# Structural analysis of sulfide containing peptides using Electron Capture Dissociation

# Structural analysis of sulfide containing peptides using Electron Capture Dissociation

**Structuuranalyse van peptiden met sulfidebindingen gebruikmakend van elektronenvangst dissociatie**

(met een samenvatting in het Nederlands)

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*Voor Thea*

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# Chapter 1

# General introduction

Our world is constructed of many small molecular building blocks. Molecules are composed of atoms that on their turn are composed of a nucleus and electrons that orbit the nucleus. Electronic interactions between atoms result in chemical bonding, which is the subject of modern chemistry. Scientific developments in Europe in the 17$^{th}$ century resulted in the present systematic and rational study of nature. Initially a fundamental distinction was made between inorganic and organic chemistry. It was thought that organic compounds could not be synthesized from inorganic compounds, a theory known as vitalism. However, in the beginning of the 19$^{th}$ century, urea, considered to be an organic compound, was synthesized by Wöhler by heating ammonium cyanate, which was considered to be an inorganic compound. Later, Berthelot routinely crossed the line between organic and inorganic chemistry with the synthesis of many more organic compounds, such as alcohol and methane, from inorganic starting materials. The distinction between organic and inorganic chemistry is still made today, however it is not based on vitality but on the fact that organic chemistry almost exclusively deals with carbon-based compounds. The defeat of vitalism paved the way for biomolecular analysis and how it is practiced today. Research focuses on structural and functional analysis of biomolecules such as proteins, lipids, DNA, carbohydrates and RNA that are all important for cell functioning and structure. With the recent elucidation of the human genome[1] a huge step was made in the structural analysis of biomolecules. However, necessary steps had to be taken to develop this information into something more applicable. Proteins carry out nearly all the important functions inside cells, while DNA “merely” contains their sequence definition. Proteins are the workhorses in cells and hence have a major influence on the properties of a cell. Cells can express DNA in very different ways. Kidney cells for instance produce different proteins compared to neuronal cells, but contain identical DNA. The analysis of proteins is hence imperative for a better understanding of cell functioning.

Proteomics studies the ensemble of proteins found in organelles, cells or complete organisms. The objective of proteomics research is to answer basic questions about how biological processes work and how they are controlled. Diseases are often protein-related. Proteins have important functions in cells that can differ in healthy and diseased states. Knowledge generated with proteomics research can result in better medical diagnosis and therapies. To obtain a better understanding of the properties of proteins

and their interactions in healthy and diseased cells it is important to know what their primary, secondary, tertiary and quaternary structural characteristics are. Several techniques have been developed to study these aspects of molecular structure and interaction. Nowadays, the most common structural analysis tools for proteins are Nuclear Magnetic Resonance (NMR),[2] X-ray crystallography[3] and Mass Spectrometry (MS). NMR and X-ray crystallography are superior to MS in the determination of three-dimensional protein structures. Main drawbacks are their time-consuming nature, the need for pure and concentrated samples and large sample volumes. NMR and X-ray crystallography are therefore hardly suitable for analysis of complex biological samples, especially when only a limited amount of material is available. Mass spectrometry offers a solution to these drawbacks. MS is a fast and highly sensitive technique: proteins can be detected even in zeptomolar concentrations.[4] Furthermore, complex biological samples can be analyzed with MS, often in combination with a separation technique. With modern mass spectrometry techniques quaternary structures and assemblies of proteins can be studied, e.g. large >1Mda biomolecular complexes and even complete viruses.[5] Therefore, today MS is a frequently used technique in the proteomics field.[6, 7]

Mass spectrometry is based on the manipulation of ion trajectories with electric and magnetic fields. Ions are separated according to an ion specific physical property: their mass to charge ratio (m/z). The determination of molecular masses following this approach has been practised for about a century. J.J. Thomson had built the first mass spectrometer early in the twentieth century. The parabolic trajectories of ions, generated in discharge tubes, were determined by passing the ions through electric and magnetic fields. Later, other analyzers were developed, such as magnetic sector, Time-of-Flight (ToF), quadrupole, ion trap and Ion Cyclotron Resonance (ICR) mass spectrometers. There are several techniques available to give molecules a charge, which is required for performing MS analysis, called ionization techniques. Some of these ionization techniques can result in dissociations as well. Beginning with Surface Ionization (SI) and Electron Impact (EI) a wide range of ionization techniques have been developed, such as Field Ionization (FI), Chemical Ionization (CI) and Fast Atom Bombardment (FAB). The drawback of these ionization techniques was that the types of molecules analyzed with mass spectrometry remained mainly restricted to small organic molecules. With the development of ElectroSpray Ionization (ESI)[8] and Matrix Assisted Laser Desorption/Ionization (MALDI)[9-11] in the late 1980s for the first time large

biomolecules could converted into gas phase ions. This was a milestone in the field of mass spectrometry. With MALDI analytes are laser-desorbed from a solid or liquid matrix. ESI will be discussed in more detail below as the research described in this thesis was performed using solely ESI as the ionization technique. Alternatively, with ESI an analyte solution is passed through a hollow needle. Applying a high potential between the conductive needle and the inlet of the mass spectrometer generates charged droplets. According to the "charge residue model"[12] the droplets become smaller via solvent evaporation and Coulombic explosions and finally bare sample ions are left that can be mass analyzed. The "ion evaporation model"[13] states that ions are ejected from the charged droplets. Compared to MALDI, ions generated with ESI generally carry multiple charges, which makes this ionization technique more suitable for studying electron capture reactions by cations, the major subject of this thesis. ESI is also suitable for bringing large non-covalent complexes in the gas phase, as it is a soft ionization technique. Furthermore ESI is applicable in high-throughput analysis, because it is readily coupled to liquid separation techniques, such as Liquid Chromatography (LC). With the development of ESI and MALDI the applications of mass spectrometry could be extended to new research areas. One of the most important new areas for MS was the field of protein structural analysis. Some structural information can be obtained for proteins by determining their accurate mass, such as the presence of posttranslational modifications. To obtain more detailed structural information, however, the employment of tandem MS is required. Typically, in tandem MS first precursor ions are isolated, then dissociated and finally the fragments are detected. The m/z values and intensities of the obtained set of fragment ions give information about the structure of precursor ions. In protein structural analysis tandem MS is mainly used to determine amino acid sequences and to identify or localize post-translational modifications. The nomenclature for protein and peptide fragment ions, which will be used throughout this thesis, was proposed by Biemann[14] (figure 1.1) and is based on the nomenclature proposed by Roepstorff and Fohlmann.[15] Only for the results in chapter 4 a slightly adapted nomenclature will be used, which will be described in that chapter.

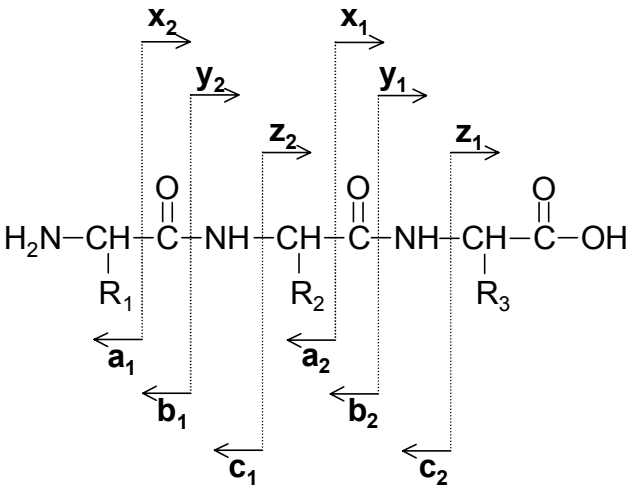



***Figure 1.1:*** *Nomenclature for peptide fragment ions. Fragment ions containing the N-terminus are a, b and c ions. Fragment ions containing the C-terminus are x, y and z ions.*

Tandem MS gives information about the primary structure of peptides and proteins in the form of fragment ion series. For instance, the difference in mass between $y_n$ ions and $y_{(n-1)}$ ions is the mass of the amino acid at the $n^{th}$ position from the C-terminus. The obtained amino acid sequences are useful for *de novo* sequencing or for identification of peptides and proteins using databases. For these purposes it is important to cleave as many interresidue bonds as possible, in other words, to obtain high sequence coverage. Several tandem MS techniques are available; the most commonly used technique is low-energy Collision Induced Dissociation (CID). Extensive series of b and y ions are typically observed when peptide ions are subjected to low-energy CID. This technique is thus widely used in proteomics research. With low-energy CID precursor ions undergo multiple collisions with gas atoms resulting in “slow heating” of the precursor ions. Because this heating process is slow compared to intramolecular vibrational energy redistribution processes, energy randomization will occur and precursor ions will fragment via the lowest-energy dissociation pathways. In peptides and proteins this will mainly result in cleavages of amide bonds (b and y ions). A disadvantage of low-energy CID is that labile post-translational modifications often dissociate from the precursor ions before backbone cleavages take place, resulting in loss of structural information about these modifications. Another drawback is that the slow heating process can lead to rearrangement reactions in the precursor ions, which results in loss of structural information as well. Other tandem MS techniques, such as InfraRed MultiPhoton

Dissociation (IRMPD) and Blackbody Infrared Radiative Dissociation (BIRD) are based on the same principle of slow heating, although the internal energy is deposited in a different manner, through the absorption of multiple photons.

In 1998 a new and promising fragmentation technique, Electron Capture Dissociation (ECD), was introduced.[16] ECD has proven to be a valuable addition to the tandem MS arsenal. In ECD multiply charged cations are irradiated with low-energy electrons. The cleavages induced by electron capture are fundamentally different from the cleavages induced by "slow heating" tandem MS methods. Electron capture leads to very rapid fragmentation reactions (<1 ps) before intramolecular vibrational energy redistribution takes place. In ECD of peptides and proteins preferentially backbone amine bonds are cleaved, resulting in the formation of c and $z^{\bullet}$ ions (figure 1.1).[16] Although fragment ions are usually low-abundant, the high sequence coverage obtained with ECD makes this a suitable tandem MS method for sequencing peptides and proteins. Labile post-translational modifications can be localized with ECD, because, in contrast to low-energy CID, ECD generally results in backbone cleavages prior to ejection of the modification. ECD offers new perspectives in the analysis of proteins and peptides containing sulfide bonds. Disulfide and other sulfide bonds are very important for protein and peptide structure. In protein folding processes disulfide bonds play a dominant role.[17] Intramolecular sulfide bonds can bring distant parts of a protein or peptide in close proximity, making the threedimensional structure more compact. Molecules with intramolecular sulfide bonds are cyclic and hence two cleavages are required for fragmentation in these regions. With tandem mass spectrometric methods such as CID it is difficult to obtain relevant structural information of peptides and proteins containing sulfide bonds.[18, 19] In contrast to CID, ECD results in selective disulfide bond cleavages in peptides and proteins, allowing their localization. Often disulfide bond cleavage is accompanied with a backbone cleavage, resulting in fragmentation in cyclic regions and thus ECD is a valuable tool to analyze peptides and proteins with these functionalities. ECD is almost exclusively combined with Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometry. Electron transfer dissociation (ETD)[20] is one recent alternative that also allows for similar protein and peptide fragments to be generated in a quadrupole ion trap. ETD uses ion-ion interactions of opposite charge rather than the ion-electron reactions used in ECD. For electron capture dissociation to occur efficiently, ions need to be immersed in a dense population of low-energy electrons and

this has only been achieved in FTICR instruments until this moment. The research described in this thesis is nearly exclusively performed using FTICR-MS. For that reason this mass analyzer is described in detail in this introductory chapter (1.1). Generally the FTICR mass analyzer is one of the best analyzers considering mass resolution and accuracy. Being an ion trap, the FTICR analyzer is extremely suitable to facilitate ion-electron interactions needed for ECD, the major subject of this thesis. In chapter 1.2 the properties and applications of ECD are described in detail. This general introduction concludes with the scope of the thesis in chapter 1.3.

## 1.1. Fourier Transform Ion Cyclotron Resonance Mass Spectrometry: theory and experiment

With a Fourier Transform Ion Cyclotron Resonance (FTICR) MS mass measurements can be done with higher accuracy and resolution than with any other mass analyzer. FTICR is derived from Ion Cyclotron Resonance (ICR) spectrometry, developed by Lawrence et al. in the 1930s.[21] The ICR technique was first incorporated in a mass spectrometer by Sommer et al. who used it for the determination of the Faraday constant by measuring the cyclotron resonance frequency of protons.[22, 23] The first FTICR mass spectrometer was built by Comisarow and Marshall.[24] In this section the principles of the FTICR technique will be explained with a focus on the experimental procedures used for the work described in this thesis. Mass measurements in FTICR are performed with an ICR cell that is placed in the central cavity of a magnet. The magnet produces a homogeneous magnetic field parallel to the z-axis of the ICR cell. Ions with non-zero kinetic energy in the x and y direction are forced into cyclotron motion in the xy-plane by the magnetic field. In the z-direction ions are confined by two opposing trap electrodes, producing an electrostatic potential well. The combination of a homogeneous magnetic field and an electrostatic potential to trap charged particles is also known as a Penning trap.[25] There are many different ICR cell designs, for instance the open-ended cylindrical cell,[26, 27] a closed cylindrical cell (figure 1.2) and the infinity cell.[28] In comparison with older cell designs like cubic cells[29, 30] and rectangular cells[31] an advantage of the infinity cell is that ion loss along the z-axis is minimized. A disadvantage is that ions are introduced through a small hole in the covering front

electrode, which makes trapping more difficult. The use of an open-ended cell eliminates this disadvantage.

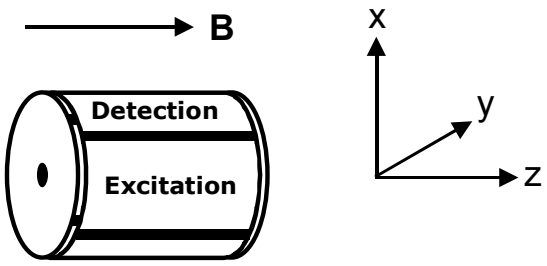



***Figure 1.2:*** *The closed cylindrical cell contains two trapping electrodes with a small inlet hole, two opposing excitation electrodes and two opposing detection electrodes.*

The use of an FTICR mass analyzer is widespread in ECD research. This is due to the fact that this is the only analyzer in which efficient ECD has been observed until now. FTICR does offer great advantages over other analyzers for performing ECD, such as the unequaled mass resolution and accuracy. This allows for instance the easy discrimination between regularly protonated peptide ions and peptide ions containing excess hydrogens that are usually observed in ECD spectra. The ICR cell as an ion trap is extremely suitable for performing ion-electron reactions. FTICR is a nondestructive mass analyzer, allowing the application of multistage tandem MS ($MS^n$) to precursor ions, such as in the ECD-CID experiments described in chapter 3. Introducing additional tandem MS events after ECD is extremely useful for confirming the identity of observed fragment ions.

## 1.1.1. Ion motion

The motion of charged particles in an ICR cell is governed by the magnetic and electric fields present. Charges in an electric field **E** and magnetic field **B** will interact with these fields and experience forces, expressed in the Lorentz force equation (eq 1.1), in which **F** is the sum of forces acting on an ion with charge q and velocity **v**.

$$\mathbf{F}_L = q\mathbf{E} + q(\mathbf{v}\mathrm{x}\mathbf{B}) \quad (1.1)$$

Because of the presence of the magnetic and electric fields, ions in an ICR cell will perform three types of motion. For FTICR the most important motion is the cyclotron motion. The cyclotron motion arises in the x and y direction from interaction of the magnetic field with the ions. The force acting on the ions, resulting from this interaction, is perpendicular to both the direction of the ions' velocities and the direction of the magnetic field and is called the Lorentz force. The Lorentz force only changes the direction of an ion's velocity, but not its magnitude. Because of the Lorentz force the ions will move in a circular orbit perpendicular to the magnetic field lines. The frequency $\upsilon_c$ and angular frequency $\omega_c$ of this cyclotron motion in absence of electric fields are expressed in equation 1.2 and 1.3 respectively.

$$\nu_c = \frac{qB}{2\pi m} \quad (1.2)$$

$$\omega_c = \frac{qB}{m} \quad (1.3)$$

As can be seen in equation 1.2 the cyclotron frequency $\nu_c$ depends only on the magnetic field strength B, which is constant due to usage of low-loss (2 ppm/year) superconducting magnets, the mass m (in kg) and charge q (in C) of the ions. It should be noted that the cyclotron frequency does not depend on the velocity of the ions and is therefore independent of their kinetic energy and their orbit radius. This is in contrast to other mass spectrometric analyzers, where the spread in kinetic energies of ions limits the resolution, and a fundamental reason for the superior resolution of FTICR MS. The orbit radius does depend on the ion's kinetic energy. The use of ultrahigh vacuum systems prevents the ions to lose kinetic energy through collisions with neutral background gas molecules and keeps the orbit radii constant.

In the z-direction, parallel to the magnetic field lines, ions do not interact with the magnetic field. To stop ions from leaving the cell in this direction trapping plates are introduced in the ICR cell[32] perpendicular to the direction of the magnetic field, which creates a potential well that traps ions in the z direction. To trap positive ions a positive potential is applied on the trapping plates and to trap negative ions a negative voltage is applied. The ions will oscillate along this z-direction. This motion is called the trapping

motion and follows equation 1.4, in which $\omega_T$ is the trapping frequency, $V_T$ the trapping potential and d the internal diameter of the trap.

$$\omega_T = \sqrt{\frac{qV_T}{md^2}} \quad (1.4)$$

The electrical field of the trapping electrodes can have a significant contribution to the cyclotron motion. This field opposes the Lorentz force. The addition of an electrostatic field thus leads to a repulsive radial potential for the ions and has two consequences for the ions' radial motion. First, the cyclotron frequency is reduced, because the electrostatic potential reduces the radial Lorentz force. Secondly, the center of the cyclotron motion will be forced into a low-frequency periodic motion, which is called the magnetron motion. The magnetron motion is in fact a mass-independent precession of an ion along a path of constant electrostatic potential and given by equation 1.5.

$$\omega_m = \frac{qB_0 - \sqrt{q^2B_0^2 - 4mqE_0}}{2m} \quad (1.5)$$

The magnetron motion arises as one of the solutions in the equation of motion of an ion in static electric and magnetic fields. This can be expressed as in equation 1.6.

$$m\omega^2 r = q\mathbf{B}\omega r - q\left(\frac{V_T r}{2d^2}\right) \quad (1.6)$$

There are two solutions to this equation, namely:

$$\omega^+ = \frac{1}{2}\left(\omega_c + \sqrt{\omega_c^2 - 2\omega_T^2}\right) \quad (1.7)$$

$$\omega^- = \frac{1}{2}\left(\omega_c - \sqrt{\omega_c^2 - 2\omega_T^2}\right) \quad (1.8)$$

Equation 1.7 gives the reduced cyclotron frequency and equation 1.8 gives again the magnetron frequency. In comparison to cyclotron frequencies, magnetron frequencies

are quite low. In general cyclotron frequencies fall in the range 5 kHz-5 MHz, whereas magnetron frequencies are in the order of 1-100 Hz.

### 1.1.2. Ion excitation and detection

In the previous section the natural motion of ions in FTICR was explained. In this section it is explained how ions are excited and detected. When the ions have entered the analyzer cell they are orbiting with randomly distributed phase and radius defined by their thermal energy. In this situation no signal can be obtained. A measurable signal can only be obtained when the ions with equal mass move spatially coherent in proximity of the detection plates. To achieve this, or to eject ions from the cell their cyclotron motion needs to be excited. This can be achieved by applying an rf pulse to the opposing excitation plates, resonant with the cyclotron frequency of the ions of interest. The oscillating phase-coherent electric field excitation has the form (eq 1.9):

$$\mathbf{E}(t) = E_0 \cos \omega_c t \mathbf{j} \quad (1.9)$$

This formula can be divided in two counterrotating components:

$$\mathbf{E}_L(t) = \frac{E_0}{2}(\sin \omega_c t + \cos \omega_c t) \quad (1.10)$$

$$\mathbf{E}_R(t) = \frac{E_0}{2}(\sin \omega_c t - \cos \omega_c t) \quad (1.11)$$

Only the component rotating with the resonant frequency and in the same direction as the ions of interest will increase the kinetic energy of the ions. The component rotating in opposite sense is so far off-resonant that it has hardly any effect on the ions' orbits. The ions absorb power and their radii increase during the excitation time ($t_{exc}$). Assuming that the absorbed power A is all converted to kinetic energy, the following equation holds (eq 1.12).

$$A(t_{exc}) = \frac{E_0^2 q^2 t_{exc}}{4m} \quad (1.12)$$

The radius of the ions becomes (eq 1.13)

$$r = \frac{E_0 t_{exc}}{2B_0} \quad (1.13)$$

As can be seen from this equation the radius is independent of the mass and the charge of the ions, thus when the excitation power over the frequency domain is constant all ions are excited to the same radius. To achieve this a Stored Waveform Inverse Fourier Transform (SWIFT) pulse[33] can be applied. In the SWIFT method the desired magnitude spectrum is specified and subsequently inverse Fourier Transform is performed to generate the corresponding excitation waveform. Another method is the application of a frequency sweep or chirp. This waveform consists of a sinusoid with constant amplitude, whose frequency changes linearly in time. All ions with a cyclotron frequency in the sweep range will be excited to large orbits.

The radii of ions entering the cell usually are small compared to the cell dimensions. When an rf voltage is applied to the excitation plates the ions with a resonant frequency will absorb power and spiral outwards (figure 1.3a). The radius of their cyclotron orbit will become larger and will reach cellular dimensions when the rf voltage is persisted (figure 1.3b).

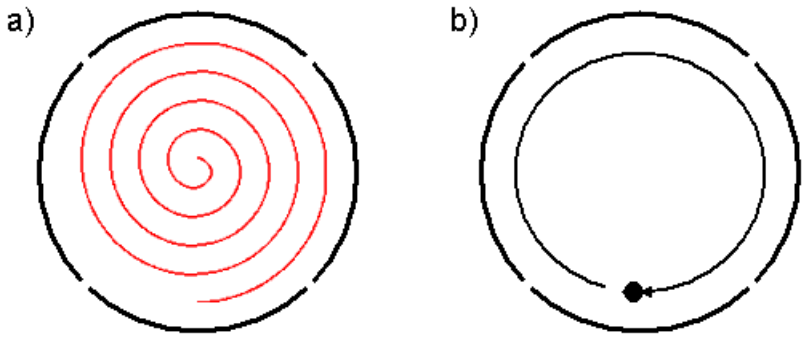



***Figure 1.3:*** *When an rf pulse is applied to the two opposing excitation electrodes, ions with a resonant cyclotron frequency will spiral outwards from the center of the cell (a). During detection a packet of ions will have a large cyclotron radius (b).*

For detection the ions are excited to an orbit diameter slightly smaller than the closest distance between the detection plates. The rotating coherent ion package induces a

sinusoidal image current on the detector electrodes. When a group of positive ions passes the detection plates electrons will be attracted. The oscillating current is converted to an oscillating voltage to give the following time-domain voltage signal f(t) (eq 1.14).

$$f(t) = \sum_{i=1}^{M} N_i \exp\left(\frac{-t}{\tau_i}\right) \cos\left(\omega_i t + \varphi_i\right) \quad (1.14)$$

Here $N_i$ is the number of ions. The intensity of the time-domain signal decreases due to collisions with gas atoms and space charge effect with the damping constant $\tau_i$. The phase of the ions is denoted with $\varphi_i$, $\omega$ is the cyclotron frequency and t is the transient length. The longer the transient is in the time domain, the greater is the mass resolution. A time-domain signal can be seen in figure 1.4.

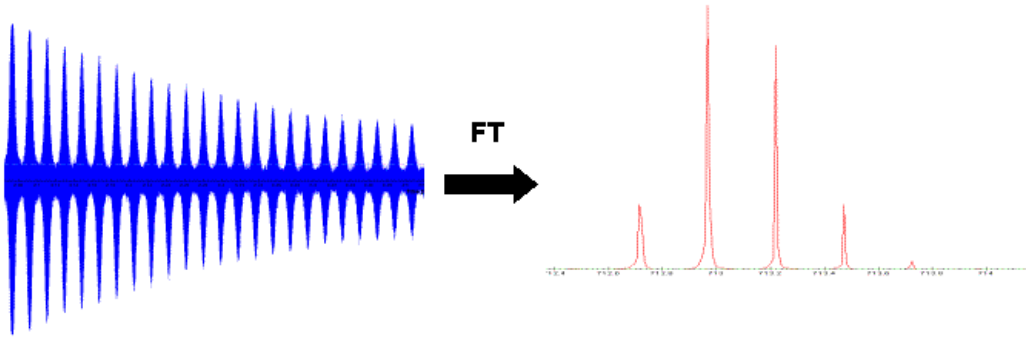



***Figure 1.4:*** *Time-domain signal (0.5 s) Fourier transformed to a baseline-resolved mass spectrum.*

The image current is composed of the sinusoidal signals of all ion packages added together. An advantage of measuring the image current is that FTICR is thus a non-destructive method and ions can be re-used after detection. Another advantage of FTICR is that the cyclotron frequencies of all the ions in the cell are detected simultaneously, which makes the detection fast. A Fourier transformation is applied to the time-domain signal to obtain a frequency spectrum. From the frequency spectrum a mass spectrum can be calculated with equation 1.3. To eject ions from the cell further excitation is needed. At a certain point ions will collide with the cell walls and will be neutralized. Ions that are not in resonance with the applied rf frequencies will not

experience a continuous outward electrical force and will remain close to the center of the cell.

