

Structural investigation of naturally occurring peptides by electron capture dissociation and AMBER force field modelling

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We present a detailed analysis of the relative yields in dissociation products of doubly protonated polypeptide cations obtained via electron capture dissociation (ECD). These experimental studies are complemented by molecular dynamics force field modelling, using the AMBER force field, to correlate with putative gas-phase conformations for these peptides. It is shown that the highest gas-phase basicity amino acid residue (i.e. arginine) is included in all the charged fragments. This is of particular use in determining the primary structure tryptic digest peptides, which will ordinarily possess a high basicity C-terminal residue (i.e. arginine or lysine). Further, these results suggest that the relative ECD dissociation pattern is related to the secondary structure of the peptide. In particular, the ECD fragmentation pattern in gonadotropin releasing hormone (GnRH) variants appears to depend on whether a β -turn or an extended α -helical structure is formed. In the peptide bradykinin, modelling suggests that the C-terminal arginine engages in much more extended solvation of the backbone than the N-terminal arginine. This strongly correlates with the observed dominance of *c* over *z* fragments. This work forms the first attempt at a systematic qualitative correlation of the low-energy structures of modelled gas-phase polypeptides, and their corresponding ECD dissociation pattern.

Keywords: Naturally occurring peptides; Structural investigation; Electron capture dissociation; AMBER force field modelling

1. Introduction

Fragmentation of polypeptides in mass spectrometers with the aim of determining their primary structure (i.e. their amino acid sequence) plays a crucial role in protein identification [1]. This fragmentation is mostly effected using vibrational excitation techniques, either through collisional activation with a gas [2] or a collision with a surface [3]; or, alternatively, peptide ions are vibrationally excited by means of infrared radiative activation with a CO₂ (10.6 μ m) laser [4] or a black-body [5]. In these techniques the peptide ion is excited above its dissociation threshold, predominantly yielding products due to cleavage across a C(O)–N peptide bond. The resulting dissociation products are notated *b* and *y* fragments depending on whether they contain the N- or C-terminal portion of the polypeptide

(figure 1). Electron capture dissociation (ECD) [6] is an electronic excitation technique of peptide cations where the recombination energy of electron capture generally leads to cleavage of a C $_{\alpha}$ –N backbone bond. In contrast to collision-induced products, this process primarily produces even-electron *c'* and odd-electron *z'* fragments [7] (figure 1). In order to explain the preference for these ECD products, a ‘hydrogen atom re-arrangement’ mechanism was proposed [8] (figure 2), where the electron is first captured at the protonated site; this leads to hydrogen atom re-arrangement to a backbone carbonyl, thus causing α -cleavage to occur on the backbone.

In proteomics applications, proteins are enzymatically digested into polypeptides which are then fragmented in mass spectrometers. The most common enzyme is trypsin, which cleaves the protein on the C-terminal side of either arginine or lysine. Since both of these residues have high gas-phase basicities one would mainly expect *z* fragments for doubly protonated peptides. This renders the determination of the primary structure from ECD fragments simpler

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than when both N- and C-terminal fragment ions are present.

While the mechanism in ECD is still controversial [9, 10] there are indications for *non-ergodic* (i.e. dissociation before energy randomization) behaviour: electron capture of a single electron in a protein leads to dissociation of a covalent bond [11, 12], which could not occur if the recombination energy is fully randomized around the molecule; further, many labile side-chain groups are not preferentially cleaved, unlike in vibrational excitation techniques where this process can dominate. This feature makes ECD particularly interesting for the identification and localization of post-translational modifications [13–16]. If ECD proceeds by a sub-picosecond mechanism it is likely that bond cleavage will occur in the vicinity of the site of electron capture (i.e. the site of protonation). The relative abundances of fragmentation products should inform on higher structure (e.g. secondary, tertiary structure) of the peptide or protein under investigation [17], as if ECD is taking a snapshot of

