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ORIGINAL ARTICLE

Investigating the inclusion properties of aromatic amino acids complexing beta-cyclodextrins in model peptides

Jolanda Valentina Caso[1] · Luigi Russo[1] · Maddalena Palmieri[1] · Gaetano Malgieri[1] · Stefania Galdiero[2] · Annarita Falanga[2] · Carla Isernia[1] · Rosa Iacovino[1]

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Abstract Cyclodextrins are commonly used as complexing agents in biological, pharmaceutical, and industrial applications since they have an effect on protein thermal and proteolytic stability, refolding yields, solubility, and taste masking. β-cyclodextrins (β-CD), because of their cavity size are a perfectly suited complexing agent for many common guest moieties. In the case of peptide-cyclodextrin and protein-cyclodextrin host–guest complexes the aromatic amino acids are reported to be the principal responsible of the interaction. For these reasons, we have investigated the inclusion properties of nine designed tripeptides, obtained permuting the position of two L-alanines (Ala, A) with that of one L-tryptophan (Trp, W), L-phenylalanine (Phe, F), or L-tyrosine (Tyr, Y), respectively. Interestingly, the position of the aromatic side-chain in the sequence appears to modulate the β-CD:peptide binding constants, determined via UV–Vis and NMR spectroscopy, which in turn assumes values higher than those reported for the single amino acid. The tripeptides containing a tyrosine showed the highest binding constants, with the central position in the Ac-AYA-NH_2 peptide becoming the most favorite for the interaction. A combined NMR and Molecular Docking approach permitted to build detailed complex models, highlighting the stabilizing interactions of the neighboring amino acids backbone atoms with the upper rim of the β-CD.

Keywords Inclusion complex · β-Cyclodextrin · Aromatic amino acids · UV–Vis spectroscopy · NMR · Molecular docking

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✉ Rosa Iacovino
rosa.iacovino@unina2.it

[1] Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, Second University of Naples, Via A. Vivaldi 43, 81100 Caserta, Italy

[2] Department of Pharmacy, University of Naples Federico II, Via Mezzocannone 16, 80134 Naples, Italy

Introduction

In recent years, peptide and protein drugs have evolved into mainstream therapeutics, representing a significant portion of the pharmaceutical market. Peptides and proteins exhibit highly diverse structures, broad biological activities as hormones, neurotransmitters, structural proteins, metabolic modulators, and therefore have a significant role as both therapeutics and biomarkers. However, the application of many of those is hindered by unfavorable solubility, aggregation, or instability. Many attempts have been undertaken to overcome these problems using cosolvents, among which cyclodextrins (CDs) represent suitable and powerful tools. CDs are natural cyclic oligosaccharide formed by six (α-CD), seven (β-CD), or eight (γ-CD) glucopyranose units joined by α-(1→4) glycosidic bonds, characterized by a truncated-cone shape. Their hydrophobic cavity confers them the ability to form inclusion complexes with a large number of compounds in aqueous solution, via non covalent interactions such as hydrogen bonding, van der Waals forces, change-transfer, electrostatic, and hydrophobic interactions (Connors 1997; Szejtli 1998). In particular, β-cyclodextrins are one of the most commonly used complexing agent in biological, pharmaceutical and industrial applications (Del Valle 2004; Szejtli 1996).

Hydration and solubility of proteins and, as a consequence, their stability and activity, can significantly change by modulating the surrounding solutes and solvent molecules, e.g., via the formation of inclusion complexes (Aachmann et al. 2012; Ekka and Roy 2013; Iacovino et al. 2013; Loftsson and Brewster 1996; Mrozek et al. 2005). However, due to the high conformational complexity of the three-dimensional structure of a protein, direct characterization of the solute-solvent influence on biological macromolecules is still very challenging.

Only a few studies investigate at a molecular level the properties of inclusion complexes between CDs and proteins or peptidic drugs giving us information about the structure of the complexes and their binding constants (Kb) (Aachmann et al. 2003; Loftsson and Brewster 1996; Saha et al. 2013). Aromatic amino acids are reported to be the principal responsible of the interaction and the formation of the host–guest complexes (Loftsson and Brewster 1996). The aim of the present work is to complement the available literature data (Kahle and Holzgrabe 2004; Rekharsky and Inoue 1998; Roy et al. 2014) by detailing the interaction of β-CD with different tripeptides designed in such a way that each of them contains a different aromatic amino acid in different position in the chain.

We have exploited spectroscopic methods such as UV–Vis and nuclear magnetic resonance (NMR) combined with molecular docking (MD) data which represent powerful tools to deeply investigate interactions of cyclodextrins with guest compounds in terms of binding constant and structural description (Iacovino et al. 2013; Schneider et al. 1998). We have synthesized nine peptides (Fig. 1), consisting of three amino acids that are composed of L-alanine (Ala, A) and one aromatic amino acid positioned, respectively, at the N-terminus, at the C-terminus and in the central position. The aromatic amino acids are L-tryptophan (Trp, W), L-phenylalanine (Phe, F), and L-tyrosine (Tyr, Y). In order to have a suitable model able to mimic the polypeptide chain of the peptide or protein, we have protected all peptide with acetyl group at N-terminus and amine group at C-terminus. For each peptide, a complex with

