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ORIGINAL ARTICLE

Interactions of cyclodextrins with aromatic amino acids: a basis for protein interactions

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Abstract Cyclodextrins (CyD) have proven effects on the stability of proteins and can be used in the formulation of aggregation prone therapeutic proteins. This effect stems from specific interactions between the CyD (preferably β-CyD) and solvent exposed amino acid residues. Here the interaction with hydrophobic aromatic amino acid residues stands out and the interaction between CyDs and these amino acid residues holds the key to understanding the observed effects, which CyDs exerts on proteins and peptides. Here we present a comparative study of the interactions between free and peptide bound aromatic amino acids and their derivatives with α, β and γ-CyDs using NMR spectroscopy. We propose a novel, quantitative means of assessing the penetration depth of guest molecules in CyD cavities, the penetration gauge Π, and apply it to the observed interaction patterns from ROESY NMR spectra. We demonstrate that the penetration depths of the aromatic rings within the CyDs rely highly on the nature of the remainder of the guest molecule. Thus the presence of charges, neighboring amino acids and the specific positioning on the surface of a protein highly influences the penetration depth and geometry of guest–CyD interactions.

Keywords Cyclodextrin · Aromatic amino acid · Peptides · Inclusion complex formation

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Introduction

A major limiting factor in the use of proteins as industrial and clinical compounds is the instability of protein formulations over extended periods of storage. A method to increase the long-term stability of the protein is to use additive compounds. Cyclodextrins (CyD) are quite interesting in this context because they have been shown to have an effect on protein solubility, thermal and proteolytic stability, refolding yields, and taste masking [1–14]. We have previously shown that CyDs interact with proteins at specific sites, preferably with solvent exposed aromatic amino acids, which offers a likely explanation for their ability to suppress aggregate formation, stabilize proteins against degradation and exert changes to their functionality [15–17]. During these studies we have observed largely different interactions of solvent exposed aromatic amino acids with CyD, indicating their dependence on their immediate surroundings.

NMR spectroscopy can be used to obtain structural information on CyD inclusion complex formation, where cross peaks in ROESY or NOESY NMR spectra occur if two hydrogen atoms are within $\sim$5 Å of each other (See Fig. 1 for the position of protons in CyD). Cross peak intensities are to a first approximation dependent on the inverse sixth power of the distance between the two atoms involved and to the concentration of the compound yielding a signal (in this case, the complex concentration). The latter can be calculated if the association constant is known. Thus, with unknown association constants, signal intensities in ROESY spectra cannot readily be converted into interatomar distances. The calculation of interatomar distances is however of great interest since it delivers information on the complex geometry.

Here, we circumvent this problem by calculating ratios of intensities of cross peaks from guest molecule atoms to

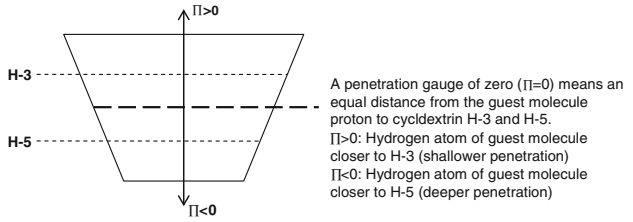

Fig. 1 The penetrationguage. The logarithm of the intensity ratios of cross peaks to H-3 and H-5, which is a direct measure of penetration depth into the CyD cavity. A value of 0 on that scale indicates that the guest molecule atom in question is equidistant from H-3 and H-5, positive values indicate a greater distance to H-5 than H-3 (less penetration), and negative values indicate a greater distance to H-3 than H-5 (deeper penetration of the atom into the CyD cavity)

H-3 and H-5 atoms inside the CyD cavity (Eq. 1). We will further refer to this quantity as the "penetration gauge" or Π.

$$\Pi = \ln\left(\frac{I_{Hg}^{H-3}}{I_{Hg}^{H-5}}\right) \quad (1)$$

where I_{Hg}^{H-3} and I_{Hg}^{H-5} refer to the intensity of the ROESY cross peaks between the investigated hydrogen atom of the guest molecule and CyD's H-3 and H-5, respectively.

Hereby we isolate the distance dependence of ROESY cross peak intensities and obtain information on the penetration depth of aromatic guest molecules (in this case, amino acids and their derivatives) into the CyD cavity. We compute the logarithm of the intensity ratios of cross peaks to H-3 and H-5, which is a direct measure of penetration depth into the CyD cavity (Fig. 1). A value of 0 on that scale indicates that the guest molecule atom in question is equidistant from H-3 and H-5, positive values indicate a greater distance to H-5 than H-3 (less penetration), and negative values indicate a greater distance to H-3 than H-5 (deeper penetration of the atom into the CyD cavity).

In this work, we present a structural study of the inclusion complex formation between CyD and three aromatic amino acids (phenylalanine, tyrosine and tryptophan) with α-, β-, and γ-CyD using NMR spectroscopy. We present a novel gauge for measuring the penetration depth of guest molecules into the CyD cavity. Furthermore, we also describe the influence of charge on the peptide backbone and its effect on the inclusion complex structure. Based on the NMR results we are able to extract information about the molecular structure for the interaction between CyD and aromatic amino acids, independent of the association constant.

Materials and methods

Pharmaceutical grade α-, β-, and γ-CyD were purchased from Wacker Chemie GmbH, Burghausen, Germany. 99.9 and 99% D_2O were purchased from Larodan Fine Chemicals AB, Malmö, Sweden. NaH_2PO_4 and Na_2HPO_4 were purchased from Bie & Bernstsen A/S, Rødovre, Denmark. 99% pure phenylalanine, 99% pure tyrosine and 98% pure tryptophan were all purchased from Sigma-Aldrich. Fmoc Ala MBHA-HMP column, Fmoc Gly PS column, Fmoc Phe PS column and reagents for the synthesis of the GGFA tetrapeptide were all purchased from PE Biosystems. 99% pure bradykinin was purchased from the American Peptide Company. Modified amino acids N-Acetyl-tyrosine amide, N-Acetyl-tyrosine, tyrosine-methylester, tryptophan-methylester, and N-Acetyl-tryptophan, all with 98% purity were purchased from Sigma-Aldrich. The molecular structures of the guest molecules used are shown in Fig. 2.