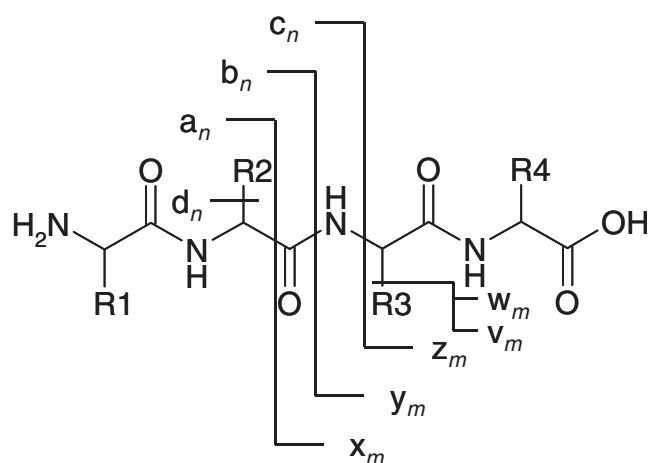


Figure 1. Nomenclature for polypeptide fragmentation based on Roepstorff and Fohlmann [51], where n is the number of residues from the N-terminus and m is the number of residues from the C-terminus.

the molecular ion. This, coupled with the low-energy nature of the ECD process, begs the question: can a relationship be found between the relative distribution of ECD fragmentation products and the ensemble of stable gas-phase structures for a given polypeptide? and, as with all studies of higher-order structures of proteins in the gas phase, *will this be pertinent to biology?*

In this study ECD is performed on a set of naturally occurring peptides where the secondary structure plays an important biological role: gonadotropin releasing hormone (GnRH) variants [18, 19], bombesin and bradykinin [20, 21]. All these systems are sufficiently small to be amenable to detailed mechanical force field modelling, which is not the case for whole proteins. In addition, we have examined the effect of altering the chirality of a single amino acid in GnRH on its ECD fragmentation behaviour; this builds on earlier work [22]. Such investigations have particular relevance to understanding the structure–function relationship of this peptide, since single amino acid changes, especially at position 6, can have marked effect on receptor binding and activity [23]—in fact, this is why such variants are readily available for study! Relative fragmentation patterns of some of these peptides are compared to the low-energy structures obtained from calculations employing the AMBER force field [24, 25].

One of the main approaches in protein identification consists in peptide ion mass isolation and fragmentation in mass spectrometers for the purpose of obtaining the primary structure (i.e. sequence) of the peptide [1, 26]. Identifying proteins from tandem mass spectra obtained from the fragmentation of digested polypeptides increasingly relies on automated analysis. Several computer algorithms have been developed for this task, (e.g. Sequest, <http://fields.scripps.edu/sequest/>) and thus correct identification of the peptide relies on an accurate prediction of the dissociation process. For vibrational excitation techniques, much systematic work has been carried out to understand the dissociation process [27], while for

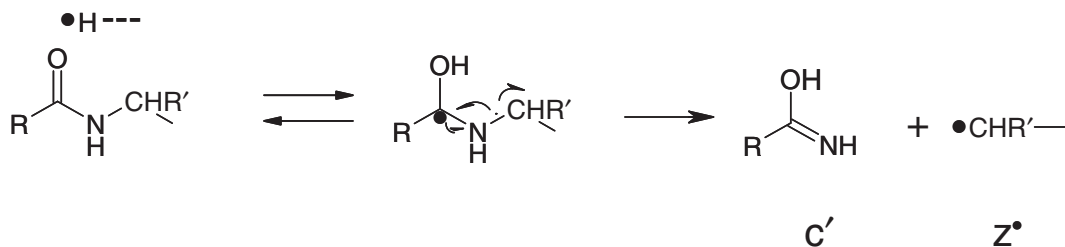


Figure 2. Schematic showing hydrogen atom re-arrangement mechanism, as proposed by McLafferty and co-workers [8]. The electron capture occurs at a protonated site leading to hydrogen atom re-arrangement to the backbone carbonyl, followed by α -cleavage on the backbone.

ECD far fewer mechanistic studies have been undertaken [28, 29]. We (and others) have demonstrated that the relative fragmentation in ECD is much more reproducible and less dependent on experimental parameters than in vibrational excitation techniques [30]. This strongly supports the development of ECD-specific computer algorithms to predict the dissociation patterns, and further verify sequence assignments. Our aim here is to explore the possibility of making predictive rules for the ECD dissociation process, which would assist identification of product ions. We also determine if ECD could reveal information on stable secondary structural conformations of peptides ions. This approach has huge potential for proteomic analysis, and could also provide a basis for assessing correlations between the stable gas-phase structure and biologically relevant structures.

