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Localization of Intramolecular Monosulfide Bridges in Lantibiotics Determined with Electron Capture Induced Dissociation

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Electron capture induced dissociation (ECD) and collisionally activated dissociation (CAD) experiments were performed on four lanthionine bridge-containing antibiotics. ECD of lantibiotics produced mainly c and z$^{\bullet}$ ions, as has been observed previously with other peptides, but more interestingly, the less common c$^{\bullet}$ and z ions were observed in abundance in the ECD spectra. These fragments specifically resulted from the cleavage of both a backbone amine bond and the thioether bond in a lanthionine bridge. ECD seemed to induce mainly cleavages near the lanthionine bridges. This fragmentation pattern indicates that lanthionine bridges play a key role in the selectivity of the ECD process. A new mechanism is postulated describing the formation of c$^{\bullet}$ and z ions. Comparative low-energy CAD did not show such specificity. Nondissociative ECD products were quite abundant, suggesting that relatively stable double and triple radicals can be formed in the ECD process. Our results suggest that ECD can be used as a tool to identify the C-terminal attachment site of lanthionine bridges in newly discovered lantibiotics.

Electron capture dissociation (ECD) was introduced in 1998 by Zubarev et al.[1] as a new tandem mass spectrometric technique for the study of polypeptides and proteins. More conventional techniques such as low-energy collisionally activated dissociation (CAD) and infrared multiphoton dissociation generally increase the internal energy of an ion in small steps until the weakest chemical bonds are cleaved[2] to yield in peptides and proteins mainly b and y″ ions.[3] With ECD, however, it is believed that the 5−7 eV energy released by neutralization during the electron capture event can cause cleavages before the energy is fully randomized.[4] Compared to CAD, ECD results in extensive fragmentation of the backbone of small proteins.[5] ECD does not seem to generate many internal fragment ions, in contrast to CAD.[6] Furthermore, it has been shown that ECD is particularly useful for the identification of posttranslational modifications, such as carboxylation,[7] glycosylation,[8] oxidation,[5,7] and phosphorylation.[5,9] ECD involves the trapping of multiply charged cations and a subsequent exposure to low-energy (thermal) electrons. The capture of an electron by a multiply protonated peptide or protein leads to the formation of odd-electron $[M + nH]^{(n-1)+\bullet}$ reduced molecular ions. The electron capture is proposed to occur at a protonated site, thereby releasing an energetic H$^{\bullet}$ atom.[10] This H$^{\bullet}$ atom can initiate a radical site reaction.[11] Common high-H$^{\bullet}$ affinity sites in peptides are the carbonyl groups.[4] This is reflected by the preferred electron capture induced formation of c and z$^{\bullet}$ ions (Scheme 1).[4,12,13]

The electron capture induced dissociation of polypeptides containing disulfide bonds has been shown to yield far more extensive fragmentation than polypeptides containing no disulfide bond.[13] Mainly the S−S bond is cleaved, which is rationalized by the fact that disulfide bridges have even higher H$^{\bullet}$ affinity than

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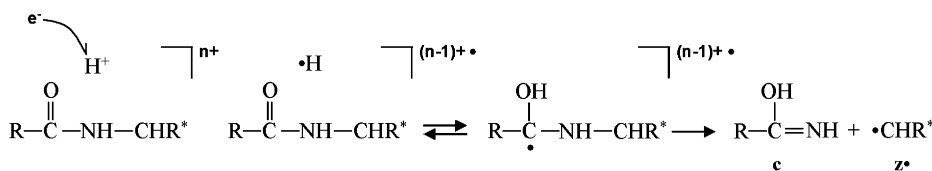
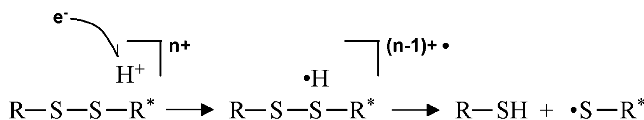
Scheme 1. Proposed Mechanism for the ECD Induced Formation of c and z• Ions[a]



[a] An electron is captured by a proton somewhere on the molecular ion. The resulting H• radical is captured by a carbonyl group. The energy released by neutralization is sufficient to produce immediate cleavage of the backbone.

