Electron capture dissociation of $b^{2+}$ peptide fragments reveals the presence of the acylium ion structure

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Electron capture dissociation (ECD) of peptides and their fragments has now been extended to $b^{2+}$ ions, where it also produced far more structural information than collisional activation. Interestingly, $b^{2+}$ ions revealed abundant loss of CO from radical monocations and the presence of $c_{m-1}^+$ fragments. The CO loss from peptide radical cations is unusual and was attributed to neutralization of the $-C\equiv O^+$ group in the acylium ion structure, supported by the observation of $c_{m-1}^+$ ions that can only be formed from an open-chain ion. These characteristic features were most prominent for $b_{12}^+$ ions of renin substrate and least prominent for $b_{10}^+$ ions of substance P ($n = 9,10$). Totally, out of seven $b_{n}^{2+}$ ions studied, CO loss above 3% level was present in all spectra as well as $c_{m-1}^+$ ions of three species, suggesting that the acylium ion structure plays a significant role for at least some $b^{2+}$ ions. This is an unexpected result in view of the literature data for small, singly charged $b$ ions, for which the protonated oxazolone structure is favoured in ab initio calculations. Apparently, more studies are required before extrapolating the small molecule results to large species. The CO loss in ECD can be used for distinguishing between $b$ and $y$ ions in the MS/MS spectrum of larger molecules. Copyright © 2000 John Wiley & Sons, Ltd.

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Vibrational (collisional or infrared) activation of protonated polypeptides produces $b$ and $y$ fragments upon cleavage of the peptide bond. While it is generally accepted that $y$ ions are simply truncated peptides, there is a great deal of disagreement in the literature about the structure of the complementary and less stable $b$ ions. Four possible structures have been suggested: the open chain acylium ion, the protonated diketopiperazine ion, the protonated oxazolone structure and the immonium ion structure (Fig. 1). Historically, the acylium ion structure was proposed first; this structure is known from the classical EI spectra. But because $b_1$ ions of simple amino acids and short peptides have not been observed, peptide acylium ions have been ruled out by many. Furthermore, calculations have indicated that the loss of CO to form the $a_1$ ions is an exothermic reaction, and thus the acylium-type $b_1$ ions are thermodynamically unstable. A cyclic dipeptide with the diketopiperazine structure has been found to produce different CAD spectra than the equivalent $b$ ions derived by collisions from a linear peptide chain. This has left the protonated oxazolone ion as the generally recognised structure of $b$ ions. Extensive ab initio calculations also seem to favour this structure. However, these calculations concerned only small ions ($b_1$ and $b_2$, and, at a lower level of theory, up to $b_9$), and the experimental evidence presented so far has mostly been circumstantial. Recently, Eckert et al. have proposed the immonium ion structure based almost solely on ab initio calculations. Harrison et al. have, however, pointed out that the protonated oxazolone structure can also account for the observations and therefore no immonium ion structure is necessary. Since the results obtained for small, singly charged ions (the biggest species studied experimentally was $b_3$) may not necessarily be applicable to large, multiply charged species, we employed a novel experimental technique to derive structural information on doubly charged $b$ ions 9–13 residues long.

The experimental techniques used so far for studying $b$ ion structures included metastable ion decay in sector instruments equipped with fast atom bombardment (FAB) and low energy collision activated dissociation (CAD) in quadrupole, hybrid, or quadrupolar ion trap instruments with electrospray interfaces. Only a few studies have used other experimental techniques, i.e. ion-molecular H/D exchange reactions and neutralization-reionization mass spectrometry (NRMS). Here we report the use of electron capture dissociation (ECD) to probe the structure of $b^{2+}$ ions.

Electron capture dissociation fragments through a different mechanism than vibrational excitation techniques, and leads to $c$, $z$ instead of $b$, $y$ products. An important feature of ECD is the preference for cleavage of just one bond near the site of charge neutralization, with S–S bonds being a possible exception. As a result, cyclic structures in the absence of disulphide bonds show in ECD spectra predominantly side-chain losses but not backbone cleavages (internal cleavage does not change the $m/z$ value of the ions). The small side-chain losses in ECD also result from single bond cleavages, in contrast to vibrational excitation, where the loss of small molecules such as H$_2$O occurs through a low-activation-energy rearrangement reaction. Furthermore, the non-ergodic cleavage in ECD is accompanied by a rather small increment in the internal
energy of fragments, suppressing secondary losses from the latter.\textsuperscript{17,18}

ECD appears to be an ideal tool for probing the structure of $b_{n}^{±}$ ions. Upon charge neutralization, acylium ions should produce extensive loss of CO:

$$R^+ - C \equiv O^+ + e^- \rightarrow R^+ - C = O^+ \rightarrow R^+ - CO$$ \hspace{1cm} (1)

while cyclic oxazolone ions should appear intact (reduced to singly charged ions) even after cleavage of an internal bond. Indeed, the cyclic amino acid proline is the only residue that appears immune to ECD. Furthermore, the presence of $c_{(n-1)}$ fragments in the ECD spectrum of $b_{n}$ ions would be an argument in favour of the open-chain structure. Here we present the results of the corresponding study.

