of Tyr residue profitably influences on entropy changes because of hydration of hydroxyl group of phenol which perturbs well-ordered water molecules creating a cavity for the hydrophobic phenyl group of Phe residue. The thermodynamic data obtained from microcalorimetric titration are more reliable than that from fluorimetric titration. In microcalorimetric measurements the thermodynamic parameters are obtained directly at constant temperature, while in the fluorimetric titration thermodynamic parameters are obtained from van't Hoff equation with assumption that the heat capacity of the solution is constant in the temperature range of the measurement. Additionally, the obtained thermodynamic parameters are apparent values because enthalpy and entropy changes are connected with inclusion complex formation as well as influence of the temperature on the fluorescence intensity of the fluorophore. However, because of the assumption made in the fitting procedure in fluorimetric and microcalorimetric titration the obtained results are only apparent values characterizing the complexation process.

## The enthalpy–entropy compensation

The compensatory enthalpy–entropy relationship has been often observed empirically [25, 57–63], while no explicit relationship between the enthalpy change and the entropy change can logically be derived from fundamental thermodynamics [51, 64]. In these papers it was shown that the diverse chemical and biochemical supramolecular systems, including cyclodextrins, can be analysed consistently using the following equations:

$$T\Delta\Delta S^0 = \alpha\Delta\Delta H^0 \quad (4)$$

$$T\Delta S^0 = \alpha\Delta H^0 + T\Delta S_0^0 \quad (5)$$

$$\Delta\Delta G^0 = (1-\alpha)\Delta\Delta H^0 \quad (6)$$

Thus, the slope (α) of the $T\Delta S^0$ versus $\Delta H^0$ plot (Eq. 4) indicates to what extent the enthalpic gain ($\Delta\Delta H^0$), which is induced by the alternations in host, guest, or solvent, is cancelled by the accompanying entropic loss ($\Delta\Delta S^0$). In the other words, only a fraction (1−α) of the enthalpic gain can contribute to the enhancement of complex stability. Moreover, the intercept ($T\Delta S_0^0$) represents the inherent complex stability ($\Delta G^0$) obtained at $\Delta H^0 = 0$, which means that the complex is stabilized even in the absence of enthalpic gain, as far as the $T\Delta S_0^0$ term is positive. The slope (α) and the intercept ($T\Delta S_0^0$) of the regression line are related to the degree of conformational changes and the extent of desolvation of both host and guest upon complexation, respectively. However, more recently it has been suggested that the actual source of the compensation effect should be related to contributions due to solvent reorganization [60, 65–67]. The thermodynamic parameters obtained from the fluorimetric and microcalorimetric titration of enkephalins studied with β-CD are presented in Fig. 9. The linear correlation for 8 data points (Nal and AcNalNH$_2$ are excluded from the correlation because those compound do not possess a peptide chain which contributes to the overall enthalpy and entropy changes in complex formation with β-CD) gives the following parameters: $\alpha = 1.01 \pm 0.13$ and $T\Delta S_0^0 = 18.7 \pm 3.7$ kJ/mol with correlation coefficient $r = 0.994$ for the fluorescence method and $\alpha = 0.94 \pm 0.17$ and $T\Delta S_0^0 = 17.2 \pm 2.9$ kJ/mol with correlation coefficient $r = 0.918$ for the microcalorimetric method. Better correlation obtained from fluorimetric data is a result of much bigger errors of the data compared to that from microcalorimetry, however, the fitted parameters obtained for both methods are in the range of the experimental error the same. These data are a little bit higher than the literature values presented by Rekharsky and Inoue [14] for β-cyclodextrin inclusion complexes ($\alpha = 0.80$ and $T\Delta S_0^0 = 11$ kJ/mol and $r = 0.89$ for 488 data points) obtained for more rigid guest molecules, however, they are comparable with data obtained for inclusion complexes of flexible guest molecules ($\alpha = 1.07$ [14]). The value of the slope indicates that the enthalpic gain induced by the system alternations is not reflected in the net increase of complex stability. A relatively large slope obtained for apparently rigid cyclodextrin suggests that the rearrangement of the peripheral hydrogen-bond network and the accompanying skeletal conformation changes should be considered as responsible for this [14, 51, 63]. Moreover, $T\Delta S_0^0 \approx 18$ kJ/mol, which is about two-fold higher than observed for more rigid N-AcTyr amides [28],