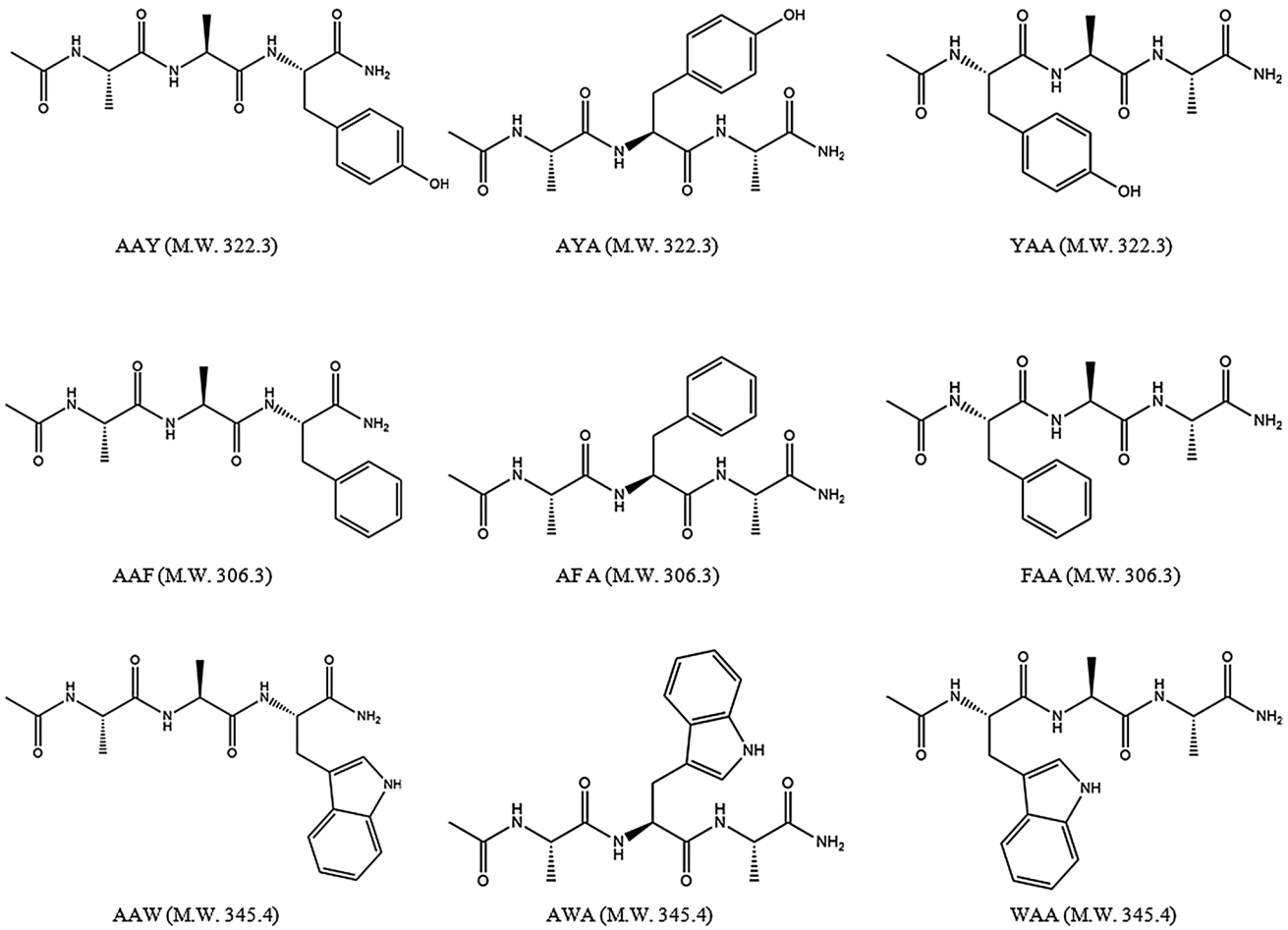


Fig. 1 Structures of tripeptides synthesized: (AAY) Ac-AAY-NH_2, (AYA) Ac-AYA-NH_2, (YAA) Ac-YAA-NH_2, (AAF) Ac-AAF-NH_2, (AFA) Ac-AFA-NH_2, (FAA) Ac-FAA-NH_2, (AAW) Ac-AAW-NH_2, (AWA) Ac-AWA-NH_2, and (WAA) Ac-WAA-NH_2

β-CD has been obtained, the different affinities of each tripeptide for the β-CD have been evaluated and the model for each interaction carefully calculated. Our findings show how the position of the aromatic side-chain in the sequence modulates the β-CD:peptide binding constants highlighting the stabilizing interactions of the neighboring amino acids backbone atoms with the upper rim of the β-CD.

Experimental section

Synthesis of peptides

Peptides were synthesized using the standard solid-phase-9-fluorenylmethoxycarbonyl (Fmoc) method as previously reported (Tarallo et al. 2011). Briefly, the Rink amide MBHA resin (substitution 0.49 mmol g^{-1}) was used as the solid-phase support, and syntheses were performed on a scale of 100 μmol. Fmoc-protected amino acids (4 equiv relative to resin loading) were coupled according to the PyBop/DIPEA method: Fmoc-amino acid (1equiv), PyBOP (0.5 M in DCM, 1 equiv), and DIPEA (2 M in DMF, 2 equiv). The Fmoc protecting group was removed with 30% piperidine in DMF (v/v).

The crude peptides were purified by RP-HPLC on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV lambda-Max Model 481 detector using a Phenomenex (Torrance, CA) C18 (300 Å), 250 mm, 21.20 mm, 5 μm column eluted with H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) from 20–80 % over 20 min at a flow rate of 20 mL min^{-1}. Purity and identity were assessed by analytical LC-MS analyses using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation San Jose, CA): C18-Phenomenex eluted with H_2O/0.1 % TFA (A) and CH_3CN/0.1 % TFA (B) from 20–80 % over 10 min at a flow rate of 0.8 mL min^{-1}. All purified peptides (purity higher than 98 %) were obtained with good yields (70–80 %). In Fig. 1 the sequences of all the synthesized peptides with their molecular weight (M.W.) are shown.

Stoichiometry determination using job plot method

For all UV–Vis spectroscopy studies, a UV-1700 Spectrometer (Shimadzu, Tokyo, Japan) was used with 1 cm matched quartz cuvettes. All measurements were recorded at room temperature in the 200–350 nm wavelength range for Ac-AAY-NH_2, Ac-AYA-NH_2, Ac-YAA-NH_2, Ac-AAW-NH_2, Ac-AWA-NH_2, Ac-WAA-NH_2, and in the 200–300 nm wavelength range for Ac-AAF-NH_2, Ac-AFA-NH_2, Ac-FAA-NH_2.

The stoichiometry of the complexes was determined using the continuous variation from Job method (Huang 1982; Job 1928). Solutions of each peptide and β-CD (0.25 mM for Ac-AAY-NH_2, Ac-AYA-NH_2, Ac-YAA-NH_2; 1 mM for Ac-AAF-NH_2, Ac-AFA-NH_2, Ac-FAA-NH_2; 0.1 mM for Ac-AAW-NH_2, Ac-AWA-NH_2, Ac-WAA-NH_2) were mixed at different concentration ratios R = [Peptide]/([Peptide] + [β-CD]) keeping the volume constant. For each complex, the stoichiometric ratio was obtained by plotting ΔA × R against R (where ΔA is the absorbance difference of the peptide without and with β-CD) and finding the R value corresponding to the extreme of this dependence.

Binding constant determination of the inclusion complex by UV–Vis spectroscopy

The evaluation of Kb by direct spectroscopic methods relies on analytical differences between the free and complexed peptide (Connors 1997). Changes in the absorption intensity of Ac-AAY-NH_2, Ac-AYA-NH_2, Ac-YAA-NH_2 (λ_{max} = 275.4 nm), of Ac-AAF-NH_2, Ac-AFA-NH_2, Ac-FAA-NH_2 (λ_{max} = 258.0 nm), and of Ac-AAW-NH_2, Ac-AWA-NH_2, Ac-WAA-NH_2 (λ_{max} = 280 nm) were monitored as a function of β-CD concentration to determine the Kb. The peptide concentration was kept constant (0.25 mM for Ac-AAY-NH_2, Ac-AYA-NH_2, Ac-YAA-NH_2; 1 mM for Ac-AAF-NH_2, Ac-AFA-NH_2, Ac-FAA-NH_2; 0.1 mM for Ac-AAW-NH_2, Ac-AWA-NH_2, Ac-WAA-NH_2) while β-CD concentration was varied (0–0.75 mM for Ac-AAY-NH_2, Ac-AYA-NH_2, Ac-YAA-NH_2 titration; 0–2.0 mM for Ac-AAF-NH_2, Ac-AFA-NH_2, Ac-FAA-NH_2 titration; 0–1.0 mM for Ac-AAW-NH_2, Ac-AWA-NH_2, Ac-WAA-NH_2 titration).

The mole ratio titration method was used to estimate the binding constants for all peptides under investigation (Benesi and Hildebrand 1949; Dotsikas et al. 2000; Yoe and Jones 1944). Various amounts of β-CD solution were added in fixed aliquots to a peptide solution. At each time, the total absorbance A in a 1 cm cell was given by:

$$A = \varepsilon_{pep.}[\text{peptide}] + \varepsilon_{\beta\text{-}CD}[\beta\text{-}CD] + \varepsilon_{pep:\beta\text{-}CD}[\text{peptide} : \beta\text{-}CD] \quad (1)$$

where [peptide], [β-CD], and [peptide:β-CD] are the respective concentrations of the species, ε_{pep}, $\varepsilon_{\beta\text{-CD}}$, and $\varepsilon_{pep:\beta\text{-CD}}$ are the molar absorptivities with units of $mol^{-1}\,cm^{-1}$

Since [peptide]tot = [peptide] + [peptide:β-CD], a combination of the Eq. 1 with the definition of the binding constant of the 1:1 inclusion complex (Eq. 2).

$$K_{1:1} = [\text{peptide} : \beta\text{-CD}]/[\text{peptide}][\beta\text{-CD}] \quad (2)$$

results in Eq. 3:

$$\Delta A = \frac{[\text{peptide}]_{tot} \cdot K_{1:1} \cdot \Delta\varepsilon_{1:1} \cdot [\beta\text{-}CD]}{1 + K_{1:1} \cdot [\beta\text{-}CD]} \quad (3)$$

where ΔA is the absorbance difference of the peptide in absence and in presence of β-CD and $\Delta\varepsilon_{1:1} = \varepsilon_{pep:\beta\text{-}CD} - \varepsilon_{pep} - \varepsilon_{\beta\text{-}CD}$. The binding constants were obtained from the titration curve data (ΔA as a function of [β-CD] fitted by non-linear regression (Eq. 3, Origin software http://www.originlab.com).

