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Electron capture dissociation of weakly bound polypeptide polycationic complexes

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We have previously reported that, in electron capture dissociation (ECD), rupture of strong *intra*molecular bonds in weakly bound *supra*molecular aggregates can proceed without dissociation of weak *inter*molecular bonds. This is now illustrated on a series of non-specific peptide-peptide dimers as well as specific complexes of modified glycopeptide antibiotics with their target peptide. The weak nature of bonding is substantiated by blackbody infrared dissociation, low-energy collisional excitation and force-field simulations. The results are consistent with a non-ergodic ECD cleavage mechanism. Copyright © 2002 John Wiley & Sons, Ltd.

Electron capture dissociation (ECD) is believed to be a non-ergodic process,[1] in which preferential fragmentation of S–S[2] and N–C_α bonds[1] in polypeptides occurs faster than the *intra*molecular vibrational energy redistribution. This is manifested by the abundant backbone cleavage in large polypeptides[3] and by the preservation in ECD fragments of groups attached by much weaker links. For example, ECD can proceed without significant losses of labile groups in polycations of phosphorylated,[4,5] γ-carboxylated,[6] O-glycosylated[7] and sulfated[6,8] peptides, in stark contrast to the facile losses of these groups in collisional and infrared excitations. If preferential cleavage of strong bonds can be achieved in weakly bound supramolecular complexes, ECD could become the mass spectrometric method of choice for studying gas-phase structures of large biomolecules and their aggregates.

In the early days of ECD, it was found that low charge states of ubiquitin polycations can capture electrons but remain undissociated; subsequent mild collisional excitation of the reduced species produced *c* and *z* ions from cleavages at markedly different places than direct ECD cleavage of the same charge states.[3] As a possible explanation, the *c,z* cleavage could occur upon electron capture without dissociation of intramolecular weak bonds that held together the reduced species until the collisional excitation induced fragment separation. Later, McLafferty *et al.* used the *absence* of ECD cleavage in a certain position in the polycations of ubiquitin as an indication of the involvement of this position in weak bonding. They have used this to derive clues about the gas-phase structure of ubiquitin ions.[9] More directly, we demonstrated the *presence* of supramolecular fragment ions in ECD of peptide cationic complexes.[8] While ECD preferentially cleaved strong *inter*molecular bonds without weak bond dissociation, collisional excitation produced dissociation of the complex into constituent species. This has been shown on non-specific dimers of a 13-residue peptide without detailed description of the experiment. Here we give such a description and extend the studies to other non-specific dimers. Force-field calculations are presented that give insight into the energetics of the process. Additionally, we demonstrate that ECD can be used to study gas-phase structures of specific, biologically relevant weakly bound complexes. As an example, polycations of vancomycin (V) and eremomycin (E) complexes were fragmented. Vancomycin (V) and eremomycin are modified glycopeptide antibiotics that produce specific complexes with the peptide diacetyl-L-Lys-D-Ala-D-Ala (KAA).[10,11]

EXPERIMENTAL

The 13-residue EA2 peptide (TTTDSTTPAPTTK) and its analogues were synthesized in-house using automated solid-phase synthesis and Fmoc (9-fluoronylmethoxycarbonyl) protection strategy on a research-scale ResPep peptide synthesizer (Intavis AG, Gladbach, Germany). Samples of V, E and KAA were obtained from collaborators.

Nano-electrospray ionization was performed on a 4.7 T Ultima FT mass spectrometer from IonSpec (Irvine, CA, USA). The peptides were dissolved in a standard electrospray mixture of water, methanol and acetic acid (49:49:2, v/v) to a concentration of 10^{-4} M. Aliquots (3–5 μL) were loaded into metallized pulled-glass capillaries (MDS Proteomics, Odense, Denmark). The electrospray-produced ions were externally accumulated in the hexapole for 0.5 s and transmitted to the FT cell by rf-only quadrupole ion guides. Capture of ions in the open-ended cylindrical cell with extra

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trapping plates was achieved by gated trapping. For performing dissociation experiments, ions of interest were isolated by application to the excitation electrodes of a pre-programmed waveform. For SORI CAD, a 500 ms rf-burst with an amplitude of 3 V was applied at a frequency ca. 2 kHz higher than the m/z value of the molecular ions together with the pulse of a collision gas (2 ms of N_2 at 20 Torr). For ECD, an indirectly heated dispenser cathode operated at 5 V and 0.34 A was employed.[8,12] The electron-emitting surface was biased to −0.5 V for 200 ms.