Stock solutions of 0.5 M NaD_2PO_4 and 0.5 M Na_2DPO_4 were prepared by dissolving NaH_2PO_4 and Na_2HPO_4 in D_2O and drying it to reduce the water signal in resulting spectra. The NMR samples of either α- or γ-CyD were prepared with either phenylalanine, tyrosine/tyrosine derivatives or tryptophan/tryptophan derivatives to a 550 μL final volume containing 25 mM CyD and 8.3 mM amino acid in 20 mM phosphate buffer at pH* of 5.9 in 99% D_2O. The NMR samples with β-CyD were prepared with phenylalanine, tyrosine/tyrosine derivatives or tryptophan/tryptophan derivatives to a final volume of 550 μL of 12 mM CyD and 4 mM amino acids in 20 mM phosphate buffer at pH* of 5.9 in 99% D_2O. The tetrapeptide GGFA was synthesised by solid-phase peptide synthesis on a Perkin Elmer ABI 432 SYNERGY peptide synthesizer and the protocol from Perkin Elmer was used. After the synthesis, the peptide was resuspended in 99% D_2O and freeze dried. The amount of synthesized GGFA peptide was estimated by measuring the absorbance at 257.4 nm on a UVIKON 934 Double Beam UV/VIS Spectrophotometer (Kontron Instruments) and assuming an extinction coefficient of 197 M^{-1} cm^{-1} [18]. After the synthesis, the peptide was resuspended in 99% D_2O and freeze dried. For the NMR samples, the tetrapeptide was mixed with a β-CyD stock solution resulting in a final concentration of 2.5 mM peptide with 7.5 mM β-CyD in 20 mM phosphate buffer, pH* 5.9. The NMR sample with bradykinin contained 550 μL of 1 mg/mL of 99% pure bradykinin in 99% D2O with 5.6 mM β-CyD and 20 mM phosphate buffer, pH* 5.9.

All ^{1}H-NMR spectra were recorded at 600 MHz on a BRUKER DRX600 spectrometer equipped with a 5 mm xyz-gradient TXI(H/C/N) probe. All spectra were recorded at 288 K. The spectra were referenced to the residual water signal (4.86 ppm at 288 K), since chemical shift standards can interact with CyDs and thereby negatively influence the inclusion complex formation between CyDs and aromatic amino acids. One-dimensional ^{1}H NMR spectra were recorded on all samples to ensure that the sample had not undergone any degradation or contamination. ^{1}H 2D

