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Investigation of the position of the radical in z_3 -ions resulting from electron transfer dissociation using infrared ion spectroscopy†

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The molecular structures of six open-shell z_3 -ions resulting from electron transfer dissociation mass spectrometry (ETD MS) were investigated using infrared ion spectroscopy in the 800–1850 and 3200–3700 cm^{-1} spectral ranges in combination with density functional theory and molecular mechanics/molecular dynamics calculations. We assess in particular the question of whether the radical remains at the C_α -site of the backbone cleavage, or whether it migrates by H-atom transfer to another, energetically more favorable position. Calculations performed herein as well as by others show that radical migration to an amino acid side chain or to an α -carbon along the peptide backbone can lead to structures that are more stable, by up to 33 kJ mol^{-1} for the systems investigated here, by virtue of resonance stabilization of the radical in these alternative positions. Nonetheless, for four out of the six z_3 -ions considered here, our results quite clearly indicate that radical migration does not occur, suggesting that the radical is kinetically trapped at the site of ETD cleavage. For the two remaining systems, a structural assignment is less secure and we suggest that a mixture of migrated and unmigrated structures may be formed.

Introduction

Electron Capture Dissociation (ECD) and Electron Transfer Dissociation (ETD) have developed into popular MS/MS methods in top-down proteomics. Advantages of ExD dissociation methods over collision-induced dissociation include overall improved sequence coverage and the retention of labile post-translational modifications (PTMs). However, ExD mass spectra are more complex and are

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often more difficult to interpret, especially because the radical-driven reaction mechanisms remain incompletely understood at a fundamental level.

ExD commonly induces cleavages of the protein backbone at the N-C α bonds, leading to N-terminal c-type fragment ions and radical C-terminal z-type sequence ions.^{1–9} The reaction mechanisms leading to the backbone cleavage and the resulting fragment structures are the subject of extensive discussion in the scientific literature.^{2,3,5,10–25} The position of the radical in the peptide and its ability to migrate along the peptide backbone or to the amino acid side chains in the open-shell fragment ions have been explored in a large number of experimental and theoretical investigations.^{26–52}

The so-called Cornell^{2,11,17,53} and Utah–Washington^{8,10,12,18–21,35,54–56} mechanisms are the best-known hypotheses describing ExD dissociation reactions. In the Cornell mechanism, electron attachment occurs at a protonated site in the ion that is hydrogen-bonded to a nearby carbonyl, whereas according to the Utah–Washington mechanism, the electron is directly captured in a π^* orbital of an amide carbonyl group, stabilized by H-bonding to a protonated site. In both mechanisms, this eventually leads to N-C α bond cleavage, although the resulting c-type fragment structures may be different. In contrast, both mechanisms agree on the C-terminal z-type ions being formed with the radical initially at the C α -position of the cleaved peptide bond, as shown in Scheme 1. However, this is in most cases not the thermochemically most favored structure and radical migration to form lower-energy isomeric species has been suggested to occur.⁵⁷ Radical migration may lead to various secondary side chain losses and backbone dissociations at sites distant from the location of the initial radical.⁵⁷ Radical migration in z-ions has therefore been investigated by CID MS/MS,^{26–30,38,58,59} and also by UV photodissociation spectroscopy (UV-PD),^{31–33,35,43,60,61} ion mobility spectrometry³⁴ and infrared ion spectroscopy.³⁶

CID methods have been employed to provide information about radical migration by activation of the z-ion and analysis of the resulting CID reaction products.^{62–64} The location of the unpaired electron in the ExD-generated z-type radical cation is assumed to be a major factor determining the cleavages of the backbone or the side chains of the peptide.⁶⁵ For example, CID has been applied on a systematically varied set of z-ions resulting from the ETD of the

Scheme 1 Schematic representation of the formation of z-type product ions formed by electron transfer dissociation and possible radical migration within the product ion. The radical can possibly migrate from the cleavage site (C α -position) to the C β - or C γ -positions of the same residue. Alternatively, it can possibly also migrate to adjacent residues (blue arrow) leading to an α -radical structure, in blue.