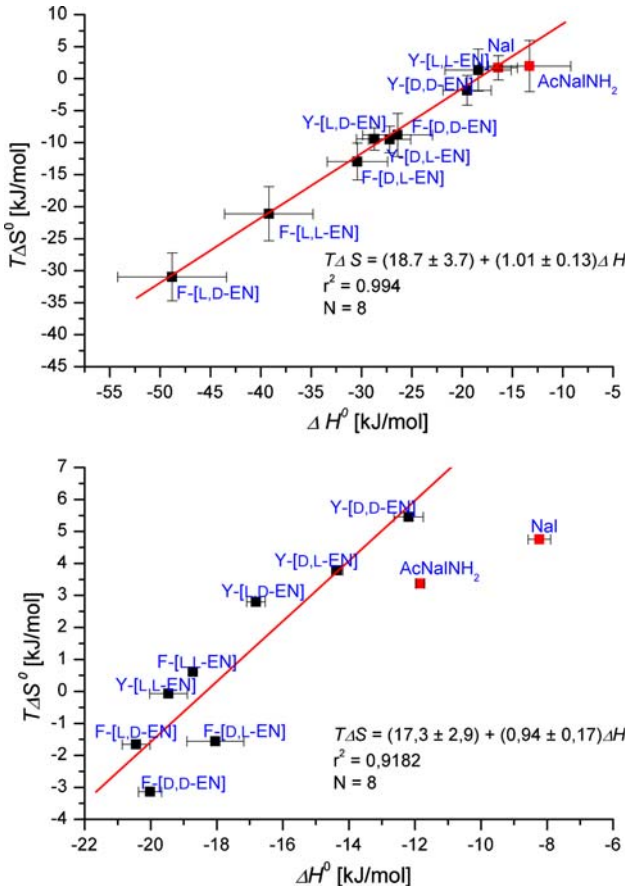


**Fig. 9** Enthalpy–entropy compensation plot for inclusion complexes of enkephalins studied with $\beta$-cyclodextrin in water (pH = 6) obtained from fluorescence titration (correlation coefficient r = 0.994, number of data n = 8) (*top*) and microcalorimetric titration (correlation coefficient r = 0.918, number of data n = 8) (*bottom*)

indicates that the entropic contribution of the solvent reorganization is the major factor governing the Leu-enkephalin-$\beta$-CD complexation.

## $^1$H NMR studies

Two-dimensional NMR spectroscopy is an essential method for the conformational studies of CDs and their complexes since one can conclude that two protons are closely located in the space if a NOE correlation is detected between the relevant protons signal in the NOESY spectrum [68]. Therefore, it is possible to estimate the orientation of the substituent moiety or guest molecule in the CD cavity using the assigned NOE correlation.

Among enkephalins studied three of them Y-[D,D-EN], Y-[L,D-EN] and F-[D,D-EN] were selected for NMR study because the binding constants of Y-[D,D-EN] and Y-[L,D-EN] differ mostly as a result of Dab residue chirality change whereas F-[D,D-EN] was selected to explain why Tyr residue substantially changes the binding constant compared to the analogue containing Phe residue. One dimensional $^1$H NMR spectrum of the solution of Y-[D,D-EN] and CD in $D_2O$ with protons assignment is presented in Fig. 10.