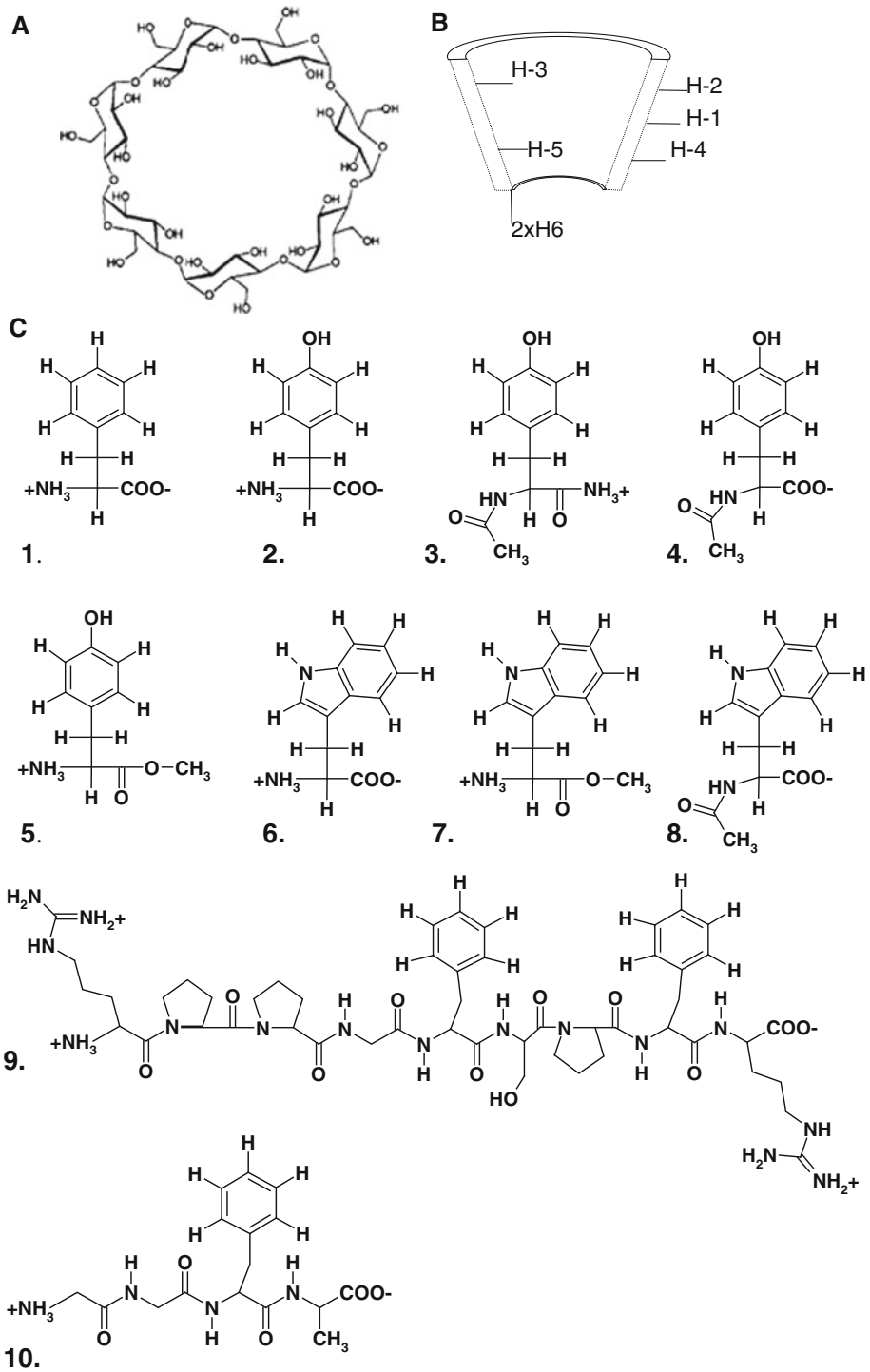

Fig. 2 **a** Molecular structure of β-CyD. **b** Sketch of the proton positions in a CyD molecule. **c** Molecular structure of the guest molecules. 1—Phenylalanine, 2—Tyrosine, 3—N-Acetyl-tyrosine amid, 4—N-Acetyl-tyrosine, 5—Tyrosine methylester, 6—Tryptophan, 7—Tryptophan methylester, 8—N-Acetyl-tryptophan, 9—Bradykinin, 10—GGFA-peptide

Rotating frame Overhauser Effect SpectroscopY (ROESY) spectra with a 250 ms continuous wave spin-lock of 6 kHz were acquired to detect any intermolecular interactions between CyDs and the aromatic amino acids. The NMR data were processed with BRUKER XwinNMR Ver. 2.5 software and the spectral analysis was done with XEASY Ver. 1.3.13 [19].

Results and discussion

The 2D ROESY spectra revealed clear interactions between phenylalanine (Phe) and α-, β-, and γ-CyDs (Fig. 3a, b). No binding stoichiometries higher than 1:1 were indicated from the data, in accordance with what was expected from the experimental setup (e.g. the low

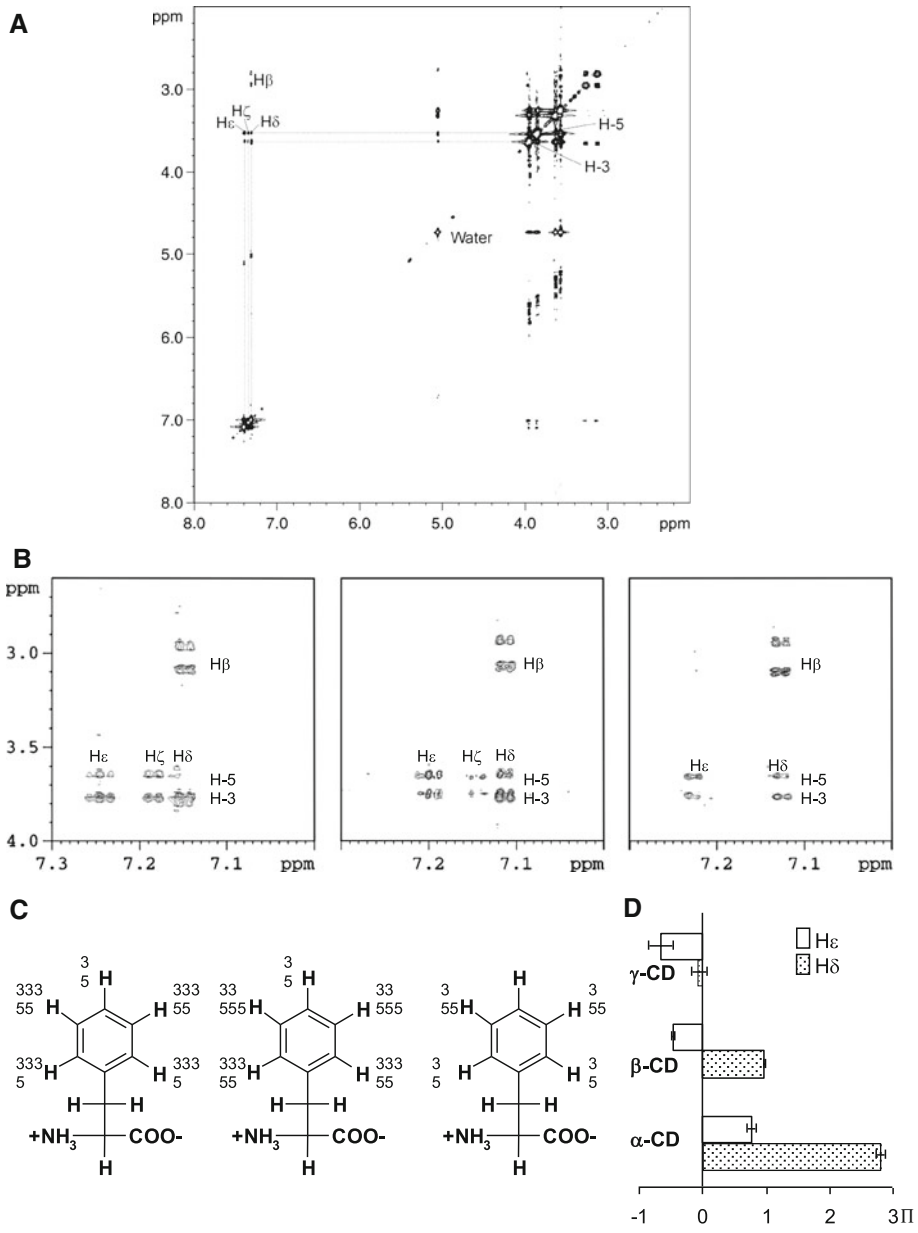

Fig. 3 CyD interaction with phenylalanine. **a** Area of 2D ROESY spectra with 250 ms mixing time on phenylalanine and CyD in 20 mM phosphate buffer of a pH* of 5.9 at 288 K. *Left* α-CyD; Center: *β*-CyD; *Right* *γ*-CyD. The cross peaks between CyDs and phenylalanine are highlighted and assigned. **b** The interaction pattern obtained from ROESY spectra; xxx: strong interaction to proton H–x of the CyD. xx: medium interaction to proton H–x of the CyD. x: weak interaction to proton H–x of the CyD. **c** *Π* as defined in Eq. 1

