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Characterization of permethylated β-cyclodextrin-peptide noncovalently bound complexes using electron capture dissociation mass spectrometry (ECD MS)

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ABSTRACT

Electron capture dissociation mass spectrometry (ECD MS) was carried out for a number of β-permethylated cyclodextrin (CD)-peptide noncovalent complexes in a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. Examined peptides included *Angiotensin II* (DRVYIHPF), *Substance P* (RPKPQQFFGLM), and *Bradykinin* (RPPGFSPFR) and its analogs (PPGFSPFR and RPPGFSPF). ECD MS for doubly protonated complexes $[M:CD+2H]^{2+}$ mainly yielded cleavage of the backbones of the constituent peptide with little disassembly of a peptide and β-CD. Analysis of ECD MS fragments indicated that a protonated basic amino-acid residue or N-terminal amino group interacted more favorably with β-CD than did aromatic group-containing amino-acid residues (inclusion complex). In contrast to the formation of inclusion CD complexes in solution, we observed no specific evidence from our ECD MS mass spectra to support the generation of phenyl inclusion complexes in the gas phase. For gas-phase peptides, we suggest that ion–dipole interaction is the main driving force for the formation of noncovalent β-CD complexes rather than phenyl inclusion interactions.

1. Introduction

Cyclodextrins (CDs) that consist of α-1-4 linked glucose units are a well-documented model system for 'host–guest' chemistry in solution (Scheme 1) [1–7]. Depending on the number of constituting glucose units, they are referred to as α(6), β(7), or γ(8)-cyclodextrins. The overall shape of a CD is torus-like, with one rim wider than the other. The rims are composed of hydrophilic hydroxyl groups, whereas the outer surface and the inner cavity are hydrophobic [4]. A guest with a hydrophobic phenyl group is known to favorably include into the cavity in solution, thus forming an inclusion noncovalently bound complex [1–8]. The ability of CDs to form inclusion complexes has led to their application in separation sciences (e.g., chiral analysis) and has drawn the interest of pharmaceutical scientists in drug formulation and delivery [7–11]. It has also provided a model system for studies of enzyme–substrate interactions.

Mass spectrometry has been widely used to probe noncovalent interactions of proteins [12]. However, there has been an ongoing debate about whether mass spectrometric detection of noncovalent complexes in the gas-phase genuinely reflects interactions in solution. For gas-phase host–guest complexes involving CDs, extensive studies have been carried out due to this system's potential for examining inclusion complexes [4,13–26]. Using electrospray ionization mass spectrometry (ESI-MS), a variety of CD–guest (e.g., drugs, steroid hormones, amino acids, and peptides) noncovalent complexes have been detected and analyzed. Many investigators have reported, on the basis of favorable 1:1 complex formation with aromatic group-containing compounds, that CD–guest noncovalent complexes formed in the gas phase were inclusion complexes that reflected the interactions in solution [14–20]. In contrast, Cunniff and Vouros reported that CD mixtures with amino acids or small peptides that might be least likely to form hydrophobic inclusion complexes in solution generally showed the most intense complex ions in their ESI-MS experiments [13]. Based on this finding, they suggested that the CD complexes were likely to be electrostatic adducts formed during the ESI process.

More rigorous studies beyond simple ESI-MS measurements have since been carried out to examine this issue in more detail [4,21–26]. For example, Lebrilla et al. showed that permethylated β-CDs formed gas-phase inclusion complexes with an amino acid in guest exchange experiments performed in a Fourier transform

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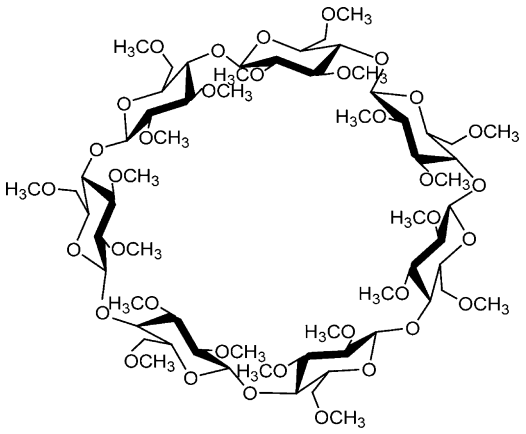

Scheme 1. Molecular structure of permethylated β-CD.

mass spectrometer [23,25,26]. Amino-acid guest exchange reactions with an alkylamine were found to be enantiospecific, and chiral selectivity was affected by both the size of the guest and the size of the cavity, which strongly suggested the formation of inclusion complexes rather than the formation of nonspecific adducts. Molecular dynamics calculations for underivatized β-CD–amino-acid complexes indicated that the ammonium and carboxylic acid groups of an amino-acid interacted with the CD cavity and rim through hydrogen bonds, while the steric interaction of the side-chain created a repulsive force.