pentapeptides [AAXAR + 2H]²⁺.^{26,27} For z-ions resulting from peptides with aromatic residues (X = F, H, Y and W), very facile radical-induced backbone cleavages were observed, which were attributed to the readily transferrable side-chain β -hydrogen atom,^{26,27} allowing for resonantly-stabilized β -radical structures to be formed. A caveat in CID MSⁿ based studies is the possibility that the radical migrates due to the collisional activation used to probe the ExD reaction, but not due to the ExD reaction itself.⁴⁶

The comparison of calculated collisional cross sections (Ω) with cross sections determined by ion mobility has been used to investigate the structures of c- and z-ions resulting from the ETD of [AAHAL + 2H]²⁺.³⁴ For the z₃ fragment, the position of the radical has been assigned as the C $_{\alpha}$ of the His residue at the cleavage site, indicating no radical migration.⁶⁶ For the z₄ ion, the Ω -values were too similar to distinguish between the different radical positions. Based on ion-mobility data for the charge-reduced but undissociated ion (ETnoD), which showed that hydrogen atom migrations were slow in comparison with the drift time, the location of the radical in the z₄-ion was determined to be the original C $_{\alpha}$ (4) position at the ETD cleavage site.

Infrared ion spectroscopy was applied to a series of radical z-ions (z₁⁻, z₂⁻, z₃⁻ and z₄-ions) generated by ETD from doubly charged [AAHAR + 2H]²⁺. In all z-ions the radical was established to be located at the α -carbon of the cleavage site, suggesting that radical migration to thermodynamically more favorable sites does not occur.³⁶ Tureček and coworkers extensively investigated radical migration in z-type ions using UV photodissociation (UVPD) spectroscopy. For the z₄ ions of [ADAAR + 2H]²⁺ and [QDAAR + 2H]²⁺, the UVPD action spectrum was attributed to a mixture of radical isomers by H-atom migration over the backbone or to the aspartic acid C $_{\beta}$ -position. Also for the z₄ ions [$\cdot$ NAAR + H]⁺, [$\cdot$ EAAR + H]⁺ and [$\cdot$ QAAR + H]⁺, the UVPD action spectra indicate that partial isomerization occurred upon ETD.³⁵ The UVPD spectra of z₄ ions formed by the ETD of [AAWAR + 2H]²⁺ and [AAFAR + 2H]²⁺ were attributed to structures with the radical at the cleavage site, although the presence of other isomers could not be excluded.³¹ Calculations indicated that radical migration from the initial cleavage site to the C $_{\alpha}$ of Arg leads to the most stable isomer for the z₄ ion of [AAWAR + 2H]²⁺.³¹ For the z₄ ion of [AAFAR + 2H]²⁺, calculations suggest that radical migration from the Ala residue at the cleavage site to the C $_{\beta}$ of Phe gives the most stable isomer. Nonetheless, the UVPD spectrum for this ion suggests an unmigrated radical structure, although this assignment is less secure.

Here we use IRMPD ion spectroscopy to explore the influence of aromatic residues at the cleavage site, with Ala serving as a non-aromatic reference. We focus on the question of whether or not radical migration occurs in the series of investigated z₃-ions, which are derived by applying ETD to heptapeptides containing an aromatic residue in the third position (counted from the C-terminus). In addition, the z₃ ion from [KAHA + 2H]²⁺ has also been investigated. Scheme 2 lists the structures of the z₃ ions that have been investigated, with possible positions of the radical (α -, β -, and γ -carbons) indicated.