Scheme 2. ECD Induced Cleavage of a Disulfide Bridge, Often Found in Proteins[a]



[a] The H• radical is captured at the disulfide bond. The following cleavage reaction leaves one of the fragments as a radical cation.

carbonyl groups. Usually major product ions in ECD are reduced $[M + nH]^{(n-1)+\bullet}$ ions, but in the ECD spectrum of a 10-kDa protein containing an S−S bond, little of this product ion remained. Dominating fragment ions resulted from the cleavage of the disulfide bridge (Scheme 2). In comparative CAD experiments, no such fragment ions were observed,[13] which indicated that ECD is far more effective in cleaving disulfide bridges than CAD.[14] In proteins, cyclized by disulfide bridges, capture of a single electron can cleave both a disulfide bridge and a backbone bond in the same ring or even both disulfide bonds holding the two peptide chains together.[13] The fact that stronger chemical bonds are cleaved during the process and not the weakest could point to a nonergodic character of ECD; alternatively, it could also result from the different energetic pathway for radical site-initiated reactions in comparison to cleavage reactions where unpaired electrons do not play a role.[15]

As electron capture seems to be preferred at disulfide bridges, we set out to study whether this specificity could also be applied to other sulfur-containing compounds. If so, ECD could be a unique tandem MS technique to analyze the specific sites of lanthionine bridges in lantibiotics. Lantibiotics contain a less common sulfur functionality, namely, lanthionine or monosulfide bridges, and are a subgroup of antibacterial peptides. The name is derived from their antibiotic action and their content of lanthionine and 3-methyllanthionine bridges (Chart 1).[16] A specific feature of these lanthionine bridges is that they consist of a thioether bond, instead of the disulfide bond.

Lantibiotics often contain a wide variety of posttranslationally modified amino acids, such as dehydroalanine (Dha), dehydroamino-2-butyric acid (Dhb), and 2-aminobutyric acid (Abu).[17] The four compounds used in this study are nisin A and Z, mersacidin, and lacticin 481 (Figure 1). They are toxic to Gram-positive bacteria even in low doses but nontoxic to humans. As such, nisin has been used as a food preservative for more than

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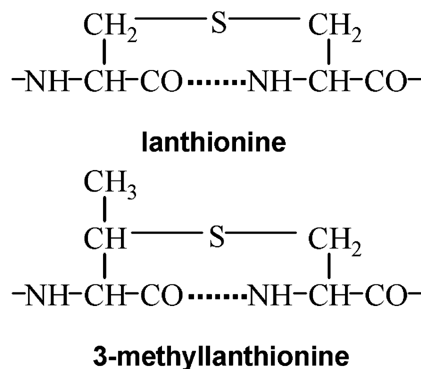
Chart 1. Chemical Structures of the Lanthionine and the 3-Methyllanthionine Bridge



50 years.[17] The structure of these lantibiotics has been studied previously, using peptide chemistry, mass spectrometry, and NMR;[18−20] more recently they have been explored as an antibiotic drug.[21]

EXPERIMENTAL SECTION

The fragmentation of the lantibiotics nisin A and Z, mersacidin, and lacticin 481 was studied using two different tandem mass spectrometric techniques, namely, ECD in a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer and CAD in a quadrupole time-of-flight (q-ToF) instrument.

The ECD experiments were performed on a modified Bruker-Spectrospin (Fällanden, Switzerland) Apex 7.0e FTICR-MS equipped with a 7-T superconducting magnet.

The ECD experiments with the lantibiotics were performed in an infinity cell, which has been described before.[22,23] To generate low-energy electrons, an indirectly heated barium−tungsten dispenser cathode (TB-198, HeatWave Labs, Inc.) was placed behind the ICR cell. It was operated using a current of 1.56 A and a potential of 5.6 V, yielding a power of 8.7 W.

All the ECD experiments were performed with 0.65-eV electrons, considering the fact that the potential in the center of the infinity cell is close to 0.3 V and that the cathode surface was at a potential of −0.35 V. A current of ~350 nA and 10-s irradiation

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Figure 1. Amino acid sequence and monosulfide bridge location of the four studied lantibiotics (A) mersacidin, (B) lacticin 481, and (C) nisin A. The only difference between nisin A and Z is that on the 27th amino acid position nisin A has a His residue and nisin Z an Asn residue.

time was used. The pressure in the ICR cell was $\sim 10^{-10}-10^{-9}$ mbar. Five stages of differential pumping were employed to bridge the atmospheric pressure conditions in the electrospray ionization source. The samples were dissolved in a 69:29:2% V methanol/water/acetic acid spray solution, containing 10−20 μM compound. The sample was pumped at a flow rate of 0.05−0.1 mL/h with a Harvard syringe pump 55-1111 (Kent, U.K.) through a fused-silica capillary. To generate positively charged droplets, a potential of ~3 kV was applied to a stainless steel spray needle. Data acquisition was performed using XMASS 4.0 (Bruker-Daltonics, Billerica, MA).