RESULTS AND DISCUSSION

Peptide homo-dimers

Non-specific proton-bound peptide aggregates can easily be obtained by electrospray ionization of a solution with increased concentration ($\geq 10^{-4}$ M).[13] The triply protonated homo-dimers of the EA2 peptide gave in low-energy collisional excitation 100% dissociation, with on-set of fragmentation of the separated monomers at higher excitations. In contrast, ECD (Fig. 1, top) yielded 11% strong bond fragmentation without *inter*molecular dissociation. The peaks at m/z 1843.53, 1944.50 and 2142.53 are due to the $[M + c_5 + H]^+$, $[M + c_6 + H]^+$ and $[M + c_8 + H]^+$ products; the complementary $z_8^{+\cdot}$, $z_7^{+\cdot}$, and $z_5^{+\cdot}$ ions are also present. The $[M + c_7 + H]^+$ and the complementary $z_6^{+\cdot}$ ions are missing due to the immunity[1] to ECD of the tertiary nitrogen in a proline residue. The obtained ECD pattern is consistent with the structure of the complex with two protons being shared between the C-terminal lysine residues and N-terminal amines. The third proton is delocalized in the region between the proline residues of the opposite chains; its neutralization gives rise to the $[M + c_x + H]^+$ and $z_{13-x}^{+\cdot}$ ions. The identity of the ions at m/z 1944.50 was confirmed when these species were isolated and collisionally activated, yielding $[M + H]^+$ ions. Retention of the proton by the intact molecule is due to the C-terminal lysine residue that is more basic than the c_6 fragment.

Similar results were obtained in ECD experiments with the R_{13} and A_{13} analogues of the EA2 peptide. The $[A_{13}]$-EA2

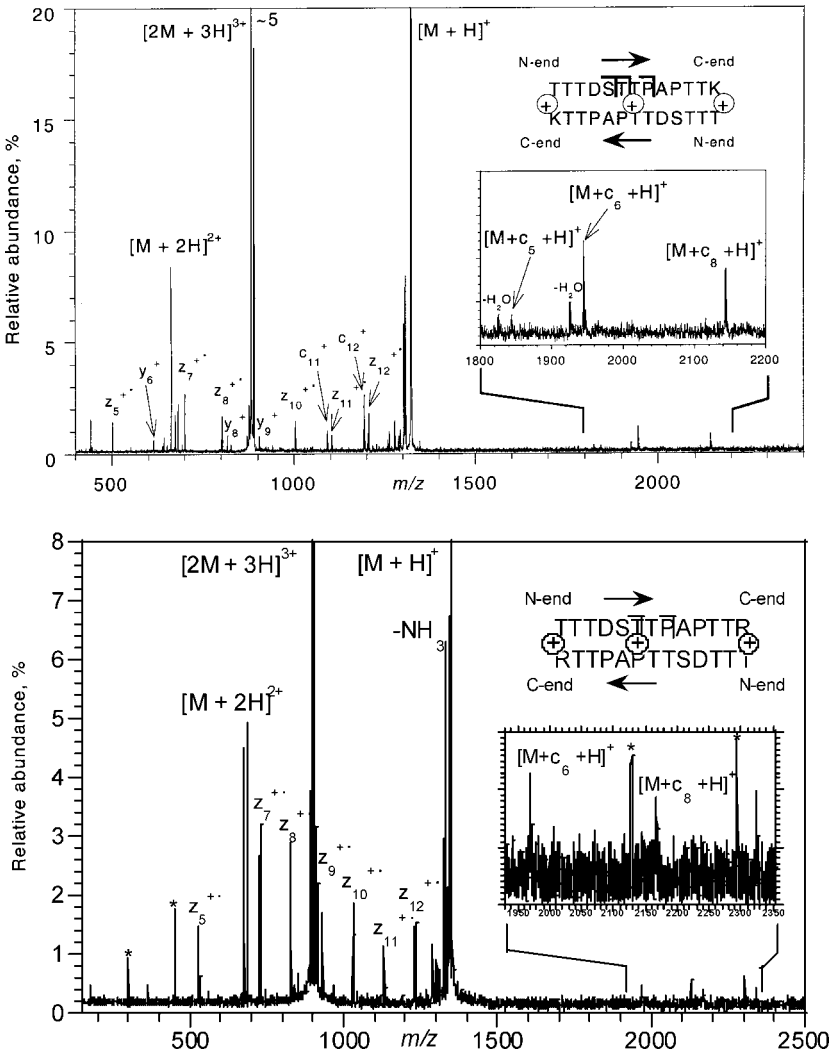


Figure 1. Mass spectrum of ECD of triply protonated homo-dimers of (a) the peptide EA2 and (b) its $[R]_{13}$ variant. The $[M + c_x + H]^+$ ($x = 5$, 6 and 8) peaks are due to supramolecular fragments resulting from N–C_α backbone bond cleavage without dissociation of the weak *inter*molecular bond.