Of these possible positions of the radical, the C $_{\alpha}$ (2)- and C $_{\alpha}$ (1)-positions in the protein backbones and the C $_{\beta}$ (3)-positions in the aromatic residues typically generate structures having the lowest energy. The radical at the C $_{\alpha}$ position in the neighboring residues is stabilized by the captodative effect,^{67–71} which can be characterized as π -electron delocalization between the adjacent electron-

Scheme 2 Structures of the investigated z_3 -ions, with the possible α , β and γ positions of the radical indicated for each z -ion.

withdrawing carbonyl group and the electron-donating amide nitrogen. This effect has for instance been extensively described and studied using ion spectroscopy for the histidine amino acid radical⁵² and is illustrated in the top panel of Scheme 3. Radical migration to the C_β position within the aromatic residue is stabilized by delocalization of the electron over the aromatic system (resonance stabilization), as illustrated in the bottom panel of Scheme 3.⁷² Finally, the $C_\alpha(3)$ -position at the cleavage site is of particular interest here. Although this isomer is usually relatively high in energy, $C_\alpha(3)$ is the initial site of the radical according to the Cornell and Utah–Washington mechanisms. If radical migration faces high barriers, the system may remain kinetically trapped in this isomer.

Experimental and computational methods

IRMPD spectroscopy

An ETD-enabled 3D quadrupole ion trap mass spectrometer (Bruker, AmaZon Speed ETD), modified to enable optical access to the ion population, was used to

Scheme 3 Resonance stabilization of a radical positioned at the C_β position in an aromatic residue (bottom) and captodative stabilization of a radical at the C_α position of the first or second residue (top).

produce the z_3 -ions and to obtain their infrared spectra.^{36,66,73–75} Precursor peptide samples were purchased from Biomatik (Canada) and were used without further purification. Doubly protonated precursor ions were generated by electrospray ionization (ESI) from $\sim 10^{-6}$ M solutions in 50 : 50 acetonitrile/water with $\sim 0.5\%$ formic acid added. The doubly charged precursor ion of interest was mass isolated and stored in the quadrupole ion trap. Fluoranthene radical anions generated in the chemical ionization source of the instrument were then guided into the trap, where the ETD reaction with the peptide dications took place for 300 ms. The fragment ion of interest is then mass-isolated for spectroscopic interrogation.

The mass spectrometer is coupled to the optical beam line of the free electron laser FELIX,⁷⁶ which enables us to record infrared multiple-photon dissociation (IRMPD) spectra in the 800–1850 cm^{-1} region; an optical parametric oscillator (OPO, Laser Vision, Bellevue, USA) is used for measurements in the 3200–3700 cm^{-1} region.⁷³ For the FELIX experiments, the z -type fragment ions were irradiated with two IR pulses at a repetition rate of 10 Hz at pulse energies of approximately 20–60 mJ. The bandwidth is around 0.5% of the center frequency. The OPO generates pulses of approximately 15 mJ at a 10 Hz repetition rate with a bandwidth of 3 cm^{-1} . The dissociation yield at each IR frequency is obtained from five averaged mass spectra and calculated as $\Sigma I(\text{fragment ions})/\Sigma I(\text{parent} + \text{fragment ions})$. By plotting the yield as a function of laser frequency, an infrared spectrum is generated. No apparent differences were noticed in the wavelength-dependent fragmentation into the individual dissociation channels; no clear evidence was therefore found for the co-existence of multiple isomers, such as for instance in some of our previous CID MS/MS studies.^{77,78} The yield is linearly corrected for the frequency-dependent change in the laser pulse energy and the infrared frequency is calibrated using a grating spectrometer (FELIX) or a wave-meter (OPO).

Computational chemistry

A Molecular Mechanics/Molecular Dynamics (MM/MD) approach was applied to z_3 ions with the radical positioned at the cleavage site $C_\alpha(3)$, at the β - and γ -positions within the terminal residue ($C_\beta(3)$ and $C_\gamma(3)$) and at the α -position of the second residue (alanine, $C_\alpha(2)$). AMBER 12 (ref. 79) was used to explore the potential energy surface of the z -ions and to find the lowest-energy conformers. After minimization within AMBER, a simulated annealing procedure using temperatures up to 500 K was used. 500 structures were obtained in this procedure and grouped based on structural similarity using appropriate rms criteria to give 20 to 30 candidate structures. Next, these structures were each optimized using Density Functional Theory (DFT) at the UB3LYP/6-31++G(d,p), the UB3LYP-D/6-31++G(d,p) and the UM06-2X/6-31++G(d,p) levels of theory. IR spectra were also predicted at these levels of theory and we use the B3LYP spectra for comparison with experiment.