The Lebrilla group further extended their research to CD–peptide complexes [4,21,22,24]. In the case of a peptide, two possible CD interaction sites, i.e., a charge site and a phenyl group, are often situated at a distance from each other. The charge (protonated) site can be mainly involved in the formation of the complex (ion–dipole complex), or a complex can be formed mainly by the inclusion of the phenyl group into the cavity of the CD (inclusion complex). Interestingly, different experimental methods have yielded intriguing results that could be interpreted in two different ways regarding whether the CD–peptide complexes in the gas phase are ion–dipole or inclusion complexes. Application of collision-induced dissociation (CID) or heated capillary dissociation (HCD) to the complexes of bradykinin (BK, RPPGFSPFR) and its analogs resulted in disassembly of the complexes [4]. The interaction strength was found to follow the order $dR^9BK < BK < dR^1BK \sim G^5G^8BK$, where dR^1BK, dR^9BK, and G^5G^8BK stand for *des*-Arg-1-bradykinin, *des*-Arg-9-bradykinin, and Gly-5-Gly-8-bradykinin, respectively. In particular, replacing both Phe (F) residues at the 5th and 8th positions in the sequence of bradykinin with glycine (G^5G^8BK) strengthened the interaction, suggesting that the Phe's destabilized the bradykinin complex. Based on the above experimental results, the authors suggested that for a CD-BK complex, a Phe residue was included in the cyclodextrin cage and the guanidinium group of the arginine residue interacted with the cyclodextrin rim, forming a 'molecular latch' to keep the inclusion complex together. On the contrary, when a more gentle method of blackbody infrared radiation was applied to BK and its analogs, complexes underwent peptide fragmentation, leading, for example, to $[b_2$:CD+H$]^+$ for the BK (RPPGFSPFR) complex, rather than the complex disassembly seen with CID and HCD [21]. The fragmentation products found in this experiment offered little support for the presence of gas-phase inclusion complexes, because the peptide fragment b_2 did not include any aromatic amino-acid residues.

To shed light on this issue with another experimental tool, we investigated CD–peptide complexes with the relatively new tandem mass spectrometry method of electron capture dissociation mass spectrometry (ECD MS). ECD MS has many useful characteristics, including high fragmentation efficiency and superior capability in characterizing post-translational modifications such as phosphorylation and glycosylation [27–46]. Furthermore, it has been shown that noncovalent interactions, both intramolecular and intermolecular, generally remain intact in ECD MS [28–34,41,42,44]. We utilized this unique property of ECD MS to study the interaction characteristics of permethylated β-CD–peptide complexes. ECD MS is expected to lead to peptide backbone fragmentation with β-CD noncovalent interactions with some preservation of a part of the peptide. Examination of *m*/*z* values of β-CD–peptide fragment peaks can help identify which amino-acid residue the β-CD is bound to. This information may allow determination of whether gas-phase β-CD complexes are ion–dipole complexes or inclusion complexes. Towards this goal, we examined *Angiotensin II* (DRVYIHPF), *Substance P* (RPKPQQFFGLM), and *Bradykinin* (RPPGFSPFR) and its analogs [dR^1BK (PPGFSPFR) and dR^9BK (RPPGFSPF)].

