Chapter 6

Peptide fragmentation by keV ion–induced dissociation

We have studied multiple ionization and dissociation of a trapped protonated peptide (leucine enkephalin) as induced by keV singly and doubly charged ions (H$^+$, He$^+$, 2$^+$) to demonstrate the potential of keV ions as a future tool for peptide identification. In contrast to conventional excitation techniques, the fragmentation pattern exhibits very strong peaks due to loss of side chains in addition to those due to backbone scission. The results can be understood on basis of the energy deposited into the peptide via electronic stopping. A pronounced dependence of the fragmentation pattern on the electronic structure of the projectile ions can be attributed to different electron capture efficiencies from localized molecular orbitals.

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6.1 Introduction

A recent series of studies on keV ion–induced ionization of isolated polyatomic molecules in the gas phase [1–4] aimed for a better insight into the molecular mechanisms underlying biological radiation damage. These studies have greatly advanced the understanding of the underlying dissociation dynamics. The experiments have been limited to smaller molecules which are stable with respect to thermal decomposition. With the advent of soft ionization techniques such as electrospray ionization (ESI [5]) and matrix assisted laser desorption/ionization [6], it has become possible to bring much larger, intact molecules such as peptides and proteins into the gas phase. Usually these molecules are in their protonated form.

Dissociation of mass–selected peptide ions is a basic technique for peptide sequencing and protein identification by means of mass spectrometric techniques. To drive the dissociation processes, excitation by multiple collisions with inert gas atoms (collision induced dissociation, CID [7]) or surfaces (surface induced dissociation, SID [7]), by electron capture (electron capture dissociation, ECD [8–10]) and by blackbody infrared radiation [11, 12] or laser light [13] has been employed. CID has been the most common method for peptide identification purposes. When CID is performed at higher peptide energies, a single collision may be sufficient to induce dissociation. Liu and co–workers have for instance investigated the fragmentation of 50 keV protonated adenosine monophosphate colliding with neutral Na or Ne atoms [14].

The reversed direct approach however, in which e.g. keV atomic ions are collided with a target of biomolecular ions has not been pursued yet. Until now sufficient target densities for such experiments could not be reached. The use of keV atomic ions would allow for fs–interaction times and higher excitation energies. The fs–interaction between keV ions and larger molecules or clusters is governed by (multiple) electron capture and electronic stopping. Electron capture leads to gentle multiple ionization of a target biomolecule and depends strongly on the charge state and electronic structure of the projectile ion. The role of the charge state and electronic structure of the projectile species has been demonstrated for collisions on nucleobases [15] and amino acids [16]. The term electronic stopping describes projectile ion–induced electronic excitations of the target molecule. Observed excitation energies after ion impact at similar energies as used here are about 10 eV for H\(^+\) on small biomolecules [17] and between 30 eV and 50 eV for H\(^+\) and He\(^+\) on C\(_{60}\) [18, 19]. Deposition of such relatively high energies on fs timescales is leading to a fragmentation regime fundamentally different from CID and SID, where the excitation energies are very close to dissociation thresholds of 2–4 eV [20], and thus opens the door to a variety of new applications.

In addition, a detailed knowledge of the molecular dynamics triggered by the interaction of energetic ions with complex biomolecules is of great relevance for understanding proton and heavy ion tumor therapy on a molecular level and assessing the risk of human space exploration due to the exposure to ions from galactic cosmic rays and solar particle events. In both cases, maximum biological damage occurs when the originally fast ions are slowed down to MeV energies and below, the so–called Bragg peak. Biological effects of ionizing radiation are known to be due to direct and indirect damage of cellular DNA. Pioneering studies on biological radiation damage on the single–molecule level have dealt with ionization
6.2 Experiment

Figure 6.1: Structure of leucine enkephalin with an indication of its 5 constituent amino acids, nomenclature of the y and b fragments as well as three side chain (immonium) fragments.

and fragmentation of isolated DNA building blocks [15, 21] and amino acids [16]. The latter are relevant since in the nuclei of most cells, DNA is wound around protein spools, the histones. The ion interaction with these proteins is biologically relevant since secondary particles formed during the radiation might in turn damage the neighboring DNA. To date, almost all experiments on radiation action on isolated biomolecules have been limited to the histones. The ion interaction with these proteins is biologically relevant since secondary beams 5–40 keV H\(^+\), He\(^+\) and He\(^{2+}\) ions were used; while as target molecule, the neurotransmitter peptide leucine enkephalin was used (leu–enk, amino acid sequence: Tyr-Gly-Gly-Phe-Leu, \(m = 555.62\), see fig. 6.1). In the following the experimental setup is briefly described. Thereafter the obtained fragmentation spectra are presented and discussed. In particular, the different influences of the electronic excitation and electron capture on the dissociation dynamics are addressed.