Nuclear magnetic resonance (NMR) spectroscopy

All ^{1}H-NMR experiments were carried out at 500 MHz using a Varian Unity 500 spectrometer located at the Department of Environmental, Biological and Pharmaceutical Sciences and Technologies in Caserta (Italy). The lyophilized peptides were rehydrated in H_2O/D_2O 90/10 (v/v) and the pH of aqueous solutions was adjusted at physiological condition (pH = 6.8). Final concentration of 0.25 mM for Ac-AAY-NH_2, Ac-AYA-NH_2, Ac-YAA-NH_2; 1 mM for Ac-AFA-NH_2; and 0.1 mM for Ac-AWA-NH_2 peptide solutions were attained. NMR experiments were performed at 298 K referenced to external TMS ($\delta = 0$ ppm). Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Mono (1D) and two-dimensional (2D) spectra were accumulated with a spectral width of 5500 Hz. 2D experiments, TOCSY, (Braunschweiler and Ernst 1983) NOESY and ROESY (Griesinger and Ernst 1987) were recorded using the States-Haberkorn method. Water suppression was achieved by DPFGSE sequence (Hwang and Shaka 1995; Pelta et al. 1998). TOCSY, NOESY, and ROESY spectra were acquired with mixing times of 70, 250, 250 ms, respectively. Typically, 64 transients of 4 K data points were collected for each of the 256 increments; the data were zero filled to 1 K in ω1. Squared shifted sine-bell functions were applied in both dimensions prior to Fourier transformation and baseline correction. PFG diffusion measurements with the PG-SLED (pulse gradient-stimulated echo longitudinal encode-decode) sequence (Gibbs and Johnson 1991) permitted to obtain the translational diffusion coefficient (D_{trans}) (Wilkins et al. 1999). Each diffusion data set contained a series of 20 monodimensional ^{1}H spectra with gradient strength from 0.5 to 30 G/cm. D_{trans} data were obtained by DOSY package of the VNMRJ software. The Kb for each complex was determined, assuming the fast exchange in the diffusion time scale, using the D_{trans} (Lin et al. 1995) by the following equation:

$$D_{\text{observed}} = D_{\text{free}}p_{\text{free}} + D_{\text{complexed}}p_{\text{complexed}} \quad (4)$$

where p_{free} and $p_{\text{complexed}}$ are the fraction of peptide free and bound to β-CD, respectively, and $p_{\text{free}} + p_{\text{complexed}} = 1$.

D_{observed} is the diffusion coefficient of the peptide in the presence of β-CD; D_{free} is the diffusion coefficient of the peptide in the absence of β-CD and $D_{\text{complexed}}$ is the diffusion coefficient of the completely complexed peptide. Therefore, considering that the diffusion of the peptide totally complexed is the same as the β-CD totally complexed ($D_{CD\text{complexed}}$) and that the diffusion observed to the β-CD partially complexed ($D_{CD\text{observed}}$) is very close to the same free diffusion ($D_{CD\text{free}}$). The Eq. 4 can be rearranged as follows:

$$P\text{complexed} = \frac{D\text{free} - D\text{complexed}}{D\text{free} - D_{\text{CD}}\text{observed}} \quad (5)$$

knowing $p_{\text{complexed}}$ and the molar concentration of each species in solution, it is possible to obtain an estimative of the Kb. Therefore, the Kb of each peptide:β-CD complex was estimated using the complexed population values previously determined as:

$$Kb = \frac{P\text{complexed}}{\{(1 - P\text{complexed})([CD]_0 - P\text{complexed}[S]_0)\}} \quad (6)$$

where $[CD]_0$ and $[S]_0$ are the concentration of substrate β-CD and peptide, respectively. Additionally, the Kb for each complex was determined using the chemical shift variations. The chemical shift displacements were calculated according to the equation: $\Delta\delta_{\text{obs}} = \delta(\text{free}) - \delta(\text{complex})$, where δ(free) is the chemical shift of the peptide without β-CD and δ(complex) is the chemical shift of the peptide complexed with β-CD. The Kb was calculated from $\Delta\delta_{\text{obs}}$ using the following Benesi–Hildebrand (Benesi and Hildebrand 1949) equation:

$$\frac{1}{\Delta\delta_{\text{obs}}} = \frac{1}{Kb \cdot \Delta\delta_c \cdot [\beta - CD]} + \frac{1}{\Delta\delta_c} \quad (7)$$

where $\Delta\delta_c$ is the difference of chemical shift between free peptide and the peptide:β-CD complex whereas [β-CD] is the β-CD concentration. In particular, in the titration experiments the concentration of the peptide was kept at 0.25 mM, while that of β-CD was increased from 0.25 to 2 mM, in order to have different peptide/β-CD molar ratios. Plot the reciprocal of $\Delta\delta_{\text{obs}}$ against the reciprocal of [β-CD] will afford a linear relationship with slope = 1/ ($Kb \cdot \Delta\delta_c$) and intercept = 1/ $\Delta\delta_c$. Kb can be determined by dividing the intercept by the slope.

Data were processed and analyzed using NMRPIPE (Delaglio et al. 1995) and CARA software (Keller 2004/2005).

Molecular docking

The Molecular docking studies of the inclusion complexes of β-CD with guests were performed using Hex software version 6.3 (Ritchie and Venkatraman 2010). The PDB files of β-CD and peptides were uploaded as inputs into Hex and treated as receptor and ligand, respectively. All input files were analyzed using the spherical harmonic surface of the Hex. Computations were performed using the shape

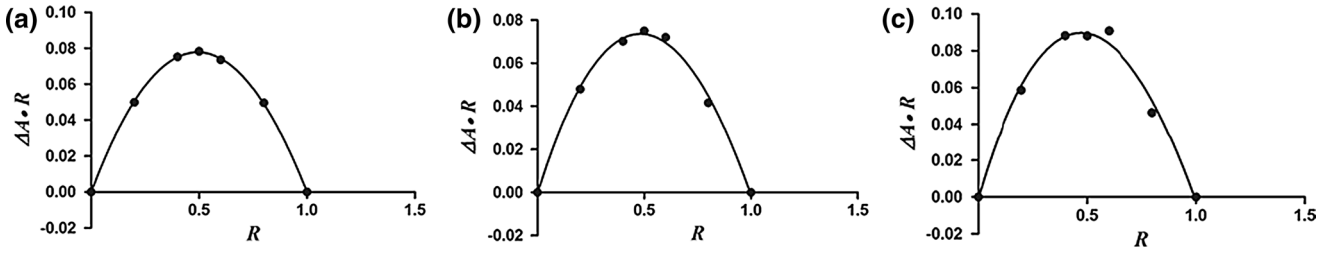


Fig. 2 Job plot at λ_{max} nm 275.4 for: **a** AAY:β-CD, **b** YAA:β-CD, and **c** AYA:β-CD

complementary scoring function, with 16 and 30 expansion orders for the initial and final search steps. The full list of parameters is given in Table 3. The β-CD molecule was kept as a fixed truncated-cone and guest were allowed to freely move. Structure refinement and energy minimization were performed with Hex itself. Docking calculation results for each guest molecule were clustered into different groups based on the root mean-square deviation values of atomic position in the inclusion complex. The lowest energy host–guest inclusion complex conformation was selected and analyzed. Each complex was analyzed and visualized using MolMol (Koradi et al. 1996), PyMOL (DeLano 2002) and CHIMERA (Pettersen et al. 2004).