2. Experimental/materials and methods

Experiments were performed using a commercial 4.7 T electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometer (ESI-FTICR MS: Varian, Lake Forest, CA, USA), equipped with ECD capability [43,45,46]. Complex cations of (peptide:β-CD) were prepared by electrospraying mixed solutions of 1 μM peptide and permethylated β-CD dissolved in a 48:48:4 (v/v/v) methanol:water:acetic acid solution. Mixture sample solutions were infused directly through a home-pulled fused silica capillary (i.d. = 100 μm) emitter at a flow rate of 0.5–1.0 μl/min, using a syringe pump (Harvard Apparatus 22, Holliston, MA, USA). A potential of 2.1–2.4 kV was applied between an electrospray emitter and the capillary entrance. The generated ions were introduced into the mass spectrometer through a heated metal capillary (i.d. = 0.75 mm, length = 23.2 cm), and then externally accumulated in a hexapole linear-trap for 300 ms before pulsing into an ICR cell. The accumulated ions were passed through a synchronized shutter and then transferred through rf-only quadrupole guides into a closed cylindrical ICR cell. Ion trapping in the cell was accomplished without the aid of a collision gas. Complex ions of interest were isolated using a single stored waveform inverse Fourier-transform (SWIFT) waveform before ECD application [47]. ECD was performed by pulsing a 300–1000 ms electron beam into the ICR cell where the isolated ions were stored. Electrons were generated by heating a dispenser cathode (Heatwave Labs, Watsonville, CA, USA) up to ~1000 °C. The cathode was mounted on a macor bracket bolted to the back trap plate. The macor bracket was placed only ~1 cm away from the back trapping plate of the ICR cell. ECD was performed with a single-pass setup, in which both the filament (rear) and quadrupole (front) trap plates were set to a potential of 10.0 V. The electron kinetic energy was set to 0.65–0.70 eV. Detection of ions was made in a broadband detection mode with 1024 kW data points and at a 2 MHz ADC rate in the range of *m*/*z* 200–2500. About 30 transients were accumulated for all the MS/MS spectra. For the mass spectra presented below, time-domain data sets were Blackman-apodized and zero-filled once. Data analysis was performed using the PeakHunter 1.1 beta software package provided by Varian. Permethylated β-CD, which was used here instead of unmethylated β-CD in order to facilitate the direct comparison of our ECD MS results with those of the Lebrilla's group, was purchased from Sejin Chemical Industry Co. (Seoul, Korea). *Angiotensin II* (DRVYIHPF), *Substance P* (RPKPQQFFGLM), and *Bradykinin* (RPPGFSPFR) and its analogs (dR^1BK and dR^9BK) (Sigma, Seoul, Korea) were used without further purification.

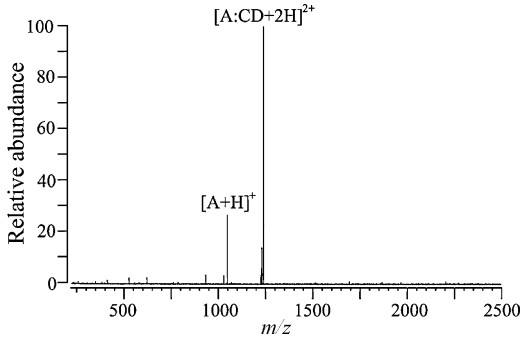

Fig. 1. A mass spectrum obtained by electrospraying mixture solutions of 1 μM peptide and permethylated β-CD dissolved in 48:48:4 (v/v/v) methanol:water:acetic acid solution.

3. Results and discussion

Fig. 1 represents a typical ESI-mass spectrum for a mixture of a peptide (in this case, *Angiotensin II*, which is denoted below as A) and permethylated β-CD in a molar ratio of 1:1 dissolved in 48:48:4 (v/v/v) water:methanol:acetic acid solution. Doubly protonated noncovalently bound complexes of *Angiotensin II* and β-CD, $[A:CD+2H]^{2+}$ found at *m*/*z* 1237.1 (monoisotopic mass), were most abundant, and singly protonated *Angiotensin II*, $[A+H]^+$ at *m*/*z* 1046.5 (monoisotopic peak), was also prevalent. A sodiated and potassiated forms of CD were initially observed, but the addition of a sufficient amount of acetic acid eliminated them. FTICR operational parameters were optimized to maximize the abundance of the $[A:CD+2H]^{2+}$ peak, which was then subject to SWIFT isolation and to the subsequent ECD MS. The generation of noncovalent complexes $[M:CD+2H]^{2+}$ has previously been well documented in the literature [13–26]. The formation of noncovalent complexes, not covalent ones, was confirmed by performing sustained off-resonance irradiation collisionally activated dissociation (SORI-CAD) on the complex, which resulted in disassembly of the complex into its constituent β-CD and peptide (data not shown here) [48].

3.1. ECD of Angiotensin II-β-CD complex, $[A:CD+2H]^{2+}$

Doubly protonated noncovalent complexes $[A:CD+2H]^{2+}$ was irradiated by a pulse of electrons, and the resulting ECD mass spectrum is shown in Fig. 2(a). For reference, the ECD mass spectrum for doubly protonated *Angiotensin II* ions alone is also given in Fig. 2(b). As shown in Fig. 2(a), ECD on $[A:CD+2H]^{2+}$ mainly yielded cleavage of the backbones of *Angiotensin II* while maintaining its complex bonding with β-CD. This finding was manifested by fragments at *m*/*z* 1716.8, 1772.9, 1816.9, 2093.2, and 2327.4, which are peptide fragment complexes of $[c_2:CD]^+$, $[a_3:CD]^+$, $[c_3:CD]^+$, $[c_5:CD]^+$, and $[c_7:CD]^+$, respectively. This result was in stark contrast to SORI-CAD experiments in which disassembly of two constituent molecules, *Angiotensin II* and β-CD, was a dominant process. Conservation of noncovalent interactions during ECD peptide backbone dissociation, as observed here, is consistent with well-known ECD characteristics [28–34,41,42,44]. In addition, a singly reduced species $[A:CD+2H]^{\bullet+}$ and its ammonia-loss counterpart $[A:CD–NH_3+2H]^{\bullet+}$ were observed at *m*/*z* 2476.9 and 2459.6, respectively. It is also interesting to note that disassembled *Angiotensin II* and its ammonia-loss ions, $[A+H]^+$ and $[A–NH_3+H]^+$, were found at *m*/*z* 1047.5 and 1030.5, respectively. The disassembly was due to the absorption of blackbody radiation emitted from the hot surface (~1000 °C) of the dispenser cathode utilized here for electron production, which was confirmed in the double-resonance ECD experiment (data not shown here) [44,49–51]. It is also noteworthy that no uncomplexed *Angiotensin II* backbone fragment was observed.