### 1.1.3. Tandem mass spectrometry in FTICR

In an FTICR several tandem mass spectrometry techniques can be employed to dissociate ions. In the research performed for this thesis only Collision Induced Dissociation (CID) and Electron Capture Dissociation (ECD) were used. The principles of ECD will be explained in detail in chapter 1.2. Before the tandem MS event usually isolation of the ions of interest is performed, which will make the interpretation of the fragment ions easier. For the isolation of ions all other ions are ejected from the cell. To realize a CID experiment in an ICR cell it is needed to provide collision partners and collision energy. The collision partners are generally provided using a neutral gas pulse from a pulsed valve system. During the gas pulse the kinetic energy of the precursor ion is increased and kinetic energy is converted into internal energy in multiple collisions.[34] When a dissociation threshold is reached, the ions will dissociate via lowest-energy pathways. The dissociation products that are generated in this manner remain trapped in the analyser cell. The timescale of CID is relatively long enabling intramolecular vibrational energy redistribution to take place prior to dissociation. The cyclotron orbit radius can be enlarged by applying the exact resonance frequency of the ions of interest to the excitation plates. In this way the cyclotron motion will be excited. This method is called on-resonance excitation. After the excitation some time is needed for relaxation of the excited ions and to pump away the collision gas. A drawback to this method is that the fragment ions are formed away from the center of the cell. Ions that are far from the center of the cell cannot be detected as efficiently as ions that are centered. Sustained Off-Resonance Irradiation (SORI)[35] overcomes this drawback of on-resonance excitation. In SORI a low-amplitude rf pulse is applied to the excitation plates, slightly off-resonant (1000-1500 Hz) compared to the cyclotron frequency of the ions of interest. This excitation frequency is alternately in- and out of phase with the cyclotron frequency, so that the ions are alternately excited and de-excited (figure 1.5). With SORI the kinetic energy of ions can be modulated for seconds, whereas on-resonance excitation takes less than half a millisecond. The cyclotron orbit will thus expand and shrink repeatedly. The amplitude of the excitation is kept low, to prevent the

fragment ions from being formed to far away from the center of the analyzer cell. Generally, in comparison with on-resonance excitation the collision energy with SORI is lower, because of the smaller kinetic energy of the ions.

***Figure 1.5:*** *Alternating excitement (solid line) and de-excitement (dotted line) of the cyclotron motion with SORI.*

## 1.1.4. Instrumental set up and experimental methods

The experiments in this thesis were mainly performed on a modified Bruker-Spectrospin APEX 7.0e FTICR MS equipped with a 7T superconducting magnet and a Bruker Infinity™ ICR Cell (figure 1.6). Argon was used as the collision gas. To generate low-energy electrons an indirectly heated dispenser cathode (TB-198, HeatWave Labs Inc., Watsonville, CA, USA) was placed approximately 30 cm behind the back trapping plate of the analyzer cell. Data acquisition and analysis were performed with in-house developed software and hardware.[36]

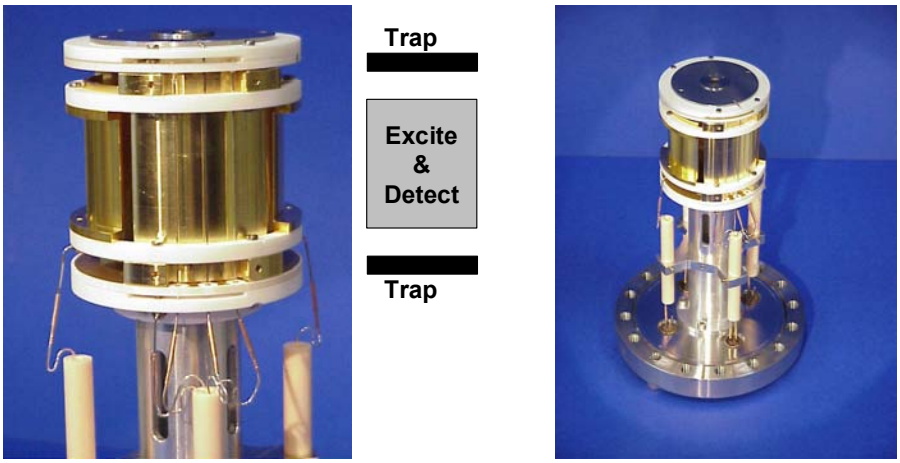



***Figure 1.6:*** *The infinity cell.*

Samples were introduced using a Harvard syringe pump model 55-1111 and were pumped through a fused silica capillary at varying flow rates from 1-100 μl/hr. Spray solutions consisted of 10-20 μM of the analyte dissolved in water with acetonitrile or methanol added to make the solution more compatible with ESI. Acetic acid (~2%) was added to make the spray solution acidic to promote sufficient protonation of the analytes. When peptides were complexed to metal ions, the acetate salt of that metal ion was added to the spray solution 25 times in excess. Positively charged droplets were generated by the application of a 1200-3000 V potential difference between the spray needle (PicoTip™ Emitter, New Objective Inc., Woburn, MA, USA) and the inlet of the mass spectrometer.

The ESI source was constructed in-house (figure 1.7).[37] The capillary is placed in a ceramic heater tube and heated to approximately 200°C to evaporate the solvent. The skimmer removes excess neutrals.

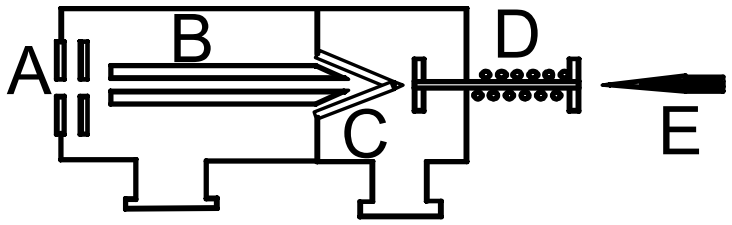



***Figure 1.7:*** *ESI source with extraction lenses (A), quadrupole (B), skimmer (C), heated capillary (D) and spray needle (E).*

The FTICR vacuum system is separated in five differential pumping stages to obtain high vacuum in the analyzer cell. The first stage is the nozzle-skimmer region, where the pressure is approximately 1 mbar. The second stage contains an rf only quadrupole and the pressure is approximately $10^{-5}$ mbar. The third region is an electrostatic optical part at a pressure of $10^{-7}$ mbar to guide ions from the exit of the quadrupole. In the intermediate region, at $10^{-8}$ mbar, ions are accelerated to 3000 eV to prevent radial ejection by the magnetic field. The ions are decelerated to ~1 eV before entering the ICR analyzer cell, which is at $10^{-10}$-$10^{-9}$ mbar.

A typical FTICR measurement sequence is shown in figure 1.8. An experiment typically lasts a few seconds. The experiment always starts with ejection of residual ions out of the cell. To do this a quench pulse is applied, for instance –100V to the front trap electrode and 100V to the back trap electrode for 10 ms. The positively charged species will be ejected along the z-axis in the direction of the front electrode and the negative species in the direction of the back electrode. After quenching, the ions are introduced into the ICR cell. All parameters in the source, the ion optic region and the analyzer cell are adjusted to let ions enter the cell. At the same time gas can be pulsed in the cell, which might be needed to trap ions efficiently. This event typically takes 50-500 ms. When a gas pulse is used to trap the ions, it takes some time to reduce the pressure again to $10^{-9}$ mbar or lower. A low pressure is needed for the next event: precursor isolation, because a high pressure would activate the ions. Usually a SWIFT pulse is calculated to eject all ions except the ions of interest, but also a chirp can be used. When the ions of interest are isolated MS/MS can be performed on these remaining ions. When ECD is performed the potential on the grid is pulsed from –100 to +100V, so that the electrons can pass the grid. At the same time potential on the surface of the cathode is pulsed from +25V to –0.15V, so that the cathode surface repels the electrons and they are ejected. Typically the cathode is operated using a current of 1.85 A and a potential of 7.9 V, yielding a power of 14.6 W. The ECD event can take 30 ms to several seconds depending on how good is the overlap between the rotating ion cloud and the electron beam, the match in kinetic energy and the capture cross section of the ions. Ions can also be activated using SORI-CID. After isolation a gas pulse is applied, during which an off-resonance rf pulse is applied to the excitation plates, in the order of 1000-1500 Hz off resonance. For SORI and ECD fragment ions will be formed near the center of the cell and can thus be efficiently detected. This can be achieved by applying a chirp to the excitation plates forcing the ions to perform coherent motion. The alternating image charge induced on the detection plates is detected. After Fourier transformation and calculation of the mass spectrum, analysis can be performed.

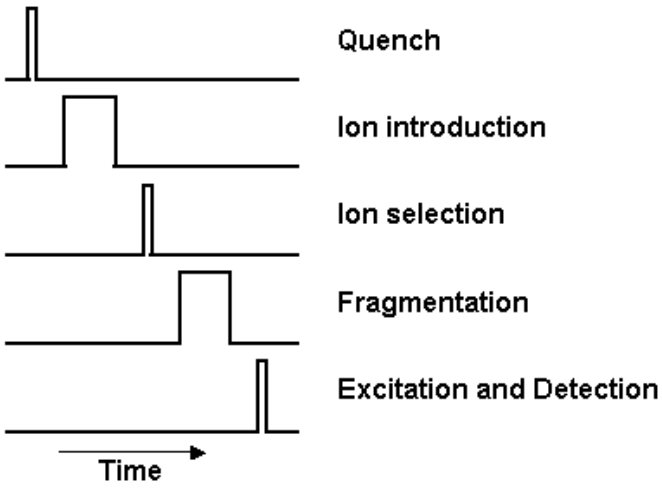



***Figure 1.8:*** *Sequence events.*

## 1.2. Introduction to Electron Capture dissociation

Tandem mass spectrometry has become an indispensable technique in proteomics, because it is fast and generates an enormous amount of information about proteins. Tandem mass spectrometry is used to identify unknown compounds, to sequence peptides resulting from protein digestion, to localize and identify post-translational modifications or even to study three-dimensional protein structure. Mostly CID is used as the tandem mass spectrometric method. As is described in this section ECD can also make a contribution as it gives complementary information about proteins compared to CID. From 1998 until now technical features in ECD have been improved. At first directly heated filaments were used to produce low-energy electrons. Later indirectly heated dispenser cathodes were used to increase the electron current and the beam diameter and to decrease the irradiation time and the spread in kinetic energy of the emitted electrons. This made ECD compatible with liquid separation techniques and thus a solid tandem MS technique, available for high throughput methods.

### 1.2.1. Electron Capture Dissociation: past, present & future

Electron Capture Dissociation[16, 38] has many similarities with a process called Dissociative Recombination (DR), which involves the capture of an electron by a positive molecular ion. Part of the recombination energy dissociates the molecule into two neutral atoms. DR takes place in nature on a large scale, such as in astrophysical

plasmas or planetary ionospheres, but also in laboratory plasmas made in storage rings. To give an example: during daytime atmospheric oxygen molecules are exposed to ultraviolet light, which creates ionized oxygen molecules. At night the oxygen ions may dissociatively recombine with electrons to form excited oxygen atoms. A prominent characteristic in the emission spectrum of these excited oxygen atoms is the green line at 557.7 nm. This green line was first detected by Campbell in the night sky.[39] Kaplan suggested that the excited oxygen atoms were the product of dissociative recombination,[40] which was confirmed by Bates and Massey.[41] This reaction is still under investigation in heavy-ion storage rings.[42] The main difference between DR and ECD is that ECD is always performed on multiply charged cations, whereas DR is usually performed on singly charged cations. The intermediate species in ECD are often radical cations that carry excess hydrogens.

#### 1.2.1.1. Birth of Electron Capture Dissociation

Guan et al.[43] performed 193 nm ultraviolet photodissociation on electrosprayed mellitin and ubiquitin ions and observed charge reduction. They also observed unusual c and $z^{\bullet}$ ions that provided additional sequence information. With other fragmentation techniques, such as collision induced dissociation[35, 44, 45] and infrared multiphoton dissociation (IRMPD),[46, 47] usually b and y ions are observed when peptides and proteins are fragmented. To trap cations and electrons simultaneously extra electrodes were added to an ICR cell by Zubarev et al.[16] Compared to the photodissociation experiment performed by Guan et al., the charge reduction effect and the formation of c and $z^{\bullet}$ fragment ions increased dramatically when electrons were trapped together with ubiquitin ions. The 10+ ions formed from $[M+11H]^{11+}$ ions surprisingly were mainly $[M+11H]^{10+\bullet}$ ions, which means that they contained an extra hydrogen atom compared to regular $[M+10H]^{10+}$ ions generated by ESI.[8] These observations led to the conclusion that the charge reduction and the c/$z^{\bullet}$ fragmentation was not a direct result from the exposure to 193 nm photons, but rather from the 193 nm photons hitting the metal surfaces in the ICR cell leading to the formation of secondary electrons, that were subsequently captured by the ions in the cell. To generate low-energy electrons directly a heated filament was implemented opposite to the ESI source and the fragmentation technique ECD was really established.

### 1.2.1.2. Overview of past ECD applications

After the successful implementation of ECD it became clear that the fragment ions produced by electron capture offered new perspectives in the analysis of several types of compounds, because they were fundamentally different from the fragment ions observed from all conventional fragmentation techniques. Especially in the field of peptides and protein analysis ECD has become extremely useful. ECD spectra generally yield more extensive sequence information than CID spectra,[48, 49] as ECD exhibits less selective backbone cleavages.[50] In CID backbone cleavage reactions are far more affected by the identity of neighbouring groups than in ECD. An additional advantage is the complementarity in backbone cleavages of ECD and CID,[16, 50] making the two techniques combined even more powerful for peptide and protein sequencing. For the localization of labile post-translational modifications ECD is superior to conventional fragmentation techniques, wherein labile modifications often dissociate from the peptide backbone before backbone cleavages can occur. Thereby the information about the location of the modification is lost. ECD preferentially involves cleavages of the amine bond while retaining the labile modifications, thus making localization possible.[51] Disulfide[38] and thioether[52] bonds on the other hand are selectively cleaved after electron capture. In addition to the sequencing of peptides and proteins, ECD has been used for sequencing polyethylene glycols,[53] polyester amide oligomers,[54] peptide nucleic acids,[55] polyglycols[56] and oligodeoxynucleotides.[57] Also protein folding and unfolding processes have been studied with ECD.[58, 59] Judging from recent literature the most promising applications for ECD seem to be peptide sequencing and localization of post-translational modifications.

### 1.2.1.3. Future outlook

In the near future certainly a lot of research will be performed on the precise mechanisms involved in electron capture dissociation. Since the first paper on ECD was published[16] this has been a matter of ongoing debate. Recently two papers were published that were dedicated to the ECD mechanism.[60, 61] This kind of fundamental research is very important to better understand the phenomena involved in ECD and to enable its application in structure analysis. Another advantage of fundamental research is that it can generate previously unconsidered new knowledge, leading to new

applications. For instance, a fundamental study resulted in more knowledge about in-source decay processes in matrix-assisted laser desorption/ionization (MALDI) as c and $z^{\bullet}$ fragments were produced.[62] A recent paper is dedicated to this phenomenon,[63] which could bring future opportunities for combining MALDI with ECD. Also instrumental developments will be important. The electron irradiation time has already been brought back from seconds to 1-100 ms[64] using an indirectly heated dispenser cathode instead of a directly heated filament. This made ECD compatible with on-line high performance liquid chromatography (HPLC)[65, 66] and applicable to complex biological samples.[67] Another instrumental development is the application of ECD in a radio frequency ion trap,[68] which is a cheaper instrument than an FTICR. If this method would be optimized ECD would become more widely available. Because of recent developments in ECD research there are a lot of opportunities for the application of ECD in the rapidly expanding field of proteomics.[6, 7] ECD together with IRMPD and high-resolution Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometry, using either the laser off axis[69] or a hollow cathode with the laser on axis,[70] could be the most powerful method for protein identification and localization of post-translational modifications. Both fragmentation techniques can be performed simultaneously with scan times less than one second.

#### 1.2.1.4. Related tandem MS techniques

In the slip stream of ECD came a range of related techniques that can cope with ions that cannot be analysed with ECD, namely negative ions and singly charged cations. Negative ions cannot be analyzed with ECD, because they have a low probability of capturing an electron due to Coulombic repulsion. When Tandem Ionization Mass Spectrometry (TIMS)[71, 72] (irradiation of ions with >10 eV electrons) was applied on anions of the sulfated peptide caerulein mainly $CO_2$ and $SO_3$ losses were observed.[73] However, also backbone cleavages resulting in the formation of a, c and z ions were observed with complete sequence coverage. Most fragment ions retained the sulfate group allowing for localization. These more energetic electrons induce electron detachment from multiply charged anions, in other words: holes are created. When a hole recombines with one of the negative charges this results in electronic excitation and subsequent backbone cleavages. When the electron energy exceeded 20 eV even more abundant backbone cleavages were observed and this technique was named

Electron-Detachment Dissociation (EDD). Haselmann et al. mainly obtained a and c ions after performing EDD on a peptide dianion, but also internal fragments were formed.[74] This indicates that the electrons deposit a lot of energy. EDD shares with ECD that labile post-translational modifications are largely retained in the fragment ions. To analyze singly charged cations Electronic Excitation Dissociation (EED)[75] can be used. EED involves the irradiation of ions with low-energy electrons, similar to ECD, but only after TIMS[71, 72] is applied. Via electron impact with >10 eV electrons ions are further ionized and hydrogen-deficient $[M+nH]^{(n+1)+\bullet}$ ions are formed from $[M+nH]^{n+}$ ions. These ions are subsequently exposed to a low-energy electron beam allowing them to capture an electron. This will result in electronically excited $[M+nH]^{n+*}$ ions and rapid dissociation. The most predominant fragments in EED spectra of substance P and angiotensin were even-electron a and c fragments, although also some radical $a^{\bullet}$ ions were observed.[76]

Multiply charged cations can also be irradiated with higher-energy electrons, compared to the low-energy electrons used for ECD, to induce fragmentation. For the capture cross section a very narrow local maximum is observed in the electron energy. This maximum is below 1 eV and corresponds to regular ECD. A second maximum was observed for electron energies around 10 eV. This electron energy range is referred to as the Hot Electron Capture Dissociation (HECD) region. In HECD[77] multiply charged cations capture hot electrons (3-13 eV), which results in regular ECD fragmentation and secondary fragmentation due to the excess energy. Because of this secondary fragmentation HECD can be used to distinguish between isoleucine and leucine residues[78] From $z^{\bullet}$ ions w ions are formed by this process; for isoleucine and leucine the w ions differ in nominal mass. With HECD as well, fragment ions largely retain their labile post-translational modifications.

## 1.2.2. Properties of ECD in protonated species

Many questions are unanswered considering the mechanism of ECD. There are several observations that led to the proposal of the “hot hydrogen” model[16] that postulates electron capture at the hydrogen of a protonated site followed by $H^{\bullet}$ transfer to a high affinity site, such as carbonyl oxygens, disulfide bonds or tryptophan residues, where

cleavage occurs. Alternatively, John Brauman has suggested that initial electron capture could form a high-n Rydberg state, expected to be long-lived,[79] that would have favorable intersystem crossings with dissociative states.

### 1.2.2.1. Initial electron capture

The first important step in ECD is to get an efficient recombination of multiply charged cations with electrons. To maximize electron capture by the cations the difference in kinetic energy between the two species should be minimized. Going from 1 eV electrons to <0.2 eV electrons leads to an increase in the capture rate of three orders of magnitude.[80] The capture cross section is proportional to the square of the ionic charge. To talk about an initial landing site on the ion is arbitrary, but the electron most probably will be captured at sites with the highest positive charge density in a cation or be transferred to those sites after capture. The capture must be primarily Coulombic because the capture cross section exceeds the ion-neutral collisional cross section by two orders of magnitude.[80] Electron capture proceeds through a series of Rydberg states and the recombination energy will be converted to vibrational energy of the reduced charge carrier or to photon emission.[81] In a protonated ion these sites with highest charge density will be the sites with the highest gas phase basicity and an excited hydrogen radical will be formed after electron capture.[16] Experimental evidence for $H^{\bullet}$ formation is the $H^{\bullet}$ loss frequently observed from ions that captured an electron.[16, 82, 83]

### 1.2.2.2. Energetic aspects

When a hydrogen radical is formed a certain amount of recombination energy will be released. This energy (RE) depends on the ionization energy of a hydrogen atom (IE ($H^{\bullet}$)), the proton affinity (PA) of ions with one proton less than the precursor ions and the hydrogen affinity (HA) of ions with one proton less than the precursor ions.

$$RE = IE(H^{\bullet}) - PA([M+(n-1)H]^{(n-1)+}) + HA([M+(n-1)H]^{(n-1)+}) \quad (1.15)$$

The ionization energy of hydrogen is 13.6 eV. The proton affinity difference lies in the range of 8-11 eV, whereas the hydrogen affinity typically is less than 1 eV. Several values have been published that estimate the energy released by proton neutralization in a typical peptide. Approximately 6 eV will be released by neutralization of a protonated carbonyl according to Zubarev et al.[16, 80] According to McLafferty et al. 5-7 eV[51] will be released by electron capture in a multiply charged protein ion and 4-7 eV according to Zubarev et al.[84] Theoretical calculations estimate that the total recombination energy of a peptide ion forming an ammonium radical in its ground electronic state for a doubly charged ion will be 4.1 eV.[85] The bond strength of an N-C bond is typically 3.0 eV so the released recombination energy will be enough for N-C bond cleavage. However, adjacent bonds will influence the bond energy. In a peptide for instance the amide bond will be weaker than the amine bond while they are both N-C bonds, because in the case of the amide bond the carbonyl oxygen will draw electron density away from this bond. In fragmentation methods where the internal energy is raised in small steps, like IRMPD and CID, the amide bond will therefore cleave before the amine bond.

#### 1.2.2.3. Primary cleavages

The neutralization of a proton by an electron leads to very fast fragmentation reactions,[16] often ECD is referred to as a non-ergodic fragmentation technique. Non-ergodicity means that cleavages occur before intramolecular vibrational energy redistribution. Modelling could only account for the observed N-$C_\alpha$ cleavages when the recombination energy was released in a very small molecular region. The time scale of this event is <1 ps, which is faster than intramolecular vibrational energy redistribution which takes at least 10 ps.[38] There is a lot of experimental evidence supporting the non-ergodicity theory, such as the extensive sequence coverage and the lack of cleavage selectivity when fragmenting large proteins, despite the large number of degrees of freedom.[51, 86, 87] The fact that electron capture leads to backbone cleavages in polypeptide polycationic complexes instead of breaking the weak non-covalent bonds between the peptide chains also points at a very local phenomenon.[88] However, concerning the non-ergodicity of ECD a theoretical study was published[81] stating that transition state theory rate constants for N-$C_\alpha$ cleavages in aminoketyl radicals indicate an extremely facile dissociation reaction. This finding makes the hypothesis for non-

ergodic behavior of ECD intermediates unnecessary. Primary cleavages induced by electron capture give an indication about the original location of the protons on a multiply charged cation. ECD of substance P at 86 K revealed selective cleavages that reflected the relatively fixed solvation sites of the protons because of the conformational homogeneity at such low temperatures.[61] This result also suggests that the extensive sequence coverage obtained with ECD at higher temperatures is the result of conformational heterogeneity and the less selective solvation of protons.

#### 1.2.2.4. Secondary cleavages

With ECD usually no internal fragments are observed,[16, 87] First, the efficiency of ECD is quite low, so the probability of an ion capturing two electrons is low. Secondly, an ion that has captured one electron will be one charge state lower, which makes the capture cross section considerably lower, because of its quadratic dependence on charge state. The lack of internal fragmentation makes ECD fragment ions easy to interpret, because one can be quite certain that the fragments in an ECD spectrum contain one of the termini. Secondary cleavages resulting from the capture of a second electron are thus minimal. However, secondary cleavages resulting from the capture of one electron have been reported for cyclic peptides[60] as well as for linear peptides.[89] These secondary losses can be: side chain loss from primary fragments, c and $z^{\bullet}$ ions originating from a proline residue or loss of part of the backbone from cyclic peptides. Secondary cleavages in ECD are usually attributed to the radical identity of product ions formed by electron capture, because odd-electron species are usually much more unstable than even-electron species.

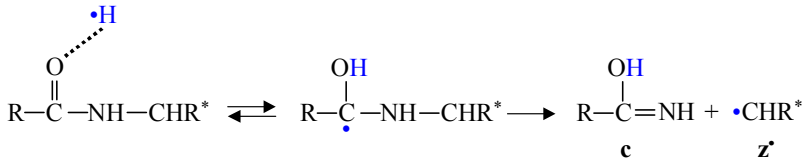
### 1.2.3. The application of ECD on peptides and proteins

Structural and functional analysis of proteins and peptides has become a very important research area at present, often referred to as proteomics. To know more about how cells function we need to identify and structurally analyze proteins from cells preferably in different states. To learn more about the molecular background of diseases, we need to analyze differential protein levels in cells, including post-translational modification patterns and even protein conformations and intermolecular complexes. Post-

translational modifications are important for regulation, structure dynamics and targeting of proteins. In particular, disulfide bonds are important for protein structure. We can use knowledge about the presence or location of these functional groups e.g. for diagnostic or therapeutic purposes. ECD has some unique features that are very useful in this proteomics field.