All DFT calculations are performed using the Gaussian09 revision D01 package.⁸⁰ The computed harmonic vibrational frequencies were scaled by 0.975 in the 800–1850 cm^{-1} region and by 0.955 for the 3200–3700 cm^{-1} region. To facilitate comparison between calculated and experimental spectra, the calculated spectra were convoluted with a 25 cm^{-1} full-width-at-half-maximum

Gaussian line shape. The computational procedure is described in more detail elsewhere⁸¹ and has for instance been applied in our studies on ETD generated w-ions and c-ions.^{66,74}

Spectral matching scores

To assess the degree of similarity between calculated and experimental infrared spectra, we employ the cosine similarity as an objective measure.⁸² The cosine of the angle θ between two n -dimensional vectors is calculated using their normalized Euclidean dot product according to

$$\text{similarity} = \cos(\theta) = \frac{\mathbf{A} \cdot \mathbf{B}}{\|\mathbf{A}\| \|\mathbf{B}\|} = \frac{\sum_{i=1}^n A_i B_i}{\sqrt{\sum_{i=1}^n A_i^2} \sqrt{\sum_{i=1}^n B_i^2}}$$

where $\mathbf{A}$ and $\mathbf{B}$ are two n -dimensional vectors with A_i and B_i as their i -th elements. This method assesses the degree to which the two vectors, representing the experimental and theoretical spectra in this case, are parallel. A cosine value closer to unity indicates that experimental and computed spectra are more similar. The intensity values in the computed spectrum are evaluated at the exact wavenumbers of the experimental spectrum, so that the two spectra have a common x -axis.

We slightly modify the above equation to make the cosine similarity index less sensitive to deviations in peak intensities between $\mathbf{A}$ and $\mathbf{B}$, and thereby more sensitive to frequency overlap between bands in $\mathbf{A}$ and $\mathbf{B}$. Both the experimental and calculated spectra are scaled to a maximum intensity of 1 and then the logarithm of these scaled values is taken

$$A_i^{\text{trans}} = \log\left(\frac{A_i}{A_{\text{max}}} + c\right)$$

where c is a constant that is identical for vectors $\mathbf{A}$ and $\mathbf{B}$. The value of c is a compromise between being sensitive to low-intensity bands in the spectrum on the one hand and avoiding experimental noise affecting the similarity on the other hand. A value of 10^{-8} was taken for c here as it gave the best results for a small test set of experimental and computational spectra. The cosine similarity is calculated for the spectra and displayed in the figures as the ‘matching score’.

Results & discussion

For each of the z_3 ions studied here, Table 1 lists the relative energies for isomers differing in the position of the radical. We adopt here a numbering of the residues and molecular groups that starts from the C-terminus. For each isomer, the B3LYP/6-31++G(d,p) relative free energy of the lowest-energy conformer identified in the conformational search is presented. For structures with the radical on the C_β atom of the second residue and on the C_α atom of the first residue, no conformational search was performed as these structures are unlikely (see below); Table 1 lists the DFT energy which may not correspond to the lowest-energy conformer. A complete overview of the relative energies including results obtained at the B3LYP-D and M06-2X levels of theory is presented in ESI Table S1.†

Table 1 Computed relative energies for the various possible isomers of the radical z₃-ions investigated at the B3LYP/6-31++G(d,p) level of theory. Residues are counted from the C-terminus

Peptide fragment	3 rd residue		2 nd residue		1 st residue	
	C _α	C _β	C _γ	C _α	C _β	C _α
AAAA AAK	+21	+63	—	0	+71	+1 ^a
AAAA FAK	+32	+3	+102	0	+64	+8 ^a
AAAA HAK	+22	+17	+153	+8	+68 ^a	0 ^a
AAAA WAK	+31	+2	—	0	+72 ^a	+56 ^a
AAAA YAK	+33	0	+111	+15	+77 ^a	+7 ^a
K AHA	+15	+66 ^a	—	0 ^a	+11 ^a	+17 ^a

^a No MM/MD conformational search was performed for these structures (see text).