The ECD mass spectrum for $[A:CD+2H]^{2+}$ can be more easily understood with knowledge of the two potential protonation sites in *Angiotensin II* 2^+ noncovalent complexes. Assuming that protonation sites do not change before and after complexation with β-CD, the two protonation sites in the 2^+ complex are likely to be two basic amino-acid residues of arginine 2 (R2) and histidine 6 (H6). This prediction is in agreement with ECD results for *Angiotensin II* 2^+ cations (see Fig. 2(b)). Fragments a_2^+, c_2^+, c_3^+, c_4^+, c_5^+, and c_7^+ can be interpreted to arise from peptide backbone dissociation initiated by electron capture at H6, while the remaining proton on R2 is utilized for detection. In contrast, in the case of fragments z_4^+ and z_6^+, electron capture must occur at R2. Our prediction is further supported by the observation that more fragmentations took place by electron capture at H6 than at R2; in other words, there were more N-terminal a_i^+ or c_i^+ fragments than C-terminal z_i^+ fragments. In general, it is known that ECD occurs much less at the most basic arginine residue than at the other less basic amino-acid residues [40].

With the above protonation scenario in mind, we further interpreted the ECD fragments shown in Fig. 2(a). Of the two fragment-CD complex types, only N-terminal fragment-CD complexes were present, including $[c_2:CD]^+$, $[a_3:CD]^+$, $[c_3:CD]^+$, $[c_5:CD]^+$, and $[c_7:CD]^+$. No C-terminal fragment-CD complex, such as $[z_i:CD]^+$, was detected. This observation suggests that some N-terminal region of *Angiotensin II* strongly interacted with β-CD to form a noncovalent complex. From the observed cleavage positions of c_i^+, it can be inferred that R2 is a very likely place for noncovalent interaction with β-CD. The protonated guanidinium group of R2 would presumably interact with β-CD through ion–dipole inter-

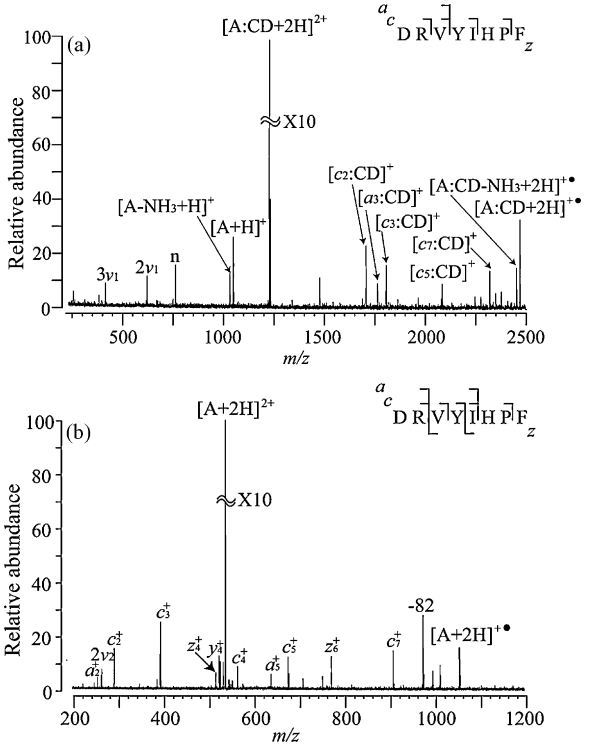

Fig. 2. ECD mass spectra of doubly protonated (a) *Angiotensin II*–β-CD noncovalent complexes $[A:CD+2H]^{2+}$, and (b) *Angiotensin II* alone $[A+2H]^{2+}$. Peptide fragmentations are summarized in the insets of the sequence map with bond cleavage notations. $2\nu_1$ and $3\nu_1$ in (a) are the second and third harmonics of the peak corresponding to $[A:CD+2H]^{2+}$, respectively, and $2\nu_2$ in (b) is the second harmonic of the peak corresponding to $[A+2H]^{2+}$. *n*: noise peak.