EXPERIMENTAL

A 4.7 Tesla Ultima (IonSpec, CA, USA) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer was used in the experiments. Samples of renin substrate (DRVYIHPFHLVYYS), bombesin (pEGRLGNQWA VGHLM) and substance P (RPKQQ FFGLM) were obtained from Sigma (St. Louis, MO, USA) and used without further purification. 2–3 $\mu$L of $10^{-5}$ M peptide solution in water/methanol/acetic acid (49:49:2, v/v) was loaded into a precoated nano-electrospray needle (MDS Protana, Odense, Denmark). Electrospray-produced ions were first activated collisionally in the nozzle-skimmer region. The resulting fragments were stored for 0.5–2.0 s in the hexapole of the electrospray interface (Analytica of Branford, MA, USA), after which they were sent to the FTICR cell. Captured there by gated trapping, the ions of interest were isolated by applying a preprogrammed waveform. Alternatively, $b_{n}^{±}$ ions were, produced inside the hexapole by multipole storage assisted dissociation (MSAD)\textsuperscript{19} or inside the cell by sustained off-resonance irradiation collision activated dissociation (SORI-CAD).\textsuperscript{20} SORI-CAD was performed by increasing the cell pressure to $10^{-3}$ Torr (4 ms pulse of N$_2$ at 20 Torr) and applying for 500 ms a 5–7 V rf burst at 20 Th higher than the $m/z$-value of the ions. Electron capture dissociation was carried out by irradiation of the isolated dications by <0.2 eV electrons from a heated tungsten filament, biased to 0.85 V, for 10 s. Between 50 and 100 scans were accumulated for every species. For comparison, SORI-CAD was carried out for the same ions.

RESULTS AND DISCUSSION

Figure 2 shows the ECD spectrum of $b_{12}^{1+}$ ($m/z$ 746) of

![Figure 1. Structures of $b$ ions suggested in the literature: (1) acylium ion, (2) diketopiperazine ion, (3) protonated oxazolone ion, (4) immonium ion.](image1)

![Figure 2. Electron capture dissociation mass spectrum of $b_{12}^{1+}$ of renin substrate. Left insert shows SORI-CAD mass spectrum of the same ion. Asterisks indicate spike peaks.](image2)

The relative abundance in percentage of the total product current is shown for the specific fragments. Data for peaks that are off by 2 Da compared to the expected mass are shown in parentheses.

Table 1. Probed b ions and their behaviour in ECD. The C-terminus of renin substrate is shown in the second column together with the corresponding residue number given below. The relative abundance in percentage of the total product current is shown for the specific fragments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>b ion</th>
<th>CO, %</th>
<th>c ion, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin</td>
<td>−1.1 Y V S</td>
<td>b12+</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Substrate</td>
<td>−11 12</td>
<td>b12+</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>Bombesin</td>
<td>−G H L M</td>
<td>b12+</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Substance:</td>
<td>−F G L M</td>
<td>b12+</td>
<td>25</td>
<td>1</td>
</tr>
</tbody>
</table>

Extensive observation (Fig. 2, right insert), although with low intensity (see Table 1). Unlike other c-fragments, this ion is an odd-electron species, 1 Da lighter than ‘conventional’ c ions.

The obtained results are inconsistent with the oxazolone structure for the b12+ ions as the precursors of these fragments. Indeed, the most likely protonation site in the oxazolone ring is the tertiary nitrogen. Neutralization of this site can cause hydrogen atom desorption (this happens in ECD of smaller peptides and carries no analytical information). Alternatively, it opens the ring between the HN–CHR bond (Fig 3, pathway 1(a)) or opens the ring between the O–CO bond with the possibility to lose CO (Fig. 3, pathway 1(b)) CO loss requires, however, additional energy. In the last reaction pathway (1(c) of Fig. 3), either loss of the R’ group happens or creation of the c(n–2) fragment. None of these losses are observed except for the CO loss in the spectrum of renin substrate. If the proton is located on the less basic carbonyl oxygen, its neutralization would cause hydrogen desorption, loss of the radical R’ (2(a) in Fig. 3), cleavage of the (−N=)C–O bond or cleavage of the N–CHR bond, which can only lead to the more unlikely nitrile formation (2(b) and 2(c) in Fig. 3). Again, the R’ loss was not observed in the spectrum of renin substrate. Since the adjacent residue to the supposed oxazoline ring is the basic histidine, the question arises which is more attractive for protonation. The gas-phase basicity of oxazoline (913.4 kJ mol−1) appears to be lower than that of histidine (925.5 kJ mol−1). Therefore, the protonation of the oxazoline ring may be thermodynamically unfavourable, which can make the CO loss and c11 ions even more difficult to account for.