### *$^1$H NMR studies of Y-[D,D-EN]*

Among the enkephalins studied by NMR method, the maximal number of cross-peaks on NOESY spectrum is observed for Y-[D,D-EN]. 2D NOESY spectra presenting cross-peaks indicating on dipole-dipole interactions between the protons of $\beta$-CD and Y-[D,D-EN] are presented in Fig. 11. The strength of the dipole-dipole interactions as a thickness of line (determined basing on a volume of the cross-peak) between the Nal, Tyr or Leu residue protons and the $\beta$-CD protons are presented in Fig. 12. Tyrosine $\delta$ and $\varepsilon$ protons interact in the same degree with internal $H^3$ and $H^5$ protons of $\beta$-CD indicating on the penetration of $\beta$-CD cavity from both wider and narrow sides. The formation of inclusion complex between Tyr residue and $\beta$-CD

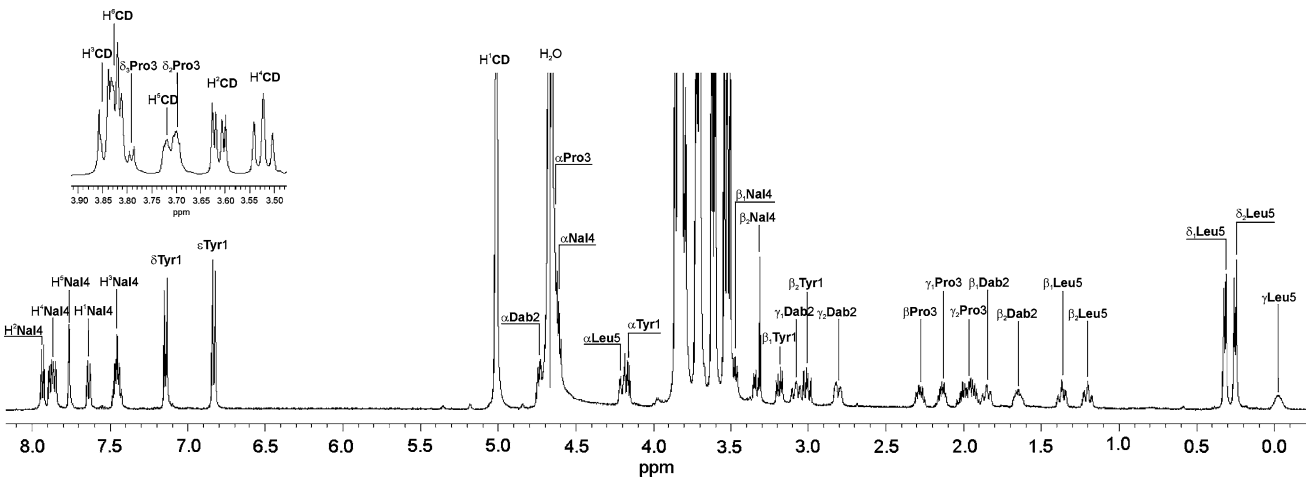


**Fig. 10** One dimensional $^1$H NMR spectrum of Y-[D,D-EN] complex with $\beta$-CD in $D_2O$

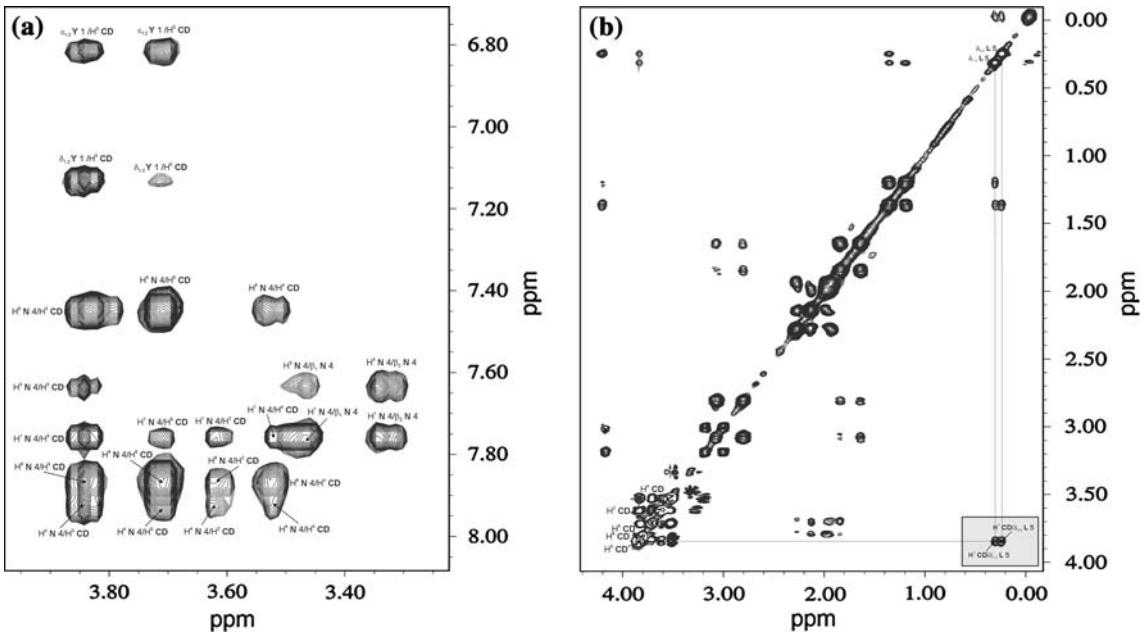


**Fig. 11** NOESY NMR spectrum of Y-[D,D-EN] complex with $\beta$-CD. The crosspeaks indicating the interaction of $\beta$-CD and Nal and Tyr (**a**), and Leu (**b**) are presented

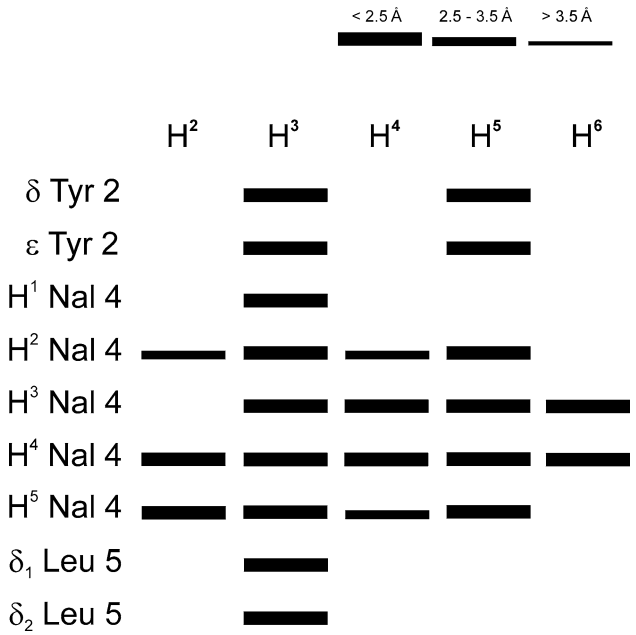


**Fig. 12** The values proportional to the interproton distances between Y-[D,D-EN] and $\beta$-CD protons

precludes the peptide chain to quench Tyr fluorescence because of the shielding effect of $\beta$-CD molecule. Also, the Nal residue can penetrate the CD cavity from both sides. However, for $H^1$ proton of the naphthyl group the interaction with $H^5$ proton of $\beta$-CD is not observed. The lack of such interaction indicates on different depth of the penetration of the naphthyl group inside $\beta$-CD cavity from the narrow side compared to the wider side. This is a