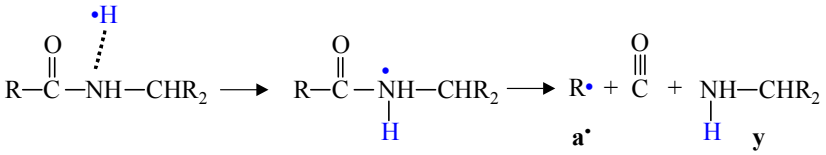
### 1.2.3.1. Fragmentation of peptides and proteins in ECD

When conventional fragmentation methods are applied on peptides and proteins this predominantly leads to the formation of b and y ions.[14, 15] Major products in ECD of peptides and proteins are usually $[M+nH]^{(n-1)+\bullet}$ ions. Actually these are precursor ions that captured one electron but did not dissociate into fragments. Also ions resulting from $H^{\bullet}$ loss from these species ($[M+(n-1)H]^{(n-1)+}$ ions) can be major product ions.[83] Very common is also the loss of $NH_3$ from $[M+nH]^{(n-1)+\bullet}$ ions, originating from the N-terminal amine or from amino acid side chains. The most intense backbone fragments are c and $z^{\bullet}$ ions, resulting from cleavage of amine bonds (scheme 1.1).[16]




***Scheme 1.1:*** *The formation of c and $z^{\bullet}$ ions.*

After the formation of a hydrogen radical via neutralization of a proton with an electron, the question is where this $H^{\bullet}$ radical is located. Carbonyl oxygens are the sites in peptides and proteins with the highest $H^{\bullet}$ affinity.[38] However, c/$z^{\bullet}$ cleavage might also proceed via subsequent $H^{\bullet}$ capture on the nitrogen atom, because of the high exothermicity of that cleavage pathway.[84] A minor fragmentation channel in ECD of peptides and proteins is the formation of $a^{\bullet}$ and y ions (scheme 1.2). It has been proposed that this fragmentation pathway is the result of direct electron capture on a protonated backbone nitrogen.[80]



***Scheme 1.2:*** *The formation of a• and y ions.*

Usually no cleavages N-terminal to proline residues are observed in ECD.[16] This phenomenon can be used for the structural characterization of proline-rich proteins.[90] However, c/z• fragmentation through the proline residue has been observed[89] In this study electron capture induced amine bond cleavage was accompanied with C-C cleavage in the proline side chain. On the contrary, C-terminal to tryptophan residues backbone bonds are preferentially cleaved with ECD.[50] The fact that ECD generates other types of fragments compared to CID means that additional information is gathered about peptides and proteins from their fragmentation. The two techniques combined are especially powerful for peptide sequencing. First, as already mentioned, ECD and CID generate cleavages between different amino acids increasing the sequence coverage. Secondly, CID mainly leads to the formation of b and y ions in peptides. As c ions are 17.027 Da heavier than b ions and z• ions are 16.019 Da less heavy than y ions it can be easily deduced which fragments are N-terminal and which are C-terminal. Finally, with ECD posttranslational modifications can be better localized, especially when combined with CID.

### 1.2.3.2. ECD of intact proteins

Due to the relatively non-specific backbone cleavages, ECD is a suitable method for top-down characterization of proteins. This has been demonstrated for several proteins. For ubiquitin 67 out of 75 possible interresidue bonds and 75 out of 103 for cytochrome c were cleaved using ECD.[80] Kruger et al. showed complete sequence coverage for two peptides of 1.3 and 2.2 kDa.[48] Also for substance P complete sequence coverage was observed,[91] except for the N-terminal backbone bond of the proline residue. Some protein ions do not show any fragmentation after electron capture but only reduced non-dissociative ions, being ions that captured one or several electrons without subsequent dissociation. It was proposed that the absence of fragmentation is due to non-covalent

interactions in the protein structure that keep the fragment ions together.[92] In such cases activated Ion (AI) ECD can be used to circumvent these problems. In AI-ECD the precursor ions are first activated with infrared photons or gas collisions to break up the intramolecular non-covalent bonds. Simultaneously or after activation the (partially) unfolded ions are exposed to a regular low-energy electron beam. With AI-ECD 99 out of 153 interresidue bonds of a 17 kDa protein were cleaved compared to ECD 33 out of 153. For larger proteins AI-ECD provides more sequence coverage than ECD and far more than CID or IRMPD.[92]

Protein folding can also be studied with ECD because of its very fast fragmentation reactions. The fragment ions are supposed to reflect the original gas phase structure of protein ions. This is in contrast to CID, which disrupts the gas phase protein structure. With CID first non-covalent bonds are broken by the slow heating of protein ions. Horn et al. investigated kinetic intermediates in the folding of gaseous protein ions and found that thermal unfolding in the 9+ ion of cytochrome c proceeds through the separate unfolding of 13 backbone regions[58] and thus melting temperatures could be determined of these separate regions. Breuker et al. performed a detailed study on the folding and unfolding of 5+ to 13+ ions of bovine ubiquitin in the gas phase. With ECD they could confirm ion mobility studies that suggest that the unfolding is a three-state process in the gas phase.[59]

### 1.2.3.3. Side chain cleavages

Besides backbone cleavages also cleavages in the side chains of amino acids are observed with ECD,[93] either primary or secondary cleavages. Side chain losses have a diagnostic value if they are unique for an amino acid. With ECD, arginine side chain cleavages result in neutral losses of $CH_4N_2$, $CH_5N_3$ and $C_4H_{11}N_3$. Histidine and asparagine/glutamine side chain cleavages result in neutral losses of $C_4H_6N_2$ and $CH_3NO$ respectively. Methionine shows a loss of $C_3H_6S$ and lysine $C_4H_{11}N$.[93] For all studied peptides and regardless of their position in the peptide chain arginine, histidine, glutamine and asparagine show diagnostic side chain losses associated with that residue, that can be used for identifying these amino acids in a peptide with ECD[93, 94]. Haselmann et al. extended these diagnostic side chain losses with $C_9H_9N$ for tryptophan and $C_7H_8O$ for tyrosine and observed an additional side chain loss for

asparagine/glutamine, namely $C_2H_5NO$.[95] Lysine and methionine did not always show typical side chain losses when they were present in a peptide, so these are of less diagnostic value than the other mentioned side chain losses.

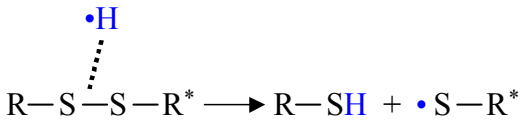
### 1.2.3.4. Labile post-translational modifications

It has been shown that ECD is useful for the identification and localization of post-translational modifications. Tandem MS of 28-residue peptides containing γ-carboxylated glutamic acid residues using either collisions (CID) or infrared photons (IRMPD) resulted in complete ejection of the γ-$CO_2$ moieties before cleavage of peptide backbone bonds. In contrast, ECD resulted in cleavages through backbone bonds without ejection of $CO_2$, allowing direct localization of this labile modification.[96] Niiranen et al.[97] applied this finding to localize γ-carboxyglutamic acid residues in osteocalcin subforms. ECD can also be used to localize sulfation in peptides. A sulfate group on the side chain of a cysteine residue was retained in ECD backbone fragments of a 21-mer peptide, although some $H_2SO_3$ loss was observed from the precursor ions. CID caused extensive $SO_3$ loss.[96] Haselmann et al. used ECD to localize a sulfated tyrosine residue in drosulfakinin.[98] Mirgorodskaya et al.[99] investigated O-glycosylated peptides using ECD. The observed c series provided direct evidence on the glycosylation sites with no glycan (GalNAc and dimannose) losses observed from these ions. Haselmann et al. could determine the locations of six GalNAc groups both in a 60-residue and a 25-residue peptide. When O-fucosylated glycopeptides were subjected to ECD the formed radical cations mainly exhibited c/$z^{\bullet}$ cleavages without loss of the labile fucosylation, allowing unambigious assignment of the glycosylation site.[100] Fragmentation of N-glycosylated peptides, using CID, typically yielded product ions that result from dissociation at glycosidic bonds, with little peptide backbone cleavages. ECD provided c and $z^{\bullet}$ backbone fragments, with no observed losses of carbohydrate residues.[69, 101] ECD tandem mass spectra also allowed facile interpretation and unambigious determination of phosphorylated amino acid residues in phosphotyrosine and phosphoserine containing peptides. ECD generated mainly c and $z^{\bullet}$ ions, without the loss of water, phosphate groups or phosphoric acid.[102] These neutral losses are often generated with CID. ECD has provided the first direct characterization of a phosphoprotein, restricting the location of 5 phosphorylation sites in the 24 kDa protein

bovine β-casein, without the need for enzymatic digestion and subsequent chromatographic separation.[103] Chalmers et al. used ECD to characterize protein kinase A phosphorylation.[104] Finally, ECD has been demonstrated to be an effective fragmentation technique for characterizing the site and structure of the fatty acid modification in ghrelin, a 28-residue peptide with an unusual ester-linked n-octanoyl modification at Ser-3. ECD cleaved 21 of 23 possible backbone amine bonds, resulting in a higher sequence coverage than obtained by CID.[105]

### 1.2.3.5. Disulfide bonds

ECD can be used to analyze peptides and proteins containing disulfide bonds. Zubarev and co-workers found that $H^{\bullet}$ capture near disulfide bonds and cleavage of disulfide bonds (scheme 1.3) are favored.[38] A polypeptide containing a disulfide bond gave far more extensive fragmentation than polypeptides containing no disulfide bond. Because the proton affinity of the disulfide group is approximately 1 eV lower than that of the carbonyl group, preferential cleavage of the disulfide bond could be explained by its higher $H^{\bullet}$ affinity. The exothermicity of both the proton neutralization and $H^{\bullet}$ capture by the disulfide bond is then used for disulfide bond cleavage, initiated by the presence of a radical site.




***Scheme 1.3:*** *Electron capture dissociation of a disulfide bond.*

Usually one of the major products in ECD spectra are the reduced $[M+nH]^{(n-1)+\bullet}$ ions. In the ECD spectra of a 10 kDa protein containing an S-S bond, these product ions were low-abundant. Dominating fragment ions were formed resulting from the cleavage of the disulfide bridge. In comparative CID experiments, no such fragment ions were observed. Generally, low-energy CID does not result in efficient cleavage of disulfide bonds.[18, 19] In search for the localization of a 16 Da addition in a peptide fragment, consisting of two chains connected with a disulfide bond, ECD was used to cleave the disulfide bond and restrict the modification to one of the two chains.[106] For S-S cyclic

proteins, capture of one electron can break both an S-S bond and a backbone bond in the same ring, or in the case of insulin even both S-S bonds holding the two peptide chains together. Also cleavages near the disulfide bond are observed.[107] Direct electron capture at uncharged S-S seems unlikely, cleavage seems to be due to high $H^{\bullet}$ atom affinity of S-S.[38] In a 26 kDa protein ECD showed the presence of two disulfide bonds and pinpointed their location, although 10 S-S combinations were possible.[87] ECD was useful in the analysis of the human atrial natriuretic peptide, because it contains a disulfide bridge, which was cleaved by ECD.[108] From all these experimental results it is clear that ECD exhibits specificity for disulfide bond cleavages but what is the explanation for this behavior? Several studies have been devoted to this subject. Electron capture is postulated to occur initially at a protonated site to release an energetic $H^{\bullet}$ that is captured at a high-affinity site, such as S-S or a carbonyl oxygen to result in very fast cleavages.[80] A theoretical study states that hydrogen atom capture by the disulfide bond in the studied molecule was calculated to be substantially exothermic. Hydrogen atom addition was followed by cleavages of S-H, C-S and S-S bonds adjacent to the hypervalent sulfur atom.[109] Another possible explanation for the preferred disulfide bond cleavage is given by Brauman [ref 23 in [38]]. The electron may form a high-n Rydberg state first, which then undergoes surface crossing involving an initial lowest energy geometry of the protonated amine and amide carbonyl to yield the final state reactants. The surface crossing to form c and $z^{\bullet}$ ions converts the carbonyl double bond to a nominal single bond, resulting in a very fast fragmentation. This crossing should even be far more favorable with disulfide bonds.

### 1.2.4 ECD on ions charged with cations, other than protons

In mass spectrometry mainly protonated ions are generated for structural analysis with tandem MS. However, many biomolecular compounds in their functional form are bound to cofactors such as metal ions. Not many studies have been performed on ECD of ions with other charge carriers than protons, thus it is not clear what the ECD mechanism is in these cases. $PEG_{24}$ charged with $2Na^+$ or $2NH_4^+$ ions yields ECD products that are analogous but of different size compared to doubly protonated $PEG_{24}$,[53] indicating that similar ECD mechanisms occur. ECD has also been performed on ions that contain a transition metal ion. When the complex of angiotensin II with $Zn^{2+}$ was subjected to ECD

it produced far less backbone cleavages than the doubly protonated ion, but the types of fragments formed were typical for ECD, namely c, z, a and y ions.[84] The metal ion probably served as a sink for electrons that were likely transferred from the original capture site to the remainder of the complex. This was different for ECD of cytochrome c 15+ ions, containing a heme group with $Fe^{3+}$. The region around the heme group containing $Fe^{3+}$ remained immune to c/z$^{\bullet}$ cleavage and there was very little heme loss. Interestingly CID of cytochrome c 14+• ions yielded 13+ ions without the heme group that were much more intense than in the CID spectrum of regular 14+ ions. Furthermore, CID of 14+• ions gave as product ions singly charged heme with m=617 Da ($[heme + H]^{+\bullet}$) and CID of 14+ ions gave m=616 Da ($[heme]^{+}$). These results are consistent with slow transfer of $H^{\bullet}$ to the less accessible heme region with subsequent fragmentation and suggest that possible electron capture on the $Fe^{3+}$ ion does not lead to ejection of the heme group.[80]

For protonated species ECD cleavages reflect the location of the protons.[61] A few studies have paid attention to the possibility of localizing metal ions in complexes with ECD. The ECD products from $[PEG_{24} + 2Na]^{2+}$ ions for instance could be rationalized with structures proposed by Bowers and coworkers,[110] which means that the ECD products could reflect the original gas phase structure of the ions.[53] Also it was found that metal chelation by retroviral zinc-finger motifs was conserved during ECD and that extensive fragmentation occurred around the residues involved in metal coordination.[111] With CID ejection of the metal ion was observed. When a series of consensus zinc finger peptides was fragmented with ECD it was found that the harder the ligand the more localized the fragmentation pattern was. This was attributed to the better ability of the zinc ion to dissipate its energy after electron capture and to accommodate the extra charge when the ligands are softer.[112] For calmodulin EF-hand 1 (EF1) there are dramatically different ECD fragmentation patterns comparing protonated ions and corresponding metal-complexes . Whereas the protonated EF1 ions present a more diffuse fragmentation pattern, the metal-complexes present extensive fragmentation in close proximity of the residues involved in metal coordination. This was observed for $Ca^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ in complex with EF1. However, in the case of the $Cu^{2+}$ complex with EF1 a lower incidence of fragmentation was observed, which was attributed to the fact that an electron may be more favorably involved in reducing $Cu^{2+}$ to $Cu^{+}$ than in

triggering ECD cleavages.[112] This mechanism, a favored reduction of the metal ion by the captured electron, could also play a role in the earlier mentioned observation that the region around $Fe^{3+}$ ions in cytochrome c remained immune to typical ECD cleavages.[80]

## 1.3 Scope of the thesis

In this introductory chapter, the theory and experimental procedures of FTICR were described and subsequently the properties of ECD and its applications. It is clear that ECD possesses some very interesting and useful features. The specificity for cleavage through and near disulfide bridges offers new possibilities for the identification and localization of these post-translational modifications with tandem MS. The localization of disulfide bonds is important for the determination of tertiary and quaternary structure in proteins. In this respect ECD is particularly useful because other tandem MS methods do not show specificity for disulfide bond cleavage.

In chapter 2 we explore this feature of ECD and investigate whether we can expand the specificity of ECD for disulfide bridges to the relatively uncommon lanthionine bridges in lantibiotics, a sub-group of anti-bacterial peptides. Therefore nisin A, nisin Z, mersacidin and lacticin 481 are subjected to ECD. Lantibiotics are toxic to gram-positive bacteria even in low doses and non-toxic to humans. Lantibiotics and derivatives have the potential to become a new generation of antibiotics.[113] The development of new antibiotics is required because (multi-)resistant bacteria are becoming a huge problem for public health. A specific feature of lanthionine bridges is that they consist of a thioether bond, instead of a disulfide bond. From theoretical calculations it was predicted that S-C bonds would be favorably cleaved after electron capture.[109] Therefore ECD could be a good method to analyze lantibiotics as opposed to the traditionally used method for peptide sequencing, Edman degradation,[114] which stops at the location of lanthionine bridges. Lantibiotics have to be chemically modified to open the lanthionine bridges[115] to complete the Edman degradation. The derivatization leads to loss of information about the precise locations of the lanthionine bridges. A fast and specific method for the localization of lanthionine bridges would thus be very welcome.

When multiply protonated peptide and protein ions are exposed to low-energy electrons often product ions are observed that captured one or several electrons without subsequent dissociation. With lantibiotics these products are remarkably intense, especially for lacticin 481 that hardly shows any fragmentation after electron capture. Lacticin 481 contains several regions where one or two lanthionine bridges link different parts of the backbone. The presence of these cyclic regions could explain the lack of fragmentation with ECD, because one cleavage in these regions will not lead to detectable fragments. Due to the stability of product ions that captured one electron without subsequent dissociation, product ions that captured two electrons possibly exhibit a stable biradical structure. Another possibility is that radical sites recombine to form a chemical two-electron bond. In chapter 3 we subject product ions of lacticin 481 that captured one and two electrons to collisional activation in order to investigate which of these scenarios is predominant.

In mass spectrometric research usually protonated species of peptides and proteins are subjected to structure-related studies. Often these species are biologically not relevant. Oxytocin for instance needs to bind a divalent transition metal ion to efficiently bind to its cellular receptor.[116] Different divalent transition metal ions have different potentiating effects on oxytocin-receptor binding, pointing at slight structural differences between these oxytocin-metal complexes. For protonated species ECD cleavages reflect the location of the charge carriers.[61] The same was observed for $PEG_{24}$ charged with sodium ions[53] and peptides in complex with transition metal ions,[111, 112] consistent with the very rapid fragmentation reactions induced by electron capture and the fact that electron capture is primarily Coulombic.[80] In chapter 4 structural differences between oxytocin complexes with $Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ ions are probed with ECD. Little is known about the ECD mechanism in peptides charged with other charge carriers than protons. Oxytocin is a good model peptide to determine whether electron capture in metallopeptides leads to the formation of typical ECD fragment ions. Between the first and the sixth amino acid of oxytocin there is a disulfide bridge and electron capture typically induces selective disulfide bond cleavages in protonated peptides.[38]

The results presented in this thesis will demonstrate that ECD as a novel tandem MS technique is a valuable addition to the field of structural analysis of peptides and proteins, especially for the identification and localization of thioether bonds and for

obtaining high sequence coverage. This thesis will also give a deeper insight in the ECD mechanism in peptides containing thioether and disulfide bonds and peptides charged with transition metal ions to supplement the existing knowledge about the ECD mechanism in protonated species.

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# Chapter 2

# Localization of intramolecular monosulfide bridges in lantibiotics determined with electron capture induced dissociation

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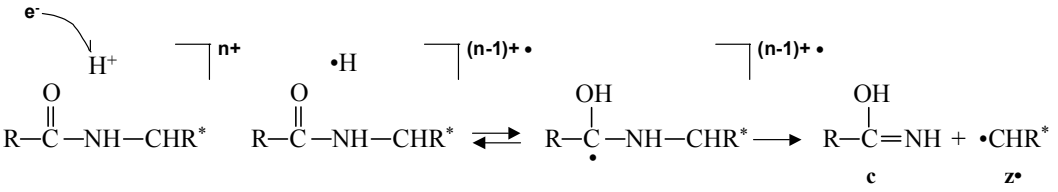
**Abstract**

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. Non-dissociative 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.

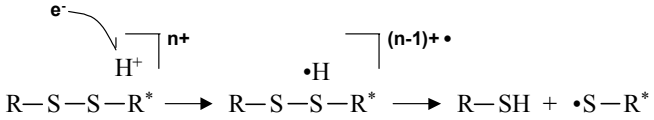
## 2.1. Introduction

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 (IRMPD) 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 post-translational 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 multiply protonated peptide or protein ions 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 2.1).[4,12,13]




***Scheme 2.1:*** *Proposed mechanism for the ECD induced formation of c and z• ions. 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.*

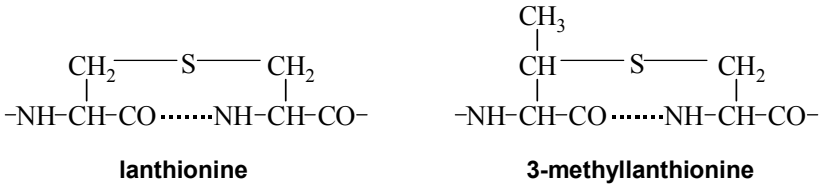



***Scheme 2.2:*** *The ECD induced cleavage of a disulfide bridge, often found in proteins. The H• radical is captured at the disulfide bond. The following cleavage reaction leaves one of the fragments as a radical cation.*

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 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 2.2). 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 non-ergodic 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 sulphur-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 sulphur functionality, namely lanthionine or monosulfide bridges, and are a sub-group of anti-bacterial peptides. The name is derived from their antibiotic action and their content of lanthionine and 3-methyllanthionine bridges (scheme 2.3).[16] A specific feature of these lanthionine bridges is that they consist of a thioether bond, instead of the disulfide bond.




***Scheme 2.3:*** *Chemical structures of the lanthionine and the 3-methyllanthionine bridge.*

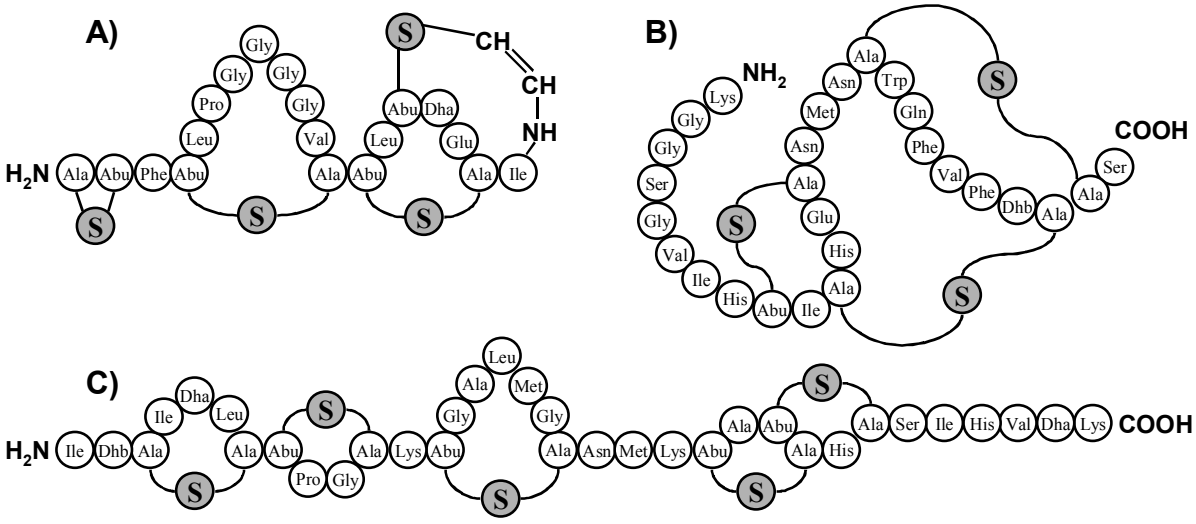



***Figure 2.1:*** *Amino acid sequence and monosulfide bridge location of the 4 studied lantibiotics A) mersacidin, B) lacticin 481 and C) nisin A. The only difference between nisin A and Z is that on the 27th amino acid position nisin A has a His-residue and nisin Z an Asn-residue.*

Lantibiotics often contain a wide variety of post-translationally 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 2.1). They are toxic to gram-positive bacteria even in low doses but non-toxic to humans. As such, nisin has been used as a food preservative for more than 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]

## 2.2. Experimental

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 tesla 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., CA) 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 approximately 0.35 eV electrons, considering the fact that the potential in the center of the infinity cell is close to zero and that the cathode surface was at a potential of -0.35 V. A current of approximately 350 nA and 10 seconds irradiation time was used. The pressure in the ICR cell was approximately $10^{-10}$-$10^{-9}$ mbar. Five stages of differential pumping were employed to bridge the atmospheric pressure conditions in the Electrospray Ionization (ESI) source. The samples were dissolved in a 69:29:2 %V methanol:water:acetic acid spray solution, containing 10-20 μM of the compound. The sample was pumped at a flow rate of 0.05-0.1 mL/h with a Harvard syringe pump 55-1111 (Kent, UK) through a fused silica capillary. To generate positively charged droplets a potential of approx. 3 kV was applied to a stainless steel spray needle. Data acquisition was performed using XMASS 4.0 (Bruker-Daltonics, Billerica, MA, USA). 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 of the 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 analysed using the MassLynx 3.4 software.

### 2.3. Results

**ECD on Lantibiotics.** For all the lantibiotics used in this study, first an MS spectrum was recorded. Under the used conditions, 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 approximately 0.3-0.4 eV electrons, yielding intense reduced pseudo-molecular 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 4 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 2.2.

The electron capture induced fragmentation of nisin A and nisin Z was quite similar (figure 2.3). 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 27th amino acid position, 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, also $c^{\bullet}$ radical ions and z ions were formed. The occurence of $c^{\bullet}$ radical ions and z ions as minor ECD products has been 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 5 available C-terminal attachment sites of lanthionine bridges; for nisin A and Z, 5 and 4 respectively were cleaved in this specific way. Mersacidin was more difficult to fragment using ECD (figure 2.3). 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 3 available sites for the earlier mentioned specific $c^{\bullet}$, z fragmentation; 2 were cleaved in this way. Finally, lacticin 481 was the most difficult lantibiotic to fragment using ECD (figures 2.3, 2.4). The ECD spectrum of lacticin 481 $[M+4H]^{4+}$ ions in figure 2.4 is dominated by non-dissociated 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.

