Structural and Mechanistic Information on c_1 Ion Formation in Collision-Induced Fragmentation of Peptides

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The formation of c_1 ions during collision-induced fragmentation of peptides with asparagine, ornithine, or glutamine at the N-terminal position 2 has been studied. For this purpose, the corresponding fragment ion spectra of a large set of synthetic peptides were investigated. It is demonstrated that the c_1 ion intensity depends on the nature of the second residue in the N-terminal dipeptide motif as well as on the peptide length. It is shown that the formation of c_1 ions proceeds by two competing mechanisms. One mechanism is the secondary fragmentation of the b_2 ion, the efficiency of which shows only a minor dependency on the complete peptide sequence. The other mechanism is the direct formation from the molecular ion, which is identified to be connected with sequence-specific c_1 ion intensities. A model for this latter mechanism is proposed based on the analysis of the formation and secondary fragmentation of the z_{max-1} ion, which is the complementary ion to the c_1 ion. Additional evidence is obtained by investigation of peptides with ornithine in N-terminal position 2, which in general exhibit c_1 ion intensities intermediate between the asparagine- and glutamine-containing species. The data presented support the reliable assignment of N-terminal dipeptide motifs using collision-induced dissociation. (J Am Soc Mass Spectrom 2010, 21, 1814–1820) © 2010 American Society for Mass Spectrometry

Peptide fragmentation by collision-induced dissociation (CID) has become one of the major techniques for the sequencing of peptides in positive ion mode mass spectrometry. It is applied in Q-TOF, Triple Quad, QTrap, and recently also in Orbitrap instruments [1, 2]. In CID of protonated peptides, the main fragmentation processes result in the formation of b and y ions, which are generated by cleavage of the amide bond between the carbonyl-carbon and the amide nitrogen. The driving force of this process appears to be protonation at the amide nitrogen, which weakens the C–N bond.

The mobile proton model [3] is based on this mechanism and, for example, can explain the formation of the extended b and y ion fragment series as experimentally observed in CID spectra of protonated peptides. Besides protonation at the amide backbones, five-centered nucleophilic attack of nitrogen atoms at positively polarized carbon atoms is another widely distributed principle in CID of protonated peptides [4]. Intuitively, it can be speculated that near the peptide termini special ion formation processes can occur, which structurally are not possible for in-chain backbone cleavages. The high abundance and stability of b_2 ions in combination with the virtual lack of b_1 ions is the most common example of a peptide terminus-specific fragmentation [5]. Due to this lack of fragmentation between the first two residues, comprehensive interpretation of low mass fragment ions in peptide CID spectra is required for unique sequence information about the N-terminal dipeptide motif, which otherwise is a blind region in peptide sequencing by CID [6, 7]. Another less extensively studied terminal fragmentation process is the formation of c_1 ions, which so far has only been reported for selected N-terminal dipeptide motifs with glutamine in position 2 [8].

In this study, 11 different peptides were used to investigate the fragmentation behavior of peptides with glutamine in position 2. Employing CID fragmentation, a c_1 ion was observed for all investigated peptides and it was hypothesized that the formation of the c_1 ion results from a further fragmentation of a b_2 ion intermediate. In the present study, c_1 ion formation dependent on glutamine, asparagine, and ornithine residues in N-terminal position 2 is demonstrated and the question is addressed if the c_1 ions are mainly first generation fragment ions from the molecular ions or second generation fragments of b_2 ions by investigating a total of 234 synthetic peptides. Finally, a mechanism is proposed for the direct formation of c_1 ions from the molecular ion.

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Experimental

All chemicals were from Sigma (Deisenhofen, Germany) of the highest purity available and solvents from Biosolve (Valkenswaard, The Netherlands) of ULC grade quality. Peptides were synthesized in house using FMOC chemistry. For every investigated amino acid in N-terminal position 2, a core sequence was synthesized and for the amino acid in position 1, instead of adding a single amino acid, a mixture of 20 common amino acids was added leading to a mixture of 20 different synthetic peptides with identical core sequences, but different N-terminal dipeptide motifs. The different core sequences used were: ILGTNTK (series 1), AEVNQLWTLR (series 2), and LIGHSQGGLTSR (series 3). These mixtures where dissolved in 2% formic acid and analyzed using nanoUPLC-MS/MS. Analyses were performed using a nanoAcquity UPLC system (Waters, Milford, MA, USA) coupled to a QTOF2 mass spectrometer (Waters Micromass, Manchester, UK). The column used was a 100 mm × 100 μm C_{18} BEH column (Waters) operated at 30 °C and a flow rate of 400 nL/min. The column outlet was connected to the inlet of the mass spectrometer using PicoTips (New Objective, Woburn, MA, USA) in combination with a pico tip sprayer (Waters Micromass, Manchester, UK). The capillary voltage was 2400 V, and the cone voltage 35 V. Peptide mixtures were loaded directly on the analytical column, washed for 24 min using 100% Solvent A (water with 0.1% formic acid), 0% Solvent B (acetonitrile with 0.1% formic acid), and then eluted with a linear gradient from 100% A, 0% B to 70% A, 30% B in 30 min. The mass spectrometer was operated in the data depended acquisition (DDA) mode acquiring one scan per s. Every survey scan was followed by up to two MS/MS scans of the most intense precursor ions. MS/MS scans were performed on doubly charged precursor ions using the following collision offset voltages: series 1, 28 V, series 2/3, 29 V and 35 V.