Results and discussion

Job plot method

According to the method of continuous variation (Huang 1982; Job 1928; Negi and Singh 2013), the maximum concentration of the complex corresponds to the molar ratio *R* that represents the complexation stoichiometry. This was identified measuring the absorbance (a physical parameter which is directly related to the complex concentration) for a series of samples having continuously varied molar fraction of its components (Huang 1982).

We determined the stoichiometry of the complexes, for each peptide using the Job method (Job 1928). Our data demonstrated that all the tripeptides have a 1:1 stoichiometry as indicated by the curve maximum at $R = 0.5$. In Fig. 2 the Job plot for the peptides containing the tyrosine residue are reported as an example of the quality of the data.

Determination of binding constant by UV–Vis spectroscopy

The binding constant between β-CD and each tripeptide has been evaluated via UV–Vis spectroscopy. The Benesi–Hildebrand method represents one of the most common strategies to determine the binding constants based on absorption spectra for inclusion complex (Benesi and Hildebrand 1949). It was demonstrated that the relative error of the Benesi–Hildebrand method (i.e., double reciprocal treatment of titration data) in measuring the association constants of cyclodextrin complexations is often poorly reliable except for $K \approx 1000\ M^{-1}$ and $|\Delta\varepsilon| \geq 150\ m^2/mol$ (Hirose 2001). Therefore, in order to have an accurate estimation of binding constants of the inclusion complexes under investigation, changes in the absorption intensity of the peptide at different wavelength, depending on the amino acid sequence, were monitored as a function of the β-CD concentration and a non-linear regression estimation of the Kb (Fig. 3) was chosen (Loukas et al. 1996; Yang et al. 2000).

Results for each tripeptide reported in Table 1 indicate that the relative errors of the estimated binding constants using UV–Vis spectroscopy are within the range of 9–27 % and in the case of AFA and AAW peptide are over 30 %. The low accuracy of the estimated binding constants may be related to the small observed absorption changes and/or to the relatively weak complexation. In spite of the low reliability of the estimated binding constants, the data demonstrate that the behavior of the three aromatic residues is quite different. In particular, among the three aromatic amino acids, the tyrosine residue is the one that appears to be more properly suited for the interaction with β-CD. The presence of the −OH group, as expected, has a crucial role in the peptide:β-CD complex stabilization as the Kb measured for all the peptides bearing the tyrosine residue are generally higher than those of the other peptides.

Moreover, the data indicate that the tyrosine affinity for the β-CD significantly increases when the residue is located in the middle of the amino acid sequence suggesting that the molecular recognition of its aromatic ring by β-CD strongly depends from the position of the side chain inside the peptide sequence. In particular, the hydrophobic aromatic ring forms an inclusion complex with the hydrophobic cavity while the flanking residues, contribute to the stabilization of the complex interacting with the hydroxyl groups of the β-CD. On the contrary, in the case of phenylalanine residue, the affinity significantly increases when the aromatic residue is positioned at the N-terminus and C-terminus, while for

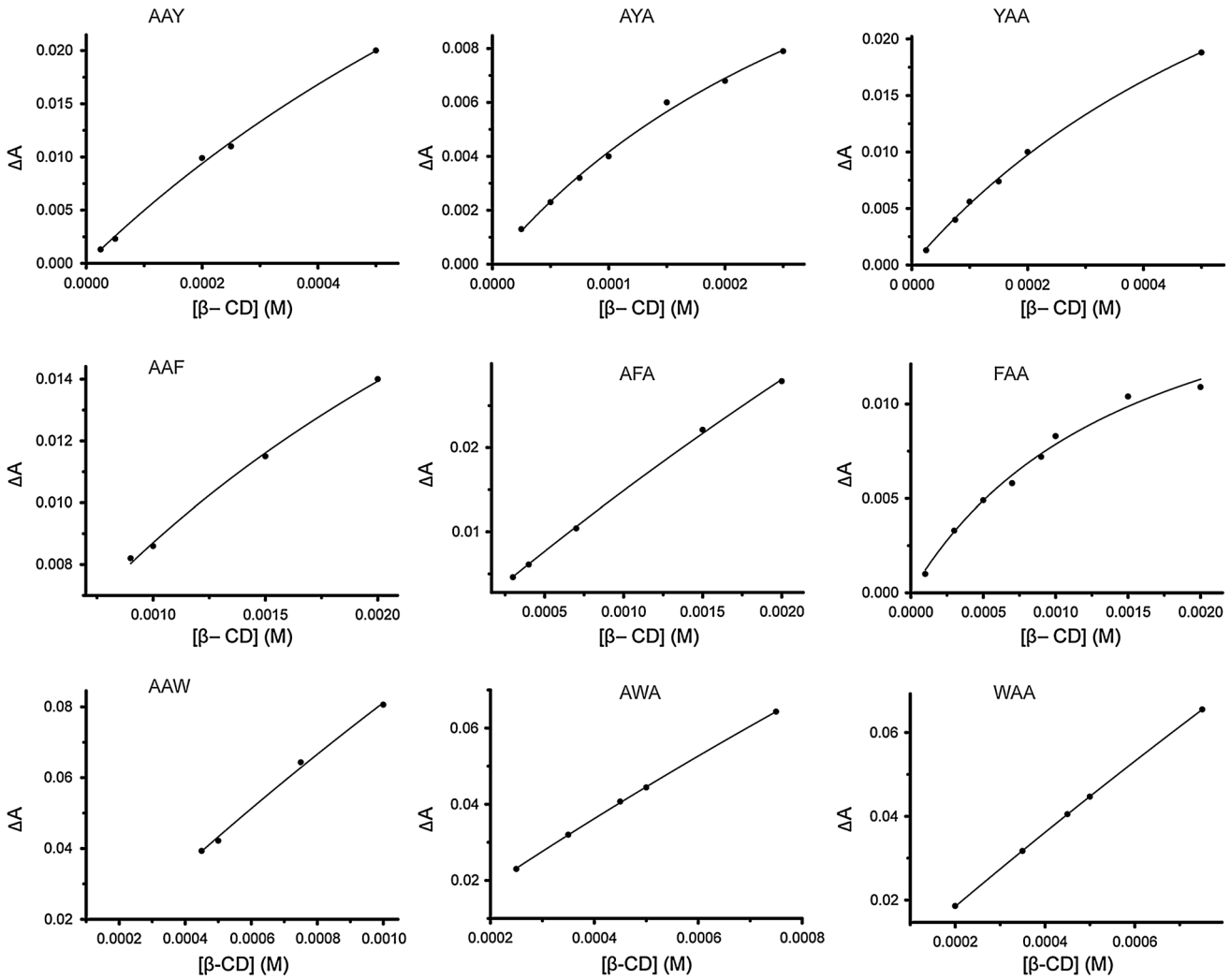


Fig. 3 Dependence of tripeptides absorbance from β-CD concentration in aqueous solutions. AAY, AYA, YAA (λ_{max}= 275.4 nm); AAF, AFA, FAA (λ_{max} = 258.0 nm); and AAW, AWA, WAA (λ_{max} = 280.6 nm)