CAD experiments were performed in a Micromass q-ToF hybrid tandem mass spectrometer, equipped with a Z-spray sample introduction system. The samples were dissolved in a 69:29:2% V methanol/water/acetic acid spray solution, containing 10 μM compound. The samples were introduced, using gold-coated glass capillaries, into the nanospray ionization source. Capillary voltages in the range of 1100−1300 V were used to spray the samples. Ar was used as the collision gas. Data were analyzed using the MassLynx 3.4 software.

RESULTS

ECD on Lantibiotics. For all the lantibiotics used in this study, first an MS spectrum was recorded. Under the conditions used, the most abundant ions in the MS spectra of lacticin 481, nisin A, and nisin Z were $[M + 4H]^{4+}$ ions, for mersacidin $[M + 2H]^{2+}$ ions. In all cases, the most abundant ions were isolated and exposed to ~0.3−0.4-eV electrons, yielding intense reduced pseudomolecular ions in addition to the fragment ions discussed below. The $[M + 4H]^{4+}$ ions are capable of capturing up to three low-energy electrons. Perhaps the $[M + 4H]^{4+}$ ions are also capable of capturing four electrons, but then the ions would be neutralized and thus not detected. The ECD spectrum of nisin A $[M + 4H]^{4+}$ ions can be seen in Figure 2.

The electron capture induced fragmentation of nisin A and nisin Z was quite similar (Figure 3). Taking the similarity of the structure into account, this is not unexpected. Nisin A and nisin Z differ only by a single substitution at the amino acid at position 27, where nisin A contains a histidine and nisin Z an asparagine. The fragmentation products were mainly c ions and $z^{\bullet}$ radical ions, resulting from cleavage of an amine bond in the backbone. This observation is in agreement with data reported on peptides.[14] More interestingly, $c^{\bullet}$ radical ions and z ions were also formed. The occurrence of $c^{\bullet}$ radical ions and z ions as minor ECD products was reported earlier.[10] The $c^{\bullet}$ and z ions described in our study, however, were the products of a different and very specific fragmentation reaction. They were formed exclusively as a result of the cleavage of both a backbone bond and a thioether bond. Most of the cleavages took place near and in the lanthionine bridges. Nisin contains five available C-terminal attachment sites of lanthionine bridges; for nisin A and Z, five and four, respectively, were cleaved in this specific way. Mersacidin was more difficult to fragment using ECD (Figure 3). The fragment ions produced were mainly c ions and $z^{\bullet}$ radical ions, but again some $c^{\bullet}$ radical ions and z ions were formed. Cleavages took place mainly near the location of lanthionine bridges. Mersacidin contains three available sites for the earlier mentioned specific $c^{\bullet}$, z fragmentation; two were cleaved in this way. Finally, lacticin 481 was the most difficult lantibiotic to fragment using ECD (Figures 3 and 4). The ECD spectrum of lacticin 481 $[M + 4H]^{4+}$ ions in Figure 4 is dominated by nondissociated reduced molecular ions, and additionally, only a few minor c ions and $z^{\bullet}$ radical ions could be detected. No $c^{\bullet}$ radical ions and z ions were observed.

Nondissociative Electron Capture and the Loss of Small Molecules. In the application of ECD on lantibiotics, it was observed that the parent ion can capture up to three electrons without a subsequent formation of fragments. In fact, the most abundant ECD products were nondissociative reduction products. Although such products have been observed more often in ECD of peptides,[14] they are remarkably intense for the lantibiotics studied here (Figure 4). With FTICR, such nondissociative products can easily be distinguished from regular molecular ions, because they carry more hydrogens (Figure 4, inset). In one case, the doubly reduced pseudomolecular ion was even higher in intensity than the isolated parent ion after irradiation with low-energy electrons. In the ECD spectra of the four lantibiotics, some

