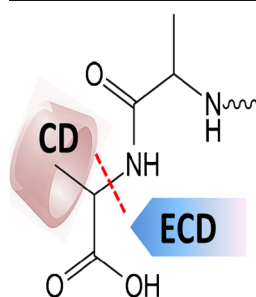


RESEARCH ARTICLE

Determining the Binding Sites of β -Cyclodextrin and Peptides by Electron-Capture Dissociation High Resolution Tandem Mass Spectrometry

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Abstract. Cyclodextrins (CDs) are a group of cyclic oligosaccharides, which readily form inclusion complexes with hydrophobic compounds to increase bioavailability, thus making CDs ideal drug excipients. Recent studies have also shown that CDs exhibit a wide range of protective effects, preventing proteins from aggregation, degradation, and folding. These effects strongly depend on the binding sites on the protein surface. CDs only exhibit weak interactions with amino acids, however; conventional analytical techniques therefore usually fail to reveal the exact location of the binding sites. Moreover, some studies even suggest that CD inclusion complexes are merely electrostatic adducts. Here, electron capture dissociation (ECD) was applied in this proof-of-concept study to examine the exact nature of the CD/

peptide complexes, and CD binding sites were unambiguously located for the first time *via* Fourier-transform ion cyclotron resonance (FTICR) tandem mass spectrometry.

Key words: Cyclodextrin inclusion complex, Non-covalent binding, Electron capture dissociation, ECD, FTICR, Differential ion mobility spectrometry

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Introduction

Cyclodextrins (CDs) are torus-like cyclic oligosaccharides with the 2- and 3-hydroxyl groups located at the wider opening of the structure and the 6-OH groups at the narrow end (Figure 1). In the liquid phase, the CD's hydroxyl groups arrange on the surface of the structure, resulting in a hydrophobic internal cone. This molecular structure enables CDs to accommodate hydrophobic guest molecules within its cavity and this complexation accounts for a variety of beneficial effects in pharmaceutical and biological applications. For example, CDs are important pharmaceutical excipients, as drugs are readily coordinated in the cavity, to increase bioavailability and to accelerate uptake in the human body [1]. CDs are also used in capillary electrophoresis for chiral separations [2], and interestingly, CDs have shown stabilization effects for proteins [3].

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Even though CDs were discovered over 100 years ago [4] and have been widely implemented in pharmaceutical products, their effects on proteins were not realized until the mid-1990s [3]. During the last decade, CDs and their derivatives have been established as a very promising class of stabilizers to protect proteins from aggregation/folding [5, 6], to increase protein stability in stress thermal/chemical conditions [7], and to maintain protein conformation during freeze-drying [8]. The exact mechanisms of CD/protein interactions have not been fully elucidated, however, as stabilization reactions occur only at certain sites on the protein surface, and binding strongly depends on the specific structure of the protein. Several different analytical techniques have been implemented to examine CD/protein interactions, including ultraviolet/fluorescence/circular dichroism spectroscopy [9], nuclear magnetic resonance [10], and mass spectrometry (MS) [11]. Most published studies have reported that CDs specifically interact with hydrophobic amino acids, in particular Tyr, Trp, and Phe [9, 11–14], suggesting that CDs form inclusion complexes with the aromatic residues. Initial mass spectrometry studies also confirmed the affinity of CDs to aromatic molecules in the gas-phase [11, 15, 16]. Subsequently, Cunniff and Vouros investigated the interaction of various amino acids and peptides to CDs and found that the CD complexes were not limited to aromatic moieties.