2. Experimental

The peptides used in this study are commercially available (Sigma, Aldrich) and are ionized using electrospray ionization (ESI) [31]. All peptides were examined without further purification and made up at a concentration of 20 μM ($\text{H}_2\text{O}:\text{MeOH}:\text{CH}_3\text{COOH}$; 0.49:0.49:0.02). The doubly protonated peptides are trapped in the Penning trap of a 9.4 Tesla Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA). This trap, known as an Infinity cell [32], consists of two circular DC trapping plates ($\text{\O} 6 \text{ cm}$) that are 6 cm apart with circular entrances ($\text{\O} 6 \text{ mm}$) to permit ions and electrons to enter. Ions trapped in the ICR cell are subjected to electron beam emission (20–50 ms) from a dispenser cathode (Heatwave, Watsonville, CA, USA) mounted on the rear flange of the instrument. Whereas the peptide ions enter the ICR cell through the aperture of the front trapping plate, the electrons enter through the rear trapping plate.

Dispenser cathodes have been shown to give the highest ECD rates due to their relatively large emission currents (here 30 μA) over a wide area (here $\sim 20 \text{ mm}^2$), thus facilitating overlap between the electron beam and the ion cloud [33]. Long electron emission times ($>200 \text{ ms}$) lead to complete neutralization of all the ions in the Penning trap, indicating a near perfect overlap between the ion cloud and the electron beam. The electron emission time in these experiments was optimized to yield maximum fragment peak abundance. In the ECD mass spectra the normalized yields of the z product dissociation channels are monitored to give the relative fragmentation patterns. These measurements are repeated eight times to provide a standard deviation.

3. Force field modelling

We have employed the AMBER force field [24] to investigate the conformational potential energy surface of doubly protonated gonadatropin releasing hormone (GnRH) variants. The sequences of these peptides differ in the identity of residue 6. We have also examined the doubly protonated form of bradykinin. Non-standard polypeptide residues are created by altering existing residues, for example the amidized glycine found at the C-terminus of GnRH is generated by subtracting a carbonyl oxygen from the standard Amber C-terminal glycine and replacing it with an NH_2 . All parameters for this group are contained within the existing force field. Charges are assigned using the restrained electrostatic potential (RESP) fitting procedure [34], with initial point charges derived from Gaussian 98 [35]. Peptides are built *in vacuo* and subjected to a simulated annealing procedure involving heating to 800 K followed by controlled cooling to 0 K to give a minimized structure. This structure also serves as the seed structure for the next annealing cycle. In total, 100 candidate minimized structures are generated with this procedure. The stabilities of the 10 lowest-energy structures are investigated by subjecting them to molecular dynamics simulations at 300 K. The most stable representative structure of each GnRH variant is presented here using the Visual Molecular Dynamics (VMD) package [36].

4. Results and discussion

ECD mass spectra of three doubly protonated peptides $[\text{M} + 2\text{H}]^{2+}$ are shown in figure 3, along with the respective amino acid sequence (in one-letter code) where a line denotes a position at which fragmentation has arisen. The corresponding fragments are labelled on the figure, according to the nomenclature shown in figure 1. For GnRH (figure 3(a)) and bombesin (figure 3(b)), the most likely sites of protonation are the amino acid side-chains of arginine and histidine. This is due to their higher gas-phase basicities (GB) ($\text{GB}_{\text{arginine}} = 992 \text{ kJ mol}^{-1}$, $\text{GB}_{\text{histidine}} = 936 \text{ kJ mol}^{-1}$) [37]. Capture of an electron results in all observed fragments to be singly charged, with the exception of those due to neutral side-chain losses. All fragments (figure 3(a) and (b)) include the most basic amino acid residue, namely arginine. Thus, there appears to be a clear correlation between the higher gas-phase basicity of arginine and the fact that all charged ECD fragments include this residue. A similar correlation is also observed in vibrational excitation techniques, where the number of ionizing protons relative to the number