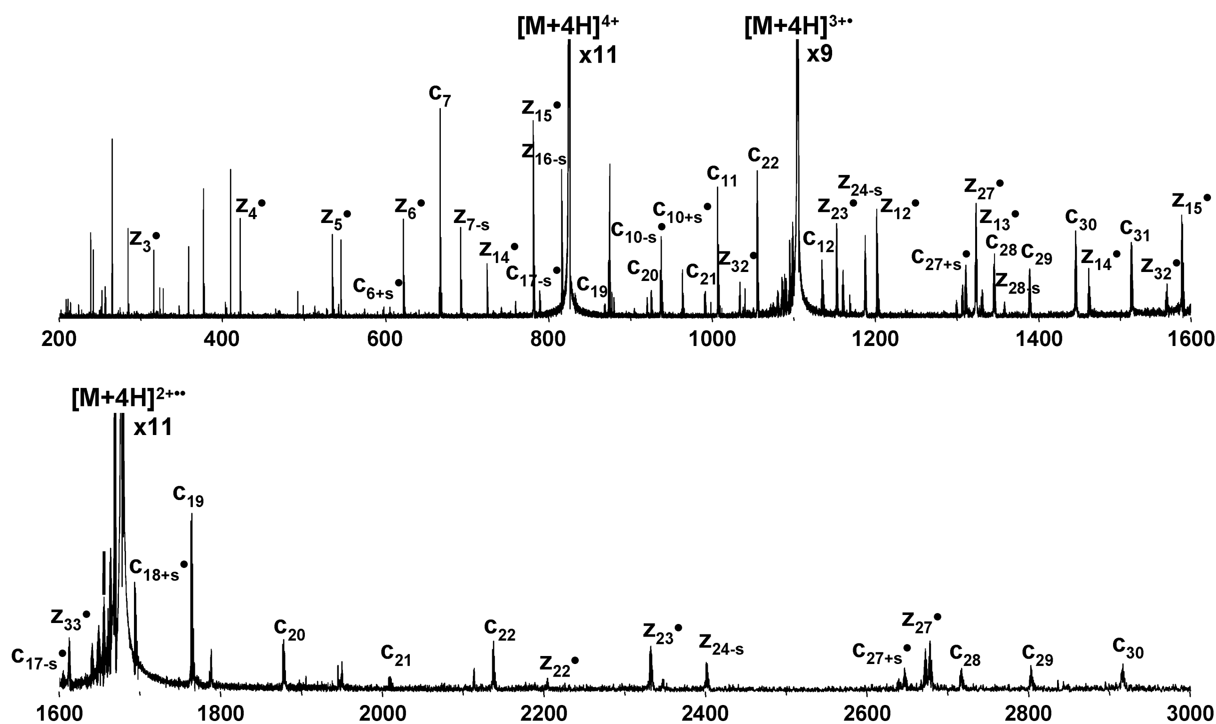

Figure 2. ECD spectrum of nisin A $[M + 4H]^{4+}$ ions. The top and bottom part of the spectrum have the same scale but were cropped to show the fragment ions. The peak intensity ratio for $[M + 4H]^{4+}/[M + 4H]^{3+\bullet}/[M + 4H]^{2+2\bullet}/[M + 4H]^{+3\bullet}$ ions was 100:86:97:11.