result of larger distance between naphthyl protons and internal $\beta$-CD protons probably caused by the presence of primary hydroxyl groups at the narrower side of CD resulting in different arrangement of the peptide chain and different spatial orientation (position) of naphthyl group compared to association from wider side. The formation of inclusion complex by penetration the cavity from both sides of $\beta$-CD torus was already described in the literature [28, 43, 46]. On the 2D NMR spectrum of Y-[D,D-EN]—$\beta$-CD intensive crosspeaks between $H^3$ and $H^4$ protons of naphthalene and $H^6$ protons of $\beta$-CD are also noticeable. These results suggest that naphthyl group deeply penetrates $\beta$-CD cavity in such a way that a part of chromophore protruding outside the $\beta$-CD cavity thereby creating a possibility to interact with a second $\beta$-CD molecule forming with the first one an association complex by hydrogen bond network. Apart from crosspeaks of Nal and Tyr and $\beta$-CD protons, relatively strong interactions between $\delta$ protons of Leu and $H^3$ protons of $\beta$-CD is observed. Taking into account the structure of enkephalin studied, it is rather impossible that $\beta$-CD cavity is simultaneously penetrated by Leu and Tyr or Nal and Leu residues. The interaction of $\delta$ protons of Leu and only $H^3$ protons of $\beta$-CD is probably a result of independent complex formation in which $\delta$ protons of Leu shallowly penetrate $\beta$-CD cavity from the wider side whereas the remaining part of enkephalin is outside $\beta$-CD molecule. Such structure of a complex gives the possibility of independent interaction of Nal or Tyr residue with second $\beta$-CD molecule and forming a complex with 2:1 stoichiometry. In spite of the low binding