concentrations used and the high ratio between CyD and the guest molecule) and structure of the guest molecule [20]. Cross peak intensities (Fig. 3c) and the penetration gauge diagram (Fig. 3d) showed a clear difference in the penetration depths for Phe in relation to the CyD cavity size. The penetration gauge for the α-CyD-Phe complex was positive for both H^{δ} and H^{ε}, which means that only a smaller part of the phenyl ring was included in the hydrophobic α-CyD cavity. The reason for this is that the cavity diameter of α-CyD (5.2–4.7 Å) was too small for a good fit with the aromatic moiety (6.5 Å) of phenylalanine. Similarly, Inoue and Miyata, (1981) concluded from relaxation time and chemical shift change studies that the inclusion of the phenyl ring into the cavity was shallow and loose in the case of α-CyD. Other studies of substituted benzenes have lead to the same conclusion that α-CyD is only capable of partly including the benzene ring [21–23]. For both *β*- and *γ*-CyD the penetration gauges indicate that the whole phenyl ring can enter into their cavities, with phenylalanine penetrating deeper into the *γ*-CyD cavity. Figure 2 shows weaker cross peak intensity for *γ*-CyD, which can be explained by a lower association constant for the inclusion complex due to the *γ*-CyD cavity being too wide (7.5–8.3 Å) for a good fit for the six-membered aromatic ring. The *β*-CyD cavity size (6.0–6.4 Å) seems to fit the phenyl moiety well. Tyrosine displays a very similar interaction pattern and penetration gauge as found for phenylalanine, which was also expected (Results not

shown). X-ray diffraction studies performed by Hamilton et al. [24] and studies of relaxation time and chemical shift changes by [24] show that the six-membered aromatic ring was located in the center of the β-CyD cavity having an optimal fit for each other. Linde et al. [12] presented equivalent results for the interaction of β-CyDs with tyrosine and phenylalanine using 2D ROESY experiments. For tryptophan, similar observations for the interaction with α- and β-CyDs were made, where the six-membered ring of the indole moiety was included into the cavity of CyD (Results not shown). Similar results showed that the indole ring did not penetrate far into the α-CyD cavity and that the interaction was further favoured by hydrogen bond formation between the backbone carboxylate and ammonium group with the secondary hydroxyl groups [25, 26]. The wider rim of the γ-CyD cavity however, was big enough to include the whole the indole ring (9.0 Å). Due to its size, the γ-CyD cavity could include tryptophan in many different conformations, which because of fast exchange were indistinguishable in the NMR spectra. Even though the γ-CyD-tryptophan complexes had different conformations, only a 1:1 binding stoichiometry was possible due to sterical hindrance. γ-CyD is more flexible than both β- and α-CyD [20], therefore it is possible for γ-CyD to make a steric fit to the guest without hindrance [27]. Based on the inclusion complex formation between free aromatic amino acids and the common CyDs, it is clear that the cavity size of β-CyD is optimal for tyrosine and phenylalanine while the γ-CyD cavity seems to fit tryptophan best. Therefore these two CyD were used further in this study.

To further study the influence of backbone charges on the penetration depth, tyrosine (Tyr) derivatives (Fig. 4) with backbones of various masking charges were investigated (NAc-Tyr-$CONH_2$ (nonionic), NAc-Tyr-COOH (anionic) and NH_2-Tyr-COOMe (cationic)) and compared to zwitterionic tyrosine (NH_2-Tyr-COOH). Based on ROESY cross peak intensities and the derived penetration gauge (Fig. 4) the elimination of backbone charges of Tyr increased the penetration depth into β-CyD. Both the nonionic and the anionic derivative exhibited similar penetration depths while the cationic derivative seemed to penetrate slightly deeper. In addition, cross peak intensities seemed to be higher for the derivatives compared to free tyrosine, indicating that the derivatives have higher association constants. The elimination of charges in the vicinity of the aromatic group has been shown by a spectrophotometrically detected competition assay to increase association constant [28], fluorescence [29], NMR and microcalorimetry [29]. The chemical groups used to mask the backbone charges might have some effect on the penetration as they can form H-bonds to both water and CyDs hydroxyl groups. Furthermore, the phenolic hydroxyl group of Tyr can make H-bonds to the primary rim of CyD and that stabilizes the inclusion complex. In addition, it has been suggested that the phenolic hydroxyl group of Tyr reaches through the cavity in order to be able to make these H-bonds with the primary rim and this accounts for the deeper penetration into the CyD cavity [30].