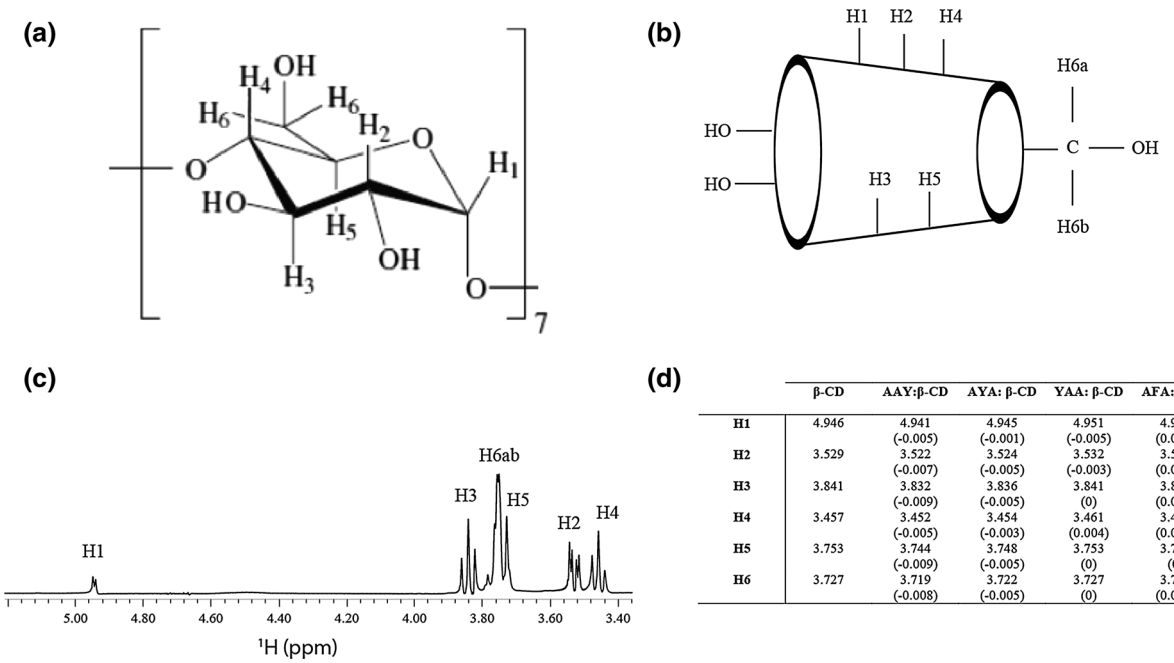
Table 1 Binding constants (Kb) of each inclusion complex obtained by UV–Vis spectroscopy

Sample	AAY:β-CD	AYA:β-CD	YAA:β-CD	AAF:β-CD	AFA:β-CD	FAA:β-CD	AAW:β-CD	AWA:β-CD	WAA:β-CD
UV–Vis Kb (M^{-1})	657 ± 180	2614 ± 429	1213 ± 117	332 ± 38	63 ± 20	640 ± 109	143 ± 66	170 ± 16	106 ± 7
R^2	0.997	0.994	0.998	0.996	0.999	0.987	0.995	0.999	0.999

the Trp residue this behavior is not observed (Table 1). Taken together, the data indicate that the stability of the aromatic amino acids:β-CD complexes is in the following order: Tyr > Trp > Phe. This finding is quite different from the results previously published in which the single amino acids alone were considered (in that case the order was: L-Trp > L-Tyr > L-Phe) (Linde et al. 2010), suggesting that the interaction is also influenced by the position that the residue under consideration occupies within the peptide chain.

Nuclear magnetic resonance assignments and chemical shifts analysis

Inclusion complexes involving aromatic compounds have been often characterized by NMR (Bergeron et al. 1978; Gelb et al. 1981; McAlpine and Garcia-Garibay 1996; McAlpine and Garcia-Garibay1998). The method relies on mutual changes in chemical shifts caused by the guest and the host interaction. In the case of aromatic compounds, some of the most important spectral changes that occur



	β-CD	AAY:β-CD	AYA: β-CD	YAA: β-CD	AFA: β-CD	AWA: β-CD
H1	4.946	4.941 (-0.005)	4.945 (-0.001)	4.951 (-0.005)	4.949 (0.003)	4.947 (0.001)
H2	3.529	3.522 (-0.007)	3.524 (-0.005)	3.532 (-0.003)	3.530 (0.001)	3.527 (-0.002)
H3	3.841	3.832 (-0.009)	3.836 (-0.005)	3.841 (0)	3.843 (0.002)	3.838 (-0.003)
H4	3.457	3.452 (-0.005)	3.454 (-0.003)	3.461 (0.004)	3.459 (0.002)	3.455 (-0.002)
H5	3.753	3.744 (-0.009)	3.748 (-0.005)	3.753 (0)	3.753 (0)	3.750 (-0.003)
H6	3.727	3.719 (-0.008)	3.722 (-0.005)	3.727 (0)	3.729 (0.002)	3.724 (-0.003)

Fig. 4 The scheme of stereo-configuration, truncated-cone of β-CD (**a**, **b**). ^{1}H NMR spectra of β-CD acquired at 298 K (**c**). Proton chemical shifts of the β-CD free and complexed to all tripeptides used in NMR analysis (**d**)

upon complexation come from the aromatic ring current effect of the host on the nearby spins of the guest. In the structure of β-CD, the hydrogens H3 and H5 are located inside the cavity (Fig. 4a, b). In particular, H3 is located near the wider rim of the cyclodextrin cavity while H5 hydrogens form a ring near the narrower rim of the methylene, H6, bearing the primary hydroxyl groups. The other hydrogens (H1, H2, and H4) are located on the exterior of the cavity (Fig. 4b).

Exploiting their higher values of Kb, as determined via UV–Vis spectroscopy, the NMR structural characterization of the tripeptides bearing the tyrosine, phenylalanine, and tryptophan residues has been carried out.

The sequential assignment of proton chemical shifts for each peptide in free and in the presence of β-CD (peptide:β-CD 1:1 ratio) was performed using the standard strategy described by Wüthrich (Wüthrich 1986) (Supplementary Table SI1). TOCSY (Braunschweiler and Ernst 1983) experiment was used to identify spin system and ROESY (Griesinger and Ernst 1987) spectra were analyzed to obtain inter-residue connectivities and to distinguish equivalent spin system.

All proton chemical shifts were assigned for β-CD in the free and complexed state for each complex in the ratio 1:1 and are reported in the Fig. 4c, d. As expected, the dynamics in all inclusion complexes are in fast-exchange regime in the chemical shift timescale, according to the measured binding constants. Therefore, the observed resonances are the average chemical shifts in the free and complex form weighted by the fractional population in each state. For this reason it was possible to follow the chemical shift changes in spectra acquired at different peptide:β-CD ratio (1:3, 1:2). Notably, the binding proprieties of the inclusion complex and the orientation of the peptide/drug molecule can be inferred by evaluating the chemical shifts variation ($\Delta\delta$). As previously reported (Greatbanks and Pickford 1987), when $\Delta\delta$(H3) is bigger than $\Delta\delta$(H5), the inclusion of the guest inside the cavities only partial. On the contrary, when $\Delta\delta(H3) \leq \Delta\delta(H5)$, a total inclusion of the guest takes place. As reported in the Fig. 4d, the H3 and H5 proton chemical shifts of the β-CD experience the larger variations upon complexation with Ac-AAY-NH_2 and Ac-AYA-NH_2, indicating that in these cases the aromatic ring results to be deeply embedded inside the hydrophobic cavity. Instead, for the tripeptide Ac-YAA-NH_2 we did not observe any difference with respect to the free β-CD form, suggesting that the side chain of the Tyr is located outside the hydrophobic cavity of the β-CD. Moreover, our analysis was fully confirmed by evaluating also the variations in the free and complexed form of proton chemical shifts of all three peptides containing the tyrosine.