The values reported in Table 1 indicate that isomers with the radical positioned at the cleavage site, C_α(3), are relatively high in energy. Radical migration to the β-position of the same residue leads to significantly lower-energy isomers for aromatic residues, due to resonance stabilization of the radical by conjugation with the aromatic moiety. Radical migration to the α-positions along the backbone (1st and 2nd residue from the C-terminus) leads to isomers that are 15 to 32 kJ mol^{−1} more stable as a consequence of captodative stabilization of the radical.

An extensive conformational search was not deemed necessary for isomers with the radical at the C_β(2) position, because this position does not enable resonance stabilization of the radical and gives high-energy structures. This is specifically verified for the z₃-ions of AAAAAAK and AAAFAK, which gave significant spectral mismatches around 1500–1700 cm^{−1}, as shown in ESI Fig. S1.†

Fig. 1 compares the experimental spectra with calculated spectra for structures in which the radical has migrated to the C_α(1) position. All the experimental IR spectra show a distinct band around 1750–1770 cm^{−1}, which is attributed to the C=O stretch of the carboxylic acid group at the C-terminus. In the computed spectra, this C=O stretch undergoes a significant red shift as a consequence of conjugation with the radical at the adjacent α-carbon, effectively reducing the C=O bond order.³⁶ Hence, the C_α(1) radicals are clearly unable to explain the experimental spectra and we can safely exclude these structures, even if they are low in energy; no conformational search has been carried out for these isomers.

Fig. 2 presents the relative energies, matching scores, fragmentation channels, and experimental and calculated spectra for the series of z₃-ions [·AAK + H]⁺, [·HAK + H]⁺, [·YAK + H]⁺ and [·AHA + H]⁺. The radical positions that are considered here are: the cleavage site, C_α(3), the β-carbon of the third residue, C_β(3), and the α-carbon of the adjacent residue, C_α(2). For the z-ions with an alanine residue in the third position ([·AAK + H]⁺ and [·AHA + H]⁺), radical migration to the C_β(3)-position does not enjoy resonance stabilization and leads to relatively high-energy structures; we therefore consider these isomers unlikely and their calculated spectra are not included in Fig. 2.

Fig. 1 Experimental IRMPD spectra (in black) of the six z_3 ions considered in this study compared to calculated spectra (blue) for z_3 ions with the radical at the C_α of the first residue (lysine, or alanine for KAHA). A striking mismatch between experiment and theory is observed for the c-terminal carboxylic $\text{C}=\text{O}$ stretch mode in the 1700–1800 cm^{-1} range; $C_\alpha(1)$ -radical structures are therefore rejected.

Fig. 2 IRMPD spectra (black) of the four z_3 ions $[\cdot\text{AAK} + \text{H}]^+$, $[\cdot\text{HAK} + \text{H}]^+$, $[\cdot\text{YAK} + \text{H}]^+$ and $[\cdot\text{AHA} + \text{H}]^+$ compared to calculated spectra with the radical positioned at $C_\alpha(3)$ in blue, at $C_\beta(3)$ in green and at $C_\alpha(2)$ in red. The presented calculated spectra are for the lowest energy conformers for each z_3 -isomer resulting from the MM/MD search.

For the $[\cdot\text{AAK} + \text{H}]^+$ z_3 -ion, the lowest-energy structure is found for the $C_\alpha(2)$ isomer (21 kJ mol⁻¹ more stable than the $C_\alpha(3)$ cleavage-site radical). A gain of 14 kJ mol⁻¹ in energy upon radical migration to the $C_\alpha(2)$ position is observed for the $[\cdot\text{HAK} + 2\text{H}]^+$ z_3 -ion, while migration to the $C_\beta(3)$ -position of the His residue gives only a marginal stabilization (−5 kJ mol⁻¹). For the $[\cdot\text{YAK} + 2\text{H}]^{2+}$ z_3 -ion, the lowest-energy isomer is that with the radical at the $C_\beta(3)$ -position of Tyr. For the $[\cdot\text{AHA} + 2\text{H}]^{2+}$ z_3 -ion, radical migration to the $C_\alpha(2)$ -position of His stabilizes the system by 15 kJ mol⁻¹. Hence, based purely on thermochemical grounds, radical

migration from the original $C_{\alpha}(3)$ -position of the ETD cleavage site to the $C_{\beta}(3)$ -position or along the peptide backbone to adjacent C_{α} -positions is plausible.