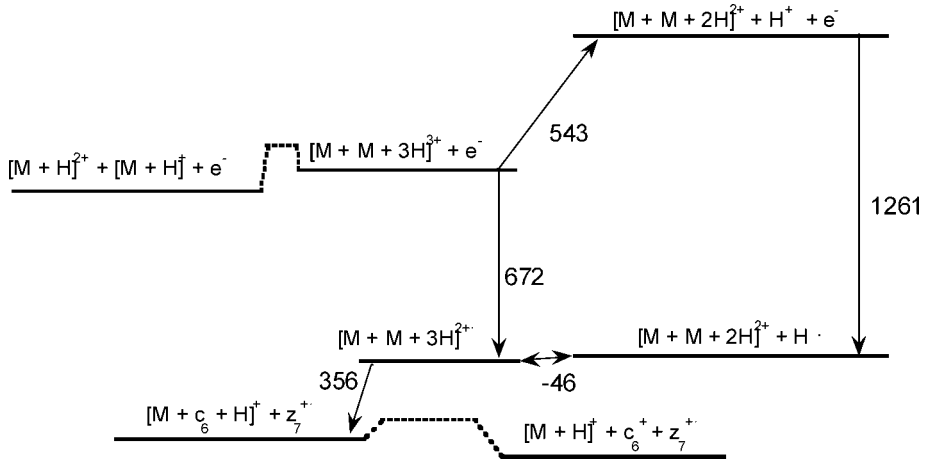


Figure 2. Energy diagram of the ECD reactions of trications of the EA2 peptide calculated using the AMBER or MMF force fields. Exothermicity is shown in kJ/mol.

analogue gave dimers at very low abundance, which is consistent with the proposed structure that the EA2 dimers cannot form without the presence of charge on the C-terminus. At the same time, trications of the homo-dimers of [R_{13}]-EA2 were abundant, explained by even higher basicity of the arginine residue compared with lysine. ECD of these trications yielded cleavages similar to that of the EA2 peptide (Fig. 1, bottom).

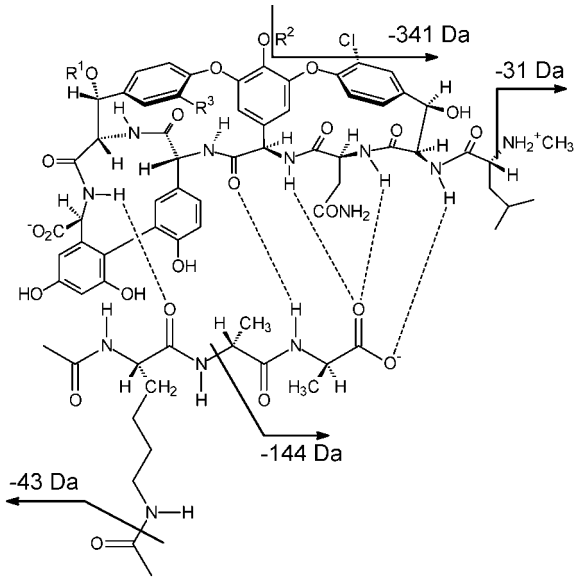
The optimal ion geometries and their energetics were found for the EA2 dimer cations by force-field simulations (AMBER or MMFF) with single point energies at PM3 (Fig. 2). The optimal dimer trication minimum-energy structure was predictably[13] unstable by 1.8 eV compared to the separated di- and monocations. The barrier for such dissociation, ≈0.6 eV, was found using the technique from Ref. 13. This low value was due to the Coulombic repulsion between the closely located charges in the trication. Electron capture by the trications was by 7 eV exothermic. Surprisingly, the fragmentation reaction $([M + M + 3H]^{2+\cdot})_{transient} \rightarrow z_7^{+\cdot} + [M + c_6 + H]^+$ was *exothermic* by 3.7 eV, more than could be accounted for by the Coulombic repulsion between the products (<2 eV). Despite the found high exothermicity of the N–C_α cleavage, no such cleavage has been reported in reaction of free hydrogen atoms at thermal energies (≤1 eV) with peptide polycations.[14] This may indicate the presence of a barrier for N–C_α bond dissociation; indeed, a barrier of ≈120 kJ/mol has recently been found by *ab initio* calculations.[15]