constant of tyrosine and leucine itself, two different types of complexes could be formed; one with Tyr residue incorporated into $\beta$-CD cavity and the other one with leucine residue. A formation of these complexes is possible because the peptide chain and $\beta$-CD hydroxyl groups interact by means of hydrogen bonds [51]. Another explanation for the presence of crosspeks of $\delta$ protons of Leu is the formation of association complex in which naphthyl chromophore interacts with external wall of $\beta$-CD cavity which is testified by the presence of crosspeaks between $H^2$, $H^4$ and $H^6$ protons of $\beta$-CD and $H^2$, $H^4$, and $H^5$ protons of naphthyl, while Leu residue shallowly penetrates $\beta$-CD cavity from the wider side. According to the literature data, if only inclusion complex is formed there is no interaction between $H^2$ and $H^4$ cyclodextrin protons and guest molecule encapsulated inside $\beta$-CD cavity because the interproton distance is higher than 5 Å [69]. Thus, the presence of crosspeaks between Nal aromatic protons and $H^2$, $H^4$, $H^6$ $\beta$-CD ones indicates that besides inclusion complex an association complexes are also present. As was shown by Al-Soufi et al. [70] using fluorescence correlation spectroscopy, an association complex is formed before inclusion complex formation. Different types of complexes (inclusion, outside and bottom association) were also suggested by Nau and Zhang for 2,3-diazabicyclo[2]oct-2-ene and $\beta$-CD [43].

### *$^1$H NMR studies of F-[D,D-EN]*

2D NOESY spectra presenting cross-peaks between the protons of $\beta$-CD and F-[D,D-EN] are presented in Fig. 13, while the strength of the dipole-dipole interactions between the Nal, Phe or Leu residue protons and the $\beta$-CD protons are presented in Fig. 14. As can be seen from Figs. 13 and 14, there is no interaction between $H^2$ protons of $\beta$-CD with naphthyl protons. Moreover, the interactions of $H^4$ and $H^6$ $\beta$-CD protons with $H^3$ and $H^4$ naphthyl protons are weak (especially with $H^6$). Also, $H^2$ and $H^5$ protons of the naphthyl group do not interact with $H^4$ protons of $\beta$-CD contrary to those of Y-[D,D-EN]. Additionally, stronger interactions of $H^2$, $H^3$ and $H^4$ naphthyl protons with $H^5$ proton of $\beta$-CD rather than $H^3$ indicate that the naphthyl group shows the preference to be incorporated into the $\beta$-CD cavity from the narrower side. Weak crosspeaks of $H^6$ protons of $\beta$-CD and $H^3$ and $H^4$ naphthyl protons confirm this assumption. Also, Phe residue seems to prefer incorporation into the $\beta$-CD cavity from the narrower side because of stronger interactions of $\delta$ protons with $H^5$ protons of $\beta$-CD than with $H^3$ ones. Rather weak interaction of $\varepsilon$ protons of Phe with $H^3$ and $H^5$ $\beta$-CD protons is also worth noticing. It indicates on different position of the phenyl group inside $\beta$-CD cavity comparing to the phenol group of Y-[D,D-EN]. For both Y-[D,D-EN] and F-[D,D-EN], $\delta$