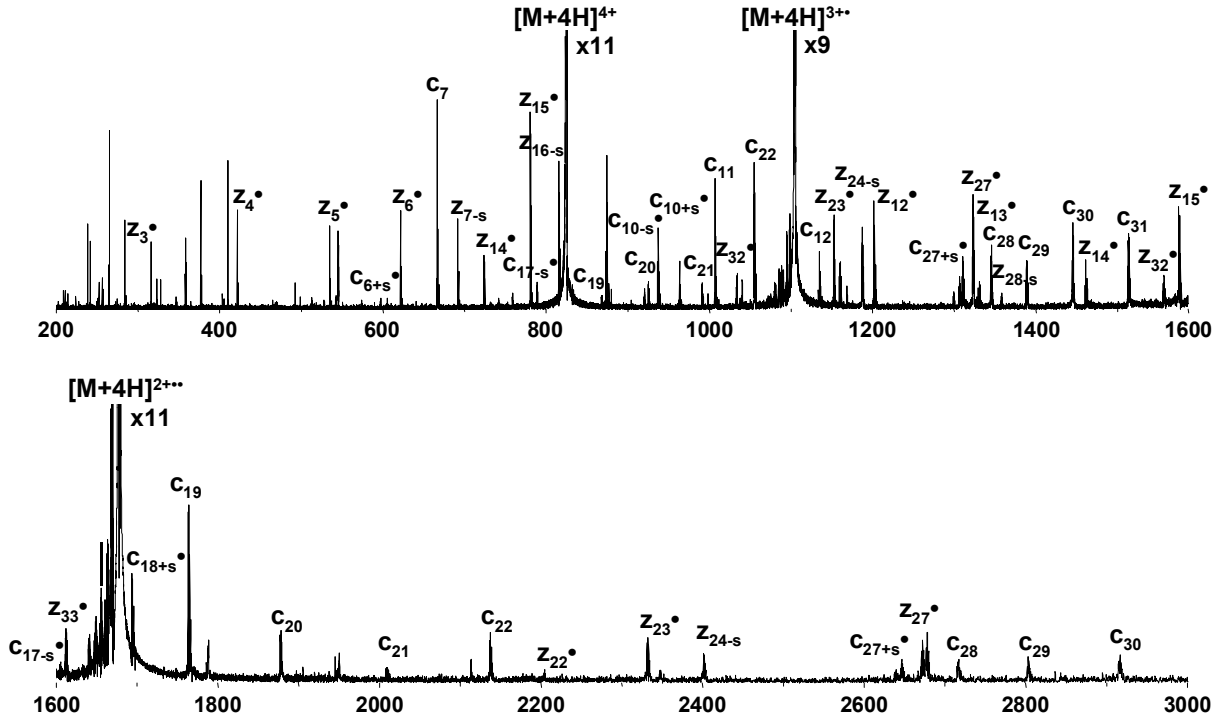



***Figure 2.2:*** *ECD spectrum of nisin A $[M+4H]^{4+}$ ions. The top and bottom part of the spectrum have the same scale, but were cropped to show the fragment ions. The peak intensity ratio for $[M+4H]^{4+}/[M+4H]^{3+\bullet}/[M+4H]^{2+\bullet\bullet}/[M+4H]^{+\bullet\bullet\bullet}$ ions was 100:86:97:11.*

**Non-dissociative 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 3 electrons without a subsequent formation of fragments. In fact, the most abundant ECD products were non-dissociative 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 2.4). With FTICR such non-dissociative products can easily be distinguished from regular molecular ions, because they carry more hydrogens (figure 2.4, inset).

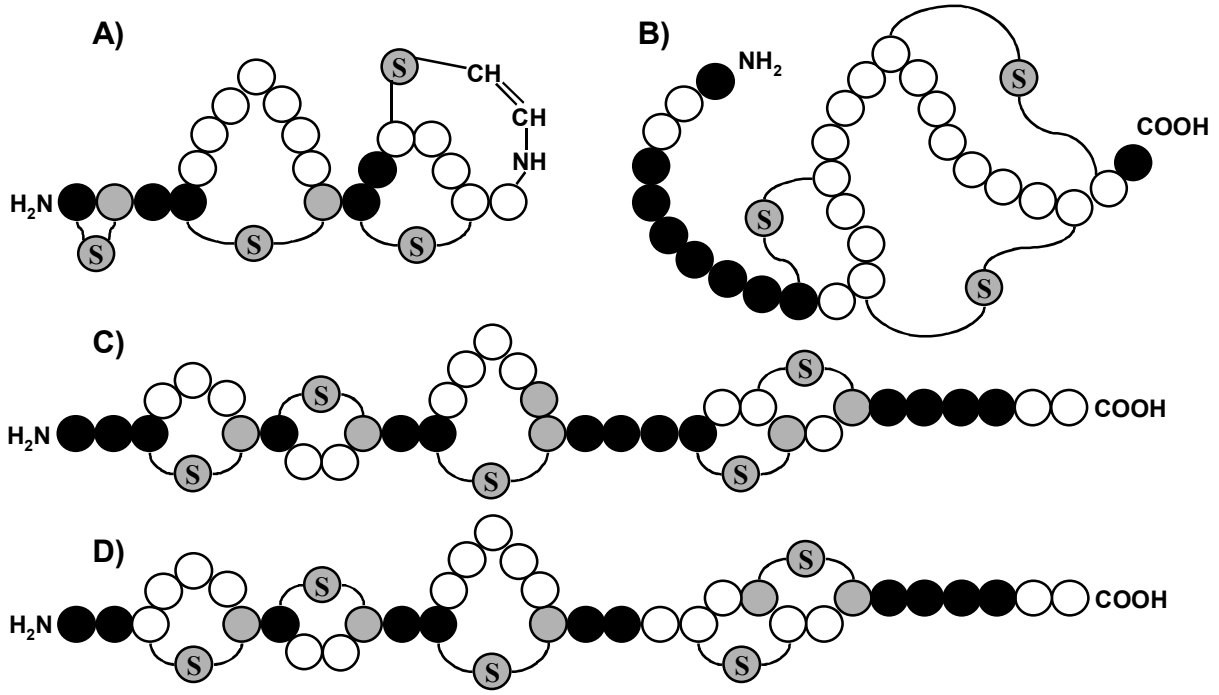



***Figure 2.3:*** *ECD induced fragmentation of A) mersacidin, B) lacticin 481, C) nisin A and D) nisin Z. The fragmentation products from black coloured sites are c and z· ions. The fragmentation products from grey coloured sites are the specific c• and z ions. c•, z fragmentation always involves the 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.*

In one case the doubly reduced pseudo-molecular 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 also some small neutral losses from the odd-electron singly reduced molecular ions were 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 using CAD. All the lantibiotic molecular ions which had captured one electron showed intense $NH_3$ loss. The ECD spectrum of nisin A contained two peaks, which can be contributed to amino acid side chain losses. Clearly visible were losses of 57 and 71. These are the masses of the side chains of Asn and Lys minus 1 Dalton. Also a loss of 28/29 was observed, which can be contributed to CO/COH loss. Mersacidin exhibited quite an intense $SH^{\bullet}$ loss and a loss of 56, which is the mass of the side chain of Ile/Leu minus 1 Dalton. Lacticin 481, finally, showed losses of 44, which could be $CO_2$ loss and losses of 72 and 129, which are likely the side chain masses of Glu and Trp minus 1 Dalton.

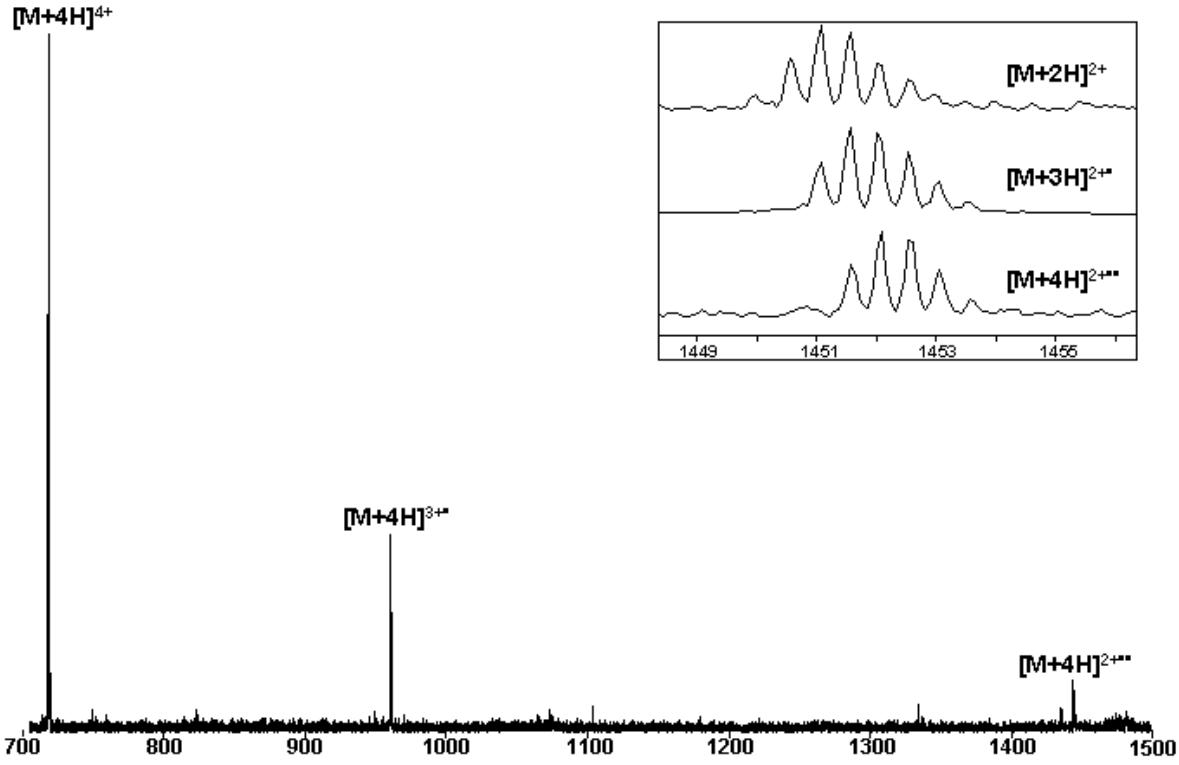



***Figure 2.4:*** *ECD spectrum of lacticin 481, showing the parent ion ($[M+4H]^{4+}$) and the singly and doubly reduced pseudo-molecular ion. The inset shows the difference in mass between a regular $[M+2H]^{2+}$ molecular ion of lacticin 481, the $[M+3H]^{2+\bullet}$ ion, formed by the capture of 1 electron by the $[M+3H]^{3+}$ ion and the $[M+4H]^{2+\bullet\bullet}$ ion, formed by the capture of 2 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.*

**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 also the neutral loss of water from the parent ion was 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 fragments ions, resulting from cleavages at the termini generally were still higher in intensity. At these higher collision energies also some fragment ions were 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 2.5.

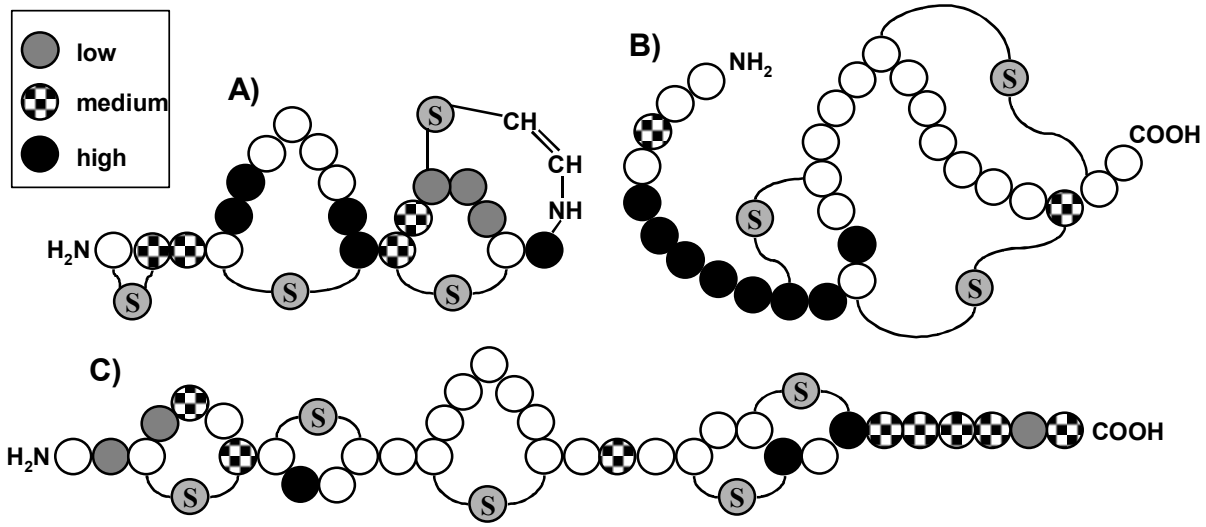
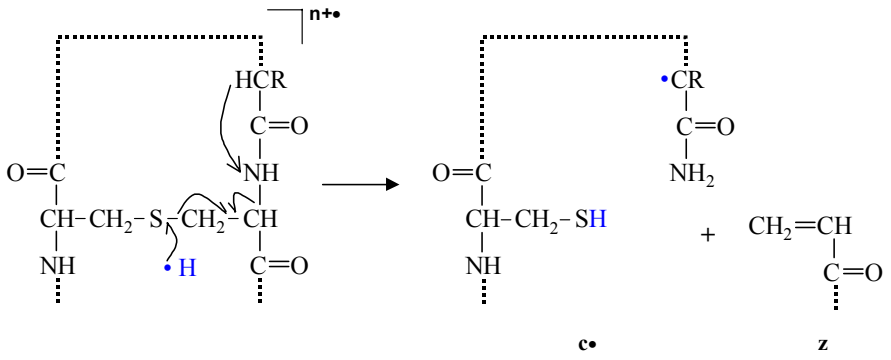



***Figure 2.5:*** *CAD induced fragmentation of A) mersacidin $[M+2H]^{2+}$, B) lacticin 481 $[M+4H]^{4+}$ and C) nisin A $[M+4H]^{4+}$. The inset displays how the colours correspond to the collision energy. To compare the collision energy of the different ions, the maximum centre-of-mass kinetic energy, gained in the collision cell, was calculated for each type of ion during the experiments. When the centre-of-mass kinetic energy of the ions did not exceed 1 eV, the collision energy was considered as low, from 1-1.4 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.*

## 2.4. 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. Via relocations of electrons, the neighboring amine bond is broken. 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• radical ions and z ions always 2 bonds are broken, namely a backbone bond and a thioether bond in one of the lanthionine bridges. A possible explanation for the formation of c• ions and z ions instead of c and z• ions could thus be that from a single electron capture, a double cleavage occurs. Probably, the radical-site initiated reaction starts at the sulphur atom in the thioether bridge. The thioether sulphur atom supposedly has a high hydrogen atom affinity, just as a carbonyl oxygen.[13] Considering the masses of the c• fragments, the thioether bond is cleaved as well as an amine bond in this way (scheme 2.4).




***Scheme 2.4:*** *Postulated mechanism of the formation of c• and z ions, resulting from the cleavage of a monosulfide bridge and a backbone bond. The H• radical is formed as a result of the capture of an electron by a proton. The location of the unpaired electron is not known.*

This c•, 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•, 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 also a thioether bond and a backbone bond were 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 2.3). 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, which 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 2.3). 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 2 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 all 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 2 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 has been observed with other types of peptide ions earlier.[14] For example, in the ECD spectrum of nisin A $[M+4H]^{4+}$ ions there is a very abundant peak present, originating from $[M+4H]^{2+\bullet\bullet}$ pseudo molecular ions. This means that the original 4 protons have captured 2 electrons, making this ion possibly a biradical cation. An explanation for the seemingly highly non-dissociative character of the reduced molecular ions could be the abundant presence of lanthionine bridges in lantibiotics. The sulphur 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 a Dalton 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.

## 2.5. 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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# Chapter 3

# Does double electron capture lead to the formation of biradicals? An ECD-SORI-CID study on lacticin 481.

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**Abstract**

We studied lacticin 481, a small lantibiotic with three lanthionine bridges, by Electron Capture Dissociation (ECD) in a Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer. Following electron capture very little fragmentation was observed, but species formed by non-dissociative single and multiple electron capture were abundant. Ions formed by double electron capture were subjected to Sustained Off Resonance Irradiation Collision Induced Dissociation (SORI-CID) to determine whether stable biradicals were formed. In the SORI-CID spectra of the ions formed by double electron capture some, but minor, $H^{\bullet}$ radical loss was observed, which was not observed at all for regularly protonated ions. A small part of the ions formed by double electron capture are thus long-lived biradicals. Apart from the observed $H^{\bullet}$ loss the SORI-CID spectra of ions that captured two electrons was similar to that of regularly protonated ions and quite different from the SORI-CID spectra of radical ions formed by single electron capture. This implies that recombination of the two radical sites is the dominant process in biradical lacticin 481 ions, at least on the time scale of our SORI-CID experiments.

## 3.1. Introduction

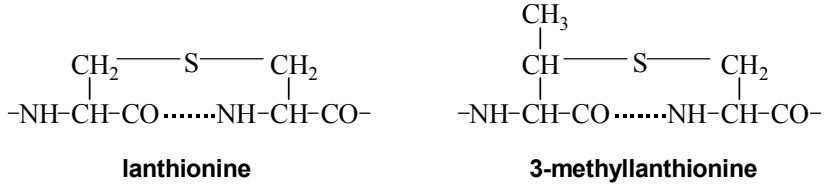
Lantibiotics are currently used as preservatives in the food industry, but have the potential to become a new generation of antibiotics.[1] The development of new antibiotics is driven by the fact that (multi-) resistant bacteria are becoming a huge problem for public health. Characteristic of lantibiotics is that they contain lanthionine bridges, consisting of a thioether bond that intramolecularly links different parts of the peptide backbone. To study the mechanism of action of lantibiotics on the molecular level it is important to perform structural analysis. Traditionally, Edman degradation [2, 3] has been used for peptide sequencing. With lantibiotics however Edman degradation can only be used to sequence the peptides from the termini until the lanthionine bridges that are closest to the termini. Lantibiotics have to be chemically modified to open the lanthionine bridges [4] to complete the Edman degradation. Nuclear Magnetic Resonance (NMR) spectroscopy can be performed on unmodified lantibiotics to retrieve information about the localization of the lanthionine bridges, but in general it is a time-consuming and insensitive analytical technique. Recently we showed that Electron Capture

Dissociation (ECD) is a specific method to rapidly localize lanthionine bridges in lantibiotics.[5]

In ECD,[6, 7] multiply charged cations are irradiated with low-energy electrons to form radical cations. A part of these ions undergoes very fast fragmentation reactions before the 4-7 eV energy,[8] released by neutralization of a proton, is fully randomized.[9] Therefore, in the case of peptides and proteins, ECD results mainly in the formation of c and $z^{\bullet}$ ions.[6, 10] ECD has been particularly useful in the analysis of even labile post-translational modifications. Fragments often retain their modifications in this fast dissociative process, in contrast to, for instance, Collision Induced Dissociation (CID). The successful application of ECD has been demonstrated e.g. in the analysis of methionine oxidation,[11] O- and N-glycosylation,[12, 13] phosphorylation,[14] sulfation[15] and carboxylation of peptides and proteins[15]

In the analysis of peptides containing disulfide bonds, ECD is a powerful fragmentation method. When peptide ions containing disulfide bonds are subjected to ECD[7, 16, 17] predominantly the S-S bond becomes cleaved first. Single electron capture can cleave both the disulfide bond and a backbone bond leading to fragmentation localized near the disulfide bond.[7] This has been rationalized by the fact that disulfide bonds have a very high $H^{\bullet}$ affinity. In complementary CID experiments no such specific disulfide bond cleavages could be observed. In a theoretical study performed by Uggerud et al.[18] it was suggested that the most plausible scenarios leading to the specific disulfide bond cleavages are either direct addition of a free hydrogen radical to the disulfide bond or addition of an electron to a disulfide bond in hydrogen-bonded contact with a proton. In the latter scenario a $H^{\bullet}$ radical is formed close to the disulfide bond. The conformation of the ions determines which scenario is predominant.[7] In another theoretical study it has been stated[19] that the electron capture induced disulfide bond cleavage is less specific than often claimed. Radicals with a hydrogen radical attached to a sulfur atom in the disulfide bond dissociated by cleavages of adjacent S-H, S-C and S-S bonds. Hydrogen atom capture by the disulfide bond in the studied molecule was calculated to be substantially exothermic, explaining the high $H^{\bullet}$ affinity of disulfide bonds.[19] More detailed information on general ECD mechanisms can be found in a recent paper by Syrstad and Tureček.[20]

Although electron capture induced dissociation can lead to extensive peptide sequence coverage, products may also arise from reduction, without subsequent dissociation.[21] These non-dissociative products are particularly dominant in the ECD spectra of larger proteins,[22] but also appeared to be dominant in the ECD spectra of <3.5 kDa lantibiotics.[5] Somehow lantibiotics produce very stable non-dissociative radicals when they are irradiated with low-energy electrons. This effect was strongest for the lantibiotic lacticin 481 (figure 3.1). This lantibiotic exhibits bactericidal activity against a wide range of Gram-positive bacteria and is produced by *Lactococcus lactis*. Lacticin 481 contains three lanthionine bridges (scheme 3.1) that link overlapping parts of the backbone. The precise locations of the lanthionine bridges were elucidated by van den Hooven et al.[23] by a combination of peptide chemistry, mass spectrometry and NMR spectroscopy. Lacticin 481 displays hardly any ECD fragmentation, hampering the localization of its lanthionine bridges with mass spectrometry. We focus this study on the non-dissociative species of lacticin 481, especially on the ions formed by double electron capture. There are two possibilities in the case of a multiply protonated ion capturing two electrons: the induced radical sites could recombine to form a new two-electron pair, or they will not recombine and two fixed or mobile radical sites will remain present in the ion. The main purpose of this study is to investigate which of these two scenarios is predominant. Because of the observed specificity of ECD for S-C bond cleavage, we expect that the radical sites in lacticin 481 are mainly localized around the lanthionine bridges. We compare the Sustained Off Resonance Irradiation Collision Induced Dissociation (SORI-CID) spectra of protonated lacticin 481 ions not exposed to electrons to the SORI-CID spectra of the ions that had captured one or two electrons and did not dissociate. The SORI-CID spectra of the species formed by double electron capture are very distinct from those of the species that captured one electron, and show quite some resemblence with the SORI-CID spectra of regularly protonated lacticin 481 ions.




**lanthionine** **3-methyllanthionine**

***Scheme 3.1:*** *Chemical structure of a) lanthionine and b) 3-methyllanthionine. The N-terminal lanthionine bridge in lacticin 481 has structure b, the other two bridges have structure a.*

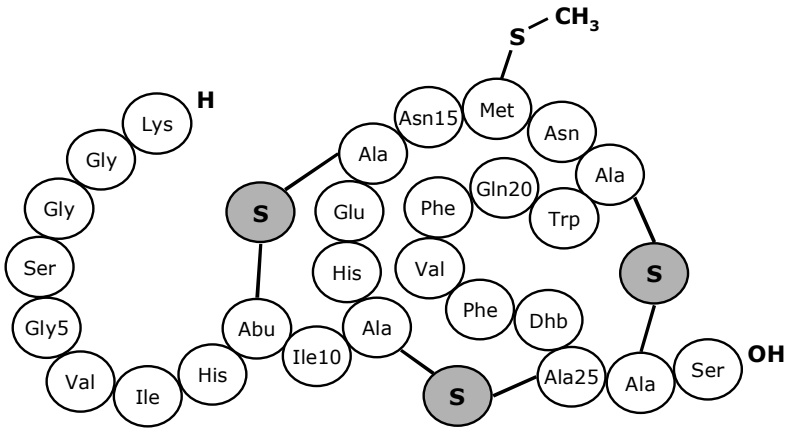



***Figure 3.1:*** *Chemical structure of lacticin 481. Abu is 2-aminobutyric acid and Dhb is dehydroamino-2-butyric acid.*

### 3.2. Experimental

The fragmentation of protonated lacticin 481 ions was studied with three different fragmentation techniques, namely SORI-CID,[24, 25] ECD and ECD followed by SORI-CID in a Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer. The FTICR instrument was a modified Bruker-Spectrospin (Fällanden, Switzerland) Apex 7.0e, equipped with a 7 Tesla superconducting magnet. The experiments were performed using an infinity cell. The electron gun was an indirectly heated barium-tungsten dispenser cathode (TB-198, HeatWave Labs Inc., Watsonville, CA, USA) placed inside the magnet approximately 30 cm behind the cell. Just in front of the surface of the cathode a copper control grid is positioned. The cathode was operated using a current of 1.85 A and a potential of 7.9 V, yielding a power of 14.6 W. Lacticin 481 and mellitin (15 μM) were sprayed from a methanol:water:acetic acid 69:29:2 solution with a flow rate of 10 μL/h and a voltage of 3 kV was applied on the spray needle (PicoTip™ Emitter, New Objective Inc., Woburn, MA, USA) to perform electrospray ionization. After isolation of the ions of interest, they were exposed to low-energy electrons for 5 seconds. A long irradiation time was required to get sufficient electron capture with lacticin 481 ions. This could have been partly due to bad overlap between the ion cloud and the electron beam. However we observed that the capture cross section of lacticin 481 ions is substantially lower than that of linear peptide ions of the same charge state and similar mass (i.e. mellitin). During the irradiation time the grid

potential was pulsed from –100 V to 80 V and the cathode surface potential from 25 V to –1.35 V. The trapping plates were held at a potential of 1 V. Reduced species were isolated again before they were subjected to SORI-CID at a frequency 1500 Hz away from the resonance frequency of the ion of interest. Argon was used for gas assisted dynamic trapping and as the collision gas. During detection, the pressure in the ICR cell was less than $10^{-9}$ mbar. Data acquisition was performed using in-house developed software and hardware.[26]

### 3.3. Results

To illustrate the predominance of non-dissociative electron capture with ECD of protonated lacticin ions, we compared the ECD spectra of the $[M+4H]^{4+}$ ions of lacticin 481 and mellitin, which has similar size but is a regular linear peptide. The $[M+4H]^{4+}$ ions of both mellitin and lacticin 481 were isolated and subsequently subjected to ECD using identical experimental conditions. The ECD spectra of mellitin and lacticin 481 are shown in figure 3.2a and 3.2b, respectively.