Similar experiments on the influence of backbone charges on the penetration depth were performed on tryptophan (Trp) derivatives (Fig. 5) with different masking of

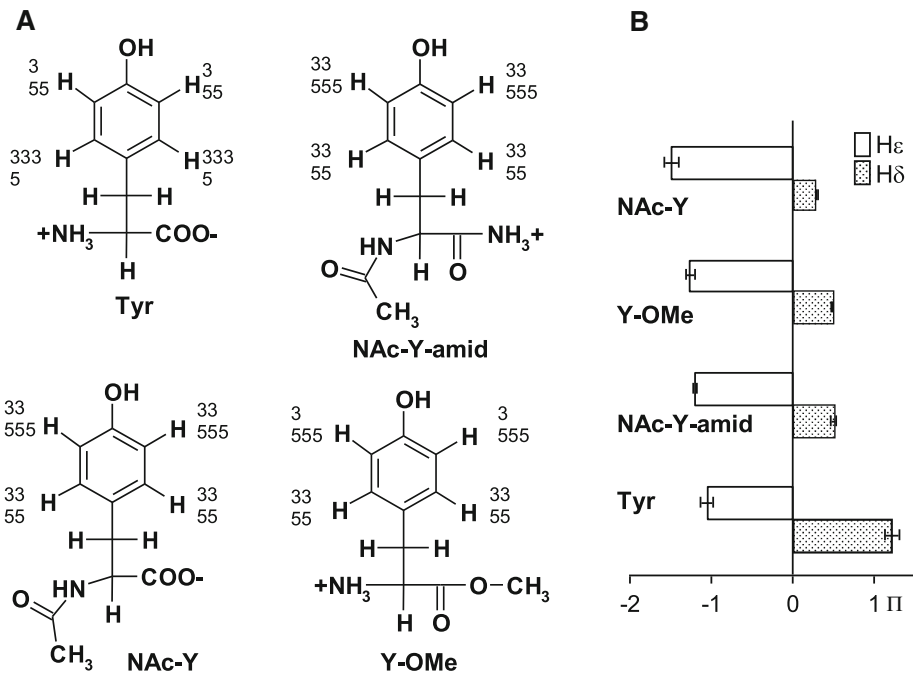

Fig. 4 β-CyD interaction with tyrosine and tyrosine derivatives with different backbone charges. **a** chemical structure of tyrosine and its derivatives (*Tyr* Tyrosine, *NAc-Y-Amid* N-Acetyl-tyrosine amid, *NAc-Y* N-Acetyl-tyrosine, *Y-OMe* tyrosine methylester) with interaction pattern obtained from ROESY spectra; xxx: strong interaction to proton H–x of the CyD. xx: medium interaction to proton H–x of the CyD. x: weak interaction to proton H–x of the CyD. **b** Π as defined in Eq. 1

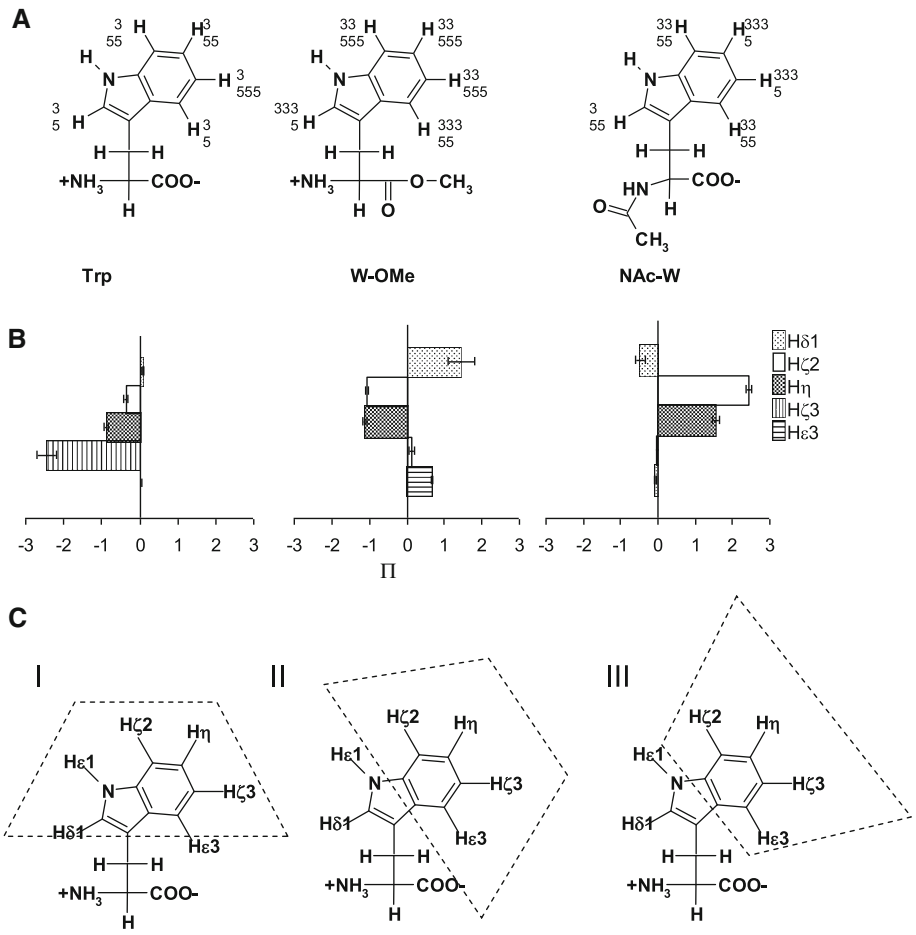

Fig. 5 β-CyD interaction with tryptophan and Trp derivatives with different backbone charges. **a** Chemical structures of tryptophan and its derivatives (*Trp* tryptophan, *W-OMe* tryptophan methylester, *NAc-W* N-Acetyl-tryptophan) with interaction pattern obtained from ROESY spectra; xxx: strong interaction to proton H–x of the CyD. xx: medium interaction to proton H–x of the CyD. x: weak interaction to proton H–x of the CyD. **b** Π as defined in Eq. 1. **c** Sketch of possible inclusion complex geometries between tryptophan and β-CyD

backbone charges: NH_2-Trp-COOH (zwitterionic), NH_2-Trp-COOMe (cationic), and, NAc-Trp-COOH (anionic). As for Tyr, the cross peak intensities derived from ROESY spectra were higher for Trp derivatives with one of their charges masked compared to zwitterionic tryptophan indicating again a higher association constant. The calculated penetration gauge diagrams clearly differs between the Trp derivatives and together with the cross peak intensity they indicate different inclusion complex geometries shown schematically in Fig. 5c. For Trp the preferred conformation seems to be II and I, for Trp-OMe mainly II and III and NAc-Trp can best be explained by geometry III. For both α- and β-CyD inclusion geometry, II is preferred for Trp (results not shown). It is thus evident, that masking the backbone charges has a substantial impact on the inclusion complex conformation and geometry. These changes may result from alterations in backbone hydration, hydrogen bonding potential, dipole–dipole and dipole–charge interactions between the guest molecule and both water and CyDs. However, there were no indications of changes in binding stoichiometry between the charge masked and non-masked amino acids used in this study. Having demonstrated the influence of the backbone charges on complex formation, we moved on to investigate the effect of the presence of neighboring amino acids on complex formation.