action. For the other basic amino-acid residue H6, if the complex was formed through an H6-CD ion–dipole interaction, uncomplexed c_i^+ ion with $i = 1$–5 or $[z_{3-7}{:}CD]^+$ should be formed upon ECD application. However, these fragment ions were not observed. Aromatic group-containing amino-acid residues such as tyrosine 4 (Y4) and phenylalanine 8 (F8) represent other interaction candidates. Indeed, these amino-acid residues have been known to form inclusion CD complexes in solution [3,18]. However, in Fig. 2(a), no fragment that supported the formation of an inclusion complex with Y4 or F8 was found.

Another plausible protonation scenario can also be suggested. For example, two protons could reside on the N-terminal amino group and R2, respectively. In this case, the protonated N-terminal group would participate in the complex formation with β-CD, and the protonated R2 would induce electron capture dissociation. We were unable to distinguish between the two scenarios based on Fig. 2 alone. However, due to the Coulombic repulsion between closely located protons, the latter complex may be less stable than the former.

To find out whether or not the above findings are generalizable, further ECD experiments were carried out for other peptide–CD complexes (see below).

3.2. Substance P–β-CD complex, $[S{:}CD{+}2H]^{2+}$

Substance P has two basic amino-acid residues (arginine (R) and lysine (K)) in the N-terminal region, and two aromatic group-containing phenylalanines (F) in the C-terminal region. A major difference between the sequences of *Angiotensin II* and *Substance P* is that *Substance P* has no basic amino-acid residue in the C-terminal region.

Fig. 3(a) shows the ECD mass spectrum for the doubly protonated *Substance P*–CD complex, $[S{:}CD{+}2H]^{2+}$. For *Substance P*–CD complexes, a multitude of N-terminal fragment-CD complexes, for example, $[c_i{:}CD]^+$, $i = 2$, 4–8, were observed, while no C-terminal fragment-CD complex was found. This pattern indicates that β-CD interacted favorably with the N-terminal region of *Substance P* where basic amino-acid residues such as R1 and K3 are located, in agreement with the results for the *Angiotensin II*–CD complex. Here, no fragment indicating the presence of inclusion CD complexes interacting with the aromatic phenylalanine residue, F7 or F8, was found (i.e., no $[z_i{:}CD]^+$ ($i = 4$–10) or c_j^+ ($j = 1$–6)). Nonetheless, the presence of the inclusion complex cannot be absolutely ruled out since it is still possible that C-terminal fragment complexes such as $[z_i{:}CD]$ ($i = 4$–10) were generated as a result of ECD but could not be detected due to the lack of a charge site in the C-terminal, although this scenario is not very plausible.

For comparison, ECD mass spectrum for uncomplexed doubly protonated *Substance P* cations is also shown in Fig. 3(b). Most of the observed fragments were N-terminal fragments such as a^+ and c^+, except for z_9^+, which again supports the supposition that two protonation sites are located in the N-terminal region, i.e., R1 and K3. A close inspection of the relative abundance of each fragment peak revealed that the ordering of the relative abundances of the *c* fragments in Fig. 3(a) and (b) are somewhat different. The ordering is $c_5^+ > c_7^+ \sim c_6^+ \sim c_4^+ > c_8^+ > c_2^+$ in Fig. 3(b), compared to $[c_6{:}CD]^+ > [c_2{:}CD]^+ \sim [c_5{:}CD]^+ \sim [c_7{:}CD]^+ > [c_4{:}CD]^+ > [c_8{:}CD]^+$ in Fig. 3(a). A similar observation was also made for the *Angiotensin II*:CD complex (see Fig. 2). Considering that the relative abundance of fragments in ECD MS often reflects the overall non-covalent interaction pattern of macromolecules under study, this finding may suggest that the overall non-covalent interactions within *Substance P* have changed after complexation with β-CD [30,31]. In general, the protonated part of a peptide usually participates, to an extended degree, in intramolecular noncovalent interactions through ionic