The differences are obvious in that the reduced ions of lacticin 481 hardly fragment, whereas the reduced ions of mellitin fragment extensively under the same conditions. This means that the reduced lacticin 481 ions are much more stable than the reduced mellitin ions. We reported before that also other lantibiotics produce very stable reduced species after electron capture.[5] We attributed this behavior to the multiple internal lanthionine bridges that are present in lantibiotics. Whereas mellitin is a regular linear peptide and one cleavage will lead to fragmentation, lantibiotics contain several regions where one or two lanthionine bridges link different parts of the backbone. One cleavage in these regions will not lead to detectable fragmentation.

A main aim of the experiments performed in this study was to find out whether long-lived biradicals are formed after double electron capture. Therefore the SORI-CID spectra of lacticin 481 ions formed from $[M+4H]^{4+}$ ions by single and double electron capture and regular triply protonated lacticin 481 ions were compared (figure 3.3).

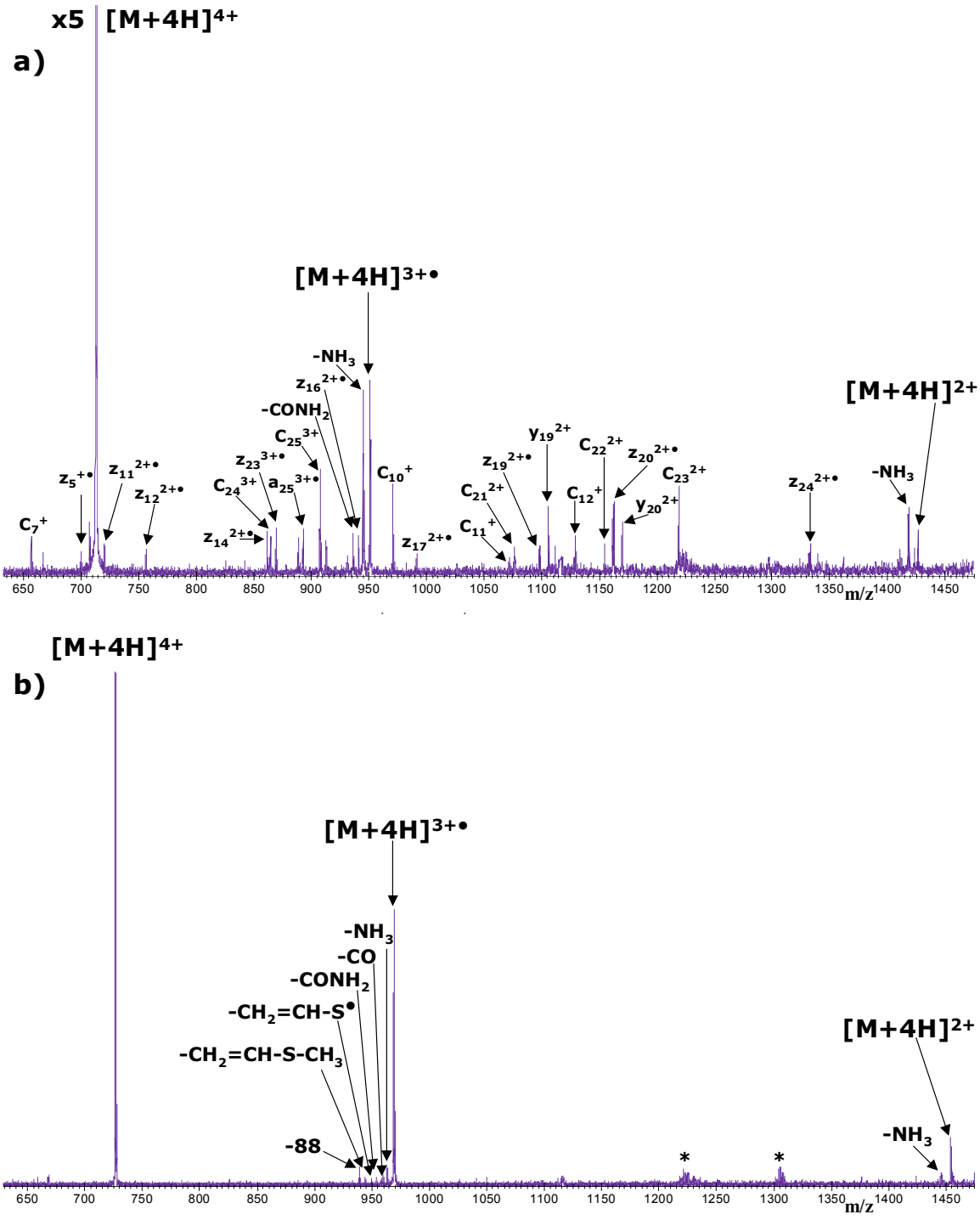



***Figure 3.2:*** *a) ECD spectrum of mellitin $[M+4H]^{4+}$ ions. The spectrum displays a wide variety of abundant electron capture induced fragment ions. b) ECD spectrum of lacticin 481 $[M+4H]^{4+}$ ions with high intensity non-dissociated reduced product ions, formed by single and double electron capture. Noise peaks are marked with asterisks.*

First, $[M+4H]^{3+\bullet}$ ions formed by single electron capture of $[M+4H]^{4+}$ ions were fragmented using SORI-CID (figure 3.3a). The loss of an $SH^{\bullet}$ radical was the most

predominant fragmentation channel. Part of the observed $SH^{\bullet}$ loss must have originated from the C-terminal lanthionine bridge (Ala18-Ala26), because when subsequently SORI-CID was applied on the $[M+3H-S]^{3+}$ ions formed by $SH^{\bullet}$ loss from $[M+4H]^{3+\bullet}$ ions, this resulted in the formation of $b_{25}^{3+}$ ions without the sulfur atom of the C-terminal lanthionine bridge (results not shown). Additionally, the SORI-CID spectra of $[M+4H]^{3+\bullet}$ ions revealed $H^{\bullet}$ loss. Very typical in the SORI-CID spectra of $[M+4H]^{3+\bullet}$ ions was an extensive series of small neutral losses. The losses of 47, 59 and 61 Da could be attributed to $CH_3S^{\bullet}$, $C_2H_3S^{\bullet}$ and $C_2H_5S^{\bullet}$ loss respectively, indicating that the $[M+4H]^{3+\bullet}$ ions have several fragmentation channels available to lose a radical. Also even-electron losses were observed, namely the losses of 74 and 131 mass units, corresponding to $C_3H_6S$ (the side chain of methionine) and the loss of the side chain of tryptophan. These electron capture induced side chain losses have been observed before in ECD spectra of peptides.[27-29] The mentioned sulfur-containing neutral losses either originate from the methionine residue or from a lanthionine bridge.

To eliminate these neutrals from a lanthionine bridge, two bonds have to be broken, of which at least one is a C-C bond. To form these neutrals from the side chain of methionine only a single cleavage is required, making this scenario more likely. Hence we attribute these neutral losses to dissociations in the methionine residue. Interestingly the spectra also displayed peaks 32.978 Da lower in mass than the peaks corresponding to the radical ions formed by non-radical tryptophan and methionine side chain losses from $[M+4H]^{3+\bullet}$ ions. These fragment species are thus able to subsequently eliminate an $SH^{\bullet}$ radical. Finally, two different y-ions were observed, namely $y_{25}$ as a 2+ and $y_{26}$, both as a 2+ and a 3+. The y-ions were 2 Da heavier than normal y ions. For comparison, also SORI-CID spectra of lacticin 481 $[M+3H]^{2+\bullet}$ ions formed by single electron capture of $[M+3H]^{3+}$ ions were acquired, but this did not lead to the formation of many fragment ions (results not shown). Very prominent was the small neutral loss of 33 Da, which again is assigned to $SH^{\bullet}$ loss. $H^{\bullet}$ loss was not observed, so the radical is preferentially eliminated from the radical cation in the form of an $SH^{\bullet}$ radical.

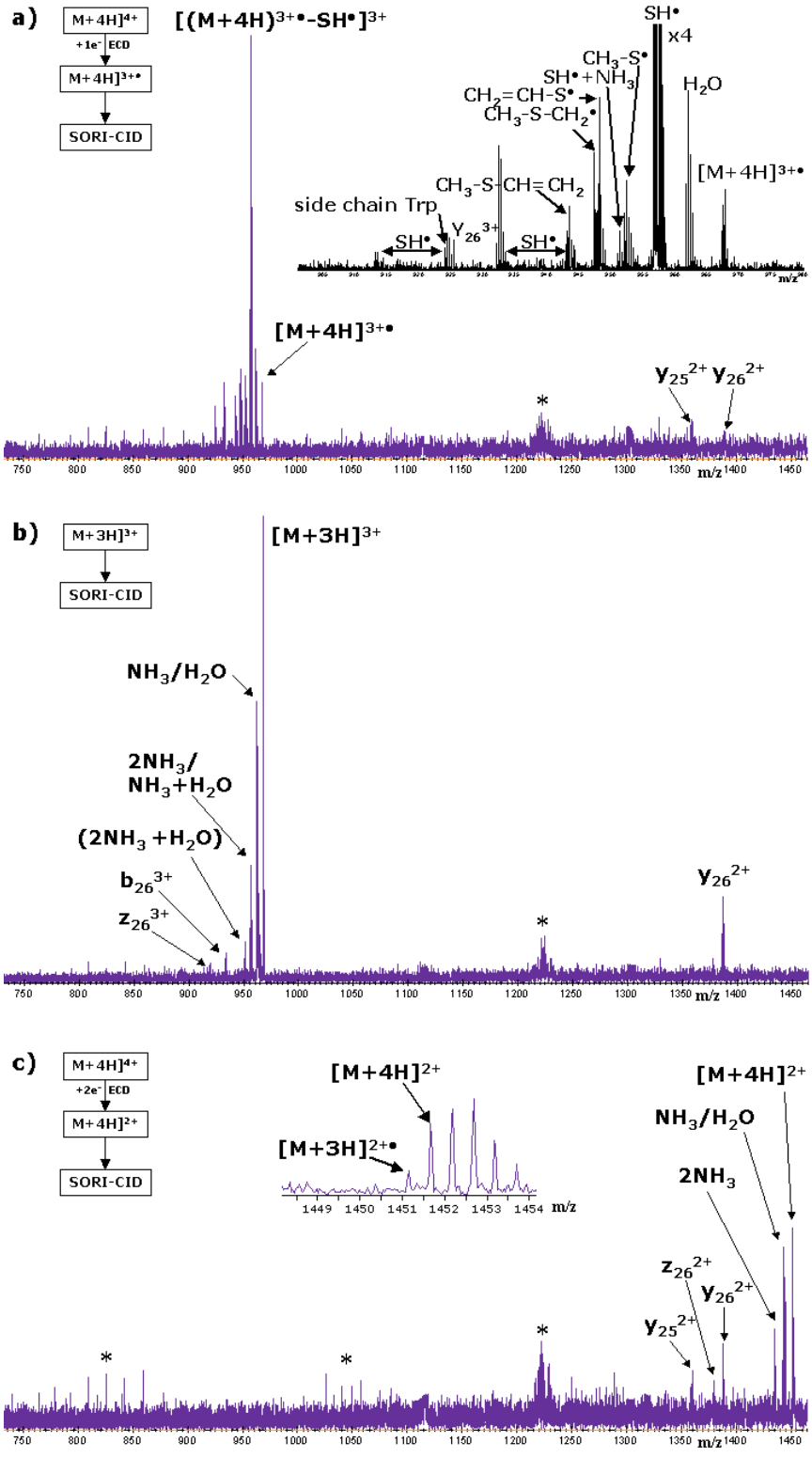



***Figure 3.3:*** *a) SORI-CID spectrum of lacticin 481 $[M+4H]^{3+\bullet}$ ions formed by single electron capture. The inset shows a rich part of the spectrum (m/z 900-980) magnified. b) SORI-CID spectrum of $[M+3H]^{3+}$ ions. c) SORI-CID spectrum of lacticin 481 $[M+4H]^{2+}$ ions formed by double electron capture. The inset shows the loss of a H• radical from the parent ion. Noise peaks are marked with asterisks.*

Next we evaluated the SORI-CID fragmentation of regularly protonated $[M+3H]^{3+}$ ions (figure 3.3b) which was very different from the SORI-CID fragmentation of $[M+4H]^{3+\bullet}$ ions and $[M+3H]^{2+\bullet}$ ions. Under exactly the same conditions, $[M+3H]^{3+}$ ions showed only $NH_3$ and $H_2O$ small molecule losses and additionally $z_{26}^{3+}$, $y_{26}^{2+}$ and $b_{26}^{3+}$ fragment ions. The combined loss of $NH_3$ and $H_2O$ gives a peak 35 Da lower in mass than the parent ion. The same type of CID cleavages were observed for lacticin 481 $[M+2H]^{2+}$ and $[M+4H]^{4+}$ ions (data not shown). There were no peaks in the CID spectra that could be assigned to $H^{\bullet}$ loss, $SH^{\bullet}$ loss, losses from the lanthionine bridges or from the side chain of methionine or tryptophan. The absence of such fragmentations was expected, as the $[M+3H]^{3+}$ ions have no radical character.

From lacticin 481 $[M+4H]^{4+}$ ions also $[M+4H]^{2+}$ ions were produced via double electron capture. The double electron capture could either induce two separate stable radical sites in the resulting ions or the two radical sites could recombine to form a new chemical two-electron bond. To investigate the nature of the $[M+4H]^{2+}$ ions, we subjected them also to SORI-CID (figure 3.3c). Remarkably, these spectra showed quite some resemblance with the SORI-CID spectra of $[M+3H]^{3+}$ ions. Quite intense is the loss of $NH_3$ and $H_2O$, furthermore $y_{25}^{2+}$, $y_{26}^{2+}$ and $z_{26}^{2+}$ ions are present in the spectra. The formed y ions were both 2 Da heavier than normal y ions. Similar as in the SORI-CID spectra of $[M+3H]^{3+}$ ions a loss of 34 Da was present, which corresponds to two $NH_3$ losses. At higher collision energies, also a 35 Da loss appeared, which corresponds to a combined $NH_3/H_2O$ loss. Interestingly there is no loss of 33 Da, like in the SORI-CID spectra of $[M+3H]^{2+\bullet}$ and $[M+4H]^{3+\bullet}$ ions, where it is the most abundant fragmentation. However, minor $H^{\bullet}$ loss from $[M+4H]^{2+}$ ions was observed leading to the formation of radical $[M+3H]^{2+\bullet}$ ions.

### 3.4. Discussion

Although non-dissociative single and double electron capture often is observed in ECD of multiply protonated peptide ions, it is usually not very abundant. In contrast, lacticin 481 does not only exhibit single electron capture but also abundant double electron capture. The predominant SORI-CID fragmentation channel observed for the radical $[M+3H]^{2+\bullet}$ ions and $[M+4H]^{3+\bullet}$ ions is $SH^{\bullet}$ loss. This $SH^{\bullet}$ loss can be explained by

assuming that the radical site, induced by the captured electron, relocates to near a sulfur atom of one of the lantionine bridges resulting in S-C bond cleavage. The selectivity for this S-C bond cleavage after electron capture is supported by our earlier research on ECD of other lantibiotics[5] and theoretically predicted to occur in ECD by Tureček et al.[19] The second S-C bond cleavage, needed for $SH^{\bullet}$ loss, has to be induced by collisional activation with argon, because $SH^{\bullet}$ loss is absent in the ECD spectra of lacticin 481 $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions. Interestingly, a study performed on the much smaller distonic radical ion of a thioether, namely $^{\bullet}CH_2\text{-}CH_2\text{-}S\text{-}CH_2^{+}$ revealed that this is a stable species in the gas phase.[30] CID spectra of this distonic radical ion are dominated by $CH_2S^{+\bullet}$ ions resulting from cleavage of an S-C bond. Thus, also in CID of this small but related distonic ion the S-C bond is cleaved. Furthermore, the described S-C bond cleavage involves a mobile radical site.[30] Also in ECD mobile radical sites can play a role because radical site initiated secondary cleavages are observed after capture of only one electron.[31] A minor CID fragmentation channel of $^{\bullet}CH_2\text{-}CH_2\text{-}S\text{-}CH_2^{+}$ distonic ions is the elimination of $SH^{\bullet}$. This means that even two S-C bond cleavages can take place in these distonic ions by collisional activation. A theoretical study performed on the same distonic radical ion was in good agreement with the earlier mentioned experimental results.[32]

If abundant stable biradicals would have been formed by double electron capture of $[M+4H]^{4+}$ ions, we would expect to see extensive collision induced $SH^{\bullet}$ losses from these $[M+4H]^{2+}$ ions as this was the most important fragmentation channel of the monoradical species. There are remarkable differences between the SORI-CID spectra of $[M+4H]^{2+}$ ions and those of the radical $[M+3H]^{2+\bullet}$ ions and $[M+4H]^{3+\bullet}$ ions. However, especially the absence of $SH^{\bullet}$ loss in the SORI-CID spectra of $[M+4H]^{2+}$ ions is striking and this could indicate that there are hardly any free radicals sites in these ions. Due to the absence of $SH^{\bullet}$ loss, we believe that the two unpaired electrons must have recombined to form two-electron bonds. One possibility is that two $S^{\bullet}$ termini, also called thiyl radicals, recombine to a disulfide bond. This is an exothermic reaction that releases about 2.2 eV energy.[33] A newly formed bond would change the structural characteristics of the lacticin 481 ions. The absence of $b_{26}^{3+}$ ions in the SORI-CID spectra of $[M+4H]^{2+}$ ions, which are present in the SORI-CID spectra of $[M+3H]^{3+}$ ions, might point at such a structural change in the C-terminal part of the lacticin 481 ions.

The reason for this structural change is a matter of speculation, but it may be that the C-terminal lanthionine bridge opened due to a captured electron and then recombined with another radical site. The remainder of the SORI-CID fragments of the $[M+4H]^{2+}$ ions were in good agreement with those of the $[M+3H]^{3+}$ ions and thus with a non-radical cation. This leads us to the conclusion that most of the $[M+4H]^{2+}$ ions must have recombined their two unpaired electrons. We say most of the $[M+4H]^{2+}$ ions, because $H^{\bullet}$ radical loss from $[M+4H]^{2+}$ ions was also observed to some extent (inset figure 3.3c). The intensity of the peaks corresponding to $[M+3H]^{2+\bullet}$ ions was approximately 25 % of the peaks corresponding to the parent ions ($[M+4H]^{2+}$ ions). This indicates that at least a part of the ion population exhibits a structure containing two separate radical sites instead of a recombined chemical bond. However, because of the absence of $SH^{\bullet}$ loss in the SORI-CID spectra of $[M+4H]^{2+}$ ions, we propose that these radical sites are not close to lanthionine bridges. This also implies that the initial biradical ion population is heterogeneous. Apparently there are other sites in lacticin 481 where stable radicals can exist, such as alpha carbons.[34] The conformation of the biradical ions is very important for bringing two radical sites in a reactive configuration. If the radical sites are not mobile, the rate of conformational changes and thermal structure fluctuations would be the limiting factors in the rate of radical recombination into two-electron bonds. Conformational changes and thermal motions take place on time scales on the order of hundreds of microseconds to seconds[35] and are slow in comparison to the actual radical recombination processes. The fact that not all the biradicals have recombined their radical sites would then be a matter of sterical hindrance. Leymarie et al. [31] have shown that the radical sites induced by electron capture can be mobile and can initiate a radical cascade. However the radicals formed in lacticin 481 leading to $H^{\bullet}$ loss do not seem to be very mobile, because of their long lifetime. Our main question was: will two captured electrons in protonated lacticin 481 ions form two separate radical sites or will they recombine to form a new chemical bond? We find that after several seconds, i.e. the time scale of our experiments, most of $[M+4H]^{4+}$ ions that have captured two electrons have become $[M+4H]^{2+}$ ions through radical recombination and a substantially smaller number of ions are still $[M+4H]^{2+2\bullet}$ biradical ions.

## 3.5. Conclusion

The most important observations from the experiments described in this article were the resemblance of the SORI-CID spectra of the $[M+3H]^{3+}$ ions and the $[M+4H]^{2+}$ ions of lacticin 481 and the large differences between the SORI-CID spectra of $[M+4H]^{3+\bullet}$ ions and $[M+4H]^{2+}$ ions. This led to the conclusion that most of the ions formed by double electron capture must have recombined their two unpaired electrons. However, the fact that some $[M+4H]^{2+}$ ions are able to lose a $H^{\bullet}$ radical points to the existence of two separated radical sites in at least part of ions formed by double electron capture. We could therefore refer to these ions as biradicals or $[M+4H]^{2+2\bullet}$ ions. In the SORI-CID spectra of the radical $[M+3H]^{2+\bullet}$ and $[M+4H]^{3+\bullet}$ ions very intense $SH^{\bullet}$ losses are present. This specificity further validates the strong involvement of lanthionine bridges in electron capture dissociation processes.

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# Chapter 4

# Atypical behavior in the electron capture induced dissociation of biologically relevant transition metal ion complexes of the peptide hormone oxytocin

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**Abstract**

Doubly protonated ions of the disulfide bond containing nonapeptide hormone oxytocin and biologically relevant oxytocin complexes with different transition metal ions were subjected to electron capture dissociation (ECD) to probe their structural features. Although, all the ECD spectra were strikingly different, typical ECD behavior was observed for complexes of the nonapeptide hormone oxytocin with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$, i.e. abundant c/z'$^{\bullet}$ and a'$^{\bullet}$/y backbone cleavages and ECD characteristic S-S and S-C bond cleavages were observed. We propose that, although in the oxytocin- transition metal ion complexes the metal ions serve as the main initial capture site, the captured electron is transferred to other sites in the complex to form a hydrogen radical, which drives the subsequent typical ECD fragmentations. The complex of oxytocin with $Cu^{2+}$ displayed noticeably different ECD behavior. The fragment ions were similar to fragment ions typically observed with low-energy collision induced dissociation (CID). We propose that the electrons captured by the oxytocin- $Cu^{2+}$ complex might be favorably involved in reducing the $Cu^{2+}$ metal ion to $Cu^{+}$. Subsequent energy redistribution would explain the observed low-energy CID-type fragmentations. Electron capture resulted also in quite different specific cleavage sites for the complexes of oxytocin with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$. This is an indication for electronic and structural differences in these complexes possibly linked to their significantly different biological effects on oxytocin-receptor binding, and suggests that ECD may be used to study subtle structural differences in transition metal ion- peptide complexes.

## 4.1. Introduction

Nowadays tandem mass spectrometry is one of the most valuable tools used in the structure analysis of peptides and proteins.[1, 2] In these experiments often peptides formed after solution-phase proteolysis of proteins are brought into the gas-phase either by matrix assisted laser desorption ionization (MALDI) or electrospray ionization (ESI). The most sensitive approach is to embed the peptides either in acidic matrices (MALDI) or acidified aqueous solutions (ESI), which enables the efficient formation of singly or multiply protonated peptides ions. Subsequently, these protonated peptide ions are fragmented in a collision cell within the vacuum of the mass spectrometer, which induces fragmentation of the peptides, often termed collision induced dissociation (CID).

Typically relatively low collision energies are used in these experiments and activation and fragmentation occurs via multiple collision events. These collision induced dissociation experiments primarily induce backbone fragmentations in the peptide, with a preference for cleavages in the "weakest" amide peptide bond. Depending on where the charge remains in the two concomitant fragments either b or y ions are formed.[2, 3] Because of this specificity tandem MS spectra of protonated peptides are relatively simple, allowing the determination of the peptide sequence with relative ease. Therefore, it is not surprising that tandem mass spectrometry is at present one of the most valuable tools used in high-throughput identification of proteins as performed in proteomics experiments.

Besides the many merits of tandem mass spectrometry of (multiply) protonated peptides by low-energy collision induced dissociation, there are also some disadvantages to this approach. It is well known that peptides often do no retain their labile modifications, such as glycosylation and phosphorylation in CID. Moreover, the relatively slow process of CID may induce substantial rearrangement reactions in the precursor ions, prior to fragmentation, a fact that for instance seriously hampers the use of CID in hydrogen/deuterium exchange mass spectrometry.[4] A decade ago a new activation method was introduced to fragment biomolecular ions termed electron capture dissociation (ECD).[5, 6] In ECD multiply charged cations stored in a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer are irradiated with low-energy electrons. As the multiply protonated peptide ions are even electron systems, the ECD process leads to the formation of odd-electron species or radical cations. It is now well established that part of these ions undergo very fast fragmentation reactions.[7] Therefore, ECD often does not result in cleavage of the "weakest" peptide bonds, but typically in formation of so-called c and z'$^{\bullet}$ ions and to a minor extent also to a'$^{\bullet}$ and y ions.[3, 5, 8] Being a complementary tool to low-energy CID, ECD has been proven to be particularly useful in the analysis of labile posttranslational modifications,[9-13] because these modifications often are retained on their initial position during the fast dissociative process. Successful application of ECD has been demonstrated in the analysis of many different peptide post-translational modifications, such as phosphorylation,[12, 14] glycosylation[10, 11, 15] and disulfide and monosulfide bonds,[6, 16, 17] all areas of structural analysis in which conventional CID often fails to produce good analytical results.