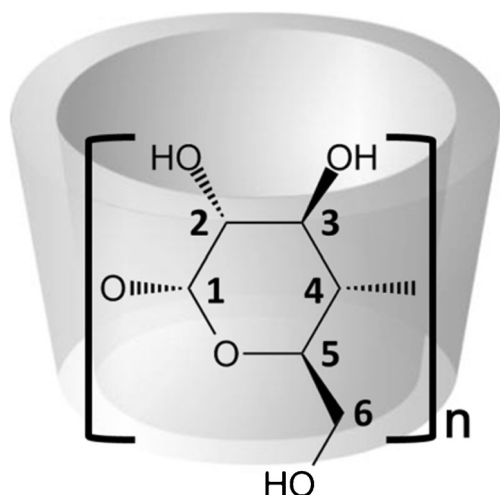


Figure 1. General structure of cyclodextrins (CD), in which $n=6, 7,$ and 8 correspond to α -CD, β -CD and γ -CD

The authors concluded that the aggregates observed in mass spectrometry originated from electrostatic interactions rather than non-covalent inclusions [17]. Since then, several groups have utilized tandem mass spectrometry (MS/MS) to explore the exact nature of CD complexes, *e.g.*, collision-induced dissociation (CID) [16], blackbody infrared radiative dissociation (BIRD) [18], and heated capillary dissociation (HCD) [19]. Unfortunately, the above dissociation techniques are based on the excitation of vibrational modes of the molecules; that is, cleavage reactions start with the weakest bond of the assembly, which is usually the non-covalent bond of the CD/protein complex [20]. For this reason, direct experimental evidence of gas-phase CD inclusion and their binding sites on proteins has never been directly obtained by tandem mass spectrometry experiments. Ramirez et al. performed computer modelling experiments and designed a guest exchange reaction in Fourier transform ion cyclotron resonance (FTICR)-MS, which proved the presence of inclusion complexes, as the amino acid guest in a CD cavity was exchanged in the gas-phase in the presence of gaseous alkyl amine [21]. A “three-point interaction” model was proposed by the authors, which attributed the CD inclusion to hydrogen bonding [22].

In recent years, ion-electron recombination techniques – in particular electron capture dissociation (ECD) [23] – have been developed, providing complementary information to conventional MS/MS methods. ECD is a non-ergodic fragmentation technique, which can preserve labile modifications and non-covalent bonds of analytes [24, 25]. For this reason, ECD was applied here to β -CD (which contains seven glucopyranose units) and gas-phase complexes formed by six different peptides composed of various amino acids. Direct evidence of hydrogen binding was observed in the experiments, which supported the “three-point interaction” theory. In addition, the labile CD modifications were preserved under ECD conditions, while the backbone of the peptide was cleaved. Finally, the CD binding sites of proteins were unambiguously identified for the first time by tandem mass spectrometry.

Experimental

Sample preparation

Standard peptides (substance P, bombesin, angiotensin I, angiotensin II, [Asn¹, Val⁵]angiotensin II, neurotensin fragment 1–8) were purchased from Sigma-Aldrich (Steinheim, Germany). β -(glucose)₇-cyclodextrin (CD), methanol and formic acid were purchased from Merck KGaA (Darmstadt, Germany). All peptides and β -cyclodextrin were diluted in aqueous solution to a molar concentration of 100 μ M and each peptide mixed with β -cyclodextrin individually at a ratio of 1:1 (v/v). The mixtures were then incubated at 37 °C for 24 h. All samples were diluted to 0.5 μ M with H₂O/methanol/formic acid (50:50:1 v/v/v) prior to MS analysis.