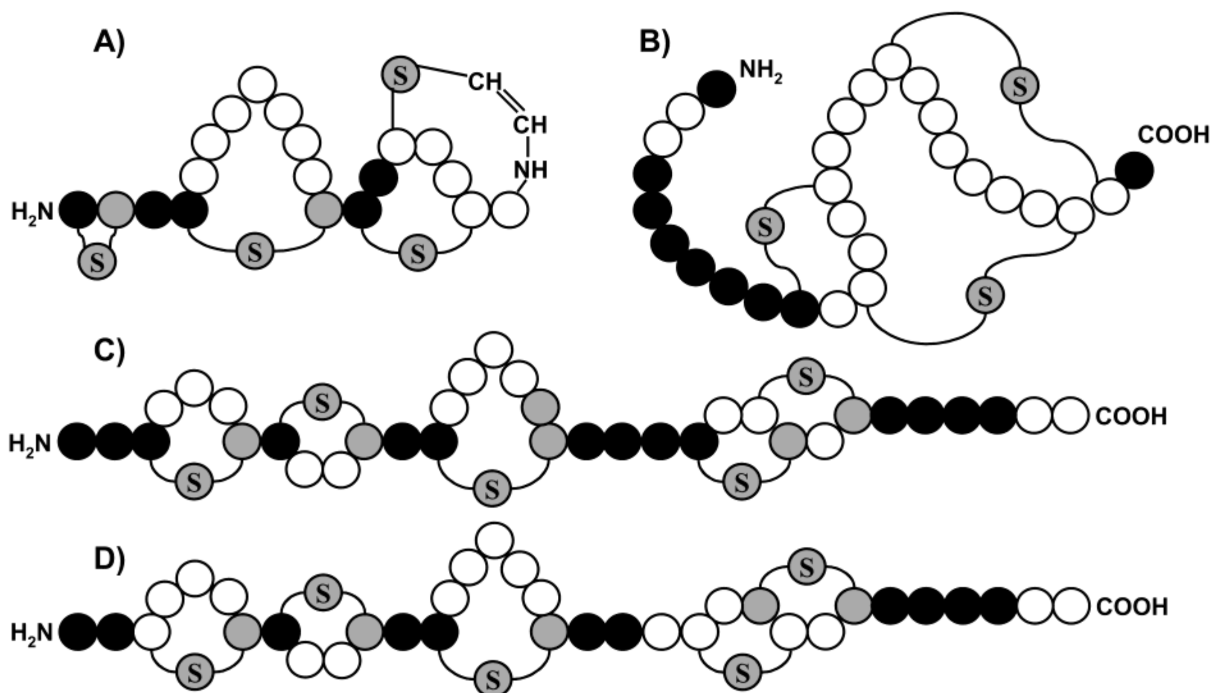

Figure 3. ECD induced fragmentation of (A) mersacidin, (B) lacticin 481, (C) nisin A, and (D) nisin Z. The fragmentation products from black-colored sites are c and z• ions. The fragmentation products from gray-colored sites are the specific c•and z ions. c•, z fragmentation always involves the cleavage of both a backbone bond and a thioether bond and nearly always results from cleavage in the amino acid attached to the C-terminal side of the lanthionine bridge.

small neutral losses from the odd-electron singly reduced molecular ions were also observed. The ions resulting from these small neutral losses were not very high in intensity, in contrast to the abundance of small neutral losses when CAD was used. All the lantibiotic pseudomolecular ions that had captured one electron showed intense NH_3 loss. The ECD spectrum of nisin A contained two peaks, which can be attributed to amino acid side-chain losses. Clearly visible were losses of 57 and 71 Da. These are the masses of the side chains of Asn and Lys minus 1 Da. Also a loss of 28/29 Da was observed, which can be attributed to CO/COH loss. Mersacidin exhibited quite an intense $SH^\bullet$ loss and a loss of 56 Da, which is the mass of the side chain of Ile/Leu minus 1 Da. Lacticin 481, finally, showed losses of 44 Da, which could be CO_2 loss and losses of 72 and 129 Da, which are likely the side-chain