As mentioned above both CID and ECD experiments are typically performed on protonated peptides formed by MALDI and ESI. However, many proteins and peptides display well-defined secondary and tertiary "native" structures in solution, which are usually lost in the acidic matrices or acidified solutions used in MALDI and ESI, respectively. Often, such "native" structures are influenced by co-factors, such as small nucleotides or metal ions. An example of that is the biologically important small peptide hormone oxytocin, produced mainly by the magnocellular neurons of the hypothalamus. It exerts various hormonal effects, but is mostly known for its ability to elicit the contraction of smooth muscle in the uterus during labor. It is also critically involved in higher cognitive functions such as memory and learning.[18]

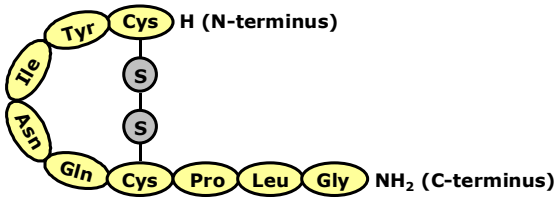



***Figure 4.1:*** *Chemical structure of the nonapeptide oxytocin.*

Oxytocin (figure 4.1) is a nonapeptide containing a disulfide bond between the Cys1 and the Cys6 residue. The presence of a specific divalent transition metal ion dramatically enhances binding of oxytocin to its cellular receptor to the nanomole range.[18] Oxytocin binding to its receptor is potentiated in increasing order by $Zn^{2+}$, $Ni^{2+}$ and $Co^{2+}$, but is negligible in the presence of $Cu^{2+}$. The affinity of bare oxytocin for transition metal ions is relatively low with $K_d$'s in the mM to μM range.[19]

Calculated molecular structures, optimized with molecular mechanics and density functional theory methods, showed that the binding of a $Zn^{2+}$ to oxytocin results in a significant change of structure.[20] Here we probed the structure of oxytocin- transition metal ion complexes using ECD. By binding divalent transition metal ions such as $Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ the oxytocin becomes automatically doubly charged in ESI, without the requirement of additional protons. We assume that the transition metal ions may act as the initial electron capture site. Fast ECD cleavages in the oxytocin- transition metal ion complexes could reflect the location and/or coordination of the metal ion in the complex. Surprisingly, the ECD spectra of each different complex are spectacularly

different, indicating that the electronic configuration of the transition metal ion in the complexes with oxytocin may lead to substantial structural differences in the ions, inducing very distinct fragmentations patterns.

## 4.2. Experimental

The fragmentation of oxytocin (Sigma) was studied with ECD in an FTICR mass spectrometer. Doubly protonated oxytocin ions and oxytocin ions complexed with $Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ ions were subjected to ECD. The ESI solution consisted typically of a 49:49:2 water:acetonitrile:acetic acid mixture and contained 20 μM oxytocin. Complexation of oxytocin to a transition metal ion was accomplished by adding the acetate salt of the metal ion (25 times in excess) to the ESI solution. The high-resolution mass spectra revealed unambiguously that complexation of the oxytocin by the transition metals ions did result in [oxytocin+$M^{2+}$] ions, without access protons or stripped hydrides, indicating that formally the transition metal is the charge carrier and the oxytocin neutral. The FTICR instrument used was a modified Bruker-Spectrospin (Fällanden, Switzerland) Apex 7.0e, equipped with a 7 Tesla superconducting magnet. The experiments were performed using an infinity cell.[21] The electron gun was an indirectly heated barium-tungsten dispenser cathode (TB-198, HeatWave Labs, Inc.) placed inside the magnet approximately 30 cm behind the cell. Just above the surface of the cathode there is a copper extraction grid. The cathode was operated using a current of 1.85 A and a potential of 7.9 V, yielding a power of 14.6 W. After isolation of the ions of interest, they were exposed to low-energy electrons. During this time the grid potential was pulsed from –100 V to 100 V and the cathode surface potential from 25 V to –0,15 V. The trapping plates were held at a potential of 1 V. For all the ion species in this study it took quite a long time of electron irradiation, namely 10 seconds, to get satisfactory ECD spectra. Mainly this must have been due to poor overlap between the electron beam and the ion cloud. Argon was used as the trapping gas. Data acquisition and analysis was performed using in-house developed software and hardware.[22]

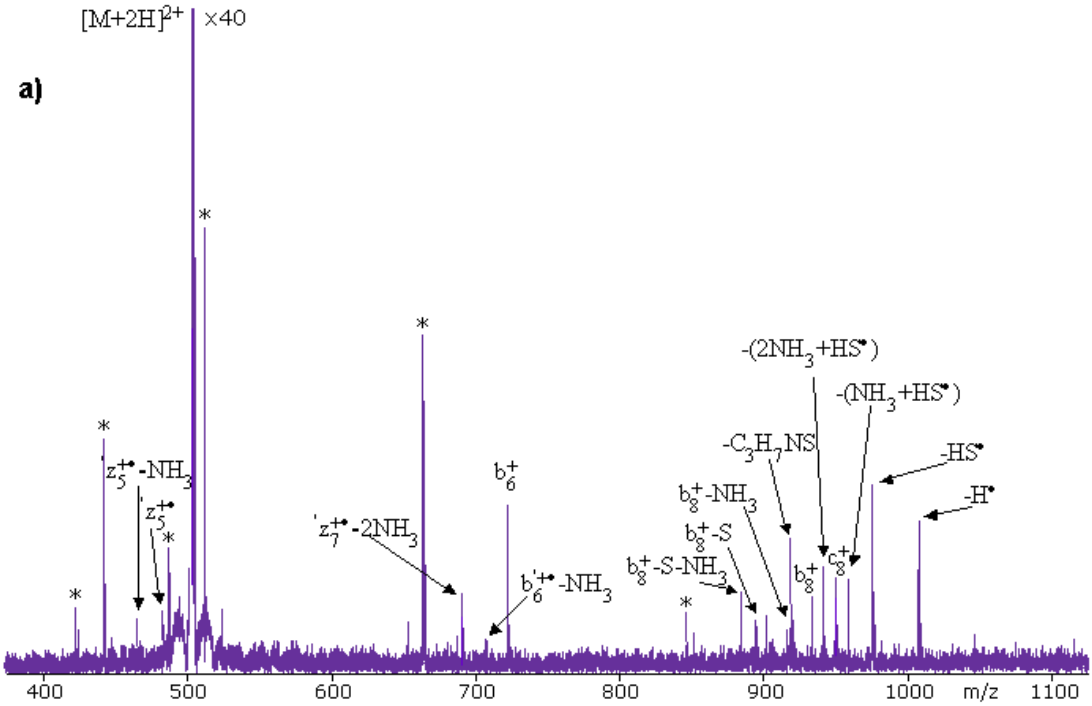
## 4.3. Nomenclature

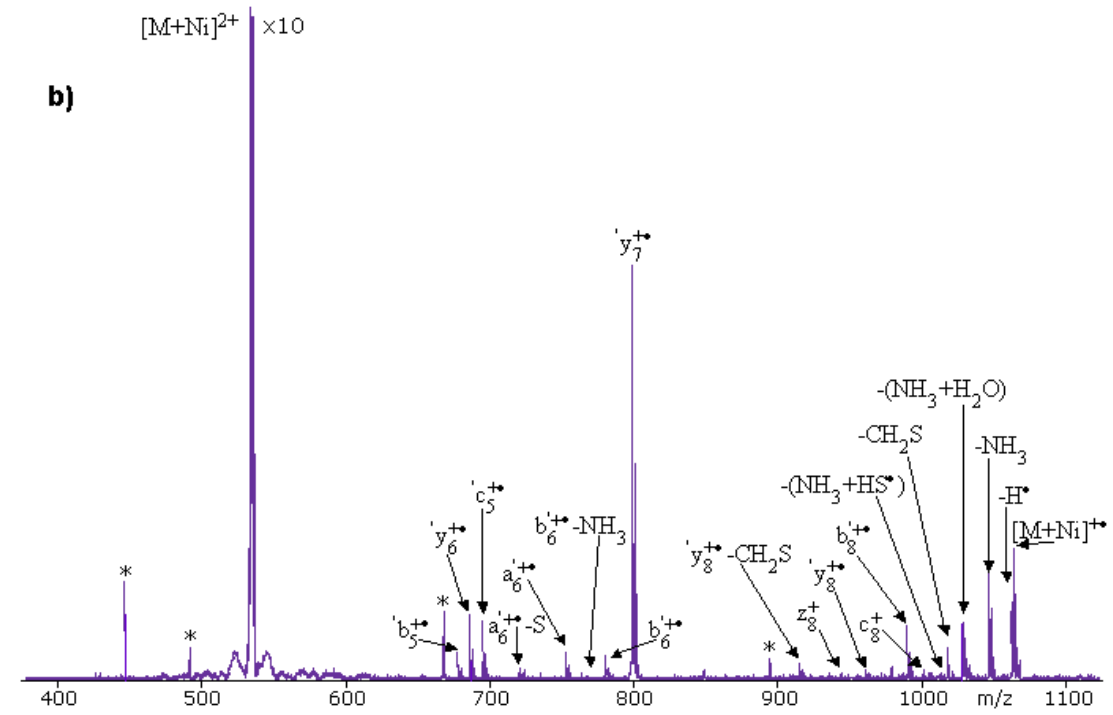
The nomenclature in this chapter for peptide fragment ions is based on the nomenclature proposed by Biemann[8] and Roepstorff and Fohlman.[3] When a fragment

has a mass 1 Da less than the normal mass of a regular peptide fragment ion, an ‘ is added before the letter annotation, when it is 1 Da heavier an ‘ is added behind the letter. A radical is annotated by adding a dot. When a backbone bond and an S-S or S-C bond are cleaved a regular fragment ion in our nomenclature includes a hydrogen atom bound to the resulting free sulfur or carbon atom in the charge carrying fragment. When a backbone bond and an S-C bond are cleaved +S or -S is added behind the letter, depending on whether the fragment ion contains two sulfur atoms or no sulfur atom. Every single fragment ion observed in our ECD spectra of oxytocin- transition metal ion complexes retained the metal ion. Therefore, we choose not to annotate the metal ion.

### 4.4. Results

In figure 4.2 the ECD spectra of doubly protonated oxytocin ions (figure 4.2a) and oxytocin complexed with $Ni^{2+}$ (figure 4.2b), $Co^{2+}$ (figure 4.2c), $Zn^{2+}$ (figure 4.2d) and $Cu^{2+}$ ions (figure 4.2e) are given, which were obtained under identical experimental conditions. Additionally, the most abundant fragment ions are summarized and annotated in table 4.1.




b)
[M+Ni]2+
×10
'y7+•
-(NH3+H2O)
-CH2S
-NH3
-(NH3+HS•)
-H•
[M+Ni]+•
'c5+
'y6+•
b'6+•-NH3
'y8+•-CH2S
b'8+•
'y8+•
z8+
c8+
'b5+•
a'6+•
-S
b'6+•
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500
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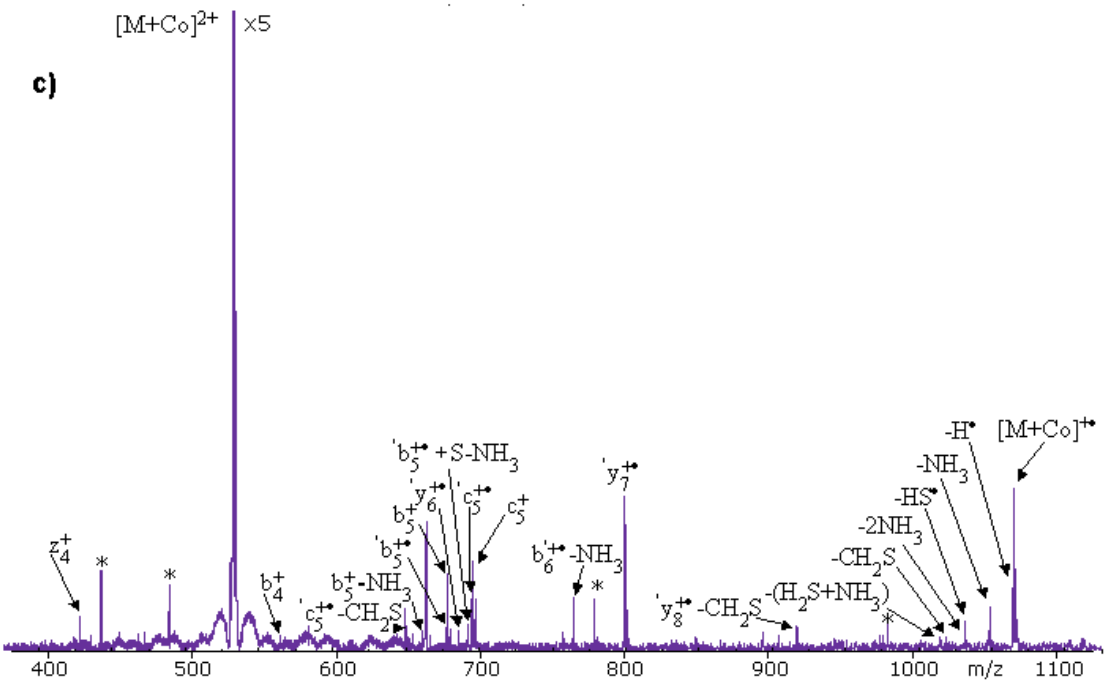
700
800
900
1000
m/z
1100


c)
[M+Co]2+
×5
'b5+• +S-NH3
'y7+•
-H•
[M+Co]+•
-NH3
-HS•
-2NH3
-CH2S
-(H2S+NH3)
'y6+•
'c5+•
c5+
'b5+•
b'6+•-NH3
'y8+•-CH2S
z4+
b4+
b5+-NH3
'c5+•-CH2S
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700
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m/z
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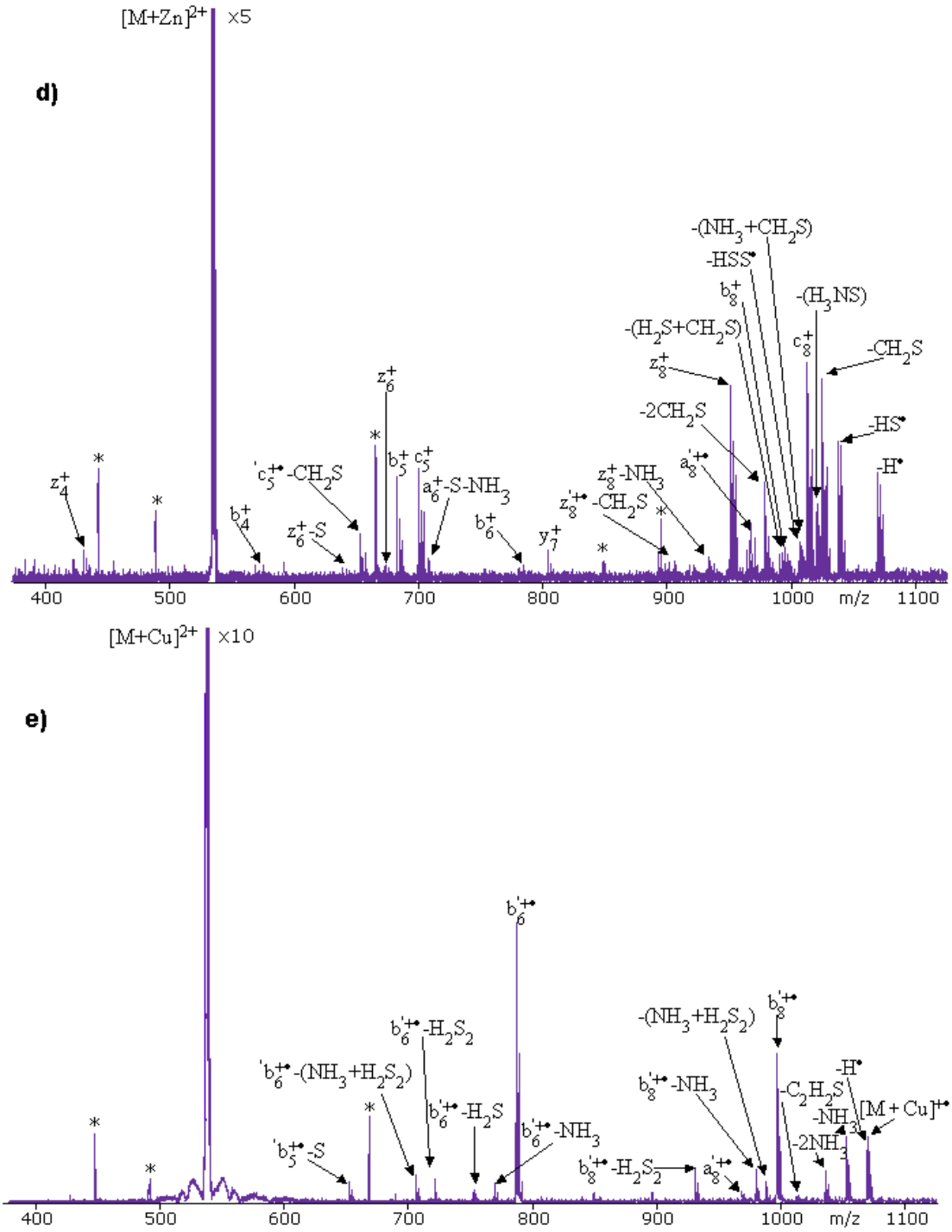


***Figure 4.2:*** *ECD spectra, taken under identical experimental conditions, of a) [oxytocin+2H]$^{2+}$, b) [oxytocin +Ni]$^{2+}$, c) [oxytocin +Co]$^{2+}$, d) [oxytocin +Zn]$^{2+}$ and e) [oxytocin +Cu]$^{2+}$ ions. The most abundant fragment ions are annotated and summarized in table 4.1. The peaks indicated with * originate from "noise" signals.*

| Parent ion | Product ion | %PII | Measured m/z | Calculated m/z |
|---|---|---|---|---|
| $[M+2H]^{2+}$ | $-H^{\bullet}$ | 15,7 | 1007,447 | 1007,445 |
| | $-SH^{\bullet}$ | 12,7 | 975,467 | 975,473 |
| | $b_6$ | 11,9 | 723,261 | 723,259 |
| | $-C_3H_7NS$ | 10,9 | 919,422 | 919,422 |
| | $-(2NH_3+SH^{\bullet})$ | 8,0 | 941,421 | 941,419 |
| | $b_8$ | 6,5 | 933,393 | 933,396 |
| | $SH^{\bullet}+NH_3$ | 5,6 | 958,467 | 958,446 |
| | $'z_7^{\bullet}-2NH_3$ | 5,4 | 691,292 | 691,300 |
| | $c_8$ | 4,6 | 950,445 | 950,423 |
| | $'z_5^{\bullet}-NH_3$ | 3,9 | 467,182 | 467,184 |
| $[M+Ni]^{2+}$ | $'y_7^{\bullet}$ | 38,8 | 798,306 | 798,300 |
| | $-NH_3$ | 10,5 | 1047,346 | 1047,345 |
| | $[M+Ni]^{+\bullet}$ | 9,2 | 1064,367 | 1064,372 |
| | $-(NH_3+H_2O)$ | 6,9 | 1029,354 | 1029,334 |
| | $-H^{\bullet}$ | 6,9 | 1063,386 | 1063,364 |
| | $'y_6^{\bullet}$ | 4,8 | 685,214 | 685,216 |
| | $b_8'^{\bullet}$ | 4,6 | 990,333 | 990,323 |
| | $'c_5^{\bullet}$ | 4,4 | 694,205 | 694,204 |
| | $-CH_2S$ | 2,5 | 1018,407 | 1018,384 |
| | $a_6'^{\bullet}$ | 2,2 | 752,189 | 752,191 |
| $[M+Co]^{2+}$ | $'y_7^{\bullet}$ | 23,0 | 799,302 | 799,297 |
| | $[M+Co]^{+\bullet}$ | 15,9 | 1065,353 | 1065,369 |
| | $c_5$ | 8,3 | 696,218 | 696,210 |
| | $-H^{\bullet}$ | 7,4 | 1064,384 | 1064,361 |
| | $b_5$ | 6,2 | 679,184 | 679,183 |
| | $'c_5^{\bullet}$ | 5,8 | 695,212 | 695,202 |
| | $NH_3$ | 5,4 | 1048,340 | 1048,342 |
| | $b_6'^{\bullet}-NH_3$ | 5,2 | 764,171 | 764,157 |
| | $'c_5^{\bullet}-CH_2S$ | 4,4 | 649,216 | 649,214 |
| | $'y_8^{\bullet}-CH_2S$ | 3,4 | 916,380 | 916,372 |
| $[M+Zn]^{2+}$ | $c_8$ | 14,3 | 1012,334 | 1012,336 |
| | $-CH_2S$ | 13,3 | 1024,369 | 1024,378 |
| | $z_8$ | 11,0 | 951,336 | 951,338 |
| | $-SH^{\bullet}$ | 10,5 | 1037,385 | 1037,386 |
| | $-H^{\bullet}$ | 7,6 | 1069,359 | 1069,358 |
| | $c_5$ | 6,6 | 701,209 | 701,206 |
| | $-2CH_2S$ | 5,3 | 978,386 | 978,381 |
| | $b_5$ | 5,1 | 684,183 | 684,179 |
| | $-H_3NS$ | 3,0 | 1021,368 | 1021,367 |
| | $a_8'^{\bullet}$ | 2,7 | 968,313 | 968,322 |

| Parent ion | Product ion | %PII | Measured m/z | Calculated m/z |
|---|---|---|---|---|
| $[M+Cu]^{2+}$ | $b_6'^{\bullet}$ | 23,0 | 785,187 | 785,181 |
| | $b_8'^{\bullet}$ | 15,9 | 995,315 | 995,318 |
| | $-H^{\bullet}$ | 8,3 | 1068,365 | 1068,359 |
| | $-NH_3$ | 7,4 | 1052,339 | 1052,340 |
| | $b_8'^{\bullet}-NH_3$ | 6,2 | 978,282 | 978,291 |
| | $b_8'^{\bullet}-H_2S_2$ | 5,8 | 929,351 | 929,358 |
| | $[M+Cu]^{+\bullet}$ | 5,4 | 1069,359 | 1069,367 |
| | $2NH_3$ | 5,2 | 1035,314 | 1035,313 |
| | $b_6'^{\bullet}-(H_3NS_2)$ | 4,4 | 704,217 | 704,210 |
| | $-(NH_3+H_2S_2)$ | 3,4 | 986,386 | 986,380 |

***Table 4.1:*** *Overview of most abundant fragment ions observed in the ECD spectra of doubly protonated oxytocin and oxytocin complexed to different transition metal ions. The m/z values in table 4.1 correspond to the first isotope of the mentioned ion species, all the peaks were isotopically resolved. PII is the abbreviation for product ion intensity, which is the sum of the intensities of all isotopic peaks of all product ions. For all the ECD product ions from oxytocin- transition metal ion complexes the same nomenclature was used. Therefore many ECD product ions from [oxytocin+$Cu^{2+}$] ions appear to be radical, whilst actually they are non-radical. When the mechanism proposed in figure 4.4 (on the right) occurs, the captured electrons are namely involved in $Cu^{2+}$ reduction instead of $H^{\bullet}$ formation elsewhere in the complex.*

In ECD of protonated peptides c and $z'^{\bullet}$ fragment ions are typically the most abundant backbone cleavages, with some minor $a'^{\bullet}$ and y fragment ions.[23-25] Commonly observed small neutral losses are $H^{\bullet}$ loss and $NH_3$ loss. In peptides containing disulfide bonds preferentially S-S and S-C bonds are cleaved, resulting in backbone cleavages near the disulfide bond.[6, 17, 26, 27]

The ECD spectra of doubly protonated oxytocin ions (figure 4.2a and table 4.1) displayed such typical ECD fragmentations. The electron capture induced backbone cleavages resulted in c and ‘$z^{\bullet}$ ions. Interestingly, also quite abundant b ions were observed, which are less often observed in ECD spectra of peptides. We hypothesize that the observed $b_n$ ions could also be $c_n-NH_3$ ions, as we observed abundant $NH_3$ losses. The most intense small neutral losses were $HS^{\bullet}$ loss, $H^{\bullet}$ loss, $C_3H_7NS$ loss and $NH_3$ loss. Overall about 60% of the product ion intensities could be attributed to fragment ions resulting from disulfide bond related characteristic ECD cleavages (fragmentation involving cleavage of at least one S-S or S-C bond), confirming previously reported findings that disulfide bonds preferentially attract the captured

electron (in the form of $H^{\bullet}$).[6, 16] Additional minor backbone cleavage sites were mainly located in the C-terminal part of oxytocin (figure 4.3a).

We reiterate, as observed previously by Wei et al,[28] that in all the studied oxytocin-transition metal ion complexes the metal ions formed complexes with oxytocin in a 1:1 ratio, without extra protonation or deprotonation giving [oxytocin+$M^{2+}$] ions. Most strikingly, as can be seen in figure 4.2 the ECD spectra of all studied oxytocin- transition metal ion complexes deviate not only from the ECD spectra obtained for the doubly protonated oxytocin, they also show distinctive variation depending on the particular transition metal ion. In the ECD spectra of the [oxytocin + $Ni^{2+}$] ions (figure 4.2b and table 4.1) radical ‘$y^{\bullet}$ ions, usually a minor ECD product, were the most abundant fragment ions. Minor backbone cleavages resulted in the formation of b’$^{\bullet}$, ‘$c^{\bullet}$ and a’$^{\bullet}$ ions. Abundant small neutral losses were $NH_3$, $H_2O$, $H^{\bullet}$ and $CH_2S$. Also minor $HS^{\bullet}$ loss was observed. Overall fragment ions resulting from disulfide bond related characteristic ECD cleavages accounted for about 60% of the product ion intensity. The most intense ‘$y_7^{+\bullet}$ fragment ion for instance, resulted from S-S cleavage and backbone amide bond cleavage between Tyr2 and Ile3 (see figure 4.3b). The identity of this ion was further confirmed with subsequent SORI-CID (data not shown).