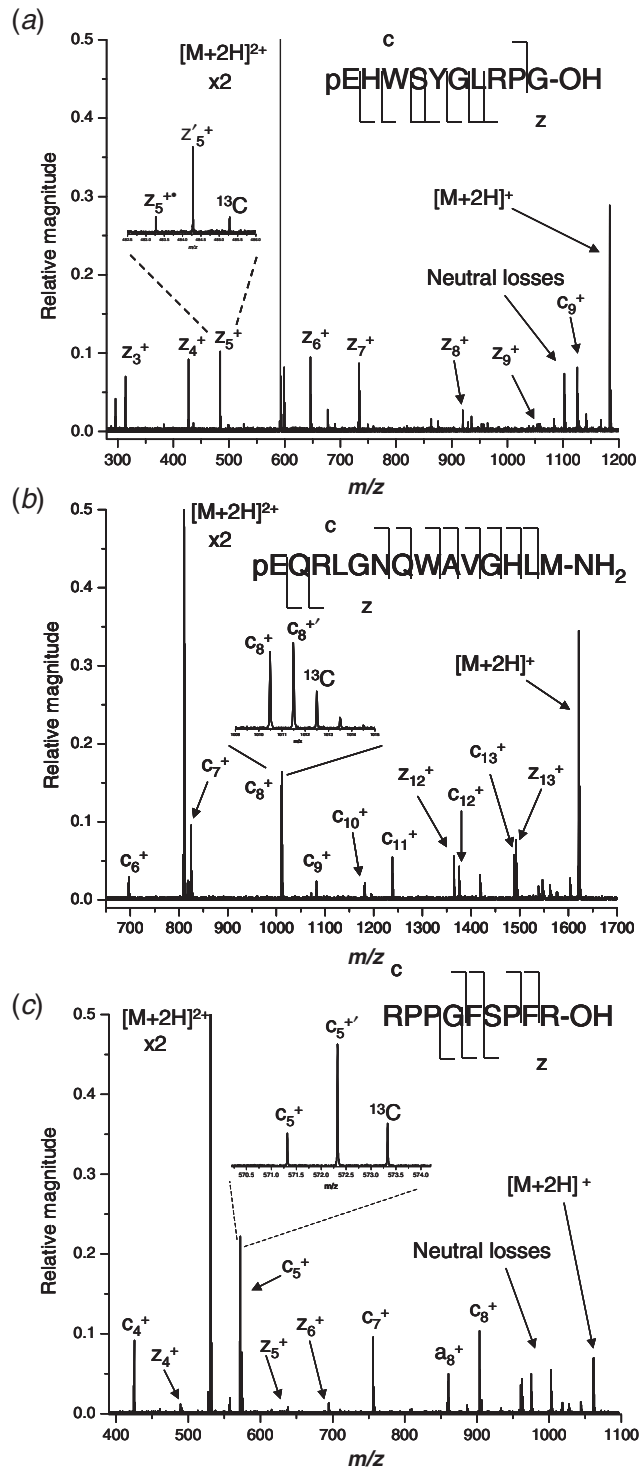


Figure 3. ECD mass spectra of doubly protonated (a) gonadatropin releasing hormone (GnRH) variant (amino acid sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly), (b) bombesin (amino acid sequence pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂) and (c) bradykinin (amino acid sequence Arg-Pro-Pro-Gly-Ser-Phe-Pro-Phe-Arg). The dissociation pattern is indicated using the one-letter amino acid code. The inserts show details of various fragments. Note that 'neutral losses' refer to loss of parts of amino acid side-chains that are also inherent to the ECD process, but that will not be considered here [52]. Note also that pGlu refers to pyro-glutamic acid, which is formed by cyclization of the N-terminus to the glutamic acid side-chain by elimination of water.

of basic sites has been shown to determine whether the dissociation pattern is selective or not [38]. This observation led to the formulation of the ‘mobile proton model’ which proposes that a labile proton is involved in the dissociation process and that this *directs* backbone cleavage [27], the mobility of the proton being inversely proportional to the gas-phase basicity of the site at which it is found. Consequently, at a highly basic site, such as the guanidine group on the arginine side-chain, the proton is sequestered more strongly and hence more energy is required to induce backbone fragmentation. In addition, if a less basic site of protonation is available, the singly protonated fragment ion observed will always contain arginine in preference. By analogy, the results here (figure 3(a) and (b)) imply that electron capture at the arginine side-chain results in inefficient peptide backbone cleavage compared with electron capture at the histidine side-chain.