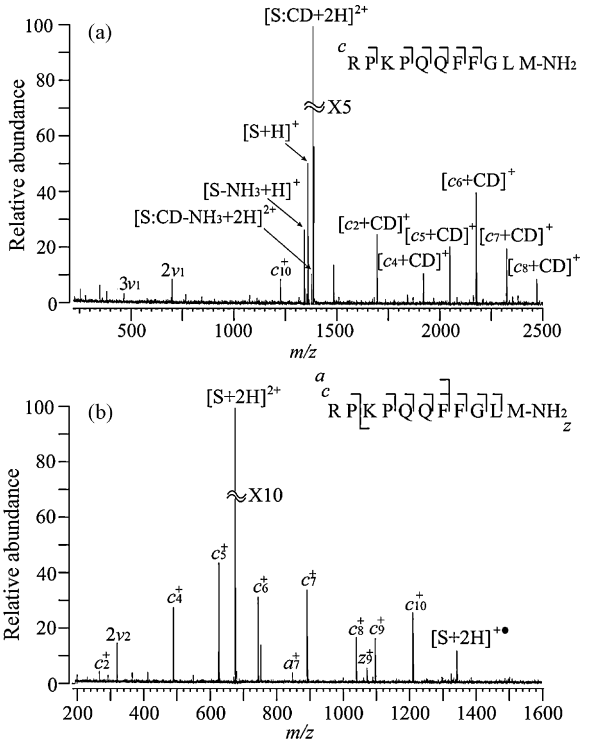

Fig. 3. ECD mass spectra of doubly protonated (a) *Substance P*–β-CD noncovalent complexes $[S{:}CD{+}2H]^{2+}$, and (b) *Substance P* alone $[S{+}2H]^{2+}$. Peptide fragmentations are summarized in the insets of the sequence map with bond cleavage notations. $2\nu_1$ and $3\nu_1$ in (a) are the second and third harmonics of the peak corresponding to $[S{:}CD{+}2H]^{2+}$, and $2\nu_2$ in (b) is the second harmonic of the peak corresponding to $[S{+}2H]^{2+}$.

hydrogen bonds. When such a protonated part is sequestered to peptide–CD ion–dipole interactions, the noncovalent interaction pattern within the peptide itself will also be substantially affected. Furthermore, the exothermicity of electron capture at such protonated sites is diminished, causing a change in the relative ECD efficiency at the other protonated site. The dissimilarity observed in the ordering of relative fragment abundances in Fig. 3 retrospectively suggests that the protonated part of a peptide is involved in the complex formation, presumably through ion–dipole interactions.

3.3. Bradykinin or its analogues–CD complex

The above ECD MS results for *Angiotensin II* and *Substance P* complexes strongly suggest that peptides tend to form a noncovalently bound complex with β-CD in the region where basic amino-acid residues such as K and R are located. This noncovalent interaction is very likely to be an ion–dipole interaction, and we found no evidence to support the presence of an inclusion complex. To further test this tendency, ECD MS was also carried out for bradykinin and its analogs-CD complexes. The following peptides were examined: bradykinin (BK: *R*PPGFSPF*R*), dR^1BK (PPGFSPFR), and dR^9BK (RPPGFSPF).

Fig. 4 shows the ECD MS mass spectra for $[M{:}CD{+}2H]^{2+}$, where M represents BK, dR^1BK, or dR^9BK, respectively. For the BK complex with two basic arginine residues located at the two termini, extensive backbone fragments appeared upon electron capture (see Fig. 4(a)). Here, both N- and C-terminal fragment-CD complexes were detected. The detected N-terminal fragment complexes were $[c_3{:}CD]^+$, $[c_4{:}CD]^+$, $[c_5{:}CD]^+$, $[c_7{:}CD]^+$, $[c_8{:}CD]^+$, and $[a_8{:}CD]^+$, while $[z_6{:}CD]^+$ was the only C-terminal fragment complex. When we