In the context of proteins, it is interesting to investigate how the interactions between aromatic amino acids and CyDs are influenced by the presence of a peptide backbone. The incorporation of amino acids into a (poly)peptide can influence complex formation in two ways; firstly, neighboring amino acids might sterically hinder complex formation. This is especially the case if the amino acid is incorporated into the hydrophobic core of the protein [16]. Secondly, the electrical charges on the amino acid vanish upon formation of peptide bonds. We have chosen two peptides, GGFA and bradykinin, which contain one and two Phe, respectively (Fig. 6a, d) to look at this effect. The 2D ROESY spectra show that the cross peak intensities were different for all Phe in the peptides (Fig. 6a, b). Furthermore, the cross peak intensities seemed to be more intense for the peptide bound Phe compared to free Phe. The penetration gauge diagram (Fig. 6c) demonstrates more clearly the difference in penetration depths for peptide bound and free Phe. Elimination of the backbone charges enhanced the penetration depths of Phe 8 in bradykinin and Phe in GGFA.

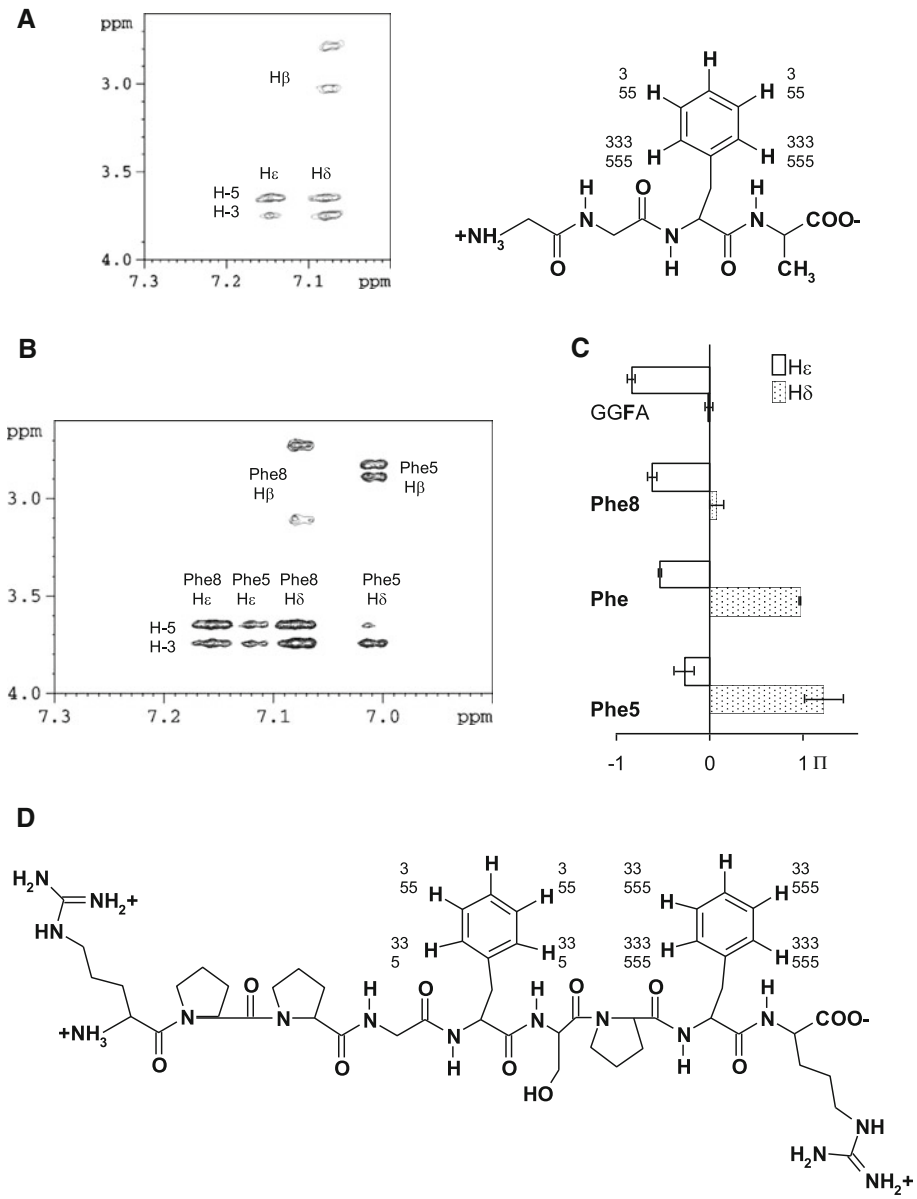

Fig. 6 β-CyDs interaction with phenylalanine as free and peptide bound amino acid. **a** Area of 2D ROESY spectra with 250 ms mixing time on GGFA and β-CyD in 20 mM phosphate buffer of a pD of 5.9 at 288 K and sketch of GGFA with the interaction pattern obtained from ROESY spectra. **b** Area of 2D ROESY spectra with 250 ms mixing time on Bradykinin (Phe5 and Phe8) and β-CyD in 20 mM phosphate buffer of a pH* of 5.9 at 288 K. **c** Π as defined in Eq. 1. **d** Sketch of Bradykinin with the interaction pattern obtained from ROESY spectra; xxx: strong interaction to proton H–x of the CyD. xx: medium interaction to proton H–x of the CyD. x: weak interaction to proton H–x of the CyD

This is in line with earlier results showing that the dislocation of the charges and deeper penetration leads to stronger interaction [28, 31] and thus to higher association constants for peptide fragments than for free aromatic amino acids.