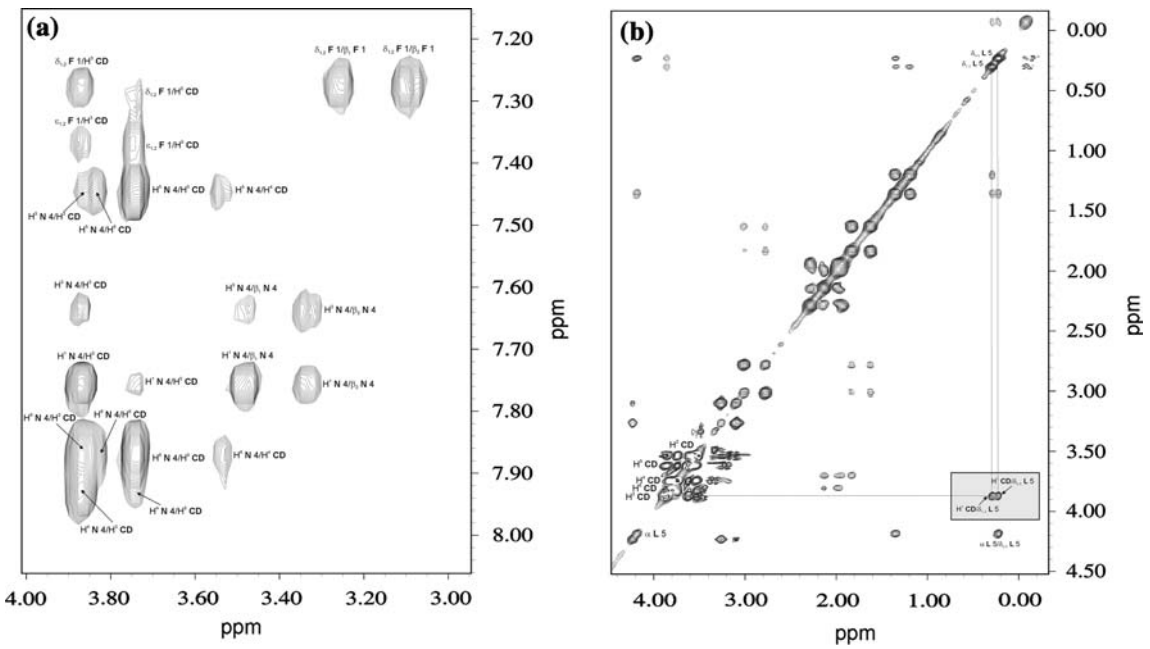


**Fig. 13** NOESY NMR spectrum of F-[D,D-EN] complex with $\beta$-CD. The crosspeaks indicating the interaction of $\beta$-CD and Nal and Phe (**a**) and Leu (**b**) are presented

protons of Leu interact only with $H^3$ protons of $\beta$-CD. Diversified pattern of crosspeaks and strength of interaction between appropriate protons of Y-[D,D-EN] and F-[D,D-EN], as a result of the presence of hydroxyl group on the side chain of the phenyl ring, indicates on the substantial influence of Tyr residue on the conformation of the peptide chain and thereby its interaction with $\beta$-CD modifying the binding constant.