The ECD fragment ions of [oxytocin + $Co^{2+}$] (figure 4.2c and table 4.1) were quite similar to the fragment ions observed for [oxytocin + $Ni^{2+}$] (figure 4.2b). The most intense small neutral losses were $H^{\bullet}$ loss, $NH_3$ loss and $CH_2S$ loss. Also minor $H_2S$ and $HS^{\bullet}$ losses were observed. Fragment ions resulting from disulfide bond related characteristic ECD cleavages accounted for approximately 65% of the product ion intensity. The main backbone cleavage sites in the [oxytocin + $Co^{2+}$] complex were the amide bond between Tyr2 and Ile3 and the amine bond in Cys6 (figure 4.3c).

The ECD fragment ions of [oxytocin + $Zn^{2+}$] (figure 4.2d and table 4.1) showed resemblance with the fragment ions observed for [oxytocin + $Co^{2+}$] (figure 4.2c). However, the most abundant backbone fragment ions were c and z ions and minor backbone fragment ions were b and a’$^{\bullet}$ ions. Abundant small neutral losses were $CH_2S$ loss, $HS^{\bullet}$ loss and $H^{\bullet}$ loss, but also less abundant $NH_3$, $H_2S$ and $HSS^{\bullet}$ losses were observed. Approximately 70% of the product ion intensity resulted from disulfide bond

related characteristic ECD cleavages. The main cleavage sites were the amine bonds in Tyr2, Cys6 and Gly9 (figure 4.3d).

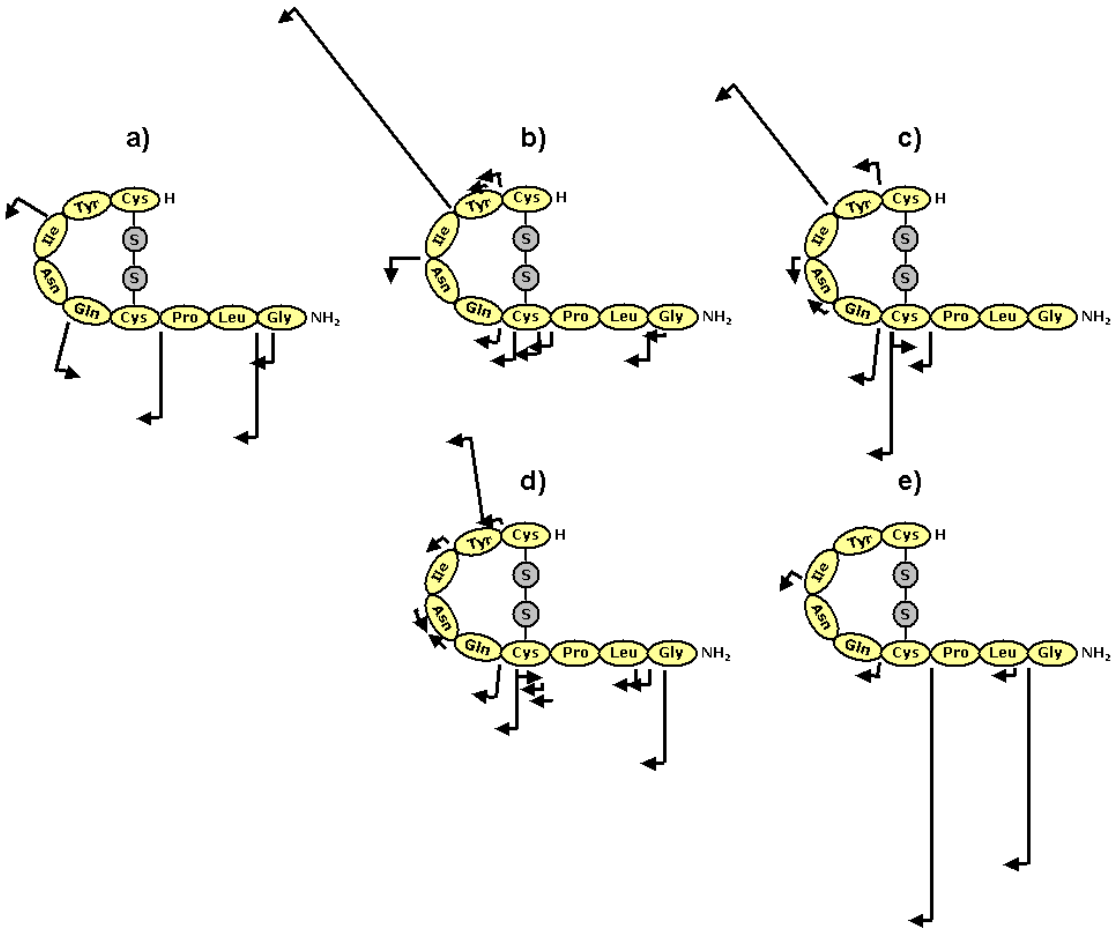


***Figure 4.3:*** *Schematic picture of the preferred localization of the electron capture induced backbone cleavages in a) [oxytocin+2H]$^{2+}$, b) [oxytocin +Ni]$^{2+}$, c) [oxytocin +Co]$^{2+}$, d) [oxytocin +Zn]$^{2+}$ and e) [oxytocin +Cu]$^{2+}$ ions.*

Summarizing the ECD results obtained for the oxytocin complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$, it can be stated that the specific transition metal ion bound plays an important role in the fragment ions formed, as the spectra of the three complexes are strikingly different (see table 4.1). However, the ECD spectra of the oxytocin complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ have in common that fragment ions resulting from disulfide bond related characteristic ECD cleavages are remarkably abundant. Furthermore, abundant $H^{\bullet}$ losses were observed from the reduced oxytocin- transition metal ion complexes. Together with the observed backbone cleavages we could therefore conclude that the oxytocin-complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ display quite typical ECD behavior.

In contrast, we observe that the ECD behavior of the [oxytocin + $Cu^{2+}$] is rather different (figure 4.2e and table 4.1). In the ECD spectra of [oxytocin + $Cu^{2+}$] $b'^{\bullet}$ ions are the most abundant fragment ions. Remarkably, no ECD characteristic c or $z'^{\bullet}$ fragment ions were formed, although electron capture was observed. $H^{\bullet}$ loss was the only observed radical loss. Fragment ions resulting from disulfide bond related characteristic ECD cleavages accounted for only 15% of the product ion intensity. This in clear contrast to the other studied oxytocin- transition metal ion complexes, where such ions accounted for 60-70% of the fragment ion intensity. The fragment ions observed in the ECD spectra of [oxytocin + $Cu^{2+}$] ions are thus rather atypical, and resemble more those typically observed in low-energy CID. In peptides low-energy CID mainly leads to the formation of backbone b and y fragments. For the [oxytocin + $Cu^{2+}$] complex the most favored backbone cleavage sites were the amide bonds between Cys6/Pro7 and Leu8/Gly9 (figure 4.3e).

## 4.5. Discussion

In this study we examined the ECD behavior of doubly protonated oxytocin and oxytocin complexed with $Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ ions. Many of these transition metal ions play a supporting role in the oxytocin-receptor binding.[19] Oxytocin is a small nonapeptide, which contains a disulfide bond in between two cysteines at positions 1 and 6 (see figure 4.1). Therefore, we hypothesized that this molecule is rather rigid, which may be even enhanced by transition metal ion binding. Surprisingly, we observed that the ECD spectra of all the four studied oxytocin- transition metal ion complexes were remarkably different, not only from the ECD spectra obtained for the doubly protonated oxytocin, but also from each other. These large differences in ECD behavior were *a priori* not expected for such a small, seemingly rigid molecule. We seek an explanation for the observed behavior in the location and the identity of the charge carrier, i.e. either the two protons or the transition metal ion. The transition metal ions all have a different electronic configuration, which may lead to different coordination towards oxytocin, resulting also in slightly different solution- and gas-phase structures. Additionally, the electron capture process may be different for the studied species, as the nature of the transition metal ion will have an effect on the released recombination energy. Moreover, the extent of charge localization/delocalization (particularly of the captured electron),

may be different for the four oxytocin- transition metal ion complexes. Below we discuss the above-mentioned factors that may play a role in the observed dissimilar ECD behavior in more detail. First we focus on the similarities observed in the ECD processes.

From experimental as well as theoretical studies [6, 17, 26, 27] it is known that S-S and S-C bonds are preferentially cleaved when ECD is performed on peptides containing disulfide bonds. Therefore, we took the abundance of S-S and S-C bond cleavages, as well as the in ECD frequently observed $H^{\bullet}$ loss and typical backbone cleavages (c/z'$^{\bullet}$ and a'$^{\bullet}$/y)[24], as a measure for "standard" ECD behavior. In ECD of doubly protonated oxytocin a high prevalence for S-S and S-C bond cleavage was observed. Fragment ions resulting from cleavage of either an S-S or an S-C bond, or both, accounted for approximately 60% of the product ion intensity. The loss of $HS^{\bullet}$ for instance, which requires the cleavage of both an S-S and an S-C bond, was very intense. Also most backbone cleavages, observed in ECD of doubly protonated oxytocin were typical for ECD, namely c and 'z$^{\bullet}$ ions. We also observed some b ions, which are typically not observed in ECD spectra of peptides. We assume, however, that the $b_n$ ions are in fact $c_n$-$NH_3$ ions. The formation of $b_6$ ions is a special case, because the formation of $c_6$ ions would require N-terminal cleavage in a proline residue. Usually N-terminal proline cleavages are not observed in ECD[5, 29] However, electron capture can lead to a free radical reaction cascade[30] and c/z'$^{\bullet}$ fragmentation through the proline residue has been observed.[31] As abundant secondary cleavages were observed for the doubly protonated oxytocin ions the $b_6$ ions could indeed be $c_6$-$NH_3$ ions. Overall the obtained ECD behavior for doubly protonated oxytocin ions is quite standard and can be used to put the ECD results obtained for the oxytocin-transition metal ion complexes into perspective.

**The oxytocin + $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ complexes display characteristic ECD behavior.** Similar as in ECD of the doubly protonated ions many fragment ions in ECD of the oxytocin complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ resulted from disulfide bond related characteristic ECD cleavages. Other typical ECD backbone fragmentations were observed ranging from mainly radical 'y$^{\bullet}$ fragments for [oxytocin + $Ni^{2+}$] ions to mainly c and z fragments for [oxytocin + $Zn^{2+}$] ions. The ECD spectra of [oxytocin + $Co^{2+}$] ions

displayed features of both [oxytocin + $Ni^{2+}$] and [oxytocin + $Zn^{2+}$] ions, with mainly 'y$^{\bullet}$ c and 'c$^{\bullet}$ backbone fragments. Generally, the ECD behavior of the oxytocin complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ leads to very specific preferences (see figure 4.2 and table 4.1) in fragmentations, albeit that they are all characteristic for ECD of disulfide containing peptide ions. An interesting question is now how does electron capture take place in peptide- metal ion complexes? It has been proposed before that in peptide- metal ion complexes the metal ion serves as an initial sink for electrons (positive charge attracts negatively charged electrons). However, the electron may be subsequently transferred from the original capture site.[32] We indeed rationalize in our ECD experiments on the oxytocin complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ the dominant characteristic ECD behavior by the occurrence of subsequent electron transfer from the metal ion to the remainder of the complex. In line with this, H$^{\bullet}$ loss, which is common in ECD,[5, 6, 33, 34] was observed from all reduced oxytocin- transition metal ion complexes, that are thus able to transfer their captured electron to a proton. Because there are no extra protons in the transition metal ion complexes of oxytocin, free H$^{\bullet}$ radicals must have been formed in the complex together with a negatively charged deprotonated oxytocin bound to the doubly charged transition metal ion (as outlined in figure 4.4 on the left). It has been well-established that such free H$^{\bullet}$ radicals have a high affinity for disulfide bonds.[6] Transfer of the free H$^{\bullet}$ radicals to the disulfide bond may thus lead to the characteristic ECD behavior observed. The [oxytocin + $Co^{2+}$] ions contain an odd number of electrons. Therefore, after electron capture an even-electron system is formed, that still displays ECD behavior! This can be explained by assuming that the [oxytocin + $Co^{2+}$] complex that captures an electron actually contains two unpaired electrons. One electron is acting as an unpaired $d^7$ cobalt ion electron. The other electron is triggering the ECD cleavages in a manner described above.

**The extraordinary oxytocin + $Cu^{2+}$ complex.** ECD of [oxytocin + $Cu^{2+}$] ions resulted in remarkably different fragmentation reactions. No c or z'$^{\bullet}$ ions were observed, as backbone cleavages almost exclusively led to the formation b'$^{\bullet}$ ions, which are atypical for ECD. Due to the complete absence of $c_n$'$^{\bullet}$ ions in the ECD spectra of [oxytocin + $Cu^{2+}$] ions we do not believe that the $b_n$'$^{\bullet}$ ions are in fact $c_n$'$^{\bullet}$-$NH_3$ ions, as was proposed for doubly protonated oxytocin ions. The disulfide bond related characteristic ECD cleavages contributed only about 15% to the total product ion intensity (see table 4.1),

whereas this number was close to 65 % for the oxytocin complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$. Evidently, the ECD process in the [oxytocin + $Cu^{2+}$] complex follows a different mechanism. We argue that the electron captured by the [oxytocin + $Cu^{2+}$] complex hardly participates in ECD processes. The electron is initially captured by the $Cu^{2+}$ to reduce it to $Cu^{+}$, i.e. the electron remains localized at the metal. A preferred use of the captured electron for reduction of the metal over transfer to the peptide can be explained by looking at the electronic structure of $Cu^{+}$. $Cu^{+}$ is an electronically stable ion, with a closed d shell, in contrast to $Ni^{+}$, $Co^{+}$ and $Zn^{+}$. If electron capture leads to the reduction of $Cu^{2+}$ ions, excited $Cu^{+}$ ions would be formed (see figure 4.4 on the right). Not the electron but the available recombination energy is subsequently transferred to the oxytocin peptide, leading to the breaking of the weakest peptide bonds, similar to those observed in low-energy CID. Although, there are not that many ECD studies on peptide- transition metal ion complexes the observed typical behavior observed here for the $Cu^{2+}$ complex is not unprecedented. For instance, Kellersberger *et al.*[31] reported a relatively low abundance of electron capture induced fragmentation for the $Cu^{2+}$ complex of a calmodulin EF-hand 1 peptide, when compared to the similar complexes with $Ca^{2+}$ and $Zn^{2+}$. Related, Zubarev *et al.*[35] reported in an ECD study on cytochrome c ions that the region around the heme group (containing $Fe^{3+}$) remained relatively immune to c/z'$^{\bullet}$ cleavage, when compared to stretches in the protein further away from the heme. Similarly, the electron captured near the iron in cytochrome c could be involved in reducing $Fe^{3+}$ to $Fe^{2+}$ not triggering typical ECD cleavages. Although, the ECD behavior of the [oxytocin + $Cu^{2+}$] complex is strikingly different from the other studied ions, the reduced [oxytocin + $Cu^{2+}$] ions did show to a minor extent $H^{\bullet}$ loss. This means that also in the [oxytocin + $Cu^{2+}$] complex the alternative mechanism can still play a role (figure 4.4 on the left). We suggest that metal ion reduction and electron transfer to the peptide are competing processes. In complexes where reduction of the metal ion leads to the formation of another stable metal ion, such as in the case of $Cu^{2+}$ and $Fe^{3+}$ ions, reduction of the metal ion can be the dominant process.

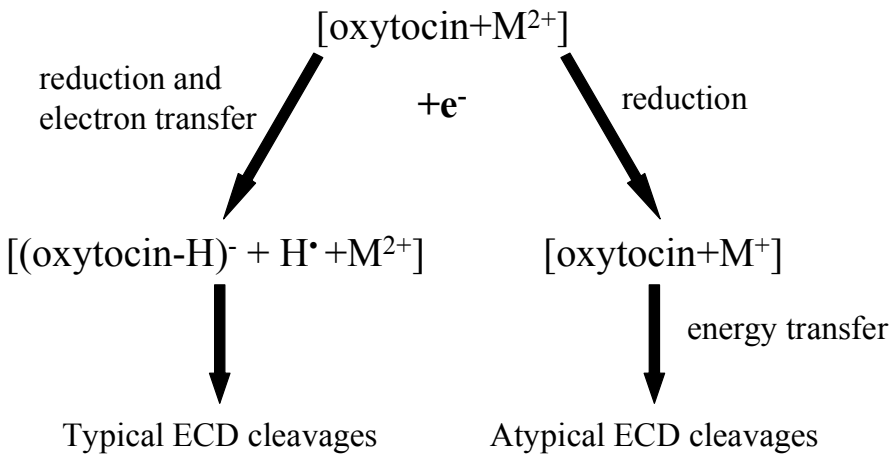


***Figure 4.4:*** *Proposed mechanisms for the electron capture process in oxytocin-transition metal ion complexes. Capture of an electron can either lead to reduction of the transition metal ion (on the right), where after energy transfer to the oxytocin peptide induces low-energy peptide bond cleavages, similar to those observed in low energy CID. Alternatively, electron capture by the transition metal ion is followed by electron transfer to the oxytocin moiety where it induces the formation a free hydrogen radical in complex with the formally negatively charged deprotonated oxytocin and the doubly charged transition metal ion (on the left). The free hydrogen radical, initiates the observed characteristic ECD cleavages, preferentially by attacking the disulfide bond present in oxytocin.*

**Structural and electronic configuration effects.** Above we focused on the abnormal behavior observed in the ECD fragmentation of the [oxytocin + $Cu^{2+}$] complex, and discussed the similarities observed in the ECD spectra of the doubly protonated oxytocin and the oxytocin complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$. These similarities are mainly in the ECD characteristic nature of the fragment ions. As can be seen in figure 4.2 and table 4.1, the ECD spectra are strikingly different when we consider the specificity of cleavages. It has been stated that ECD can be used to localize charge carriers in ions. For instance, Kellersberger *et al.*[36] reported that metal chelation during ECD is conserved, as evidenced by the fact that transition metal ion complexes of calmodulin EF-hand 1 with $Ca^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ displayed extensive fragmentations in residues involved in metal coordination. Therefore, the remarkable differences in backbone cleavage sites we observe for the oxytocin- transition metal ion complexes could point at structural differences, likely linked to the different potentiating effects these transition metal ions have on the binding of oxytocin to its receptor.[19] The earlier mentioned study by Seuthe *et al.*[20] on the structure of the gas-phase oxytocin complex with $Zn^{2+}$ suggests that this complex adopts a helical shape, in which the backbone

oxygen atoms of six residues (Tyr2, Ile3, Gln4, Cys6, Leu8, Gly9) solvate the $Zn^{2+}$ ion in an octahedral coordination. This means that the $Zn^{2+}$ ion is tightly embedded in the structure of oxytocin. As illustrated in figure 4.3e the [oxytocin + $Zn^{2+}$] ions display preferred cleavage sites at the amine bonds of Gly9, Cys6 and Tyr2. These residues are all involved in coordinating $Zn^{2+}$. Furthermore no c/z'$^{\bullet}$ cleavages were observed in amino acids that are not involved in coordinating the $Zn^{2+}$ ion. With these observations it seems that ECD reflects the coordination of the metal ion in the oxytocin- $Zn^{2+}$ complex. Without further knowledge of the three-dimensional structures of the complexes of oxytocin with $Ni^{2+}$, $Co^{2+}$and $Cu^{2+}$, however, we can only speculate whether we can extrapolate this observation to the other complexes. It is, however, likely that the coordination of the metal ion, and thus the interactions of the metal ion with specific residues of oxytocin, is dissimilar for the different transition metal ion complexes. To illustrate this further we discuss finally the possible effect of the reduction of the $Cu^{2+}$ ion to $Cu^{+}$ ion on the coordination and binding to the oxytocin. Normally, $Cu^{2+}$ forms highly distorted octahedral $d^9$ complexes. The unpaired electron can either occupy the $d_x2\text{-}_y2$ or the $d_z2$ orbital. Distortion to square-planar coordination, with paired electrons in the $d_z2$ orbital removes the degeneracy and lowers the energy of the complex. This can be regarded as an extreme case of the Jahn-Teller effect. When, however, $Cu^{+}$ is formed the electronic configuration becomes $d^{10}$ for which the preferred coordination is octahedral. In some cases also trigonal or digonal coordination is observed for $Cu^{+}$ ions.[37] It has been reported that reduction of the $Cu^{2+}$ ion in a complex with a protein to $Cu^{+}$ leads to significant structural changes.[38] Similarly, the specific electronic configurations of the used transition metal ions will have a distinct effect on the coordination of the metal ion by the oxytocin. We speculate that this may largely explain the strikingly different ECD behavior reported here. High-level theoretical calculations on the gas-phase structures of the initial and reduced oxytocin- transition metal ion complexes would be needed to validate our proposal.

### 4.6. Conclusion

Oxytocin complexes with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ showed typical ECD behavior with disulfide bond related characteristic ECD cleavages accounting for 60-70% of the product ion intensity. In contrast, the oxytocin-$Cu^{2+}$ complex showed mainly low-energy CID type cleavages. Disulfide bond related characteristic ECD cleavages accounted for

only 15% of the product ion intensity. Therefore we propose that in this complex the captured electron is more favorably involved in reduction of the $Cu^{2+}$ ion to $Cu^{+}$. Subsequent transfer of the recombination energy, instead of electron transfer, would explain the atypical ECD cleavages we observed. All the oxytocin- transition metal ion complexes showed $H^{\bullet}$ loss, indicating that the captured electron can be transferred to a proton. Due to the absence of free protons in the complexes this must be accompanied by the formation of a negatively charged site. The remarkable differences in the observed backbone cleavage sites could point at structural differences between the oxytocin- transition metal ion complexes.

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Summary

# Summary

In the classical biochemical approach to sequence peptides and proteins Edman degradation is used,[1] especially for linear peptides. The sequence analysis of more complicated peptides such as those containing thioether or disulfide bonds however is problematic.[2] The Edman degradation reaction stops when thioether or disulfide bonds are encountered, hampering further identification of the peptide sequence and other structural elements. Nowadays, tandem mass spectrometry has become the tool of choice in peptide sequencing. Therefore, peptides are first dissolved in matrix or aqueous solution and then ionized with Matrix Assisted Laser Desorption Ionization (MALDI) or ElectroSpray Ionization (ESI). Peptide ions of interest can be isolated and subjected to tandem MS to generate sequence information. The most common fragmentation method, Collision Induced Dissociation (CID), often fails to specifically cleave thioether and disulfide bonds.[3, 4, 5, 6] Therefore in both methods, Edman degradation and CID tandem MS, thioether and disulfide bonds have to be reduced and alkylated prior to analysis, which is time-consuming and reduces the sensitivity. Within the MS field another technique for sequence analysis of peptides has arisen, namely Electron Capture Dissociation (ECD), which for instance does result in specific cleavage of disulfide bonds in peptides and proteins.[3, 7, 8, 9, 10] To investigate if this specificity could be expanded to thioether bonds, lantibiotics were subjected to ECD.

Lantibiotics are bacterial antibiotic peptides that contain intramolecular thioether bonds or so-called lanthionine bridges. This class of compounds is particularly interesting, because lantibiotics have the potential to become a new generation of antibiotics.[11] We used ECD[12] as a new method to sequence lantibiotics and specifically localize their thioether bonds without the need for derivatization. ECD experiments were performed on four different lantibiotics: nisin A, nisin Z, mersacidin and lacticin 481. The ECD fragmentation was compared to the fragment ions obtained with the more traditional CID. ECD of lantibiotics produced mainly c and $z^{\bullet}$ ions[13, 14] resulting from backbone amine bond cleavages, as has been observed previously for other peptides.[12] In general, cleavages mainly took place near the location of lanthionine bridges. More interestingly, less common $c^{\bullet}$ and z ions were observed to be abundant in the ECD spectra. These fragment ions specifically resulted from the cleavage of both a backbone

amine bond and a thioether (S-C) bond in a lanthionine bridge. A new mechanism was postulated describing the formation of $c^{\bullet}$ and z ions. The $c^{\bullet}$/z cleavages mainly took place in amino acids attached to the C-terminal side of a lanthionine bridge and could thus be used as a tool to localize the C-terminal attachment site of lanthionine bridges to the peptide backbone. The ECD fragmentation pattern indicated that lanthionine bridges are selectively cleaved in the ECD process. Lacticin 481 was the most difficult lantibiotic to fragment using ECD, there were no $c^{\bullet}$ radical ions and z ions present in the ECD spectra. Lacticin 481 contains 3 lanthionine bridges for which no specific cleavages were observed. This was attributed to the fact that lacticin 481 contains several regions held together by several lanthionine bridges, so that sometimes three cleavages are required for complete dissociation. Comparative low-energy CID did not reveal the specificity for lanthionine bridge cleavages as observed for ECD. Globally the CID fragmentation patterns of the lantibiotics used in this study were quite similar. Another striking observation was the complementarity of the two tandem MS techniques, ECD and CID. The sequence coverage for nisin A with low-energy-CID was 38% and with ECD 59%, but when the cleavage sites of the two techniques were combined a sequence coverage of 74% was obtained. Therefore the combined use of CID and ECD is strongly recommended in the analysis of lantibiotics.