Furthermore, the interactions with peptide-bound residues of the same type do not need to be identical; for bradykinin, Phe 8 displayed a stronger interaction with β-CyD and a deeper penetration than Phe 5. Factors like the conformation of the peptide backbone as well as steric hindrance from other amino acids will affect both the association constant and penetration depth [32] and it was found that both Phe 5 and Phe 8 in bradykinin interacted with β-CyD. These results also indicated an interaction with arginine residues in bradykinin. By ESI–MS they were able to show an interaction between free arginine and β-CyD. The present study does not show interactions to anything other than the phenylalanine side-chains of bradykinin.

In our study, no interaction between H^{ζ} of phenylalanine and H-3 and H-5 of β-CyD can be identified. This could be caused by the larger distance of H^{ζ} to the CyD molecule compared to H^{δ} and H^{ε}. While there are a number of crystal structures of proteins with CyD in the PDB database, there are no NMR structures of protein-CyD complexes available. We have measured NOESY spectra of protein: CyD complexes on three proteins: insulin, S6 and CI2 [16]. In order to learn more about the inclusion complex formation between aromatic amino acids and CyD we have calculated the penetration gauge for those amino acids with well-resolved NMR resonances (Fig. 7). The penetration gauges for tyrosine in both insulin and S6 (Fig. 7a, b) are similar to

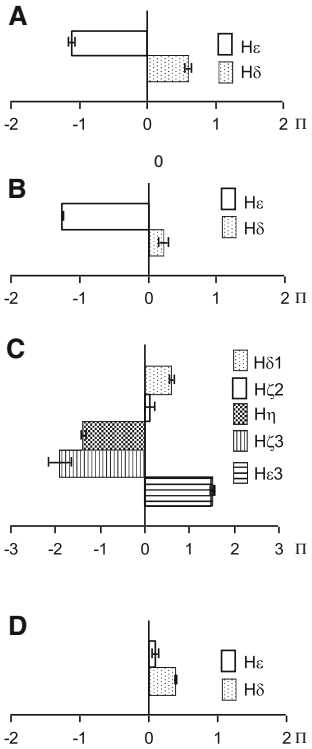

Fig. 7 The logarithm intensity ratio between H-3s and H-5s protons to the aromatic protons amino acid in protein and CyDs. **a** Insulin Tyr14 and *β*-CyD. **b** S6 Tyr49 and *β*-CyD. **c** CI2 Trp65 and *β*-CyD (Strong interaction between Hζ3and H-5). **d** S6 Phe97 and *β*-CyD

results obtained for NAc-Tyr-NH_2. However for tryptophan (CI2) and phenylalanine (S6) (Fig. 7c, d) the penetration gauges cannot be directly compared to those of the investigated derivatives. This means that complex geometry in inclusion complexes of CyD with protein-bound amino acids is also dictated by sterical demands of neighboring amino acids of the protein.

Conclusion

We have introduced here a penetration gauge as a means for the straightforward evaluation of the penetration depths of aromatic rings into the CyD independently of the association constant. We determined the penetration gauges of several inclusion complexes of different CyDs with aromatic amino acids, their derivatives and peptides containing these amino acids. Based on the penetration gauges we can conclude that the backbone charges on aromatic amino acids reduced the penetration depth of the aromatic ring and masked the charges, either by modification or incorporation into a peptide chain, which lead to increased penetration depths. We also demonstrated that identical amino acid residues within the same peptide or protein can have largely different inclusion complex geometries and complex stabilities.

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References

1. Branchu, S., Forbes, R.T., York, P., Nyqvist, H.: The Effect of Cyclodextrins in Monomere Protein Unfolding. Biocalorimetry Applications of Calorimetry in the Biological Sciences, vol. 1. Wiley, West Sussex (1998)
2. Cooper, A.: Effect of cyclodextrins on the thermal stability of globular protein. J. Am. Chem. Soc. **114**(23), 9208–9209 (1992)
3. Cooper, A., McAuley-Hecht, K.E.: Microcalorimetry and the molecular recognition of peptides and proteins. Philos. Trans. R. Soc. A **345**, 23–35 (1993)
4. Brewster, M.E., Hora, M.S., Simpkins, J.W., Boder, N.: Use of 2-hydroxypropyl-beta-cyclodextrin as a solubilizing and stabilizing excipient for protein drugs. Pharm. Res. **8**, 792–795 (1991)
5. Charman, S.A., Mason, K.L., Charman, W.N.: Techniques for assessing the effects of pharmaceutical excipients in the aggregation of porcine growth hormone. Pharm. Res. **10**, 954–961 (1993)
6. Izutsu, K.-L., Yoshioka, S., Terao, T.: Stabilization of beta-galactosidase by amphiphilic additives during freeze-drying. Int. J. Pharm. **90**, 187–194 (1993)
7. Karuppiah, N., Sharma, A.: Cyclodextrin as protein folding aids. Biochem. Biophys. Res. Commun. **211**(1), 60–66 (1995)
8. Katakam, M., Banga, A.K.: Aggregation of proteins and its prevention by carbohydrate excipients: albumins and gamma-globulin. J. Pharm. Pharmacol. **47**, 103–107 (1995)
9. Lee, M.-J., Fennema, O.R.: Ability of cyclodextrins to inhibit aggregation of beta-casein. J. Agric. Food Chem. **39**, 17–21 (1991)
10. Sigurjónsdóttir, J.F., Loftsson, T., Másson, M.: Influence of cyclodextrins on the stability of the peptide salmon calcitonin in aqueous solution. Int. J. Pharm. **186**, 205–213 (1998)
11. Linde, G.A., Laverde, A., de Faria, E.V., Colauto, N.B., de Moraes, F.F., Zanin, G.M.: Taste modification of amino acids and protein hydrolysate by alpha-cyclodextrin. Food Res. Int. **42**(7), 814–818 (2009)
12. Linde, G.A., Laverde, A., De Faria, E.V., Colauto, N.B., De Moraes, F.F., Zanin, G.M.: The use of 2D NMR to study beta-cyclodextrin complexation and debittering of amino acids and peptides. Food Res. Int. **43**(1), 187–192 (2010)
13. Yamamoto, T., Fukui, N., Hori, A., Matsui, Y.: Circular dichroism and fluorescence spectroscopy studies of the effect of cyclodextrins on the thermal stability of chicken egg white lysozyme in aqueous solution. J. Mol. Struct. **782**(1), 60–66 (2006). doi:10.1016/j.molstruc.2005.01.024
14. Yoshikiyo, K., Takeshita, R., Yamamoto, T., Takahashi, T., Matsui, Y.: The effects of cyclodextrins on the thermal denaturation and renaturation processes of bovine pancreatic ribonuclease A in an aqueous solution studied by circular dichroism and fluorescence spectroscopy. J. Mol. Struct. **832**(1–3), 96–100 (2007)
15. Otzen, D.E., Knudsen, B.R., Aachmann, F., Larsen, K.L., Wimmer, R.: Structural basis for cyclodextrins' suppression of human growth hormone aggregation. Prot. Sci. **11**(7), 1779–1787 (2002)
16. Aachmann, F.L., Otzen, D.E., Larsen, K.L., Wimmer, R.: Structural background of cyclodextrin–protein interactions. Prot. Eng. **16**(12), 905–912 (2003)