6.2 Experiment

All experiments were carried out at the ZernikeLEIF facility of the KVI (University of Groningen, The Netherlands). Recently, we have developed a new apparatus (for a sketch, see fig. 6.2) in which a home–built electrospray ionization (ESI) source is interfaced with an electron cyclotron resonance (ECR) ion source. A brief description of the experimental set up follows, which is described in more detail in chapter 3.

The singly protonated pentapeptide leucine enkephalin ions were generated in the in–house built ESI source from a \(~30 \mu M\) methanol solution with 1% formic acid. The ions were then guided by a collisionally focusing RF–only quadrupole and an RF–quadrupole
mass analyzer through the end cap into a quadrupole ion trap.

The base pressure inside the trap chamber was $1 \times 10^{-9}$ mbar. For collisional cooling of the peptide ions, a He–buffer gas pulse was applied. The estimated pressure inside the trap increased to $1 \times 10^{-3}$ mbar, and the trap was typically loaded with protonated peptides over a period of 300 ms. The ESI beam was then blocked by means of a 100 V skimmer bias, and at the same time the solenoid valve, controlling the He buffer gas flow, was closed. A delay of about 500 ms allowed the pressure in the trap to decrease to about $1 \times 10^{-6}$ mbar, necessary to keep charge–changing collisions by the keV ions below 1% before reaching the trap center. A $\sim 100$ nA beam of keV $\text{H}^+$, $\text{He}^+$ or $\text{He}_2^+$ projectile ions extracted from the ECR ion source intersected the Paul trap through the ring electrode.

The protonated peptides were then exposed for about 100 ms to the ion beam. The conditions were chosen such that a total of about 10% of the trapped protonated peptides were dissociated by collisions with projectile ions. Directly after the projectile ion pulse, a second He–buffer gas pulse was applied to the trap, to cool energetic dissociation products.

Trapped protonated peptides and their cationic dissociation products were then extracted into a linear time–of–flight (TOF) mass spectrometer ($M/\Delta M=200$) by applying a bias voltage ($U_{\text{bias}}=\pm 200$ V, duration: 5 $\mu$s) to the RF–trap end caps. The ions were detected by a silhouette–type micro–channel–plate detector with the front plate biased to -5 kV and the anode kept at ground potential. The detector signal was recorded by a 1–GHz digitizer.

Despite the low background pressure and a liquid nitrogen cooled cryo–trap close to the RF–trap, contamination of the buffer gas or neutral molecules from the ESI source may contribute to the mass spectra. To extract the mass spectrum due to peptide fragmentation only, the data acquisition was divided into successive cycles of three mass scans. In each cycle, first the TOF spectrum resulting from keV irradiation of trapped protonated peptides and neutral residual gas was recorded (inclusive scan). To obtain the net effect of keV ion irradiation upon the trapped protonated peptides, in a second scan the ECR source was switched off and a TOF spectrum of the initial trap content only was recorded. For the third scan, the ESI source was switched off and the TOF spectrum resulting from the ion–induced ionization of residual gas molecules was recorded. The latter two spectra were then subtracted from the inclusive scan. A three–scan cycle took about 3 s. To obtain the final mass spectra a series of 2000–6000 cycles was accumulated, which assures that long term fluctuations of peptide– and projectile–ion current were averaged out.