The ECD studies of lantibiotics in chapter 2 pointed out that abundant product ions were formed that even captured several electrons without subsequent dissociation. These reduced species are common in ECD of peptides and proteins,[15] but were remarkably intense in the case of lantibiotics. This was attributed to the presence of lanthionine bridges in the lantibiotics. Especially lacticin 481 exhibited highly abundant non-dissociative reduced species. In chapter 3 we studied these species of lacticin 481 in more detail, especially the ions that captured two electrons without subsequent dissociation. The main question was: will the capture of two electrons by protonated lacticin 481 ions result in the formation of stable biradicals or is radical recombination the major process? Therefore Sustained Off Resonance Irradiation (SORI) CID spectra of $[M+4H]^{2+}$ ions ($[M+4H]^{4+}$ ions that captured two electrons) were compared with those of regularly protonated $[M+3H]^{3+}$ ions and radical $[M+4H]^{3+\bullet}$ ions. The obtained results indicated that recombination of the two radical sites is the dominant process in lacticin 481 $[M+4H]^{2+}$ ions, at least on the timescale of our SORI-CID experiments. However,

from the lacticin 481 ions that captured two electrons also minor $H^{\bullet}$ loss was observed leading to the formation of radical $[M+3H]^{2+\bullet}$ ions. A small part of the ions formed by double electron capture were thus long-lived $[M+4H]^{2+2\bullet}$ biradicals. Another interesting observation was the abundant $SH^{\bullet}$ loss from lacticin 481 $[M+4H]^{3+\bullet}$ ions, indicating that radical sites were favorably located near lanthionine bridges.

Native structures of peptides and proteins are often influenced by co-factors, including metal ions. This is the case for the biologically important small peptide hormone oxytocin, mostly known for its ability to elicit the contraction of smooth muscle in the uterus during labor.[16] The presence of a specific divalent transition metal ion dramatically enhances binding of oxytocin to its cellular receptor.[16] Oxytocin binding to its receptor is potentiated in increasing order by $Zn^{2+}$, $Ni^{2+}$ and $Co^{2+}$, but is negligible in the presence of $Cu^{2+}$.[17] In chapter 4 doubly protonated oxytocin ions and oxytocin complexes with different transition metal ions were subjected to ECD to probe their structural features. It is well established that with ECD part of the precursor ions can undergo very fast fragmentation reactions, even before the energy gained by electron capture is fully randomized.[18] Furthermore, oxytocin contains a disulfide bond between Cys1 and Cys6, which should be selectively cleaved with ECD. We assumed that the transition metal ions act as the initial electron capture site.[19] In principle fast ECD cleavages in the transition oxytocin-metal complexes would then reflect the location and/or coordination of the metal ion in the complex.

The ECD spectra of doubly protonated oxytocin ions displayed typical ECD fragmentations. The electron capture induced backbone cleavages resulted in the formation of b, c and ‘$z^{\bullet}$ ions. Overall about 60% of the product ion intensity could be attributed to disulfide bond related characteristic ECD cleavages (fragmentation involving the cleavage of at least one S-S or S-C bond). Although all the ECD spectra were strikingly different, typical ECD behavior was observed for complexes of oxytocin with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$. Overall abundant disulfide bond related characteristic ECD cleavages were observed, accounting for about 60-70% of the product ion intensity. We proposed that, although the metal ions serve as the main initial capture site in the oxytocin- transition metal ion complexes, the captured electron is transferred to other sites in the complex to form a hydrogen radical, which drives the subsequent typical

ECD fragmentations. In contrast, the complex of oxytocin with $Cu^{2+}$ displayed noticeably different ECD behavior. The formed fragment ions were similar to fragment ions typically observed with low-energy CID. Remarkably, no ECD characteristic c or z'$^{\bullet}$ fragment ions were observed. Overall disulfide bond related cleavages accounted for only 15% of the product ion intensity. This was in clear contrast to what was observed for the other studied oxytocin species. We proposed that the electrons captured by the oxytocin- $Cu^{2+}$ complex might be favorably involved in reducing the $Cu^{2+}$ metal ion to $Cu^{+}$. Subsequent energy redistribution would explain the observed low-energy CID-type fragmentations.

Finally, some general conclusions can be drawn from the results that were presented in this thesis regarding structural ECD studies on peptides. It is important to stress again that with ECD high sequence coverage can be obtained for peptides and proteins, because of its low c/z$^{\bullet}$ cleavage selectivity for different interresidue bonds. Especially in combination with complementary CID or infrared multiphoton dissociation (IRMPD) and high resolution Fourier transform ion cyclotron resonance (FTICR) MS, ECD could be the most powerful technique for high-throughput identification and *de novo* sequencing of peptides and proteins. Proteins that still have secondary and tertiary structure in the gas phase often display little fragmentation in ECD, presumably because intramolecular non-covalent bonds prevent separation of the cleavage products.[15] To obtain a high sequence coverage in these cases ECD should again be combined with CID or IRMPD, a technique known as activated ion (AI) ECD,[15] to activate the ions and break remaining non-covalent interactions. Furthermore ECD offers perspectives in the localization of posttranslational modifications on peptides. This had already been illustrated for modifications such as glycosylation, phosphorylation and disulfide bridges; in this thesis it was shown that lanthionine bridges could be localized with ECD. In general, bonds near sulfur atoms, whether in the form of a thioether bond, like in lantibiotics, or in the form of a disulfide bond, such as in oxytocin, are preferentially cleaved in ECD. These sulfide bond cleavages often are accompanied with peptide backbone cleavages. Therefore the application of this novel tandem MS technique should always be considered in the structural analysis of peptides, especially when they contain thioether or disulfide bonds.

The selectivity of ECD for thioether and disulfide bonds indicates that, after the electron capture event, radical sites favorably relocate to sites that are in close proximity to thioether and disulfide bonds in peptides. Another observation that supports this conclusion is the abundant $SH^{\bullet}$ loss from collisionally activated protonated lacticin 481 ions that captured one electron. Thioether and disulfide bonds thus act as a "radical trap," probably as a result of their high $H^{\bullet}$ affinity, preventing the occurrence of secondary cleavages. These are required to obtain fragmentation in cyclic regions. Therefore $MS^3$ could be used, for instance an additional SORI-CID event, to break a sufficient number of bonds. The preferred collision induced loss of $SH^{\bullet}$ for lacticin 481 radical ions however indicates that not in all cases additional structural information is gathered with ECD-SORI-CID studies compared to regular ECD experiments. Introducing a second ECD event to break additional bonds is not recommended. When two electrons were captured by protonated lacticin 481 ions the dominant process seemed to be recombination of radical sites, which would lead to structural changes in the ions. Therefore it should be considered instead to (partially) digest peptides, specifically in the cyclic regions, before performing ECD. In this way the structural changes will be known and the susceptibility of these peptides to fragmentation will be improved.

Although thioether and disulfide bonds can act as radical traps, often secondary cleavages were observed in the sulfide containing peptides that were studied in this thesis. This indicates that ECD is not a single process and not purely non-ergodic. Primary cleavages, for instance those resulting in the formation of c and $z^{\bullet}$ ions or direct side chain losses, could still be very fast reactions that take place before intramolecular vibrational energy redistribution. Secondary cleavages, however, seem to involve free radical rearrangements[20] and could explain the observed multiple cleavages after the capture of only one electron.

Similar to protonated oxytocin ions, transition metal ion complexes of oxytocin with $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ displayed typical ECD behavior, expressed by $c/z^{\bullet}$ cleavages, disulfide bond related cleavages and $H^{\bullet}$ loss. These results suggest that the ECD mechanism in these species is comparable to that in protonated species and that hydrogen radicals also play a major role in ECD processes in transition metal ion complexes of peptides.

Summary

This observation opens up perspectives for the application of ECD in tandem MS structural analysis of metallopeptides and -proteins as an alternative to the more commonly used CID. The complex of oxytocin with $Cu^{2+}$ displayed atypical ECD behavior. The low-energy CID type of cleavages observed after electron capture indicate that the captured electron is rather involved in reduction of $Cu^{2+}$ to $Cu^{+}$ instead of $H^{\bullet}$ radical formation. This process could also take place in peptide or protein complexes with other metal ions, such as $Fe^{3+}$ that could form stable $Fe^{2+}$ ions after electron capture. The identity of the metal ion should be taken into account when considering the analysis of metal-complexes with ECD as electron capture will not always lead to the desired typical ECD fragmentation.

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# Samenvatting

Om de aminozuurvolgorde (sequentie) van peptiden en eiwitten te bepalen wordt in de biochemie van oudsher Edman degradatie gebruikt, vooral voor lineaire peptiden. De sequentiebepaling van complexere peptiden, die bijvoorbeeld thioether- en disulfidebindingen bevatten, is echter problematisch. De Edman degradatie reactie stopt zodra thioether- of disulfidebindingen worden bereikt, waardoor de verdere identificatie van de peptidesequentie en andere structuurelementen verhinderd wordt. Tegenwoordig wordt vaak voor tandem massaspectrometrie gekozen bij de sequentiebepaling van peptiden. Daarvoor worden peptiden eerst opgelost in matrix of waterige oplossing en dan geïoniseerd met behulp van matrix geassisteerde laser desorptie/ionisatie (MALDI) of electrospray ionisatie (ESI). Peptide-ionen kunnen daarna worden geïsoleerd en geanalyseerd met tandem MS, zodat sequentie-informatie gegenereerd wordt. De meest algemene tandem MS techniek, botsingsgeïnduceerde dissociatie (CID), levert net als Edman degradatie weinig specifieke structuurinformatie op over thioether- en disulfidebindingen. Daarom worden de thioether- en disulfidebindingen voor de analyse vaak gereduceerd en gealkyleerd, hetgeen tijdrovend is en de gevoeligheid verlaagt. De laatste jaren is er een nieuwe tandem MS techniek opgekomen om peptidesequenties te analyseren, namelijk ECD. Bij deze techniek worden langzame elektronen gevangen door meervoudig geladen kationen, hetgeen zeer snelle fragmentatiereacties oplevert. Met ECD kunnen disulfidebindingen in peptiden en eiwitten specifiek gebroken worden. Om te onderzoeken of dit ook geldt voor thioetherbindingen werd ECD toegepast op lantibiotica.

Lantibiotica zijn bacteriële, antibiotische peptiden die thioetherbindingen bevatten, ook wel lanthioninebruggen genoemd. Deze klasse van verbindingen is zeer interessant, omdat lantibiotica potentie hebben om uit te groeien tot een nieuwe generatie antibiotica. Wij hebben ECD gebruikt als een nieuwe methode om de sequentie van lantibiotica te bepalen en hun thioetherbindingen specifiek te localiseren zonder ze te derivatiseren. ECD experimenten werden uitgevoerd met vier lantibiotica: nisine A, nisine Z, mersacidine en lacticine 481. De ECD fragmenten werden vergeleken met de fragmentionen die werden verkregen met CID. Zoals dat al eerder werd waargenomen bij andere peptiden, resulteerde ECD van lantibiotica voornamelijk in de vorming van c

en $z^{\bullet}$ ionen, door het breken van aminebindingen in de peptidehoofdketen. Veel gebroken bindingen bevonden zich dichtbij de lanthionine bruggen. Interessanter is dat ook minder algemene $c^{\bullet}$ en z ionen in hoge intensiteit werden waargenomen in de ECD spectra. Deze fragmentionen waren specifiek het resultaat van het breken van een aminebinding in de hoofdketen en een thioetherbinding (S-C) in een lanthioninebrug. Er werd een nieuw mechanisme voorgesteld dat de vorming van deze $c^{\bullet}$ en z ionen beschrijft. De $c^{\bullet}$ en z ionen werden voornamelijk gevormd door fragmentaties in aminozuren die verbonden waren aan de C-terminale kant van lanthioninebruggen en konden dus gebruikt worden om de locaties van C-terminale verbindingspunten van lanthioninebruggen aan de peptidehoofdketen te bepalen. Het ECD fragmentatiepatroon wees erop dat lanthioninebruggen een sleutelrol vervullen in het ECD proces. Lacticine 481 was het moeilijkst te fragmenteren lantibioticum, er werden geen $c^{\bullet}$ en z ionen aangetroffen in de ECD spectra. Lacticine 481 bevat 3 lanthioninebruggen, waarvan geen enkele specifiek werd gebroken. Een oorzaak hiervoor zou kunnen zijn dat lacticine 481 verschillende gedeelten bevat waar 2 lanthioninebruggen overlappen, zodat soms 3 bindingen verbroken moeten worden om fragmentionen te verkrijgen. Vergelijkende CID experimenten lieten niet dezelfde specificiteit als ECD zien voor het breken van thioetherbindingen. Een andere belangrijke observatie in deze studie was dat de twee gebruikte tandem MS technieken, ECD en CID, complementair zijn. Nisine A is een goed voorbeeld: met lage-energie CID werd 38% van de bindingen tussen aminozuren verbroken, terwijl dat met ECD 59% was. Als de ECD en CID fragmentaties gecombineerd worden, is de dekkingsgraad 74%. Daarom wordt het gecombineerde gebruik van CID en ECD sterk aangeraden bij de analyse van lantibiotica.

De ECD studies van lantibiotica in hoofdstuk 2 lieten zien dat intense productionen werden gevormd die wel één of meerdere elektronen hadden gevangen, maar daarna niet waren gefragmenteerd. Deze “gereduceerde” ionen zijn algemeen bij ECD van peptiden en eiwitten, maar waren opvallend intens bij lantibiotica. De aanwezigheid van lanthioninebruggen in de lantibiotica zou hiervoor een verklaring kunnen zijn. Vooral bij lacticine 481 waren de niet-gefragmenteerde, gereduceerde ionen zeer intens. In hoofdstuk 3 bestudeerden we deze ionsoorten van lacticine 481 gedetailleerder, vooral ionen die 2 elektronen hadden gevangen zonder te fragmenteren. De belangrijkste

vraag was: leidt de vangst van twee elektronen door geprotoneerde lacticine 481 ionen tot de vorming van stabiele biradicalen of wordt recombinatie van de radicalen geprefereerd? Daarom werden $[M+4H]^{2+}$ ionen ($[M+4H]^{4+}$ ionen, die 2 elektronen hebben gevangen), $[M+3H]^{3+}$ ionen en radicale $[M+4H]^{3+\bullet}$ ionen geactiveerd met SORI-CID. Hierbij worden ionen langdurig afwisselend geëxiteerd en gedeëxiteerd door een frequentie op de exitatieplaten toe te passen, die 1000-1500 Hz naast de resonantiefrequentie van de ionen zit. Door tegelijkertijd gas in de cel te pulsen, worden de ionen geactiveerd door de vele botsingen met gasatomen. De verkregen resultaten wijzen erop dat de twee radicale sites in lacticine 481 $[M+4H]^{2+}$ ionen recombineren, op de tijdschaal van onze SORI-CID experimenten. Er werd echter ook een klein $H^{\bullet}$ verlies waargenomen uit deze ionen, hetgeen resulteerde in de vorming van $[M+3H]^{2+\bullet}$ ionen. Een klein deel van de ionen die twee elektronen hadden gevangen waren dus langlevende $[M+4H]^{2+2\bullet}$ biradicalen. Een andere interessante observatie was het abundante $SH^{\bullet}$ verlies uit lacticine 481 $[M+4H]^{3+\bullet}$ ionen, hetgeen erop wees dat radicale sites voornamelijk bij lanthioninebruggen gelocaliseerd zijn.

Natieve structuren van peptiden en eiwitten worden vaak beïnvloed door cofactoren zoals metaalionen. Dit geldt ook voor het kleine peptide oxytocine dat vooral bekend is vanwege het veroorzaken van weeën tijdens de bevalling. De aanwezigheid van een specifiek divalent overgangsmetaalion zorgt voor een versterkte binding van oxytocine aan zijn cellulaire receptor. Deze binding verbetert in toenemende mate met $Zn^{2+}$, $Ni^{2+}$ en $Co^{2+}$, maar is te verwaarlozen in de aanwezigheid van $Cu^{2+}$. In hoofdstuk 4 werden de structurele eigenschappen van dubbel geprotoneerde oxytocine-ionen en oxytocinecomplexen met verschillende overgangsmetaalionen bestudeerd met ECD. Het is algemeen bekend dat een deel van de precursorionen zeer snelle fragmentatiereacties ondergaat met ECD, zelfs voordat de recombinatie-energie volledig gerandomiseerd is. Verder bevat oxytocine een disulfidebinding tussen Cys1 en Cys6, die selectief gebroken zou moeten worden met ECD. We namen aan dat elektronen door de overgangsmetaalionen zouden worden gevangen. Daardoor zouden de snelle ECD fragmentaties een indicatie kunnen geven over de locatie en/of coördinatie van metaalionen in oxytocinecomplexen.

Samenvatting

De ECD spectra van $[oxytine+2H]^{2+}$ ionen toonden typische ECD fragmentaties. Er werden voornamelijk b, c en 'z$^{\bullet}$ ionen gevormd. Circa 60% van intensiteit van de productionen kon worden toegeschreven aan disulfidegerelateerde karakteristieke ECD fragmentaties, waarbij tenminste 1 S-S of S-C binding werd gebroken. Alhoewel de ECD spectra van de oxytocinecomplexen met overgangsmetaalionen zeer verschillend waren werd karakteristiek ECD gedrag waargenomen voor de complexen met $Ni^{2+}$, $Co^{2+}$ en $Zn^{2+}$. Over het geheel genomen vonden er veel disulfidegerelateerde ECD fragmentaties plaats, circa 60-70% van de intensiteit van de productionen. Alhoewel de metaalionen in de oxytocinecomplexen kunnen dienen als locatie waar de elektronen worden gevangen, stelden we voor dat een gevangen elektron zich daarna verplaatst naar andere locaties in het complex om H$^{\bullet}$ te vormen. Dit waterstofradicaal drijft dan de typische ECD fragmentaties aan. Het ECD gedrag van het oxytocinecomplex met $Cu^{2+}$ was geheel anders. De gevormde fragmentionen leken het resultaat te zijn van fragmentaties die normaal gesproken voorkomen bij lage-energie CID. Opvallend genoeg werden er geen karakteristieke c en z'$^{\bullet}$ fragmentionen waargenomen. In totaal kon maar 15% van de intensiteit van de productionen worden toegeschreven aan disulfidegerelateerde fragmentaties. Dit staat in scherp contrast met de andere oxytocine- overgangsmetaalion complexen. We stelden voor dat de elektronen die gevangen worden door het oxytocine-$Cu^{2+}$ complex voorkeur hebben voor het reduceren van het $Cu^{2+}$ metaalion naar $Cu^{+}$. De daaropvolgende overdracht van energie zou de waargenomen lage-energie CID fragmentatie kunnen verklaren.

Ten slotte kunnen er nog wat algemene conclusies worden getrokken uit de in dit proefschrift gepresenteerde resultaten. Het is belangrijk om te benadrukken dat met ECD veel structuurinformatie kan worden verkregen over peptiden en eiwitten, vanwege de lage selectiviteit voor het breken van bindingen tussen aminozuren. Zeker in combinatie met complementaire tandem MS technieken zoals CID en infrarood multifoton dissociatie (IRMPD) en hoge resolutie FTICR-MS, zou ECD de meest krachtige techniek kunnen zijn voor *high-throughput* identificatie en structuuropheldering van peptiden en eiwitten. Eiwitten die nog een secundaire en tertiaire structuur bezitten in de gasfase vertonen vaak weinig fragmentatie met ECD, waarschijnlijk omdat intramoleculaire non-covalente interacties het scheiden van de fragmentatieproducten beletten. Om in deze gevallen een hoge dekking van de sequentie te verkrijgen, zou

ECD gecombineerd moeten worden met CID of IRMPD. Dit om de ionen eerst te activeren en de overgebleven non-covalente interacties te breken. Verder biedt de toepassing van ECD perspectieven bij de localisering van posttranslationele modificaties op peptiden. Dit is al aangetoond voor modificaties zoals glycosylering, fosforylering en disulfidebruggen; in dit proefschrift werd aangetoond dat ook lanthioninebruggen kunnen worden gelocaliseerd. In het algemeen worden met ECD bij voorkeur bindingen dichtbij zwavelatomen gebroken, of het nu een thioetherbinding is, zoals bij lantibiotica, of een disulfidebinding, zoals bij oxytocine. Het breken van deze sulfidebindingen gaat vaak gepaard met het breken van bindingen in de peptidehoofdketen. Daarom zou het gebruik van deze nieuwe tandem MS techniek altijd overwogen moeten worden bij de structuuranalyse van peptiden, vooral als zij thioether- of disulfidebindingen bevatten.

De selectiviteit van ECD voor thioether- en disulfidebindingen wijst erop dat gevormde radicale sites bij voorkeur relocaliseren naar thioether- en disulfidebindingen in peptiden. Een andere observatie die deze conclusie ondersteunt is het abundante $SH^{\bullet}$ verlies dat werd waargenomen uit botsingsgeactiveerde geprotoneerde lacticine 481 ionen die een elektron hadden gevangen. Thioether- en disulfidebindingen werken dus als een val voor radicalen, waarschijnlijk vanwege hun hoge affiniteit voor $H^{\bullet}$, en voorkomen zo secundaire fragmentaties. Deze zijn echter wel nodig om fragmentionen te verkrijgen uit cyclische gedeelten van een ion. Daarom zou in deze gevallen $MS^3$ kunnen worden gebruikt, bijvoorbeeld een extra SORI-CID stap, om voldoende bindingen te breken. Het geprefereerde botsingsgeïnduceerde $SH^{\bullet}$ verlies van radicale lacticine 481 ionen wijst er echter op dat niet in alle gevallen extra structuurinformatie wordt verkregen met ECD-SORI-CID experimenten, in vergelijking met ECD. De introductie van een tweede ECD stap om meer bindingen te breken wordt niet aangeraden. Als er namelijk twee elektronen door geprotoneerde lacticine 481 ionen worden gevangen, lijkt recombinatie van de radicale sites het dominante proces te zijn. Dit zal ook leiden tot structurele veranderingen in de ionen. In plaats hiervan moet daarom overwogen worden peptiden (gedeeltelijk) te digesteren voordat ECD uitgevoerd wordt en dan specifiek in cyclische gebieden. Op deze manier zijn de structurele veranderingen bekend en is het gemakkelijker om deze peptiden te fragmenteren.

Alhoewel thioether- en disulfidebindingen kunnen fungeren als een val voor radicalen werden er toch vaak secundaire fragmentaties waargenomen in de sulfidebevattende peptiden die werden bestudeerd in dit proefschrift. Dit wijst erop dat ECD geen enkelvoudig proces is en niet volledig non-ergodisch. Primaire fragmentaties, zoals degene die leiden tot de vorming van c en $z^{\bullet}$ ionen of directe zijketenfragmentaties in aminozuren, kunnen nog steeds zeer snelle reacties zijn die plaatsvinden voor intramoleculaire herverdeling van vibratie-energie. Secundaire fragmentatiereacties lijken echter veroorzaakt te worden door de herschikking van vrije radicalen. Dit zou de meervoudige fragmentaties kunnen verklaren terwijl er maar één elektron is gevangen.

Net zoals geprotoneerde oxytocine ionen lieten de oxytocinecomplexen met de overgangsmetaalionen $Ni^{2+}$, $Co^{2+}$ en $Zn^{2+}$ typisch ECD gedrag zien, hetgeen zich uitte door de vorming van c en $z^{\bullet}$ ionen, de abundante disulfidegerelateerde fragmentaties en $H^{\bullet}$ verlies. Deze resultaten suggereren dat het ECD mechanisme in deze ionsoorten vergelijkbaar is met dat in geprotoneerde ionen en dat waterstofradicalen ook een grote rol spelen in ECD processen in peptidecomplexen met overgangsmetaalionen. Deze observatie opent perspectieven voor de toepassing van ECD in tandem MS structuuranalyse van metallopeptiden en –eiwitten als een alternatief voor het algemener gebruikte CID. Het complex van oxytocine met $Cu^{2+}$ liet atypisch ECD gedrag zien. De lage-energie CID type fragmentaties, die werden waargenomen, wijzen erop dat het gevangen elektron meer betrokken is bij de reductie van $Cu^{2+}$ naar $Cu^{+}$ in plaats van de vorming van $H^{\bullet}$. Dit proces zou ook plaats kunnen vinden in peptide- of eiwitcomplexen met andere metaalionen zoals $Fe^{3+}$, waaruit het stabiele $Fe^{2+}$ gevormd zou kunnen worden. Het is dus belangrijk om het type metaalion in acht te nemen als men erover denkt om ECD te implementeren in de analyse van metaalcomplexen, want het vangen van een elektron zal niet altijd leiden tot de gewenste typische ECD fragmentaties.

# Curriculum Vitae

Anne Kleinnijenhuis werd op 15 februari 1978 geboren in Den Ham. Na het behalen van het VWO-diploma in Aalten in 1996 werd in september van dat jaar begonnen aan de opleiding scheikunde aan de Universiteit Utrecht. Tijdens deze opleiding werd de afstudeerstage uitgevoerd bij de sectie Biochemie van Lipiden: een functionele studie van het fosfatidylcholine transporterende eiwit PC-TP. Het doctoraalexamen werd behaald in augustus 2000. In januari 2001 begon hij als onderzoeker in opleiding bij de stichting Fundamenteel Onderzoek der Materie onder begeleiding van prof. dr. Albert J.R. Heck en prof. dr. Ron M.A. Heeren. Het onderzoek werd uitgevoerd bij de sectie Biomoleculaire Massaspectrometrie van de Universiteit Utrecht en bij het Instituut voor Atomaire en Moleculaire Fysica in Amsterdam. Het onderwerp van het promotieonderzoek was de structuuranalyse van peptiden met sulfidebindingen, gebruikmakend van elektronenvangst dissociatie (ECD), resulterend in dit proefschrift. Sinds juli 2005 is hij als postdoc werkzaam in de sectie Biochemie en Moleculaire Biologie van de Universiteit van Zuid-Denemarken.

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Kleinnijenhuis AJ, Mihalca R, Heeren RMA, Heck AJR. Atypical behavior in the electron capture induced dissociation of biologically relevant transition metal ion complexes of the peptide hormone oxytocin (*in preparation*).

# Dankwoord

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