17. Matilainen, L., Larsen, K.L., Wimmer, R., Keski-Rahkonen, P., Auriola, S., Jarvinen, T., Jarho, P.: The effect of cyclodextrins on chemical and physical stability of glucagon and characterization of glucagon/gamma-CD inclusion complexes. J. Pharm. Sci. **97**(7), 2720–2729 (2008)
18. Creighton, T.E.: Proteins: structures and molecular properties, 2nd edn. W. H. Freeman and Company, New York (1993)
19. Bartels, C., Xia, T.-H., Billeter, M., Günter, P., Wüthrich, K.: The program XEASY for computer supported NMR spectral of biological macromolecules. J. Biomol. NMR **5**, 1–10 (1995)
20. Szejtli, J.: In: Szejtli, J., Osa, T. (eds.) Comprehensive Supramolecular Chemistry, vol. 3. Pergamon, Exeter, UK (1996)
21. Inoue, Y., Hoshi, H., Sakurai, M., Chujo, R.: Geometry of cyclohexaamylase inclusion complexes with some substituted benzenes in aqueous solution based on carbon-13 NMR chemical shifts. J. Am. Chem. Soc. **107**, 2319–2323 (1985)
22. Inoue, Y., Okuda, T., Miyata, Y., Chujo, R.: N.M.R. Studies of cycloamylose inclusion-complexes with p-substituted phenols. Carbohydr. Res. **125**, 65–76 (1984)
23. Komiyama, M., Harai, H.: Carbon-13 NMR study on the cyclodextrin inclusion complexes in solution. Bull. Chem. Soc. Jpn. **54**(3), 828–831 (1981)
24. Hamilton, J.A., Sabesan, M.N., Steirauf, L.K., Geddes, A.: The crystal structure of a complex of cycloheptaamylose with 2,5-diiodobenzoic acid. Biochem. Biophys. Res. Commun. **73**, 659–664 (1976)
25. Lipkowitz, K.N., Raghothama, S., Yang, J.: Enantioselective binding of tryptophan by alfa-cyclodextrin. J. Am. Chem. Soc. **114**, 1554–1562 (1992)
26. Kahle, C., Holzgrabe, U.: Determination of binding constants of cyclodextrin inclusion complexes with amino acids and dipeptides by potentiometric titration. Chirality **16**(8), 509–515 (2004)
27. Hembury, G., Rekharsky, M., Nakamura, A., Inoue, Y.: Direct correlation between complex conformation and chiral discrimination upon inclusion of amino acid derivatives by beta- and gamma-cyclodextrins. Org. Lett. **2**(21), 3257–3260 (2000)
28. Horský, J., Pitha, J.: Inclusion complexes of proteins: interaction of cyclodextrins with peptides containing aromatic amino acids studied by competitive spectrophotometry. J. Incl. Phenom. Mol. **18**, 291–300 (1994)
29. Mrozek, J., Banecki, B., Karolczak, J., Wiczk, W.: Influence of the separation of the charged groups and aromatic ring on interaction of tyrosine and phenylalanine analogues and derivatives with beta-cyclodextrin. Biophys. Chem. **116**(3), 237–250 (2005)
30. Waibel, B., Scheiber, J., Meier, C., Hammitzsch, M., Baumann, K., Scriba, G.K.E., Holzgrabe, U.: Comparison of cyclodextrin-dipeptide inclusion complexes in the absence and presence of urea by means of capillary electrophoresis, nuclear magnetic resonance and molecular modeling. Eur. J. Org. Chem. **18**, 2921–2930 (2007)
31. Bekos, E.J., Gardella, J.A., Bright, F.V.: The binding of free oligopeptides to cyclodextrins: the role of the tyrosine group. J. Incl. Phenom. Mol. **26**(4), 185–195 (1996)
32. Camilleri, C., Haskins, N.J., New, A.P., Saunders, M.R.: Analysing the complexation of amino acids and peptides with beta-cyclodextrin using electrospray ionization mass spectrometry. Rapid Commun. Mass. Spec. **7**, 949–952 (1993)