![Experimental setup](image-url)
6.3 Results

Fragment cation spectra obtained for interactions of He\(^{2+}\), H\(^{+}\) and He\(^{+}\) with leu–enk are displayed in fig. 6.3, 6.4, 6.5 respectively. In these figures we compare the dissociation patterns obtained for different projectile ion kinetic energies ranging from 5 to 40 keV. The spectra are normalized to the intensity of the m/z=107 amu peak which is most often the strongest peak in the spectra. Masses smaller than 50 are not trapped under the present experimental conditions. In general, the spectra are dominated by fragments with m/z 80–140 amu with the peaks 86, 120 and 136 being the intact immonium groups of leu, phe and tyr (see fig. 6.1). The peaks at 107 and 91 amu are common fragments of these groups. Fragment ions with masses exceeding 350 are not observed. The peaks at masses smaller than 86, e.g. the group between 75 and 80, are due to common amino acid fragments (strong peaks in this region after electron impact ionization of amino acids are 74 (gly, leu, phe) and 77 (tyr) [22]).

The ion–induced fragmentation spectra are fundamentally different from those observed in CID [23], SID [20] and also e.g. IR multiphoton absorption [24] in which fragments with m/z exceeding 350 dominate the spectra and side chain fragments are weak or very weak. In these cases, the backbone scission leading to the heavy fragments is described in the framework of the mobile proton model [25]. Upon an increase of vibrational excitation energy, the proton attached to the peptide becomes mobile and samples various protonation sites within the molecule. Attachment to a backbone N atom can for instance weaken the backbone such, that a b/y fragment is formed directly.

In the KID spectra peaks belonging to masses larger than 170 amu are of much lower intensity. For He\(^{+}\) projectiles, the peaks in this range are barely observable (fig. 6.5). For He\(^{2+}\) and H\(^{+}\) (fig. 6.3 and 6.4) the high m/z peaks can be easily assigned to fragments resulting from single or double scission of the peptide backbone. The peak at 278/279 amu is probably due to the b\(_3\) and y\(_2\) fragments (for the notation, see fig. 6.1). A small contribution from the doubly charged parent peptide cannot be excluded. At 221 amu, the b\(_2\) fragment is clearly visible. An internal gly–phe fragment that is due to loss of b\(_2\) and y\(_1\) from leu–enk shows up at 205 amu. The fragment with m/z=295 is likely to originate from an a\(_4\) fragment (see fig. 6.1) as will be discussed in the context of the He\(^{2+}\) spectra. Note that the internal gly–phe fragment could alternatively also be formed in a sequence involving the cyclic intermediate, as will also be discussed later.

6.3.1 He\(^{2+}\)

For He\(^{2+}\) projectiles, reducing the energy gives rise to the most pronounced change in relative peak intensities. Formation of the intact immonium fragment ions tyr (m/z =137) and phe (m/z=120) is getting suppressed with decreasing projectile energy, as is the yield of the side chain fragment tyr/phe (m/z=91). At 10 keV, the dominant tyr side chain fragment at m/z=107 dominates over all other peaks in the spectrum by at least a factor of 2. The decrease of the phe immonium ions may be rationalized by the fact that its formation requires a double backbone scission which becomes energetically unfavorable at lower projectile kinetic energy. Phe side chain ion formation is also reduced.

Interestingly, the intensities of the internal fragments at m/z=205 (gly–phe) and 295 (phe–tyr–gly minus CO) both increase. The formation of these fragments also requires a double
Figure 6.3: Mass spectra of the product cations for He\(^{2+}\) with leu–enk at different projectile energies: (a) 40 keV, (b) 30 keV, (c) 20 keV and (d) 10 keV. Arrows indicate the direction of change of (b)–(d) with decreasing energy.
6.3 Results

backbone scission. Their increase at lower projectile kinetic energies is non–intuitive. Both fragments originate from an intermediate \( a_4 \) or \( b_4 \) fragment. These fragments are most abundant when established dissociation techniques are employed but not observed here. According to Laskin [20] and DFT calculations by Jalkanen [26] protonation of leucine enkephalin occurs at the N–terminal amino group. This \( \text{NH}_3^+ \) then forms a bifurcated hydrogen bond between the oxygen of the fourth carbonyl group and the hydrogen of the OH group of the (C–terminal) COOH group. Similarly, Polfer and co–workers [27] identify energetically favored structures, where the N–terminal \( \text{NH}_3^+ \) is solvated by the COOH group. In any case, the fragmentation of these secondary structures follows entropically favored pathways and can lead to the formation of cyclic \( a_4 \) or \( b_4 \) intermediates which subsequently reopen and for instance the gly–phe fragment (m/z=205) can be formed. Loss of the glycine residue, \( \text{NH}_3 \) and \( \text{CO} \) from the reopened \( a_4 \) intermediate lead to the formation of the m/z=295 fragment [28]. The increase of the two peaks with decreasing projectile energy suggests, that the described process requires relatively little activation energy.