Mass spectrometry

Samples were ionized using electrospray ionization (ESI) and mass spectra recorded using a 7 Tesla FTICR-MS instrument described elsewhere [26]. For each spectrum, 40–80 individual transients were collected and co-added to enhance S/N [27]. In MS/MS mode, precursor ions were isolated first in the quadrupole and externally accumulated for 0.1–0.5 s. For CID, 5–20 V collision voltage was applied. For ECD, the accumulated ions were transferred into the ICR cell and irradiated with 1.5 eV electrons from a 1.5A heated hollow cathode dispenser for 30–100 ms. All spectra were internally calibrated (see peak lists in Supplementary Material). Due to H transfer between c'/z ions in ECD experiments [28, 29], peak assignment was based on matching both the theoretical mass and the isotopic pattern. The differential ion mobility spectrometry (DMS) experiments were performed on an AB Sciex (Concord, ON, Canada) QTRAP 5500 quadrupole linear ion trap (QqLIT) mass spectrometer equipped with a AB Sciex SelexIon differential ion mobility spectrometry device [30]. The QqLIT instrument was operated in positive ion mode. Analyses were carried out using the Turbo-V ESI source at +5.5 kV potential, with the heat injectors turned off. Nitrogen was used as curtain gas at 18 psi pressure, nebulizer (GS1) at 30 psi, auxiliary gas (GS2) at 10 psi; DP was set to 80 V, EP to 10 V. The SelexIon DMS device was operated using the following parameters: temperature (DT), low; resolution enhancement (DR), off; DMS offset (DMO), –3; separation voltage (SV), 4 kV; no chemical modifier.

Results and Discussion

In the experiments described here, β -CD was incubated individually with six different peptides (Table 1) and the formed complexes were subsequently directly ionized under regular ESI conditions at a nebulizing gas temperature of 200 °C. The observed CD complexes were generally stable and intact CD/peptide ions detected for all six samples. Based on previous studies, we assumed that CD interactions with peptides were either non-covalent [21] or electrostatic [17]. CID analysis was

Table 1. Peptide cleavage maps of β -CD inclusion complexes (CD binding fragments are marked with #)

| Peptide | Cleavage Map | Binding Sites |
|---|--|--------------------|
| Angiotensin I | $\begin{array}{cccccccc} \# & \# & \# & \# & & \# & \# & \# \\ \text{D} & \text{R} & \text{V} & \text{Y} & \text{I} & \text{H} & \text{P} & \text{F} & \text{H} & \text{L} \\ & \# & \# & \# & & & & & & \end{array}$ | Asp1, Tyr4 |
| Angiotensin II | $\begin{array}{cccccccc} \# & \# & \# & \# & & \# & & & & \\ \text{D} & \text{R} & \text{V} & \text{Y} & \text{I} & \text{H} & \text{P} & \text{F} & & \\ & \# & & \# & & & & & & \end{array}$ | Arg2, Tyr4 |
| [Asn ¹ , Val ⁵]- Angiotensin II | $\begin{array}{cccccccc} \# & \# & \# & \# & \# & & \# & & & \\ \text{N} & \text{R} & \text{V} & \text{Y} & \text{V} & \text{H} & \text{P} & \text{F} & & \\ & \# & \# & \# & \# & & & & & \end{array}$ | Asn1, Tyr4 |
| Neurotensin Fragment 1-8 | $\begin{array}{cccccccc} & & & & \# & & \# & & & \\ \text{pE} & \text{L} & \text{Y} & \text{E} & \text{N} & \text{K} & \text{P} & \text{R} & & \\ & \# & \# & \# & \# & & \# & & & \end{array}$ | Asn5, Lys6 |
| Substance P | $\begin{array}{cccccccc} \# & & \# & \# & \# & \# & \# & \# & \# & \# \\ \text{R} & \text{P} & \text{K} & \text{P} & \text{Q} & \text{Q} & \text{F} & \text{F} & \text{G} & \text{L} & \text{M} \\ & \# & & & & & & & & & \end{array}$ | Arg1 or Pro2, Lys3 |
| Bombesin | $\begin{array}{cccccccc} \# & \# & \# & \# & \# & \# & \# & \# & \# & \# & \# \\ \text{pE} & \text{Q} & \text{R} & \text{L} & \text{G} & \text{N} & \text{Q} & \text{W} & \text{A} & \text{V} & \text{G} & \text{H} & \text{L} & \text{M} \\ & \# & \# & \# & \# & \# & \# & \# & \# & \# & \# & \# & \# & \# \end{array}$ | Lys4, Gln7 |

applied initially to investigate the binding stabilities. Not surprisingly, however, CID failed to provide useful information on the CD complexes, considering the very weak chemical interactions. Figure 2 illustrates that the CD/peptide bond already started to cleave at an energy of 5 eV under CID conditions,