In addition to the relative energies of the isomers (see above) and the IR spectral information (see below), the observed IR-induced fragmentation channels may also contain information on the structures of the z_3 -ions. However, one should keep in mind that IR multiple-photon excitation is a slow process and that rearrangements such as H-atom migration may occur upon activation prior to dissociation. A similar reservation was made concerning CID probing of radical migration.⁴⁶ For the systems in Fig. 2, no radical side-chain loss is observed for $[\cdot\text{AAK} + \text{H}]^+$, $[\cdot\text{AHA} + \text{H}]^+$ and $[\cdot\text{YAK} + \text{H}]^+$ suggesting that the radical does not migrate to the side chains. For $[\cdot\text{HAK} + \text{H}]^+$ and $[\cdot\text{YAK} + \text{H}]^+$ the formation of a z_2 -ion is observed, suggesting that the radical is able to migrate to the $C_{\alpha}(2)$ position; as mentioned, we cannot exclude that this happens only upon IR activation.

We now inspect the IR spectra in Fig. 2. For systems with an aromatic residue in position 3, a $C_{\beta}(3)$ radical z_3 -ion structure is conceivable and their calculated spectra are shown in green. Both computed spectra predict an intense peak around 1650 cm^{-1} (C=O stretching in the Tyr or His amino acid residue, combined with NH_3 bending) and around 1550 cm^{-1} (NH_3 bending). Both of these features are poorly reproduced in the experimental spectrum. Based on this severe mismatch, the resonance-stabilized $C_{\beta}(3)$ structure is rejected for the $[\cdot\text{YAK} + \text{H}]^+$ and $[\cdot\text{HAK} + \text{H}]^+$ z_3 -ions.

Next we consider the $C_{\alpha}(2)$ radical structures that are stabilized by the captodative effect, whose calculated spectra are shown in red in Fig. 2. Again, the spectral region between 1650 and 1550 cm^{-1} containing the C=O stretches is poorly reproduced by the calculations. Also, for $[\cdot\text{AHA} + \text{H}]^+$ the doublet feature between 1700 cm^{-1} and 1800 cm^{-1} is not correctly reproduced by the calculation. Finally, for all systems in Fig. 2 except $[\cdot\text{AHA} + \text{H}]^+$, the bands near 1400 cm^{-1} are slightly red-shifted compared to the experimental spectra. Based on these observations the possibility of radical migration to the C_{α} -position of a neighboring residue is unlikely.

Computed spectra for structures with the radical positioned at $C_{\alpha}(3)$, the ETD cleavage site, are colored blue in Fig. 2. Of the three types of isomers considered in this figure, these structures provide the best match for the experimental feature around 1675 cm^{-1} , especially for $[\cdot\text{HAK} + \text{H}]^+$, assigning this peak as an amide C=O stretch. Overall, the calculated spectra for the $C_{\alpha}(3)$ structures that have not undergone radical migration show the best agreement in the amide 1400 – 1650 cm^{-1} region. Especially for the experimental spectra of $[\cdot\text{AAK} + \text{H}]^+$ and $[\cdot\text{YAK} + \text{H}]^+$, the C=O stretch band of the second amide linkage around 1600 cm^{-1} is reproduced better than by the alternative structures. It is also striking that the predicted spectra for the $C_{\alpha}(3)$ radical reproduce the experimental spectra accurately in the entire 1000 – 1400 cm^{-1} region, whereas the alternative $C_{\beta}(3)$ and $C_{\alpha}(2)$ spectra fail to do so. The $[\cdot\text{AHA} + \text{H}]^+$ z_3 -ion is an exception in the present series of investigated systems in that the $C_{\beta}(2)$ -position is of interest for radical migration, due to stabilization through resonance with the imidazole group of the His residue. The computed spectrum for this isomer is overlaid onto the experimental spectrum in Fig. S2,[†] and the agreement between the computed and experimental spectra is seen to be comparable to that for the $C_{\alpha}(3)$ isomer. Radical migration to the $C_{\beta}(2)$ -position can thus not be excluded for the $[\cdot\text{AHA} + \text{H}]^+$ ion.