For the doubly protonated peptide bradykinin, the most likely protonation sites are the side-chains of both arginines. Given the argument above it might be expected that this would result in a low fragmentation yield. However, ECD of this peptide (figure 3(c)) results in abundant backbone dissociation, all fragments containing an arginine residue. It is worth noting here that the absence of certain fragments for both the GnRH peptides and Bradykinin can be attributed to the fact that dissociation of the C_{α} -N bond on the N-terminal side of proline will not result in backbone fragments due to the cyclic structure of proline.

Results from these three peptides show that electron capture at the arginine side-chain can result in efficient backbone fragmentation, but that given the choice between a higher and lower basicity site in the ECD process the molecule somehow ‘knows’ where the most basic site is, and the proton will be retained there. In figure 3(c), c fragments are much more abundant than z fragments (by a factor of 17:1). For a fully extended peptide, possessing no permanent secondary structure, both arginines should have almost identical gas-phase basicities. However, these results here indicate that the N-terminal arginine is considerably more basic, and the reason for this discrepancy, and its implications for the gas-phase structure of the peptide, will be addressed later in this paper.

The inserts in figure 3(a)–(c) show the detailed annotation of c and z fragments, which demonstrate the presence of c' and z' fragments, as well as more unusual c^{\bullet} and z^{\bullet} fragments. These rarer fragments are not often considered, despite the fact that they have been reported by others [8, 39]. They are not easily explained by a straightforward hydrogen atom re-arrangement mechanism as shown in figure 2, although one way to rationalize them is by invoking

a hydrogen atom transfer after C_{α} -N cleavage [40]. The relative abundances of z' and z^{\bullet} (z_3 to z_9) fragments for three GnRH variants are summarized in figure 4. These are well reproduced for each product ion, and, furthermore, are exceptionally robust to slightly different experimental conditions (note small values for average standard deviations SD_{avg} in figure 4). Conversely, the relative product yield in vibrational excitation techniques is highly dependent on many parameters, particularly on the total energy deposited in the molecule and the time-scale of excitation [41]. The GnRH peptides differ in the identity of residue 6: Gly (figure 4(a)), D-Trp (figure 4(b)) and L-Trp (figure 4(c)). In all three figures there is a striking change in that z' ECD products are favoured for smaller fragments (especially in figure 4(a)), whereas z^{\bullet} ions are favoured for larger fragments ($>z_5$). Relative dissociation patterns in figure 3(a) and (b) are more similar, compared with figure 3(c) (see table 1). The largest difference between these spectra is that the abundance for z'_3 in figure 3(c) is much reduced compared with figure 3(a) and (b). In fact, omitting the standard deviation for z'_3 in these average standard deviations renders all of them very comparable. The appearance of z_3 is due to C_{α} -N cleavage on the C-terminal side of residue 7 (i.e. leucine). It has been postulated that ECD cleavage is favoured on the C-terminal side of some amino acid residues, such as tryptophan [28], and that ECD is a ‘reducible’ dissociation technique therein. Cleavage efficiency is related to the immediate chemical vicinity and not to the rest of the molecule [29]. Our findings support this, and the data shown in figure 3 illustrates how ECD may be used to probe subtle single amino acid differences in peptide ions.

In these peptides, the identity of residue 7 is the same for all and yet there are differences in the relative abundances of z'_3 . The fact that replacing (D)tryptophan with (L)tryptophan in position 6 makes such a difference to the abundance of z'_3 suggests that this is due to an *inherent* conformational differences exhibited by these peptides. ECD studies on a small protein by Adams *et al.* have also shown that a replacement of one amino acid by its D counterpart leads to a change in the relative dissociation pattern, which they attributed to a change in conformation [42]. A separate study of ECD on colder peptide ions (86 K) resulted in fewer backbone cleavages than at room temperature [43], suggesting that fewer dissociation channels are accessible due to a reduced conformational heterogeneity at lower temperatures.