Following the indications obtained by the UV–Vis analysis, the contribution of the tyrosine −OH group in the peptide:β-CD complex stabilization has been evaluated by investigating the peptide in which a Phe residue is located in the middle of the peptide sequence (Ac-AFA-NH_2). In this case, the small or absent variation of H3 and H5 chemical shifts of the β-CD and of the protons belonging to the

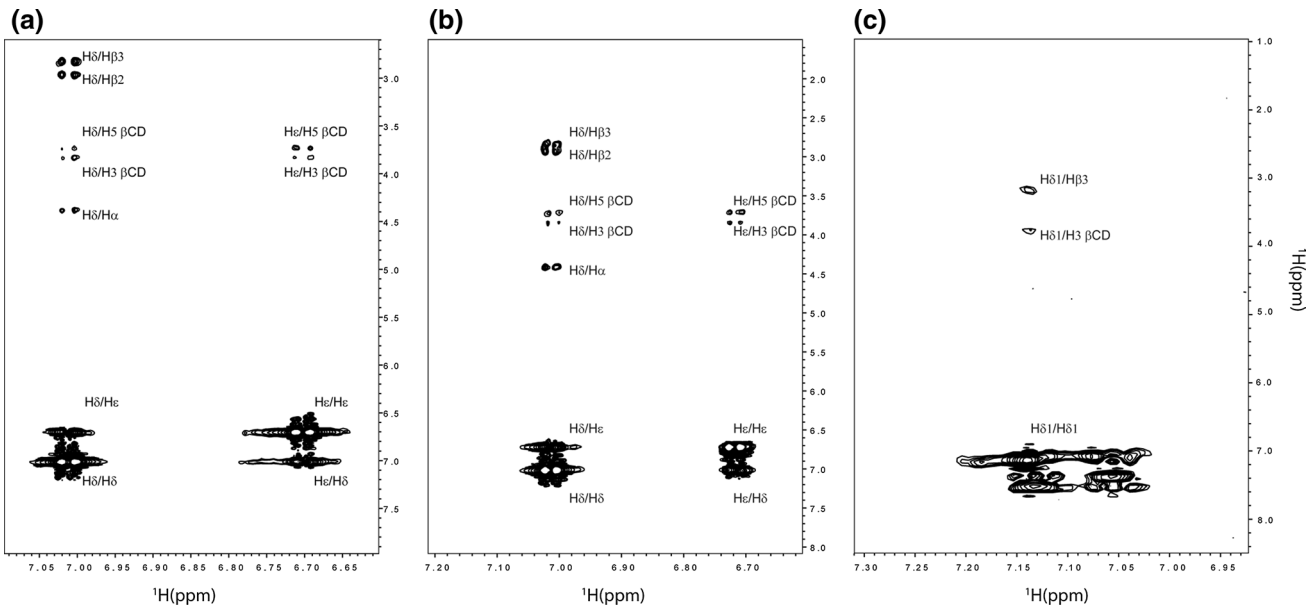


Fig. 5 A portion of the 2D ^{1}H-^{1}H ROESY spectrum of AAY:β-CD (**a**), AYA:β-CD (**b**), and AWA:β-CD (**c**). The intra- and inter-molecular NOEs are labeled

phenylalanine aromatic ring clearly indicate that the side chain is partially embedded inside the β-CD macrocyclic ring. These data further outline the importance of the −OH group in the formation of the complex. Finally, in order to evaluate also the steric effect on the complexation we have characterized the peptide bearing the bulkier tryptophan in the middle position. In this case, the chemical shift variations upon interaction clearly indicate that the aromatic ring is partially buried inside the cyclodextrin cavity. In particular, the chemical shifts of ε3CH (from 7.324 to 7.339) and ζ2CH protons (from 7.542 to 7.532) experience the larger chemical shift variation with respect to the other side-chain protons that appear mostly unperturbed. This indicates that the part of the indole ring bearing ε3CH and ζ2CH protons is embedded in the CD cavity while the rest of the side chain lays on the CD surface.

NOEs evaluation of β-CD inclusion complexes

In principle, a guest can penetrate the CD cavity in several ways: through the wide or the narrow rim, in a parallel or perpendicular orientation with respect to the cavity long axis or in any orientation in between.

Here, in order to gain insight into the structural details of the peptide:β-CD inclusion complexes we exploited the information provided by the two-dimensional NMR experiments NOESY (Kumar et al. 1980) and ROESY (Griesinger and Ernst 1987). Despite challenges associated with binding dynamics, which may interfere with the development of cross-relaxation interactions, the nuclear overhauser effect (NOE) is one of the most widely used methods (Alderfer and Eliseev 1997; Rüdiger et al. 1996). 2D-NOESY and 2D-ROESY experiments give rise to cross peaks between dipolarly coupled spins (Neuhaus and Williamson 1989), indicating the close proximity between atoms in the two components of the complex. Under favorable conditions, 2D NOESY and 2D ROESY experiments provide an upper limit (*ca*. 5Å) on the distance between protons that produce cross peaks. Therefore, we performed both experiments on tripeptides Ac-AAY-NH_2, Ac-YAA-NH_2, Ac-AYA-NH_2, Ac-AFA-NH_2, and Ac-AWA-NH_2 complexed to the β-CD. In our experimental conditions no intermolecular NOEs were detected in 2D-NOESY experiment while moderate NOEs were observed in the 2D-ROESY spectrum. These results can be explained in terms of the dynamics of the β-CD complex which modulate the evolution and the sign of the NOE (Bax and Davis 1985; Bothner-by et al. 1984). The 2D-ROESY spectrum acquired on Ac-AAY-NH_2 and Ac-AYA-NH_2 complexed to the β-CD is shown in Fig. 5. Cross peaks between the aromatic protons of the tyrosine and H3, H5 hydrogens of the β-CD indicate their proximity (Fig. 5a, b). These findings, together with the variations of proton chemical shifts of the β-CD upon binding, provide further evidences that the aromatic ring of the tyrosine is deeply embedded inside the hydrophobic cavity of the β-CD, thus stabilizing the complex. On the other hand, the 2D-ROESY acquired on the Ac-YAA-NH_2:β-CD complex does not show any through-space connectivity with the H3 and H5 hydrogens located inside the cavity, confirming that, in this case, the side chain of the tyrosine is outside

Table 2 Tripeptides diffusion coefficient free and in the presence of β-CD. The translational diffusion coefficient of the β-CD free is 2.706 ± 0.332 10^{-10} m^2 s^{-1}

Sample	$D_{peptide}$ ($/10^{-10}$ m^2s^{-1})	$D_{\beta CD}$($/10^{-10}$ m^2s^{-1})	$P_{complexed}$ (%)	Kb_{dosy} (M^{-1})	$Kb_{chemical\ shifts}$(M^{-1})
AAY_{free}	5.128 ± 0.532	–			
AAY:β-CD (1:1)	4.778 ± 0.590	2.918 ± 0.280	15.8	893	787 ± 15
AYA_{free}	5.448 ± 0.458	–			
AYA:β-CD (1:1)	4.560 ± 0.201	2.840 ± 0.332	33.0	2940	2566 ± 33
YAA_{free}	4.956 ± 0.720				
YAA:β-CD (1:1)	4.399 ± 0.131	2.168 ± 0.200	20.0	1250	1140 ± 20
AFA_{free}	4.773 ± 0.376	–			
AFA:β-CD (1:1)	4.742 ± 0.237	3.145 ± 0.423	1.0	41	43 ± 1
AWA_{free}	5.029 ± 0.376	–			
AWA:β-CD (1:1)	4.999 ± 0.217	3.088 ± 0.277	2.0	208	162 ± 15

Values of percentages of complexed tripeptides with β-CD and binding affinity constants of each inclusion complex are reported

The binding constants obtained using the chemical shifts differences are also reported

the cavity. Accordingly, the 2D ROESY spectrum acquired on the Ac-AWA-NH_2:β-CD (1:1) complex (Fig. 5c) indicates that the aromatic ring of the tryptophan is located inside the hydrophobic pocket of the β-CD, while the same spectra registered on the Ac-AFA-NH_2:β-CD complex does not show intermolecular NOEs suggesting that the phenylalanine side-chain is predominantly outside the cavity.