The spectra recorded in H-stretching range generally show strong and sharp OH-stretch features and NH-stretch bands that are often weaker than predicted; this is likely due to the fact that OH moieties are free in the computed structures, whereas NH moieties, especially on the Lys side chain, engage in strong hydrogen bonds.^{83–88} In any case, most pertinently to the present discussion, the spectra in this range provide little or no diagnostic information that would allow us to distinguish between isomers.

We finally inspect the cosine similarity (matching) scores between the calculated and experimental spectra. The spectra predicted for the $C_\alpha(3)$ structures yield significantly higher values for all four systems, except for $[\cdot\text{AAK} + \text{H}]^+$, for which $C_\alpha(3)$ and $C_\alpha(2)$ yield virtually equal scores. In conclusion, based on visual inspection of the spectra as well as on the cosine similarity value, the $C_\alpha(3)$ radical structures provide the best matches, suggesting that radical migration does not take place after ETD for these systems under our experimental conditions. As the $C_\alpha(3)$ is not the lowest-energy structure, this also implies that the radical is kinetically trapped.

Fig. 3 presents the experimental spectra for the two remaining z_3 -ions studied in this work, $[\cdot\text{FAK} + \text{H}]^+$ and $[\cdot\text{WAK} + \text{H}]^+$. We again compare these with calculated spectra for isomeric structures with the radical positioned at $C_\alpha(3)$, $C_\beta(3)$ or $C_\alpha(2)$. For these ions, radical migration from the cleavage site $C_\alpha(3)$ to the $C_\beta(3)$ - or $C_\alpha(2)$ -position yields similar energy gains of about 30 kJ mol^{-1} , so that based purely on thermochemical arguments no distinction can be made between these two. Earlier studies on ETD fragments containing Phe and Trp report calculations indicating that for $[\cdot\text{AFAR} + \text{H}]^+$ z_4 -ions, radical migration to the C_β position of Phe gives the lowest energy structure, while for the $[\cdot\text{AWAR} + \text{H}]^+$ ion, placing the radical at the C_α of the Arg residue gives the lowest-energy structure.³¹

Based on the cosine similarity scores between the experimental spectra and the calculated spectra, the $C_\alpha(2)$ isomer gives the most favorable match for both structures, but it is only marginally better than those for the alternative structures. A visual inspection of experimental and computed spectra for the $C_\alpha(2)$ isomer shows a mismatch for both species at 1600 cm^{-1} (combined NH_3 bending, $\text{C}=\text{O}$ stretching and $\text{N}-\text{H}$ bending in the first amide bond). Also, the band around 1675 cm^{-1} is not reproduced in the calculated spectra for both species. However, calculated spectra for the alternative isomers do not reproduce this band satisfactorily either. A point in favor of the $C_\alpha(2)$ isomer is the observation of z_2 -ions as IRMPD fragmentation channels, which may be evidence for radical migration to the $C_\alpha(2)$ position.

The calculated spectra for the $C_\beta(3)$ -isomers in green show a doublet of peaks at about 1600 and 1650 cm^{-1} , similar to the $C_\beta(3)$ -calculated spectrum for $[\cdot\text{YAK} + \text{H}]^+$, which is not observed in the experimental spectra. This consistent mismatch appears to disqualify the $C_\beta(3)$ -isomer for $[\cdot\text{FAK} + \text{H}]^+$ and $[\cdot\text{WAK} + \text{H}]^+$, although the IRMPD reaction channels for $[\cdot\text{WAK} + \text{H}]^+$ include loss of the radical side chain, which may be evidence for the $C_\beta(3)$ -isomer.