The molecular mechanics modelling performed here reveals that GnRH peptides with Gly and D-Trp in position 6 adopt a β -turn type structure (figure 5(a) and (b)), while the GnRH peptide with L-Trp in position 6

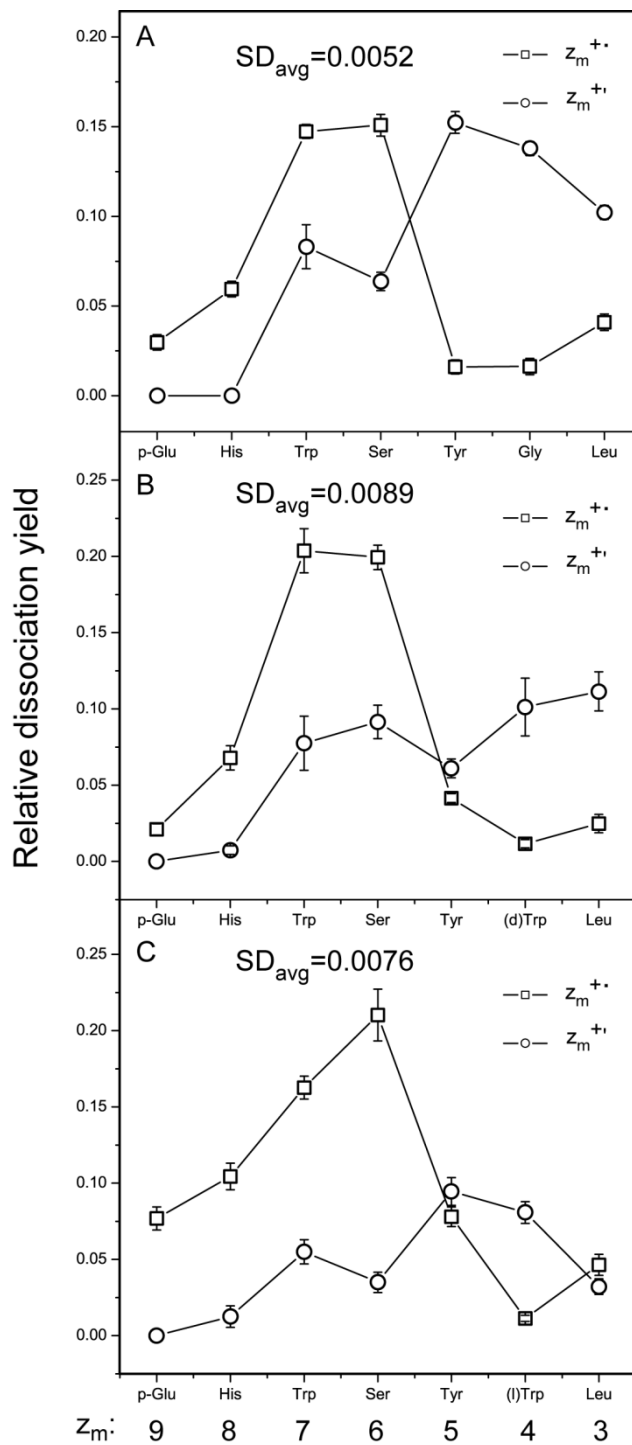


Figure 4. Relative fragment yield of z_m^* and z_m' ions for the GNRH variants (a) pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly, (b) pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly and (c) pGlu-His-Trp-Ser-Tyr-L-Trp-Leu-Arg-Pro-Gly. On the x scale is given the amino acid residue (in three-letter code), on the C-terminal side of which α -cleavage must occur for that z_m fragment to appear. The mean and standard deviations of each dissociation channel are given, as well as the average standard deviation SD_{avg} of all the z_m fragment yields.

Table 1. Average standard deviations of the relative z fragment yields between figure 4(a), (b) and (c), showing that the dissociation patterns in figure 4(a) and (b) are most similar. The dissociation patterns in figure 4(b) and (c) are less similar, and the patterns in figure 4(a) and (c) are most different.