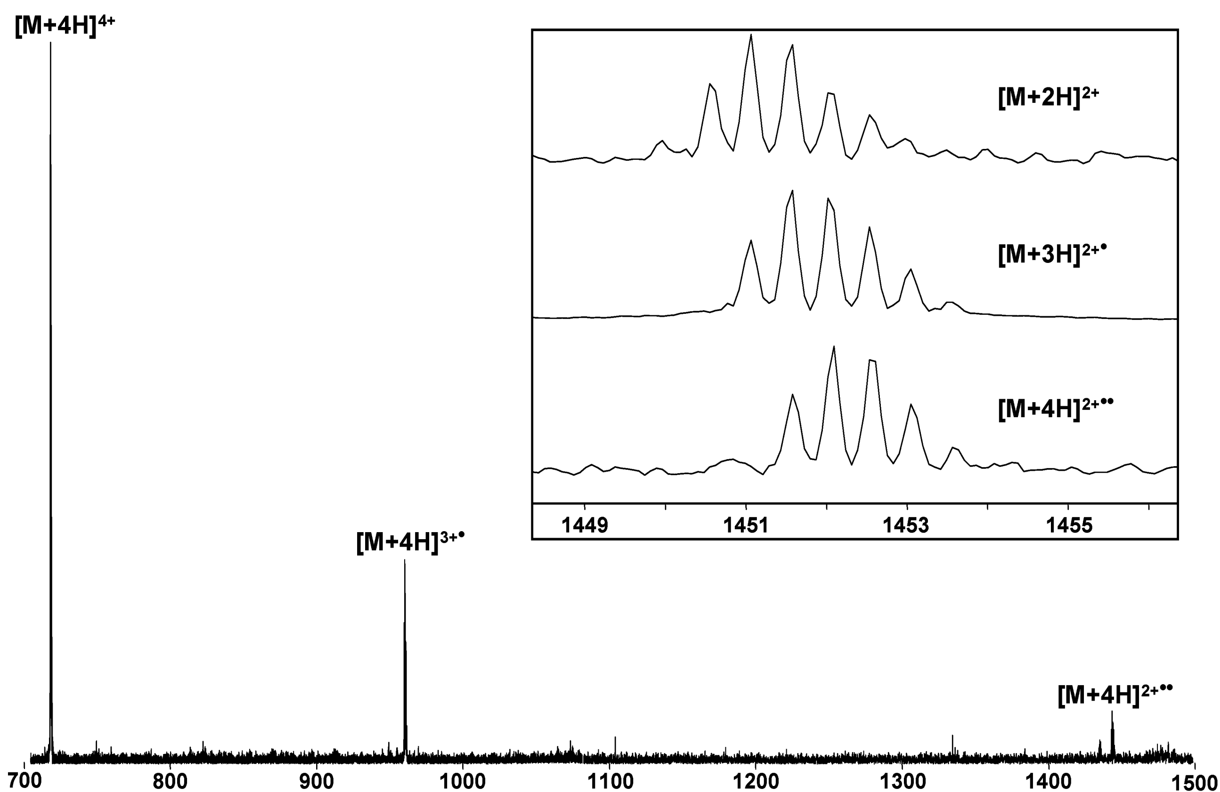

Figure 4. ECD spectrum of lacticin 481, showing the parent ion ($[M + 4H]^{4+}$) and the singly and doubly reduced pseudomolecular ion. The inset shows the difference in mass between a regular $[M + 2H]^{2+}$ molecular ion of lacticin 481, the $[M + 3H]^{2+\bullet}$ ion, formed by the capture of one electron by the $[M + 3H]^{3+}$ ion, and the $[M+4H]^{2+2\bullet}$ ion, formed by the capture of two electrons by the $[M + 4H]^{4+}$ ion. These ECD products can easily be distinguished from regular molecular ions by FTICR, because they carry more hydrogen atoms.