Despite its higher energy, the $C_\alpha(3)$ -isomer with the radical at the cleavage site cannot be dismissed, certainly in light of our conclusions for the three systems in Fig. 2. The main mismatch between the computed and experimental spectra is observed for the intense experimental band around 1500 cm^{-1} , which is likely due to $\text{N}-\text{H}$ bending in both amide bonds. This mismatch appears to be somewhat similar to that observed for the $[\cdot\text{AAK} + \text{H}]^+$ ion in Fig. 2, which otherwise gives

Fig. 3 IRMPD spectra (black) of the $[\cdot\text{FAK} + \text{H}]^+$ and $[\cdot\text{WAK} + \text{H}]^+$ z_3 ions compared to calculated spectra for isomers with the radical at the $C_{\alpha}(3)$ cleavage site (blue), at the $C_{\beta}(3)$ atom of the aromatic side chain (green) and at the $C_{\alpha}(2)$ atom enjoying captodative stabilization. The calculated spectra shown are the lowest-energy conformers after an MM/MD search for each of the isomers. In addition, for structures with the radical positions indicated, the relative free energies in kJ mol^{-1} , IRMPD induced dissociation channels, and spectral matching scores based on the cosine similarity are given.

a convincing match. Also, the band near 1200 cm^{-1} in the experimental spectra of $[\cdot\text{FAK} + \text{H}]^+$ and $[\cdot\text{WAK} + \text{H}]^+$ is not reproduced in the $C_{\alpha}(3)$ calculations, but all the remaining peaks show a reasonably good agreement. We must conclude that a definitive assignment for these two ions remains insecure, but we tentatively assign a mixture of $C_{\alpha}(3)$ and $C_{\alpha}(2)$ isomers for the z_3 -ions presented in Fig. 3.

Conclusions

The position of the radical in z_3 ions generated by the ETD of a set of six peptides has been investigated using a combination of infrared ion spectroscopy and quantum-chemical calculations. It is well known that (CID) MS/MS product ions may often be kinetically trapped species, so that an outright comparison of computed relative energies for different isomers has only limited value in predicting the structure of the product ions. Also in the present study, the z_3 -ions with the radical positioned α -carbon of the third residue are higher-energy isomers, with radical migration to the $C_{\alpha}(1)$, $C_{\alpha}(2)$ or $C_{\beta}(3)$ atoms leading to lower-energy isomers as a consequence of resonance stabilization, including captodative stabilization, of the radical. Nonetheless, we find that under the

experimental conditions of our Bruker AmaZon ETD instrument, radical migration to form lower-energy isomers often does not occur for the z_3 ions investigated. Radical migration to the α -carbon of the C-terminal residue, $C_\alpha(1)$, can securely be ruled out based on the position of the carboxylic C=O stretch band. Further spectral comparison for the $[\cdot\text{AAK} + \text{H}]^+$, $[\cdot\text{HAK} + \text{H}]^+$, $[\cdot\text{YAK} + \text{H}]^+$ and $[\cdot\text{AHA} + \text{H}]^+$ z_3 -ions leads us to the conclusion that the radical remains kinetically trapped at the cleavage site; for $[\cdot\text{AHA} + \text{H}]^+$ radical migration to the His $C_\beta(2)$ position cannot be excluded based on the IR spectrum. For the $[\cdot\text{FAK} + \text{H}]^+$ and $[\cdot\text{WAK} + \text{H}]^+$ z_3 -ions, the current experimental and theoretical spectra do not allow us to make a clear structural assignment; partial isomerization by radical migration to the adjacent amino acid residue, $C_\alpha(2)$, appears to occur. We tentatively attribute the experimental spectra to a mixture of isomers with the radical at the C_α -positions of the third and second residues.

Conflicts of interest

There are no conflicts to declare.

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