	Figure 4(a)	Figure 4(b)	Figure 4(c)
Figure 4(a)	N/A	0.0175	0.0249
Figure 4(b)	0.0175	N/A	0.0212
Figure 4(c)	0.0249	0.0212	N/A

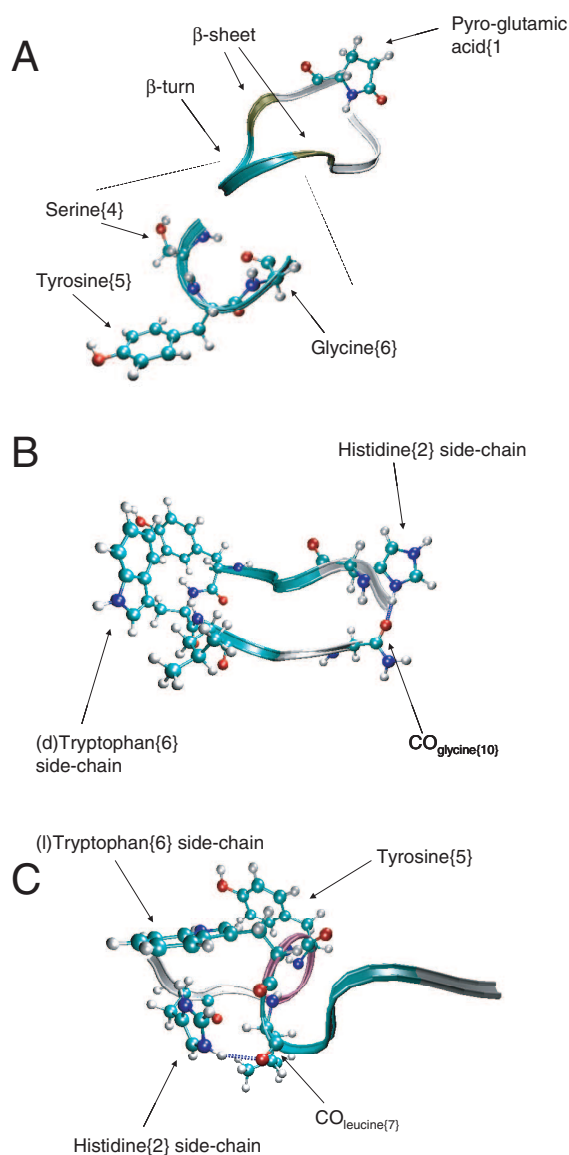


Figure 5. Molecular dynamics (300 K) ribbon structures for the GNRH variants (a) pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly (insert shows amino acids involved in β -turn), (b) pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly and (c) pGlu-His-Trp-Ser-Tyr-L-Trp-Leu-Arg-Pro-Gly.

favours an extended structure with an α -helix around residues 3–7 (figure 5(c)). Note that these represent the lowest-energy structures for each variant which is also found to be stable at higher internal energies (300 K dynamics). Solution-phase modelling of some GnRH variants by Guarnieri *et al.* [44] and in our recent work [22] also predicted a propensity for β -turn structures, which have been experimentally confirmed by solution-phase nuclear magnetic resonance (NMR) studies [45]. Further, the identity of the residue in position 6 is known to play a very important role in the activity of these peptides [19]. The presence of the achiral glycine here will favour a β -turn structure, which has long been considered key to binding at the mammalian receptor [46]. The differences in gas-phase conformations could thus account for the differences in the ECD dissociation patterns. We have demonstrated the importance of this in our related study of GnRH analogues where both ECD and ion mobility have been used to probe secondary structural differences that arise via single amino acid changes in another set of GnRH analogue peptides. These findings are particularly intriguing given that this peptide binds to a membrane bound receptor, and at such a binding site the dielectric constant is likely to be much lower than in aqueous solution, and much less dominant to the conformation. This underlies the potential of gas-phase structure determination to biology.