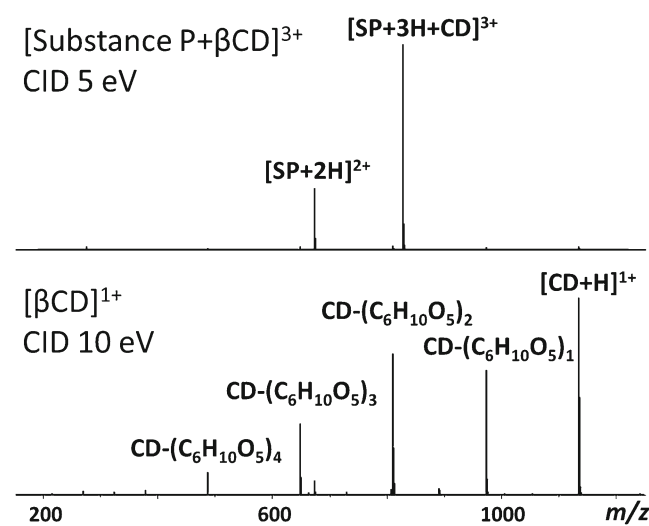


Figure 2. Top: CID spectrum of β -CD and substance P complex ([SP+3H+CD]³⁺) at 5 eV. Bottom: CID spectrum of the β -CD precursor ion at 10 eV, showing dissociation of glucopyranose (C₆H₁₀O₅) groups

while the peptide backbone remained intact. CID experiments on β -CD alone showed that the attached glucopyranose units on the CD ring dissociated at ~ 10 eV. To break backbone amide bonds, typically >20 eV are required [31]. Therefore, the experimental results indicate that CD binding to peptides was much weaker than amide bonds and any information on CD/Peptide interaction was lost under the applied CID conditions.

To better understand the nature of the CD/peptide complexes, electron-capture dissociation (ECD) was therefore implemented. The ion-electron dissociation technique generated rich product ion spectra with low peak intensities, which required significant manual efforts for data interpretation (Figures 3-5). For example, ECD of the triply-charged bombesin/CD (B+CD) precursor ion (Figure 3a) induced cleavages of both the CD/bombesin bond as well as the peptide backbone's N-C α bond (the full peak list is available in Table S1, Supplementary Material). More importantly, however, the ECD data also demonstrated that some of the peptide fragments were still attached to the intact CD molecule. To our knowledge, this is the first time that an intact CD modification has been preserved in MS/MS experiments. Except for the peptide fragment ions, intact peptide and CD/peptide complex ions were also observed with lower charge states. Interestingly, the isotope distribution of the singly-charged bombesin species was different from its theoretical distribution (Figure 3b). A closer look at the spectrum revealed that the isotope pattern was actually a