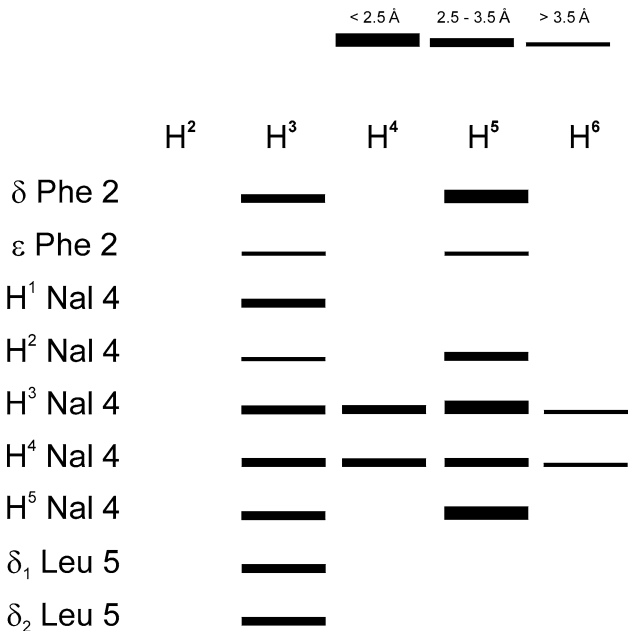


**Fig. 14** The values proportional to the interproton distances between F-[D,D-EN] and $\beta$-CD protons

### *$^1$H NMR studies of Y-[L,D-EN]*

For Y-[L,D-EN] analogue, 2D NOESY spectra are presented in Fig. 15, while the strength of the dipole-dipole interactions between the Nal, Tyr or Leu residue protons and the $\beta$-CD protons are presented in Fig. 16. The pattern of crosspeaks and strength of interaction between appropriate protons of this enkephalin is quite different than observed for Y-[D,D-EN] indicating that the chirality of Dab substantially modifies the peptide chain conformation of the enkephalins studied. First of all, there is no crosspeaks between Tyr and $\beta$-CD protons indicating on the lack of interaction between them. It can be rationalized assuming sticking interactions

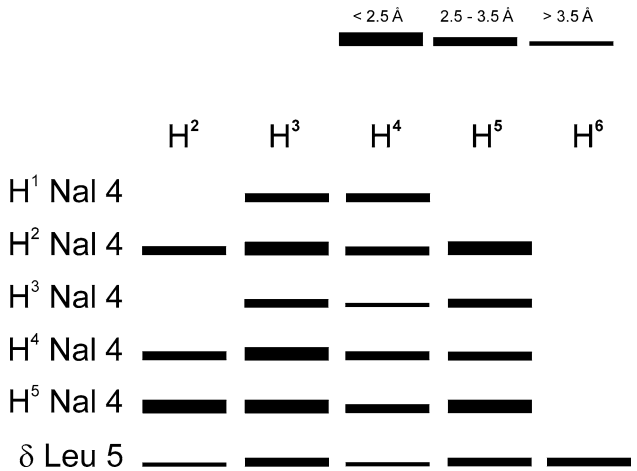


**Fig. 16** The values proportional to the interproton distances between Y-[L,D-EN] and $\beta$-CD protons

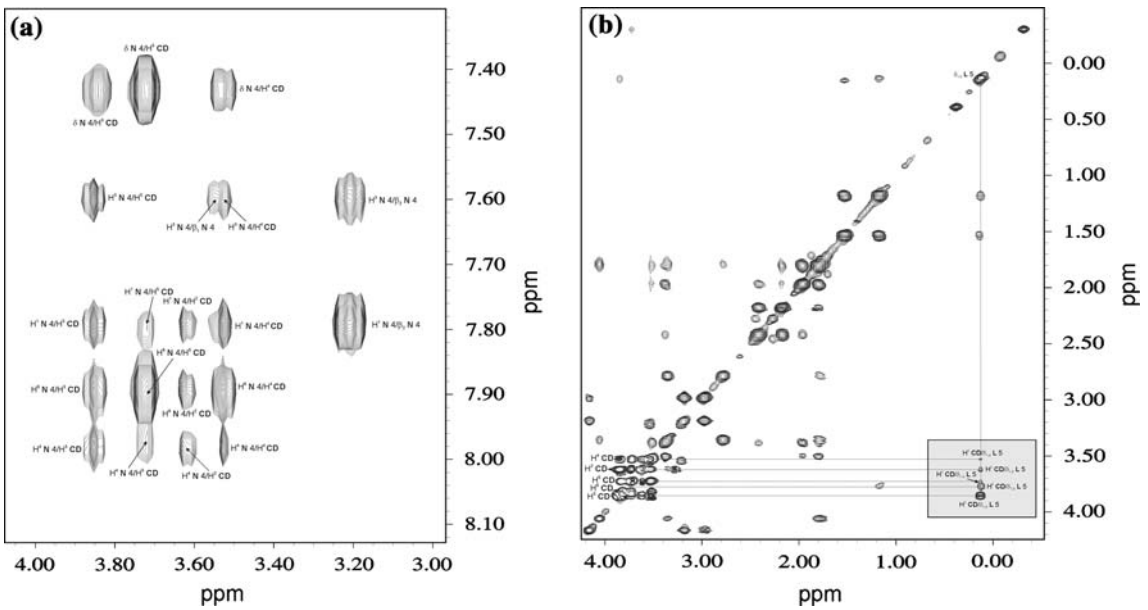


**Fig. 15** NOESY NMR spectrum of Y-[L,D-EN] complex with $\beta$-CD. The crosspeaks indicating the interaction of $\beta$-CD and Nal (**a**) and Leu (**b**) are presented