6.3.2 \( \text{H}^+ \)

The fragmentation patterns of leu–enk obtained with \( \text{H}^+ \) are very similar to the spectra for \( \text{He}^{2+} \), in particular when comparing identical projectile velocities (see 10 keV \( \text{H}^+ \) in fig. 6.4(c) and 40 keV \( \text{He}^{2+} \) in fig. 6.3 (a)). Compared to the \( \text{He}^{2+} \) case, the relative intensity of the strongest peak, the N–terminal immonium fragment at m/z=107, is less pronounced and the intensities of the higher–mass fragments are also lower. Decreasing the energy of the \( \text{H}^+ \) projectile from 20 keV to 5 keV reveals only little effect on the immonium ions and a small increase of the heavier fragments. It is worth mentioning that the intact tyr and phe immonium ions at m/z=136 and m/z=120 increase or stay constant with decreasing projectile velocity, at variance to what is observed for \( \text{He}^{2+} \).

6.3.3 \( \text{He}^+ \)

As mentioned before, large m/z fragments are either strongly or fully suppressed for \( \text{He}^+ \) projectiles (fig. 6.5). For comparable beam intensities, the total fragment yield is much lower for \( \text{He}^+ \) compared to the other projectiles as can also be seen from the signal to noise ratios of the spectra. It is interesting that for this projectile ion at 5 keV two new fragmentation channels appear at 177 and 285. These fragments are not observed at higher \( \text{He}^+ \) kinetic energy or for other projectile ions. The peak at m/z = 177 can be assigned to the additional loss of CO from the internal fragment gly–phe [29]. The peak at m/z = 285 remains unassigned.

The trend of the relative intensities of the immonium ions is not monotonically changing with the \( \text{He}^+ \) kinetic energy. However, at 5 keV the intensity of all immonium ions is increased again and reveals the strongest fragmentation of the peptide, with the phe/tyr side chain fragment (m/z=91) as the strongest fragmentation channel. In summary all the KID spectra differ from established techniques in such that strong side chain features show up while peaks to backbone scission are weak. The differences to established techniques (and between different) keV projectiles must be due to the larger amount of excitation energy deposited during KID by electronic stopping or by capture of one or more electrons from the peptide.
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Figure 6.4: Mass spectra of the product cations for $H^+$ with leu–enk at different projectile energies: (a) 20 keV, (b) 15 keV, (c) 10 keV and (d) 5 keV. Arrows indicate the direction of change of (b)–(d) with decreasing energy.
Figure 6.5: Mass spectra of the product cations for He\(^{+}\) with leu–enk at different projectile energies: (a) 20 keV, (b) 15 keV, (c) 10 keV and (d) 5 keV. Arrows indicate the direction of change of (b)–(d) with decreasing energy.
Figure 6.6: Isosurface \((0.006 \text{ a.u.}^{-3})\) of the leu–enk valence electron density obtained from density functional theory calculations (see text).

### 6.4 Discussion

#### 6.4.1 Electronic stopping

As mentioned before, the term electronic stopping describes the electronic excitations of the target molecule induced by the projectile ion. For a quantitative analysis of the electronic stopping, here, the molecular valence electrons are treated as an electron gas. We determined the valence electron density distribution of protonated leu–enk by summing up the electron densities of all valence molecular orbitals obtained by density functional theory (DFT) calculations (B3LYP level, 6-31+G(d,p) basis set) using Gaussian03 [30]. This was done for the lowest energy conformer as determined by IR spectroscopy and quantum chemical calculations [27]. Figure 6.6 shows an isosurface \((0.006/\text{a.u.}^{3}, r_s = 3.4 \text{ a.u.})\) of the leu–enk valence electron density.