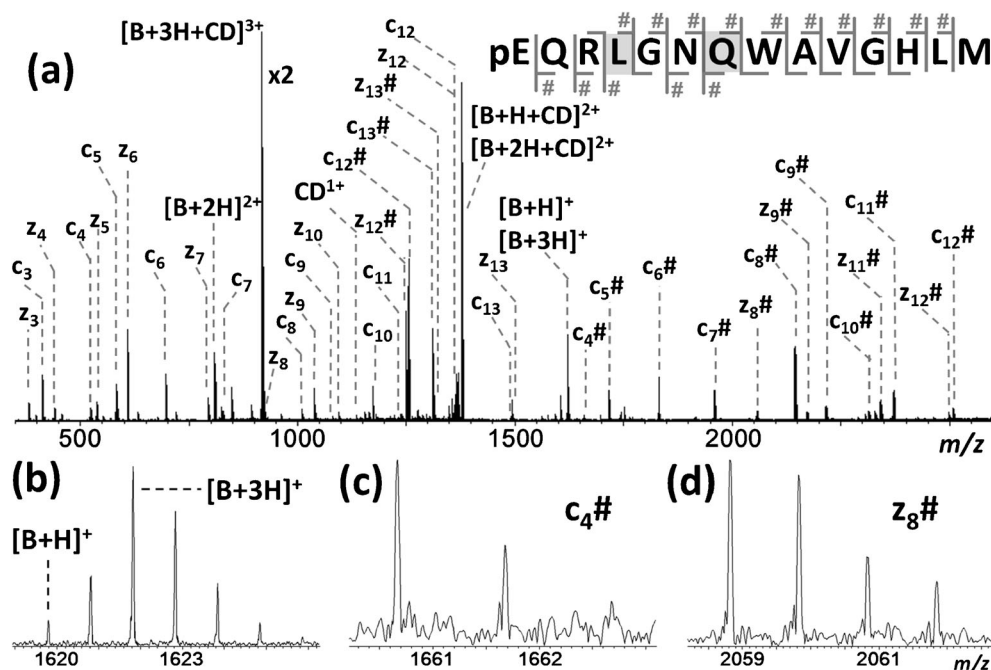


Figure 3. (a) ECD mass spectrum of the triply-charged β -CD+bombesin precursor ion along with the peptide cleavage map. The *c/z* fragments are labeled and β -CD binding fragments marked with # (full peak assignment is available in Table S1, Supplementary Material); (b) mass scale expansion of the singly-charged bombesin ions showing the $[B+H]^+$ and $[B+3H]^+$ species; (c-d) mass scale expansion of the areas of characteristic fragment ions corresponding to the CD binding site

combination of three bombesin species with a different number of hydrogens attached; *viz.*, $[B+H]^+$, $[B+2H]^+$, and $[B+3H]^+$. Of these three species, the first two are commonly observed in ECD experiments of small peptides: $[B+H]^+$ is a CID-type product ion induced by voltage and heating, and $[B+2H]^+$ is the charge-reduced species from electron capture [23]. Here, the $[B+3H]^+$ species of the bombesin could be explained by hydrogen migration between CD and the peptide during ECD cleavage; those species were also observed in the ECD spectra of the other five investigated CD/peptide complexes. The existence of the $[B+3H]^+$ ion suggests that a hydrogen bond was formed between the amino acid side chain and the -OH groups of the CD molecule.

The observation is consistent with our theory that the CD inclusion complexes in the gas-phase are due to non-covalent binding [21] rather than electrostatic forces. In theory, double electron capture and the CD molecule simply stabilizing the intermediate long enough that the second electron capture occurs before fragmentation may also be considered as potential mechanism. In our experiments, we did not detect double electron capture species $[\text{peptide}+\text{CD}+n\text{H}]^{(n-2)+}$ however. Further confirmation of our proposed mechanism for $[B+3H]^+$ formation by hydrogen transfer from CD to B could be achieved, for example, by deuterium labeling experiments, which were beyond the scope of the present work