Determination of the binding constants by NMR

Molecular diffusion in solution, a phenomenon related to the molecular dynamics in biological and chemical systems, can be measured by NMR. The translational diffusion coefficient (Dt) provides important information about the molecular organization as it can be used to determine the stoichiometry and binding constants of complexes. In fact, the diffusion coefficient depends on the size of the molecule and it can be calculated from the Stoke-Einstein equation $Dt = (K_BT)/6\pi\eta r$ where K_B is the Boltzmann constant, T is the temperature in Kelvin, η is the viscosity of the solution in $Pa{\cdot}s$ and r is the hydrodynamic radius. Since in the case of interaction between the host and the guest, the observed Dt is a weighted average of the free and complexed values, the population of the guest involved in the complexation process and the Kb can be calculated from the Dt observed values in the free and complex forms (Lin et al. 1995). Therefore, using the diffusion coefficients measured in the DOSY (gradient compensated stimulated echo with spin lock) (Pelta et al. 1998) spectra as reported in the experimental section, the relative populations and the obtained binding constants, assuming a fast exchange on the NMR time scale, were calculated. All the experimental Dt for the peptides, the β-CD, the complexes, the calculated populations and the Kb are reported in Table 2.

As expected, the diffusion coefficient of peptides complexed to β-CD shows smaller values with respect to the free form thus reflecting the interaction with the β-CD. Interestingly, all the calculated populations and the binding constants estimated by NMR spectroscopy are in excellent agreement with the data obtained via UV–Vis experiments (Table 1). In order to compare, considering the relative errors, the estimated binding constants by UV–Vis spectroscopy measurements with respect to the values obtained by NMR experiments we evaluated the binding affinity using the proton chemical shift differences of the peptide upon binding to the β-CD. In particular, we monitored the chemical shift variations of the aromatic protons (Table SI2) by titrating each peptide with an increasing amount of β-CD. The binding constants estimated as reported in the Materials and methods section are listed in the Table 2. Notably, using the chemical shift differences the accuracy of the binding constants, ranging from 2 to 9%, is considerably improved with respect to UV–Vis results. All the NMR data, in according to the UV–Vis analysis, indicate the AYA:β-CD complex as the most populated one and shows the higher binding constant confirming the importance of the flanking residues backbone atoms in the stabilization of the interaction.

Molecular docking

In order to deeply describe the structural details of the molecular recognition of amino acids by β-CD we performed a series of MD studies. Molecular docking aims at predicting the binding mode and affinity of a complex formed by two or more constituent molecules with known structures. The analysis has been restricted to the Ac-AAY-NH_2, Ac-AYA-NH_2, and Ac-AWA-NH_2 peptides as, according to the NMR and UV–Vis results, these are the only

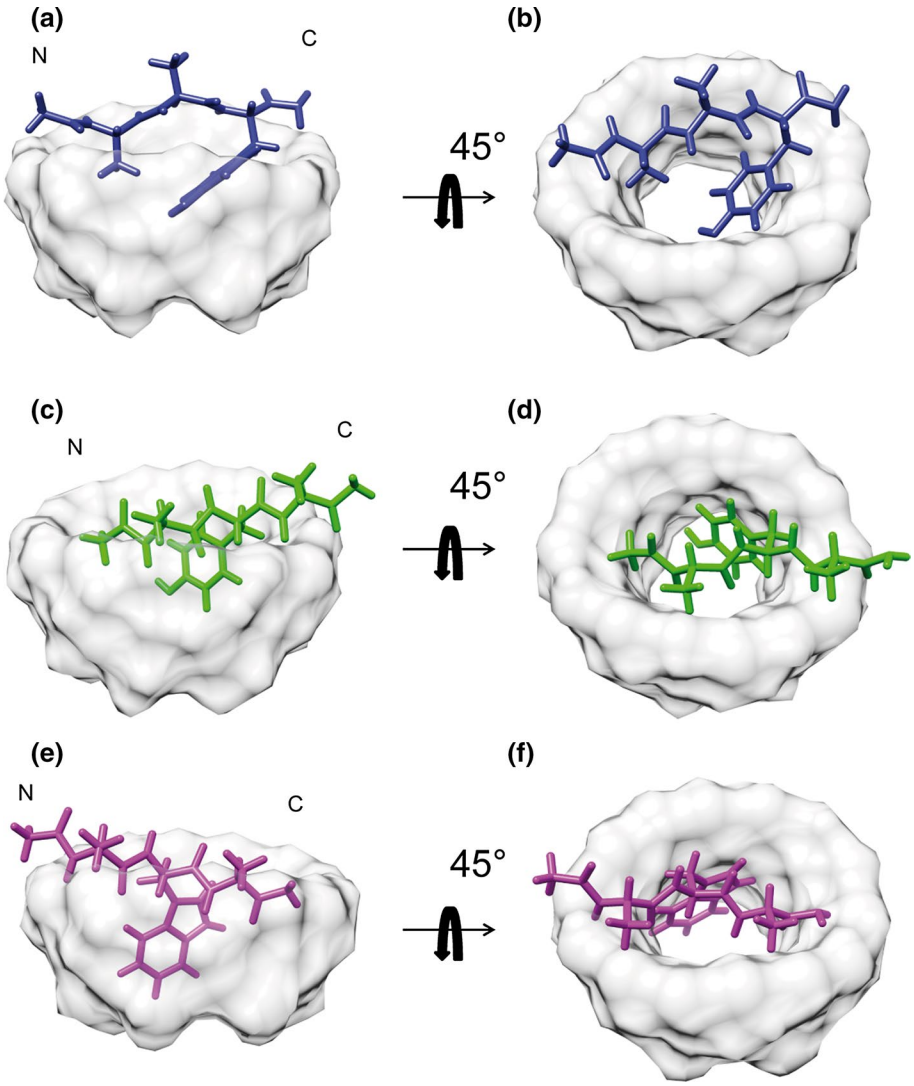


Fig. 6 Molecular docking models of AAY:β-CD (**a**, **b**), AYA:β-CD (**c**, **d**), and AWA:β-CD (**e**, **f**), in two orientations rotated by 45° around *x* axes

three cases in which the aromatic ring is either fully or partially embedded in the β-CD cavity.

The inclusion complexes of β-CD with guests were generated using Hex software version 6.3 (Ritchie and Venkatraman 2010), which is an interactive molecular graphics program for calculating and displaying feasible docking modes of pairs of protein or peptide molecules. Molecular docking studies involved the following step: the 3D structure of the β-CD in the protein data bank (PDB) format was downloaded and protonated using the software Reduce (Word et al. 1999), all peptides were drawn in ChemDraw Ultra software (Li et al. 2004) and the minimum energy configuration was set for the 3D structure. The 3D optimized structures were stored as PDB file and finally the docking simulations were run, as reported in the Materials and methods section, using the parameter listed in the Table 3.