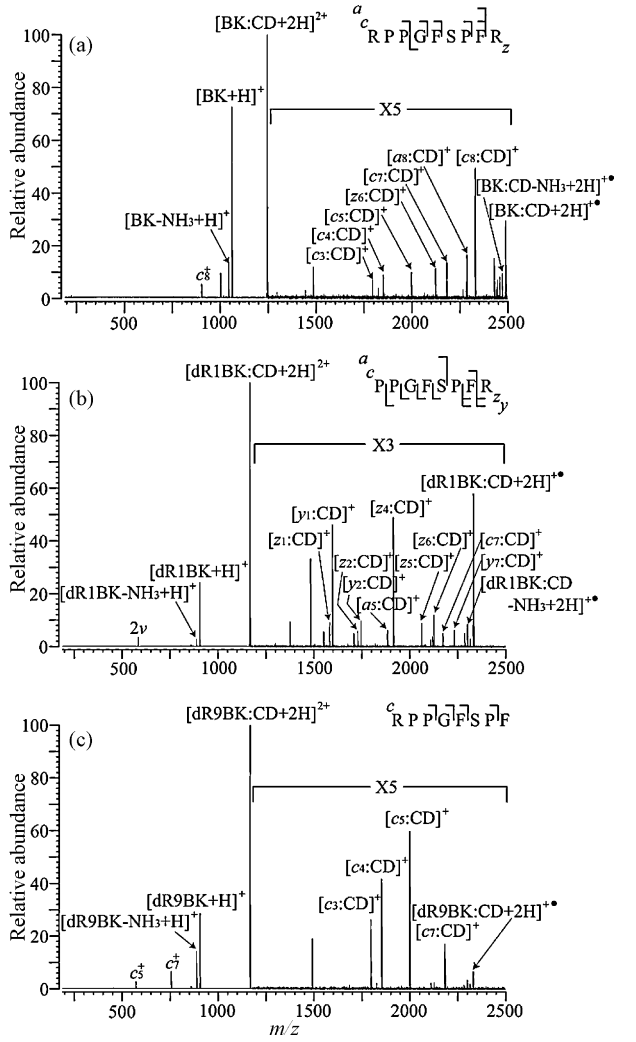

Fig. 4. ECD mass spectra of doubly protonated (a) *Bradykinin* (BK), (b) *des*-Arg-1-bradykinin (dR^{1}BK), and (c) *des*-Arg-9-bradykinin (dR^{9}BK) β-CD noncovalent complexes. Peptide fragmentations are summarized in the insets of the sequence map with bond cleavage notations. *v*: the second harmonic of the peak corresponding to $[dR^1BK:CD+2H]^{2+}$.

assume that the two protons were sequestered to the two basic arginine residues (R1 and R9), the backbone cleavage sites of the N-terminal fragment complexes indicate that the protonated R1 was the amino-acid residue that mainly participated in the interaction with β-CD. For the C-terminal fragment complex $[z_6:CD]^+$, a couple of interacting candidates can be suggested. In view of dominant ion–dipole interactions over inclusion interactions in the gas phase, R9 may interact with β-CD. In contrast, when F5 or F8 were included into the cavity of β-CD, electron capture at R1 could also induce $[z_6:CD]^+$. However, the occurrence of the fragments $[c_5:CD]^+$, $[c_7:CD]^+$, and $[c_7:CD]^+$ makes the latter less plausible. Furthermore, the uncomplexed c_8^+ ion found at *m*/*z* 902.5 also provides evidence that R9 interacted with β-CD.

To further investigate which amino acid in bradykinin participated in complex formation, ECD MS was also carried out for doubly protonated dR^{1}BK–CD complexes (Fig. 4(b)). We interpreted Fig. 4(b) based on the assumption that two protons are likely to reside on the N-terminal amino group and R8 in the C-terminus of dR^{1}BK. The ECD MS spectrum for dR^{1}BK–CD complexes showed a cleavage pattern quite different from that of the BK complex. For example, a multitude of C-terminal fragment complexes such as $[z_1:CD]^+$, $[y_1:CD]^+$, $[z_2:CD]^+$, $[y_2:CD]^+$, $[z_4:CD]^+$, $[z_5:CD]^+$, and $[y_7:CD]^+$ were detected, while only two N-terminal fragment complexes, such as $[a_5:CD]^+$and $[c_7:CD]^+$, were observed. The absence of an arginine residue in the N-terminus from the BK sequence was responsible for this large change in the ECD cleavage pattern. The observed C-terminal fragment complexes indicate that the most basic arginine R8 participated in the complex formation with β-CD in a protonated form, presumably through ion–dipole interaction. In particular, of the many fragment complexes, close attention should be paid to $[z_1:CD]^+$ and $[y_1:CD]^+$. The presence of these two fragments clearly shows that there must be some population of complex isomers in which R8 was an interacting site. In Fig. 4(b), two N-terminal fragment complexes of $[a_5:CD]^+$and $[c_7:CD]^+$ were also observed. These fragment complexes were probably induced from complex isomers in which the protonated N-terminal amino group was noncovalently attached to β-CD. It is also possible that there were some isomers in which the aromatic group of F5 or F7 was included in the cavity of β-CD, but no specific evidence in support of such isomers was observed in Fig. 4(b).

Another BK analog, i.e., dR^{9}BK, in which R9 is missing from BK, was also subjected to ECD MS (Fig. 4(c)). As expected, the absence of the basic R9 gave rise to an ECD cleavage pattern different from that of the dR^{1}BK and BK complexes. Only N-terminal fragment complexes were found at *m*/*z* 1795.9, 1853.0, 2000.0, and 2184.1, corresponding to $[c_3:CD]^+$, $[c_4:CD]^+$, $[c_5:CD]^+$, and $[c_7:CD]^+$, respectively. The N-terminal fragment complexes further support the supposition that the protonated N-terminal amino group or the protonated arginine R1 was responsible for the formation of β-CD noncovalent complexes. For the dR^{9}BK complex, no specific evidence was found indicating the presence of an inclusion complex. ECD MS experiments were also repeated for other peptide–CD complexes, but failed to yield specific evidence indicating the presence of an inclusion complex (data not shown here).