The $[M + c_6 + H]^+$ product was unstable by 0.9 eV, with ca. 1.0 eV barrier for dissociation. Despite the total exothermicity of 10.7 eV of reactions (2) and (3), a significant fraction of the $[M + c_6 + H]^+$ ions did not overcome this barrier and remained stable, possibly due to collisional or radiative relaxation (the exchange rate ca. 10 eV/s[16] for 2.5 kDa ions). The increase of the internal temperature was still sufficient to cause dehydration[8,17–19] in some of the supramolecular $[M + c_x + H]^+$ fragments. The less pronounced water loss for the larger (x = 8) ions compared with the smaller (x = 5, 6) species was due to the redistribution of a fixed amount of the excess energy over a larger number of degrees of freedom.

Antibiotic peptide complexes

The electrospray-produced dications of the V-KAA complex (Fig. 3) have yielded[20,21] upon collisional activation 100% dissociation without *intra*molecular fragmentation. The blackbody infrared dissociation (BIRD)[22] technique confirmed that dication separation into two molecular cations is the lowest-energy channel, and gave low (0.9 ± 0.2 eV) activation energy for such dissociation. The solution binding constant (K_A) for the V-KAA complex is approximately 8×10^5 M^{-1}.[23] Among the ECD products (Fig. 4), 27% were due to *intra*molecular fragmentation without weak bond dissociation, 23% due to fragmentation combined with dissociation, 11% due to non-dissociative reduction ($[V + KAA + 2H]^{+\cdot}$ ions) and the rest (39%) due to weak bond dissociation without fragmentation (see fragmentation assignment in Fig. 3). The 31-Da loss was observed from both V-KAA complex and V dications, but not from those of *N*-desmethyl-vancomycin (Fig. 4, right insert), indicating the loss of CH_3NH_2 from vancomycin. The 43-Da loss from the reduced dimer and the absence of such a loss from the V monomer revealed fragmentation of the ligand. Since the corresponding mass difference increased by 3 Da when the ligand with deuteriated[20,21] acetyl groups was used instead, the −43-Da product was attributed to the cleavage of the R—CH_3CO bond. The 341-Da loss was undoubtedly from V, as it occurred in both dimer and monomer ECD spectra (Fig. 4, left insert) and was not mass-shifted for deuteriated ligand. The observed mass difference and the isotopic pattern are consistent with the simultaneous elimination[24] of the glycan group and a chlorine atom.

Trications of the three-molecule specific E-E-KAA complex[25,26] (Fig. 5) dissociated in low-energy collisional activation into protonated E-E and KAA species. ECD of the same ions yielded predominant fragmentation (73%) with no dimer dissociation. Ten percent of the product ion current was due to dimer-tripeptide bond dissociation and the rest due to non-dissociative reduction. Noticeably, a loss (-AA) corresponding to N-C_α bond cleavage between the Lys and Ala residues (z_2) in the ligand was present in ECD of the E-E-KAA trications, but not in ECD of E-E trications (Fig. 5).



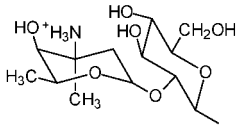
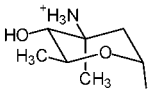
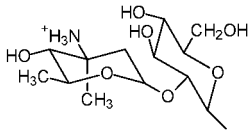
Molecule	R^1	R^2	R^3
Vancomycin	H		Cl
Eremomycin			H

Figure 3. Solution structures of the complex formed between the antibiotics vancomycin and eremomycin and the cell-wall precursor analogue tripeptide diacetyl-L-Lys-D-Ala-D-Ala (adapted from Refs 10 and 11). Dotted lines indicate hydrogen bonds, solid lines indicate ECD cleavages.