between Tyr residue and the peptide chain which do not allow the phenol group to penetrate into $\beta$-CD cavity. This type of interaction explains the lack of tyrosine fluorescence which is quenched by the peptide bond and hydrogen bond network formation of the phenol hydroxyl group. Contrary to the two previously described enkephalins, the change of the peptide chain conformation caused by the different Dab chirality allows Leu residue to be in the proximity to both $H^3$ and $H^5$ protons of $\beta$-CD. Moreover, the volumes of both crosspeaks are comparable, thus the Leu residue can penetrate the $\beta$-CD cavity equally from both sides. Also, Nal residue can penetrate the $\beta$-CD cavity from both sides (strong corsspeaks between $H^2$, $H^3$, $H^4$, and $H^5$ of naphthyl group and $H^3$ and $H^5$ of $\beta$-CD are observed). Besides inclusion complexes, as for previously described enkephalins, association complexes between $\beta$-CD and enkephalin are observed.

Concerning the Leu residue, there are some differences between Y-[L,D-EN] and the other two enkephalins studied. Contrary to Y-[D,D-EN] and F-[D,D-EN] enkephalins, the orientation of the peptide chain of Y-[L,D-EN] allows Leu $\delta$ protons to be in close proximity to all external $\beta$-CD protons ($H^2$, $H^4$, and $H^6$). Moreover, the crosspeak of $H^6$ $\beta$-CD and Leu $\delta$ protons is bigger than these with $H^2$ and $H^4$ indicating on the preference of the bottom association.

Low solubility of $AcNalNH_2$ and relatively low binding constant with $\beta$-CD, do not allow to obtain a good 2D NMR spectrum of this complex to unequivocally confirm whether or not the second $\beta$-CD molecule may interact with the part of the naphthyl group protruding $\beta$-CD cavity.

Unfortunately, fluorescence measurements as well as NMR studies are not able to give unequivocally information about structure of complexes present in the solution. It seems that for such complicated systems all aforementioned types of complexes (inclusion complexes from both sides formed by different side chains present in enkephalin as well as an association complexes) are present in different extents.

## Conclusion

Incorporation of Dab and/or Pro residues with different chirality into a peptide chain of cyclic enkephalin changes the spatial conformation of enkephalin itself as well as changes the mutual orientation of Tyr/Phe and Leu side chains. Different chirality of Dab and/or Pro in enkaphalin indicates on the different interaction with cyclodextrin which is manifested by the diversified binding constant as well as by the different mutual orientation of host–guest molecules. Beside a small binding constant of Tyr/Phe and Leu amino acid alone, interaction of the peptide chain with $\beta$-CD molecule increases stability of a complex formed by enkephalin. The presence of two side chains which are able to penetrate $\beta$-CD cavity as well as possibility of hydrogen bonds formation between CD and a peptide chain and hydrophobic interaction of the peptide side chains lead to a formation of mixed complexes. The analysis of 2D NMR spectra of complexes of enkephalins studied with $\beta$-CD allows to determine the spatial and mutual differences between host and guest molecules as well as the spatial orientation of Tyr or Phe and Leu residues caused by different Dab and Pro chirality. However, the binding of enkephalins studied with $\beta$-cyclodextrin is relatively weak and not sufficiently diversified to consider it as a drug carrier or enantiomeric discriminator. Application of different experimental methods (microcalorimetry, fluorescence and 2D NMR spectroscopy) allows to determine stoichiometry, binding constant and thermodynamic parameters of guest-host interaction in solution. However, binding constants determined by fluorescence spectroscopy and microcalorimetry for complex guest molecules do not necessarily have to coincide, because the observables for the two techniques are quite different and the guest/host concentration ratio for all applied methods are different.

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