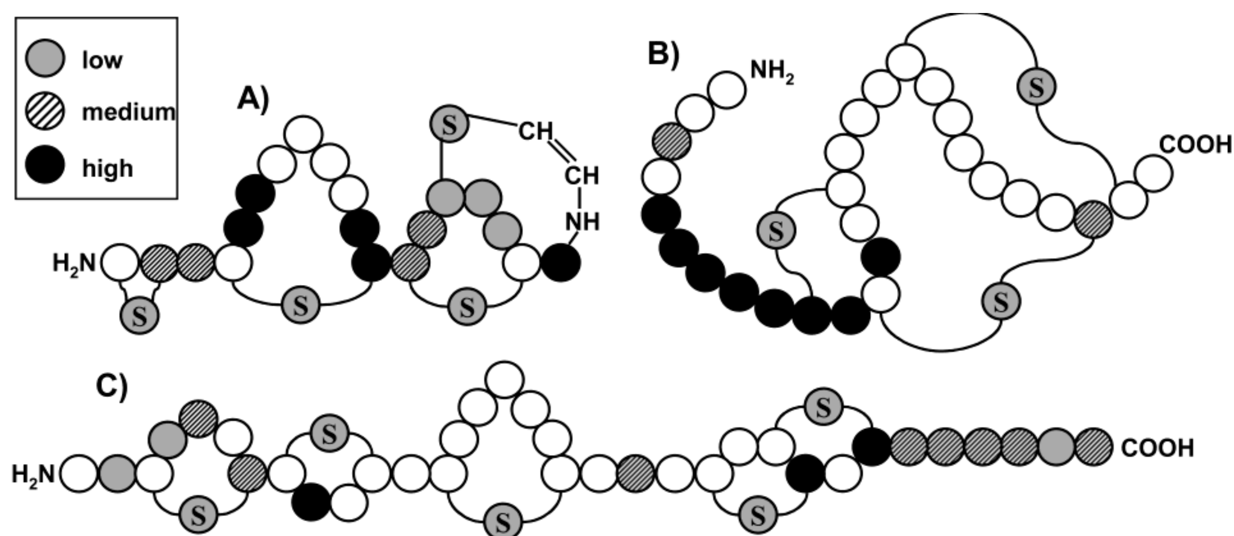

Figure 5. CAD induced fragmentation of (A) mersacidin $[M + 2H]^{2+}$, (B) lacticin 481 $[M + 4H]^{4+}$, and (C) nisin A $[M + 4H]^{4+}$. The inset displays how the colors correspond to the collision energy. To compare the collision energy of the different ions, the maximum center-of-mass kinetic energy, gained in the collision cell, was calculated for each type of ion during the experiments. When the center-of-mass kinetic energy of the ions did not exceed 1 eV, the collision energy was considered as low, from 1 to 1.4 eV as medium, and above 1.4 eV as high. In the case of b and y″ ions, the amino acid at the right of the cleavage site is indicated.

masses of Glu and Trp minus 1 Da.

CAD on Lantibiotics. To compare the results of ECD and CAD, the lantibiotics were fragmented in a q-ToF instrument using different collision energies and Ar as the collision gas. Roughly speaking, the CAD fragmentation patterns of the lantibiotics used in this study were quite similar. All the fragment ions found in a SORI-CAD FTICR study of nisin A by Lavanant et al.[20] were also found in the CAD q-ToF spectrum with some additional fragments. The most abundant fragments were b, y″, and z ions, and the neutral loss of water from the parent ion was also prominent. At a low collision energy, cleavages mainly took place at the termini of the lantibiotics; cleavages of lanthionine bridges were rare. At higher collision energies, more backbone bonds in the central parts were cleaved and more internal fragments were formed, but

Scheme 3. Postulated Mechanism of the Formation of $c^{\bullet}$ and z Ions Resulting from the Cleavage of a Monosulfide Bridge and a Backbone Bond[a]

[a]The $H^{\bullet}$ radical is formed as a result of the capture of an electron by a proton. The location of the unpaired electron is not known.

fragment ions resulting from cleavages at the termini generally were still higher in intensity. At these higher collision energies, some fragment ions were also formed, which resulted from the cleavage of both a backbone bond and a lanthionine bridge, but these fragment ions were generally quite low in intensity. With low-energy CAD, the sequence coverage obtained for nisin A was 38%. At higher collision energies, more and more internal fragments were formed, so this number did not improve. For comparison, the sequence coverage obtained with ECD was 59%. The two techniques combined yielded a sequence coverage of 74%. This result demonstrates the complementarity of the two techniques, which has been shown previously.[11] The CAD-q-ToF results are given schematically in Figure 5.

DISCUSSION

The formation of c and $z^{\bullet}$ ions from peptides has been observed previously with ECD.[14] This radical site-initiated reaction starts at the carbonyl oxygen. The neighboring amine bond is broken via relocations of electrons. After the cleavage, the $z^{\bullet}$ radical ion is left with the unpaired electron and hence contains an odd number of electrons. More importantly, the formation of $c^{\bullet}$ and z ions is observed. In this case, after cleavage, the $c^{\bullet}$ ion is left with an odd number of electrons and the z ion with an even number. It can be deduced that in the formation of $c^{\bullet}$ radical ions and z ions two bonds are always broken, namely, a backbone bond and a thioether bond in one of the lanthionine bridges. A possible explanation for the formation of $c^{\bullet}$ and z ions instead of c and $z^{\bullet}$ ions could thus be that, from a single electron capture, a double cleavage occurs. Probably, the radical site-initiated reaction starts at the sulfur atom in the thioether bridge. The thioether sulfur atom supposedly has a high hydrogen atom affinity, just as a carbonyl oxygen.[13] Considering the masses of the $c^{\bullet}$ fragments, the thioether bond is cleaved as well as an amine bond in this way (Scheme 3).