In the keV energy range collective electron excitations only contribute weakly to the electronic stopping. Individual electron excitation due to long range coupling to electron–hole pairs [31] prevails, implying that the deposited energy can be approximated as linear with projectile velocity [32]. Interactions with the target nuclei (nuclear stopping) are negligible for light ions at keV energies. The stopping \(S\) being the energy loss \(dE\) per trajectory length \(dR\) can then be written as a friction force

\[
S = \frac{dE}{dR} = \gamma(r_s)v \tag{6.1}
\]

with \(\gamma\) the friction coefficient depending on the local molecular electron density parameter (one–electron radius):

\[
r_s = \left(\frac{4}{3} \pi n_0(\overline{r}^3)\right)^{-\frac{1}{3}} \tag{6.2}
\]
and \( v \) the projectile velocity [33]. This concept has been successfully applied to keV ion-induced multifragmentation of \( C_{60} \) [18, 34]. For \( H^+ \) and \( He^+ \), \( \gamma(r_s) \) has been calculated by Puska and Nieminen [35] and can be approximated by the exponentials

\[
\gamma(r_s) = 0.53e^{-(r_s-0.41)/2.13} \tag{6.3}
\]

and

\[
\gamma(r_s) = 0.755e^{-(r_s-1.5)/0.88} \tag{6.4}
\]

respectively. The electronic stopping of a projectile ion along a straight line trajectory through \( n_0(\vec{r}) \) can now be integrated. Fig. 6.7 shows the results for \( 5 \times 10^6 \) random trajectories of 5 keV/amu \( H^+ \) and \( He^+ \) through protonated leu–enk. For both ions there is a local maximum at \( S \approx 34 \text{ eV} \) due to trajectories traversing the peptide backbone or side chains once and under a relatively steep angle. The strong intensity increase at low stopping (I) is due to grazing trajectories through the tails of the peptide electron density. Electronic stopping even exceeding 100 eV (II) is due to the few trajectories which traverse the peptide structure more than once or which have long pathways through high electron density regions. The cross section for trajectories with electronic stopping \( S \geq 34 \text{ eV} \) (shaded area in fig. 6.7) amounts to 165 \( \text{Å}^2 \) and 140 \( \text{Å}^2 \) for \( H^+ \) and \( He^+ \) respectively which is close to the geometric cross section of 162 \( \text{Å}^2 \) as determined by CID [36]. The region II class of trajectories will in most cases lead to multifragmentation of the peptide into small fragments which are not trapped for the trap parameters used. For the region I class of trajectories, little excitation due to electronic stopping occurs and the interaction is dominated by relatively gentle resonant electron capture from the protonated molecule. Due to the deposition of relatively little energy in the peptide, these grazing trajectories lead to less extensive fragmentation and are thus most relevant for the fragmentation patterns observed.

Figure 6.7: Electronic stopping for 5 keV \( H^+ \) and 20 keV \( He^+ \) trajectories through randomly oriented protonated leu–enk.
6.4.2 Electron capture

For $\text{H}^+$ and $\text{He}^+$ the electronic stopping is almost identical (see fig. 6.7). Therefore the differences in fragmentation pattern for the two projectiles must be due to electron capture from the molecule. In KID, electron capture will most likely involve electrons from the highest occupied molecular orbitals (HOMOs) of the peptide since those electrons can be resonantly captured at large impact parameters. Using the classical over-the-barrier model [37] for symmetric systems such as $\text{C}_{60}$, in an earlier study we have already reported critical impact parameters for resonant capture into $\text{He}^+$ and $\text{He}^{2+}$ [38].

We determined the spatial distribution of the HOMOs of the protonated leu-enk and the spin density of protonated leu-enk dication (see fig. 6.8) by DFT calculations as mentioned before. For the region I class of trajectories, little excitation due to electronic stopping occurs and the interaction is dominated by relatively gentle resonant electron capture from the protonated molecule. The calculated HOMO and HOMO–1 have $\pi$ character and are located on the phe side chain ring. The HOMO–2 is a $\pi$–orbital located on the tyr side chain ring.
Also the spin density of the protonated leu–enk dication after non–adiabatic (because of fs–ionization) electron removal resides mainly on these side chains. The fact that we observe strong tyr and phe side chain fragments indicates dissociation close to these side chains and little to no distribution of excitation energy over the whole molecule. Such non–ergodicity is also believed to be the basis of ECD [8] where a low energy electron is captured by the peptide, leading predominantly to formation of c and z fragments (see fig. 6.1) [9].