3.4. Ion–dipole and inclusion complex

The results described above suggest that protonated gas-phase peptides tend to form a complex with a permethylated β-CD through ion–dipole interactions rather than inclusion interactions. This finding is consistent with molecular dynamics/modeling calculations performed by the Lebrilla group [21]. In their calculations for $[BK:CD+2H]^{2+}$, they projected that low energy structures involved inclusion of R1 into the cavity. In particular, the protonated guanidinium group of R1 was found to interact with the lower rim. In the lowest energy structure, R9 interacted with the upper rim, while there were little interaction between F5/F8 and the β-CD. Even when the beginning structure of the complex involved the inclusion of F5 into the cavity, R1 displaced F5 during the simulation. Unfortunately, our ECD MS experimental results offered few clues about the detailed interaction structures between a peptide and β-CD. For example, we could not deduce whether the protonated guanidinium group of R1 of BK was included into the cavity of the β-CD or simply adducted to the outer rim.

As mentioned before, the Lebrilla group observed some experimental evidence in support of the formation of an inclusion complex in their heated capillary dissociation and collision-induced dissociation experiments for peptide–CD complexes, though their blackbody radiation dissociation experiments also indicated the formation of ion–dipole complexes [4,21]. We are unable to clearly explain these contradictory results. It may be possible, however, that a variety of isomers exist with different interaction structures. Certain ensembles of inclusion complex isomers might somehow be more responsive to specific dissociation methods such as heated capillary dissociation and collision-induced dissociation, providing evidence in support of the existence of inclusion complexes,

while other ensembles of ion–dipole complex isomers may be more responsive to ECD MS, thus providing evidence in favor of the presence of ion–dipole complexes.

Extensive research evidence supports the favorable formation of CD–host inclusion complexes in solution, particularly for aromatic group-containing compounds. In solution, the protonated part of a molecule, such as an arginine residue, favorably interacts with the hydrophilic solvent, while the hydrophobic aromatic group stabilizes the complex by being inserted into the cavity. However, in the gas-phase where no solvation effect is expected, a protonated part must compete for β-CD interactions with a hydrophobic part that mainly involves van der Waals interactions with the cavity. In view of thermodynamics, an ion–dipole interaction is more favorable than a van der Waals interaction. Such energetic competition could allow a protonated part to interact more favorably with a β-CD than an aromatic group. It is also interesting to note that for another host–guest system of an 18-crown-6-ether noncovalent complex with a peptide, it was found that a protonated amino-acid residue, for example, lysine, mainly participated in the complex formation through ion–dipole interactions [52–54].

In the present experiments, β-CD–peptide complexes formed in solution were transferred into the gas phase through ESI processes. The gas-phase complexes formed were then transferred into an ICR cell through a heated capillary, hexapole ion accumulator, and a quadrupole ion guide. Since these transfer processes took place in a time scale of 1–2 s, there was sufficient time available for the inclusion complexes favorable in solution to shift to the ion–dipole complexes favorable in the gas phase, though it is still unclear exactly where such relaxation occurred during transfer.

We would also like to emphasize that our experimental results should not be extended to other CD complexes with small molecules for which extensive experimental evidence in support of inclusion complexes has been previously established. A small molecule like an amino acid can be easily included in the cavity of a CD, and its protonated part can still interact with its rim through ion–dipole interactions.

4. Conclusions

ECD MS was carried out for a number of doubly protonated gas-phase peptide–CD complexes. Examination of fragments in the ECD MS spectra suggested that noncovalently bound peptide–CD complexes were likely to be ion–dipole complexes. It appears that hydrophobic interactions (van der Waals interactions), which are a main driving force for noncovalent interactions in solution, do not prevail in the gas-phase. Instead, ion–dipole interactions, which are more favorable in terms of thermochemistry, play a dominant role in forming noncovalent complexes in the gas phase. Our experimental results also suggest that caution should be used when employing a simple ESI mass measurement to determine the capability of a certain molecule (at least for peptides) to form an inclusion complex with a CD in solution. The formed gas-phase CD complex could easily be a simple ion–dipole complex rather than a phenyl inclusion complex. The present study also demonstrated that ECD MS is a very useful tool for characterizing noncovalent complexes in the gas phase.

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