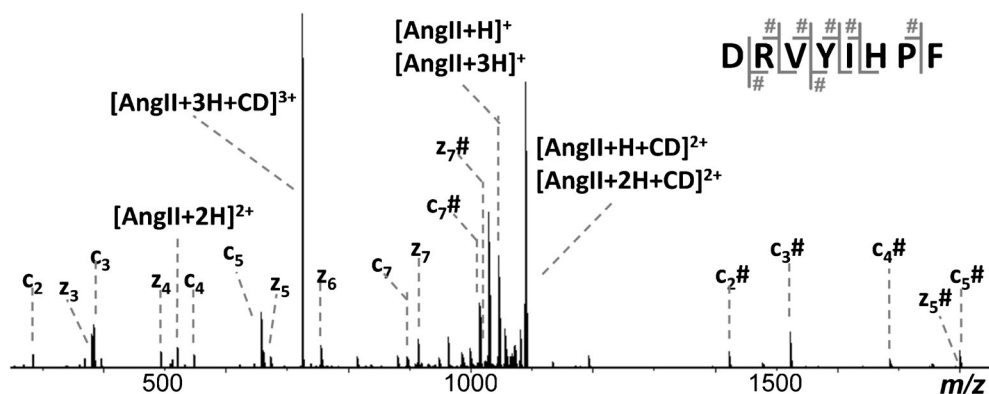


Figure 4. ECD mass spectrum of the triply-charged β -CD+angiotensin II precursor ion along with the peptide cleavage map. The *c/z* fragments are labeled and β -CD binding fragments marked with # (full peak assignment is available in Table S3, Supplementary Material)

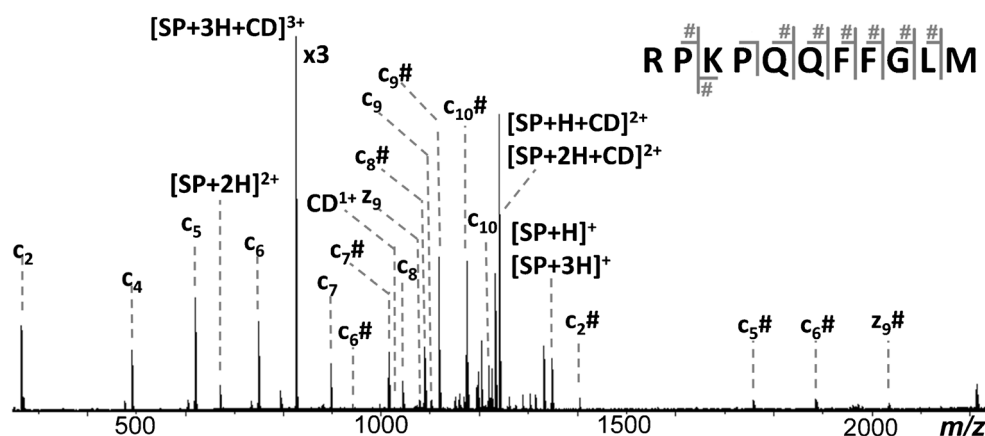


Figure 5. ECD mass spectrum of the triply-charged β -CD+substance P precursor ion along with the peptide cleavage map. The c/z fragments are labeled and β -CD binding fragments marked with # (full peak assignment is available in Table S6, Supplementary Material)

Detailed interpretation of the mass spectrum provided the peptide's cleavage map shown in Figure 3a, which illustrates that all inter-residue bonds of bombesin were cleaved and 100% sequence coverage was achieved *via* ECD (the symbol “#” in the figure designates peptide fragments attached to CD). Observation of $c_n\#$ and $z_n\#$ ions (Figure 3c and d) further demonstrates that CD binds to both Lys4 and Gln7 residues of bombesin. Interestingly, the isolated precursor ion for the ECD experiments was bombesin with only one CD attached; however, the discovery of two binding sites implied that the CD binding was not specific for a certain amino acid of the peptide; therefore, the isolated precursor ions are potentially a complex mixture of ions of the same elemental composition, but different CD modification sites. This is not surprising, however, as Lys and Gln possess ammonium groups and carboxylic hydrogen in their side chains, respectively, both of which can form hydrogen bonds with the hydroxyl groups of CD. In order to further examine this hypothesis, the

monoisotopic peak of the triply-charged bombesin/CD species was subjected to differential ion mobility spectrometry (DMS)-MS experiments on a low resolution QqLIT instrument, and at least two isomers were resolved in the gas-phase separation (Figure 6). Considering the flexibility of the molecule and the various possibilities for hydrogen bonding, there are likely more isomers formed; the slightly skewed and broad peaks in the DMS separation may also indicate the presence of further species (Figure 4). We therefore suggest that β -CD forms non-covalent inclusion complexes with peptides *via* non-specific hydrogen bonding; for bombesin, the favored binding sites appear to be Lys4 and Gln7.