DFT is well–known for usually underestimating the true ionization energies of the calculated HOMOs. We therefore assume that the singly protonated peptide leu–enk has an ionization energy IE of 10.9 eV according to the empirical formula by Budnik et al. [39]. The DFT calculations do tell us however, that the energies of the 3 HOMOs lie within an interval of less than 1 eV. This implies that for H$^+$ and He$^{2+}$ [21]: electron capture into the \( n = 1 \) (H$^+$) and the \( n = 2 \) state respectively is a (near) resonant process (see fig. 6.9) already occurring at large impact parameters for which electronic stopping is small. For ion–atom collisions, electron capture distances can be estimated from the classical over–the–barrier model [40]. For a fixed ionization energy of the target, the capture distance depends on the ion charge state and for the leu–enk ionization potential of 10.9 eV, capture distances would be \( \sim 7 \) a.u. for H$^+$ and \( \sim 10 \) a.u. for He$^{2+}$. Since here we are dealing with a large molecule instead of a target atom, the situation is more complex. However, we can translate the information from the ion–atom system and assume that electron capture sets in at a distance of \( \sim 7 \) a.u. (H$^+$) and \( \sim 10 \) a.u. (He$^{2+}$) from the leu–enk constituent atom closest to the incoming ion. The capture distance defines the class of trajectories for which the traversed electron density and thus the molecular excitation due to electronic stopping is minimum. It is thus obvious that for He$^{2+}$ with its larger capture distance the interaction is more gentle than for H$^+$. This might also be the reason for the larger yield of intact tyr and phe immonium ions (m/z=136, m/z=120) at low projectile energies observed for H$^+$ as compared to He$^{2+}$: For formation of a non–terminal intact immonium ion, two bonds have to be broken whereas for the related ions at m/z=120 and m/z=91 only single bond scission is required. For He$^+$, the n = 1 state at -24.6 eV only becomes resonant in close collisions in which electronic stopping is high. This is consistent with the experimental findings: Large fragments due to backbone scission are strongly suppressed in the He$^+$ case because of the too high excitation energy. Furthermore, for He$^+$ the phe side chain peaks at m/z = 120, 91 are relatively stronger. This is due to the fact that for production of side chain ions from the non–terminal amino acid phe, the backbone has to be broken twice whereas for the N–terminal tyr side chain, only a single bond scission and thus less excitation energy is necessary. For H$^+$ and He$^{2+}$ an increase of the projectile ion velocity and thus an increase in deposited energy leads to a relative decrease of the backbone scission peaks (m/z = 150–350) with respect to the side chain peaks. For the non–resonant case of He$^+$ the projectile velocity has only little influence on the fragmentation.

### 6.5 Conclusion

To conclude we have shown the keV ion–induced fragmentation of a free protonated peptide. In contrast to the findings for conventional peptide dissociation techniques, amino acid side chains dominate the fragment mass spectrum. Backbone scission is a relatively weak
channel. This can be qualitatively explained in terms of peptide excitation due to electronic stopping and resonant electron capture from the peptide. KID is a promising new tool for peptide dissociation, not only because fundamentally different dissociation dynamics occur, but also because ion kinetic and potential energies can be varied over a wide range. Efficient dissociation of very large peptides and small proteins is feasible due to the deposition of large amounts of kinetic energy on fs–timescales. The use of trapped complex molecular ions as a target facilitates a whole new realm of collision experiments, for instance in the field of biomolecular radiation damage and physics of size selected clusters.

In the context of biological radiation damage on the molecular level, our results show that the large amounts of excitation energy deposited in complex biomolecules upon keV ion impact lead to very severe fragmentation. This is in line with previous results obtained with nucleobases [15], deoxyribose [21] or amino acids [16]. The question remains, how the solvation layers that surround biomolecules in living cells affect the fragmentation dynamics. Being based on target production by means of electrospray ionization, the developed technique allows investigation of nanosolvated biomolecules [41]. In the future we plan to exploit this potential by studying keV ion–induced dissociation of nanosolvated oligonucleotides as nano–models of the situation in the nucleus of a living cell.

**Figure 6.9:** Relevant energy levels of the projectile ions in comparison with ionization energy of leu–enk. The dashed rectangle indicates the levels available for resonant electron capture (see text).
**References**