This $c^{\bullet}$, z cleavage is very specific. It was observed almost exclusively when cleavage took place in an amino acid attached to the C-terminal side of a thioether bridge. The electron capture induced $c^{\bullet}$, z cleavage can thus be used as a tool to reveal the C-terminal attachment site of lanthionine bridges in newly discovered lantibiotics. Note that two exceptions were observed, namely, with the third bridge (from the N-terminus) in nisin A and with the fourth bridge in nisin Z. In these cases, a thioether bond and a backbone bond were also cleaved. However, the amino acid in which the cleavage took place was not attached to the C-terminal side of a thioether bridge but located one position further in the direction of the N-terminus (Figure 3). Assigning the C-terminal attachment site of the third lanthionine bridge in nisin A is quite straightforward, because the amino acid attached to this bridge was also cleaved, so this location can still be deduced. Assigning the C-terminal attachment site of the fourth lanthionine bridge in nisin Z was more difficult, because fragments resulting from cleavage in the amino acid at this location are absent in the ECD spectrum. Fortunately, CAD of nisin Z provided a fragment that could be used to identify this location. This example demonstrates nicely the complementarity of the two tandem mass spectrometric techniques.

It was observed that ECD of lantibiotics mainly occurs near the location of the lanthionine bridges (Figure 3). In contrast, CAD of lantibiotics shows no such specificity. CAD at low energy induces primarily cleavages at the termini of the ions; only by increasing the collision energy may some of the more distal lanthionine bridges be cleaved. A problem with raising the collision energy, however, is the increasing abundance of internal fragment ions. The fact that with ECD lanthionine bridges are easily cleaved could point to a high hydrogen atom affinity of these bridges and the specificity of ECD for cleaving thioether bonds. Fragments from cleavages in amino acids between the two attachment sites of a lanthionine bridge were hardly ever observed with CAD. When the backbone is cleaved at such a location, the lanthionine bridge remains intact and therefore no fragments are formed.

Not all the C-terminal attachment sites of lanthionine bridges were cleaved using ECD. One in nisin Z, one in mersacidin, and none of the three in lacticin (that is, 5 out of 16 in total) did not show this specific cleavage. For four of these five bridges there is a good explanation, in that these sites are located in a region where two lanthionine bridges overlap. To get detectable fragments from these sites, two lanthionine bridges and one backbone bond must be cleaved, for which two dissociative electron capture events are needed. The probability of an ion capturing two electrons and undergoing the required cleavages for fragmentation is not very high. It was also observed that the application of ECD on lantibiotics gave rise to singly and multiply reduced ions, which did not exhibit dissociation. This was observed earlier with other

types of peptide ions.[14] For example, in the ECD spectrum of nisin A $[M + 4H]^{4+}$ ions, there is a very abundant peak present, originating from $[M + 4H]^{2+2\bullet}$ pseudomolecular ions. This means that the original four protons have captured two electrons, making this ion possibly a biradical cation. An explanation for the seemingly highly nondissociative character of the reduced molecular ions could be the abundant presence of lanthionine bridges in lantibiotics. The sulfur atom in this bridge is able to capture an electron or a $H^\bullet$ radical, and the radical then initiates cleavage of the lanthionine bridge. In this cleavage, however, no backbone bond needs to be broken, so the ion will remain intact. However, it is possible that there is sufficient internal energy left in the ion to yield small neutral losses. Apart from the frequently observed loss of NH_3, amino acid side-chain losses were also observed. These neutrals were 1 Da less in mass than the side chain, suggesting that the $C_\alpha-C_\beta$ bond is cleaved and that a double bond is formed, somewhere in the side chain, during the fragmentation process. Strikingly, these side-chain losses arise from amino acids in close proximity to the location of lanthionine bridge attachment sites to the backbone. This specificity points again to the importance of lanthionine bridges in electron capture processes.

CONCLUSION

ECD fragmentation can be used as a tool to localize the C-terminal attachment site of lanthionine bridges, because of the specific $c^\bullet$, z fragmentation in which a backbone amine bond and the thioether bond of a lanthionine bridge are cleaved. Lanthionine bridges play an important role in the electron capture process in lantibiotics. One indication for this is the fact that ECD cleavages mainly take place near the location of lanthionine bridges, whereas low-energy CAD cleavages mainly take place near the termini of the molecular ions. A second indication for the important role of lanthionine bridges as an electron capture site is that side-chain losses were observed from amino acids next to lanthionine bridge attachment sites. In the future, ECD can be used in combination with CAD for structural analysis of newly discovered lantibiotics.

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