The higher abundance of c (c' and c^{\bullet}) compared with z (z^{\bullet} and z') product ions in the ECD mass spectrum of doubly protonated bradykinin (figure 3(c)) referred to earlier in this paper could not be explained based on differences in gas-phase basicity of both arginine residues. For this peptide, two configurations are possible: either the N- and C-terminus are neutral (figure 6(a)) or the N-terminus is protonated and the C-terminus is de-protonated (zwitterionic structure, figure 6(b)). Whether the neutral-termini or zwitterionic structure is favoured has not been conclusively proven [47–49]. Molecular dynamics studies of both configurations demonstrate a propensity for the C-terminal arginine to engage in hydrogen bonding with carbonyls on the backbone, whereas the N-terminal arginine is hydrogen bonded to the C-terminus as shown in figure 6. Recent *ab initio* calculations have shown that intramolecular hydrogen atom transfer is favoured when the electron capturing protonated site is in hydrogen bonding contact with the backbone carbonyl to which hydrogen atom transfer takes place [50]. This would explain why electron capture at the C-terminal arginine gives rise to predominant backbone fragmentation, whereas electron capture at the N-terminal arginine results in less efficient hydrogen atom transfer to the backbone and hence less efficient backbone cleavage.

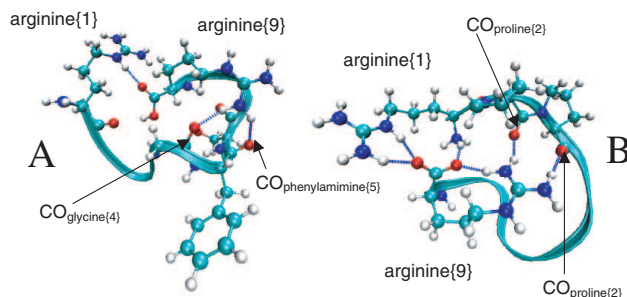


Figure 6. Molecular dynamics (300 K) ribbon structures for bradykinin (amino acid sequence Arg–Pro–Pro–Gly–Ser–Phe–Pro–Phe–Arg): (a) neutral termini form and (b) zwitterionic form.

The higher abundance of c versus z fragments could thus be accounted for by conformational differences in arginine side-chain solvation.

5. Conclusions

These results have shown that the dissociation pattern in ECD is largely dependent on the relative gas-phase basicities of the protonated sites *and* is related to the secondary structure of the peptide. ECD experiments are performed over a long time period, and a population of different structures of these small peptides must be present, however the reproducibility of the fragmentation patterns for each peptide, coupled with the noticeable variation on changing one chiral centre, points to a structurally directed successful cleavage process. Additionally, it suggests that the processes involved in ion creation and subsequent transmission through the spectrometer do not significantly disturb the population of stable gas-phase structures. The fact that gas-phase peptide structures are stable is bourn out by extensive molecular dynamics runs at elevated temperatures, wherein the hydrogen bonding dictating the form of low-energy structures is maintained. The results for bradykinin also suggest that more extensive hydrogen bonding of the protonated site to backbone carbonyls results in enhanced backbone cleavage. A detailed analysis of preferred hydrogen-bonded structures found by modelling could be quantitatively correlated to enhanced dissociation channels. However, given the controversy surrounding the ECD mechanism, this could be difficult to interpret. What is clear is that there are two distinct contributions to the production of ECD fragments, the first is capture of an electron at a given site, and the second is the hydrogen directed cleavage of the polypeptide backbone. The success of cleavage to give the reproducible distribution of fragment ions we see here will depend on the proximity of this radical hydrogen

to the cleavage site. This must be decided by its ability to accept hydrogen bonding and in analogy to the mobile proton model for vibrational dissociation by the lability of the 'hot' hydrogen.

Our findings here for the GnRH peptides are of particular significance since it is known that single amino acid changes, or even just switching chirality, will greatly affect the activity of these peptides [19]. These ECD and molecular modelling measurements both point to appreciably different stable structures for these ions, the challenge is now to relate these gas-phase conformational preferences to those found *in vivo* at the active site.

The dependence of the ECD dissociation pattern on the conformations of the peptides (as shown for the GnRH variants and bradykinin) make a prediction of the relative fragmentation pattern more difficult for large-scale proteomics applications. However, this also enables secondary structure information on gas-phase peptides to be gained from ECD studies. The work presented here makes the first attempt at a systematic study of the relative ECD fragmentation behaviour of small peptides. As FT-ICR MS becomes more widely available, allowing the use of ECD as a sequencing tool for proteomic samples, the procedure outlined above will have increasing relevance. It also provides food-for-thought on the use of ECD as a novel gas-phase structural probe.

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