The docking simulations, in agreement with the results reported above, revealed that the three guest peptides are able to form a 1:1 complex with the β-CD. The optimized structure of each peptide:β-CD complex is reported in Fig. 6a–f. In all cases, the aromatic ring is deeply embedded into the hydrophobic cavity of the β-CD. Furthermore, as expected, the aromatic side-chain enters the β-CD hydrophobic cavity from the wider rim. Interestingly, in according to the NMR data (NOEs and Chemical Shifts),

Table 3 Hex parameters used in this study

Correlation type	Shape only
FFT mode	3D fast life
Post processing	MM minimization
Receptor range angle	180
Ligand range angle	180
Twist range	360
Distance range	40
Docking main scan	18
Docking main search	25

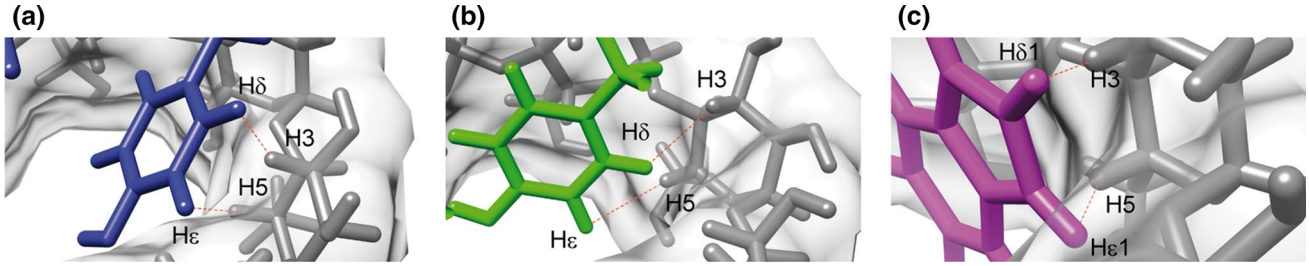


Fig. 7 Aromatic ring close view of the AAY (**a**), AYA (**b**), and AWA (**c**) complexed with β-CD. The hydrogens of the peptide:β-CD giving rise to NOEs cross peaks are labeled

the obtained models for each complex clearly indicate that the two alanine residues, stabilizing the interaction, are leaned on the β-CD surface and exposed to the solvent bulk. In the case of the Ac-AYA-NH_2 (Fig. 6c, d) the interaction appears to be facilitated by the presence of the two flanking alanines that allow the right orientation of the aromatic ring inside the cavity. On the contrary, for the Ac-AAY-NH_2 (Fig. 6a, b) with both alanines at the N-terminus the aromatic ring is not aligned along the longitudinal axis of the β-CD. This finding clearly indicates that Ac-AAY-NH_2 and Ac-AYA-NH_2 are characterized by a different binding mode and are likely to explain the variation of the binding affinity observed for the two peptides. Finally, for the Ac-AWA-NH_2 (Fig. 6e, f) the Trp side chain is deeply buried inside the cavity with an orientation similar to that observed for the aromatic ring of the tyrosine in the Ac-AYA-NH_2. Overall, the data suggest that the molecular recognition of an aromatic residue by β-CD depends from its position along the peptide sequence as well as by the structure and the chemical proprieties of the aromatic side-chain that contribute to stabilize the complexation. Finally, to validate the molecular docking data we used the NMR data (NOEs) that were not considered in the peptide:β-CD complex generation. As reported in Fig. 7(a–c) for all calculated docking models the distances between the aromatic protons and the H3/H5 hydrogens located inside the β-CD cavity are in excellent agreement with the NOEs cross-peaks observed in the 2D ROESY spectra (Fig. 5). Therefore, the combination of the NMR data and docking models represents a powerful strategy for investigating inclusion systems.

Conclusion

The complexation ability of CDs has been largely proved to be useful for the development of new carriers with improved performances in term of patience compliance, protection of encapsulated substances, solubility enhancement, control of delivery rate and bioavailability. Moreover, cyclodextrin have many advantages as buffer additives for protein refolding. They are biocompatible, no toxic, stable, and relatively poorly expensive. They not only enhance protein folding but also act as anti-aggregating agents for native proteins that have the tendency to aggregate in solution. In addition, cyclodextrins can stabilize protein prone to degradation during storage. The aim of the present study is to provide further insight into the structural details of the molecular recognition of amino acids by β-CD using experimental and theoretical approaches in order to develop a strategy able to increase the protein solubility using β-CD as co-solvent. We have examined how the position of an aromatic amino acid in model tripeptides affects the strength of interactions with β-CD. We demonstrate that the affinity depends on the type of aromatic side-chain and from its position in the tripeptide.

The 1:1 stoichiometry of the complex in all the cases was established by Job plot method and the binding constants for each inclusion complex were obtained using UV–Vis and NMR techniques. The NMR analysis demonstrate that the binding constants obtained using two different methods are in a good agreement indicating that the estimation of the binding affinity by diffusion order spectroscopy NMR experiments represent a suitable alternative methodology with respect to the use of the chemical shift titration method for weak host–guest inclusion complexes showing small chemical shifts variations upon binding. The highest Kb has been found for the Ac-AYA-NH_2:β-CD interaction while in the peptides containing the phenylalanine residue, the affinity significantly increases when the aromatic residue is positioned at the N-terminus and C-terminus.

The presence of the −OH group in the phenolic ring of the tyrosine residue has a crucial role in the peptide:β-CD complex stabilization as the Kb measured for all the peptides bearing the tyrosine residue are generally higher than those of the other peptides. This important contribution in the complex stabilization is further confirmed by comparing the lower binding constants estimated for all peptide containing the Phe residue.

Although the −OH group should disfavor the interaction because reduces the overall hydrophobicity of the aromatic side-chain and increases its volume with respect to the

benzenic side chain of the Phe residue (Tang et al. 2006), its hydrophilic nature allows the Tyr phenolic ring to more deeply penetrate the CD cavity. This behavior fosters the flanking residues to establish interactions with the hydroxyl groups of the upper rim of the host resulting in a further stabilization of the complex. This, also, explains why the incorporation of the aromatic amino acids into tripeptides results in a change in selectivity with respect to the corresponding monomeric amino acids (Linde et al. 2010).

The data regarding the binding constants obtained for the peptide with the Trp amino acid, considering the reduction of the binding affinity with respect to Tyr, strongly suggest that the steric hindrance of the indolic side chain play a significant role in the complex stability.

Overall, our study demonstrates that the chemical properties of the aromatic side-chain can structurally shape and enhance the β-CD:peptide binding proprieties. Moreover, we have outlined the role of the backbone atoms of neighboring amino acids in stabilizing the complex through their interaction with the upper rim of the β-CD. In particular, NMR and Molecular docking data demonstrate that in the case of the tyrosine the orientation of the aromatic ring with respect to the hydrophobic cavity long axis of the β-cyclodextrin is strongly influenced by the flanking residues. This interaction could be likely modulated using properly functionalized CDs (Di Fabio et al. 2012; Hanessian et al. 1995; Stancu et al. 2014). Additionally, our study demonstrate that the combination of NMR data with Molecular docking analysis provide a powerful method to deeply investigate at atomic resolution β-CD inclusion complexes. Hence, the findings explained and discussed in this paper can be useful applied in various fields as for example drug delivery or pharmaceutical chemistry. In fact, this knowledge on the recognition mechanisms of amino acids by CDs might be particularly useful to engineer cyclodextrin-binding sites adding highly exposed aromatic side-chains into protein that need to be stabilized against aggregation, degradation, or temporarily inactivated. Finally, our data might be used to create new cyclodextrin-based materials for more specific binding to proteins.

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Conflict of interest The authors declare that there are no conflicts of interest.

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