To test the hypothesis, the same ECD experiments were performed using five other peptides: angiotensin I, angiotensin II, [Asn¹, Val⁵] angiotensin II, neurotensin fragment 1–8, and substance P. The experimental results are summarized in Table 1; ECD spectra and cleavage maps of triply-charged β -CD+angiotensin II and β -CD+substance P are shown in

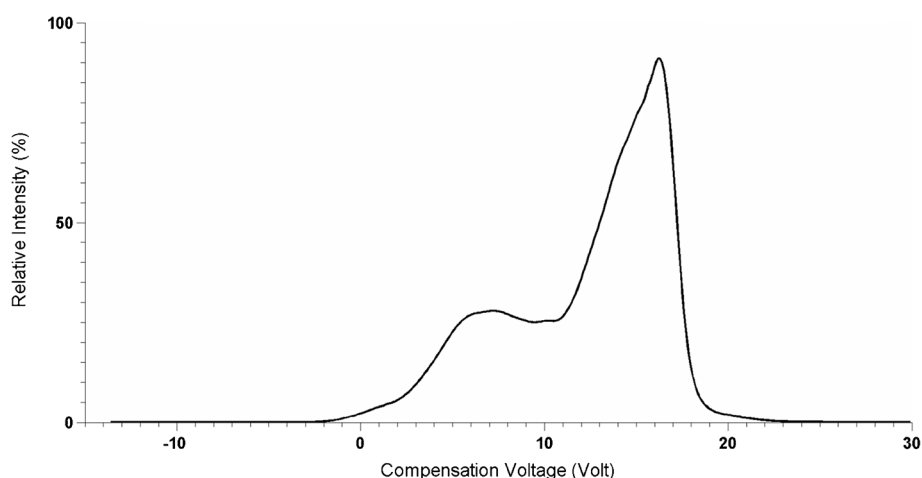


Figure 6. Differential ion mobility spectrometry separation of the monoisotopic species of the triply-charged β -CD-bombesin ion, demonstrating the existence of a mixture of at least two isomers. Note: the two species were not baseline resolved, because they have virtually the same structures differing only in the CD modification site; as well, the slightly skewed and broad peak shapes possibly indicate the presence of further isomers

Figures 4 and 5. The peptide cleavage maps illustrate that almost all the peptide inter-residue bonds can be cleaved *via* ECD, except for the N-terminal side of Pro (note that Pro is 100% resistant to ECD cleavage due to the tertiary nitrogen of the side chain). Even though all precursor ions selected for ECD were peptides with only one β -CD molecule attached, each of the peptides exhibited two possible binding sites. In this work, the CD/peptide complexes contained seven different amino acids; namely Tyr, Asp, Asn, Gln, Lys, Arg, and Pro. These seven amino acids exhibit different side chains: acidic, basic, aromatic, and amide chains. They have one characteristic feature in common, however, namely that their side chains contain either an ammonium or carboxylic acid group, which both exhibit the ability to form hydrogen bonds. On the other hand, of the seven amino acids, only Tyr contained an aromatic residue, while the widely reported CD interactions with Phe and Trp [3, 6, 12, 32] did not occur in the CD complexes described here, even though these amino acids were part of the sequence of most of the investigated peptides.

Conclusions

Our proof-of-concept experiments have demonstrated that cyclodextrins formed stable inclusion complexes with amino acids regardless of whether or not the amino acid included an aromatic moiety. The results further suggested non-covalent interactions between CD and the investigated peptides. The results are in agreement with experimental and theoretical data reported by Cunniff and Vouros [17] and Lebrilla and co-workers [21]. ECD fragmentation provided detailed insights into CD/peptide complexes, which enabled us to precisely locate CD binding sites for different peptides. For future work, the described approach will be extended to wider range of different CD derivatives and protein structures.

Acknowledgments

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