The observed fragmentation is most readily explained by the acylium ion structure. In general, CO loss is unusual for ECD of peptides, including y ions, as opposed to the loss of ammonia typical for ECD that was also present in the spectrum of b12+ (9% of the total abundance). The observation of the c11 ion is also in accordance with the acylium structure (Fig. 3, pathway 4). Normally, c ions incorporate the neutralized proton, which is missing in the acylium ion. This is consistent with the odd-electron nature of the observed c11 ion. All other c ions present in the ECD spectrum of renin substrate were even-electron species. Their presence was either due to intramolecular hydrogen rearrangement (such rearrangement has been observed in ECD of oxygenated dications containing no protons) or the presence of other b-ions. When these and other results are summarized in Table 1. Noticeably, all investigated species lost CO via the corresponding radical cations. This makes ECD suitable for differentiating between b- and y-polyatomic fragments of larger molecules. The probed b ions of substance P showed the lowest loss of CO. In b9 ions of that molecule, the C-terminal residue is glycine, and thus there is no side chain to stabilize an a9 ion. The last column in Table 1 shows the relative abundances of the odd-electron c(n–1) fragment. Here, the picture is more complicated. Three out of seven (43%) of the probed b12+ ions gave a significant fragment and the rest either did not show any detectable peak at the corresponding mass or the peak was off by 2 Da (such abundances are in brackets). In general, formation of a c(n–1) fragment from an acylum cation is not expected to be a favoured process, since no charge solvation near that bond is possible due to steric reasons. Furthermore, odd-electron ions are less stable than even-electron species and may undergo radical-site initiated fragmentation to e.g. smaller even-electron c ions after hydrogen rearrangement. Therefore, the absence of c(n–1) fragments cannot be considered direct evidence against the acylium ion structure.

b12+ and b11+ of bombesin showed the loss of 81.03 Da which corresponds to the side chain of histidine. Such a loss has not been reported earlier for histidine-containing peptides. Since in these ions, histidine is close to the C-terminus, neutralization of the nearby acylum site can account for this single-bond cleavage.

SORI CAD of b12+ of renin substrate also produced a minor loss of CO to form an a12+ ion (Fig. 2, left insert). This loss is a result of a rearrangement reaction in an even-electron dication and is unrelated to the ECD loss from the radical monocations formed upon electron capture. Indeed, the CO loss due to recombination energy release in the oxazolone structure would be preceded by hydrogen atom desorption to create an even-electron monocation. This would result in the total loss of 29 Da from the MH+ species instead of the observed 28 Da loss. In other words, CAD produces even-electron a ions, while CAD generates a radical species. Similarly, the NH3 loss in ECD resulting from neutralization of a N–H group and single bond cleavage in the radical cations (Fig. 2) is unrelated to the rearrangement-based ammonia loss often observed in CAD. Also, the extensive CAD-induced water loss, (Fig. 2, left insert), which is also due to a rearrangement reaction, is absent in the ECD spectrum (Fig. 2).

From the analytical point of view, ECD once again provided much more structural information than CAD. For b12+ of renin substrate, all inter-residue bonds except for the prohibited -Pro bond were cleaved by ECD, as opposed to just two peptide bonds in CAD (Fig. 2). ECD spectra of other b+ ions also contained a wealth of structural information.

Concerning the ion energetics, ab initio simulations of
Figure 3. Possible decomposition pathways after electron capture for both acylium ion and protonated oxazolone structures.
smaller cations were clearly in favour of the oxazolone structure and against the acylium ion structure.\cite{1,2,3} On the other hand, Williams et al. provided evidence that \textit{b, y} fragmentation in larger, multiply charged polypeptides can proceed via direct bond cleavage without prior rearrangement (cyclization).\cite{4} Such a cleavage must necessarily proceed through an acylium ion, which then may rearrange to other structures, e.g. protonated oxazolone. This rearrangement almost certainly involves an activation barrier, and this may cause relative kinetic stability of large acylium ions. Even though the oxazolone structure may be energetically most favourable, it does not have to dominate in the population of ions produced under certain experimental conditions. Another difference between the small and large polypeptides is that charge solvation plays an important role in the latter and is maybe absent in the former. The acylium ion structure is more compact than the cyclic oxazolone structure, and thus can be solvated more effectively. The acyclic acylium isomer was found to be 1.5 eV higher in energy than the protonated oxazolone;\cite{4} charge solvation may reduce this difference significantly. In any case, conclusions on the energetics of larger structures must be drawn on the basis of full-scale simulations of the whole molecule. Such calculations, which are themselves not a trivial task, are currently being initiated.

CONCLUSIONS

Electron capture dissociation has once again proved its utility for structural analysis of labile species, this time for \textit{b}$_{2+}$ ions. The obtained results are consistent with the suggestion that at least a fraction of the population of studied \textit{b}$_{2+}$ ions have acylium ion structure. While this is unexpected, as the dominant view favours the oxazolone structure in general, such a result is not a direct contradiction to the \textit{ab initio} calculations that have been made for small molecules. Rather, the ECD results point out the danger of extrapolation from small model molecules to larger species and the necessity to perform calculations on realistic gas-phase structures.

The most significant fingerprint of ECD of \textit{b} ions found in this work is the loss of CO from neutralized radical cations. This feature can be used to distinguish N-terminal \textit{b} ions from C-terminal \textit{y} fragments in MS/MS experiments.

Acknowledgements

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REFERENCES