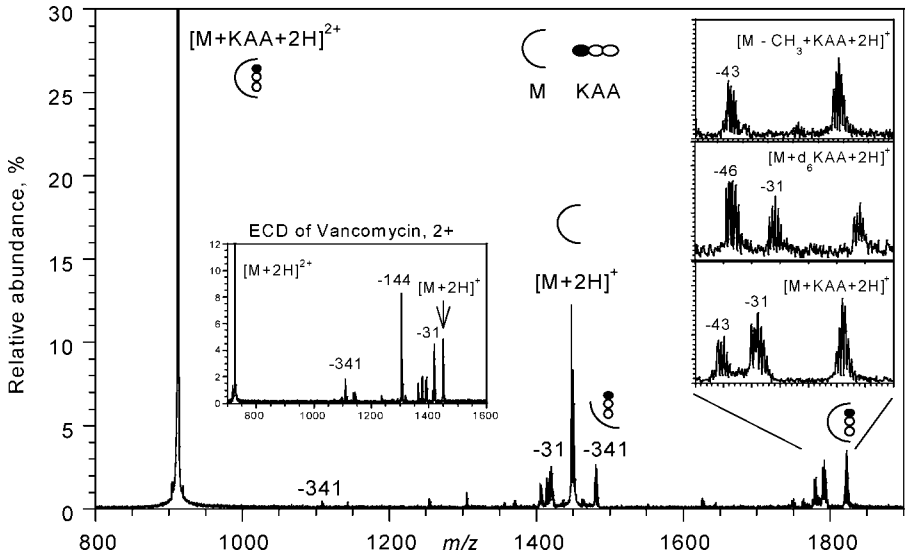


Figure 4. Mass spectrum of ECD of the doubly protonated complex of vancomycin (M) and diacetyl-L-Lys-D-Ala-D-Ala (KAA). Inserts show the control experiments when vancomycin dications were fragmented (left) and when one component in the complex was modified (right). The −31, −43 and −341 Da peaks above the $[M + 2H]^{+\cdot}$ ion peak correspond to covalent bond cleavage without dissociation of the complex.

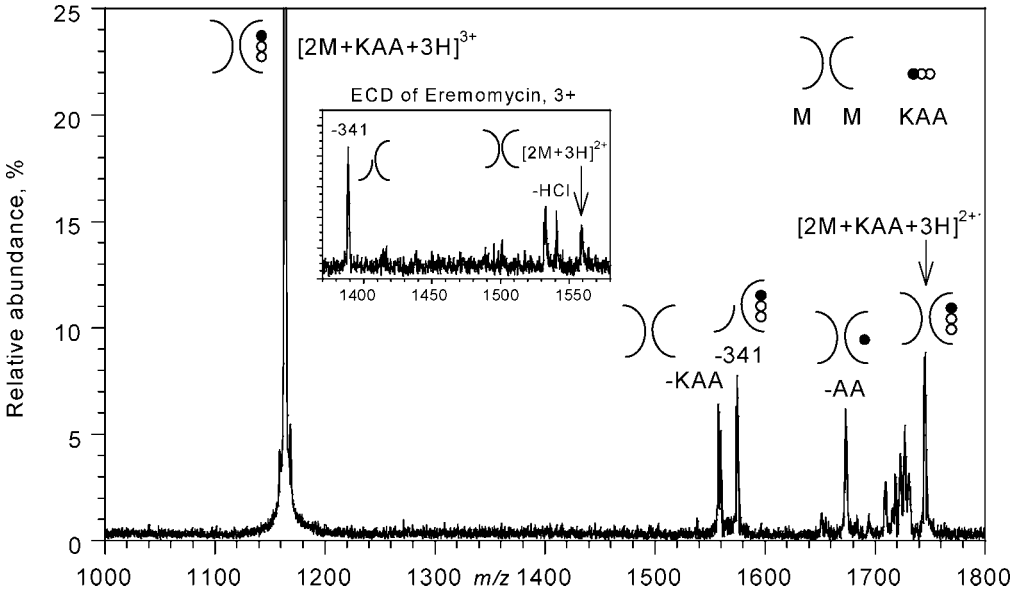


Figure 5. Mass spectrum of ECD of the triply protonated complex of two eremomycin molecules and diacetyl-L-Lys-D-Ala-D-Ala (KAA). Insert shows the control experiments when eremomycin trications were fragmented. The -341 Da peak above the $[M + 2H]^{+\cdot}$ ion peak corresponds to covalent bond cleavage without dissociation of the complex. Dimerization (E-E) of antibiotics of the vancomycin group enhances their affinity for the bacterial cell wall.[27]

However, in the insert of Fig. 4 a loss of 144 Da is seen, that would correspond to the z_2-fragment of KAA. This is only observed in ECD of V itself, we believe its origin is different and instead connected to a sugar moiety loss.

CONCLUSIONS

It has been confirmed in direct experiments that ECD can cleave strong covalent bonds without dissociation of weak bonding present nearby. We predict that this feature will be very useful in studying the gas-phase structure of large biomolecules and their aggregates. Apparently, exothermicity of the ECD cleavage can be quite large; therefore, larger ($\geq$3 kDa) aggregates are expected to be more suitable for such studies. Complete localization and characterization of non-covalent binding sites could however in some cases be inadequate, as ECD in general cleaves near protonation sites.

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