Pseudo MS^3 experiments were carried out using static nanoESI-MS/MS. Pure synthetic peptide samples were synthesized using FMOC chemistry, dissolved in 50% acetonitrile 2% formic acid and sprayed from in-house-produced gold coated spray needles. Static electrospray was established by applying a capillary voltage of 1000 V. Then peptides were fragmented in the source of the mass spectrometer using a cone voltage of 120 V. The resulting fragment ions of interest were further fragmented in the collision cell using an additional collision offset of 3 V for c_1 ions and 30 V for z_{max-1} ions. Acquired datasets were interpreted manually using MassLynx 4.1 (Waters).

Results and Discussion

In CID of peptides, the formation of c_1 ions has been mentioned only sporadically. The formation of c_1 ions for peptides with Q in N-terminal position 2 was described using a set of 11 synthetic peptides representing 10 different sequences for the N-terminal dipeptide motif [8]. In this study, the authors propose a mechanism for the formation of c_1 ions by secondary fragmentation of the b_2 ion. This model accounts for the formation of c_1 ions, but is not able to explain variable signal intensities of the c_1 ion relative to the b_2 ion. In a recent study by our group, the presence of c_1 ions was observed upon fragmentation of peptides containing a Q or N residue in N-terminal position 2 for nine synthetic peptides [6]. We also observed that c_1 ions were constantly accompanied by their complementary high mass fragments, the z_{max-1} ions. This led to the conclusion that the formation of c_1 ions does not solely proceed via a secondary fragmentation of the b_2 ion but also by direct fragmentation of the molecular ion.

To investigate further the formation of c_1 ions, a set of synthetic peptides was used consisting of three core sequences (ILGTNTK, AEVNQLWTLR, and LIGHSQGGLTSR) and varying residues for the additional two N-terminal amino acids. The N-terminal dipeptide motifs consisted of N and Q in positions one or two and all 19 unmodified amino acids in the corresponding position (L and I counted as one residue as they cannot be discriminated by CID). This resulted in 228 different synthetic peptides in total. Mixtures of these synthetic peptides were separated using UPLC. Baseline separation was achieved for all different combinations of synthetic peptides used due to the high separation power of UPLC [9] leading to pure compounds eluting, which were fragmented by CID. The fragment ion spectra were interpreted manually. For all peptides with N and Q in position 2, c_1 ions were observed with differing intensities. For peptides with N and Q in position 1, no c_1 ions were observed. The intensity for c_1 ions originating from peptides with Q in position 2 were consistently higher compared to peptides with N in position 2 but otherwise identical sequences. As an example, the low mass regions of the fragment ion spectra of four synthetic peptides with the N-terminal dipeptide motifs QY, YQ, YN, and NY are shown in Figure 1.

As shown in Figure 1, the fragmentation of the peptide with Q in position 1 leads to the formation of a b_2 ion, an intensive a_2 ion and neutral losses of −17 and −18 both from the b_2 and a_2 ion (see Figure 1a). In contrast, the fragmentation of the peptide with Q in position 2 results in a b_2 ion, an a_2 ion of low intensity, an intense c_1 ion and a neutral loss of −17 from the b_2 ion (see Figure 1b). Both sequences lead to an intense Y immonium ion. The differences indicate that the b_2 ion with the sequence QY and YQ exhibit different structures in the gas-phase. The structure of the b_2 ion QY is probably an oxazolone or diketopiperazine structure [10], while the b_2 ion YQ presumably is of the structure proposed [8]. Apart from this, the fragmentation behavior of the synthetic peptides with N in position 1 and 2 is nearly identical (see Figure 1 c/d). The fragmentation of both sequences results in a b_2 ion, an a_2 ion, an a_2-17 ion and a Y immonium ion. Additionally, the peptide

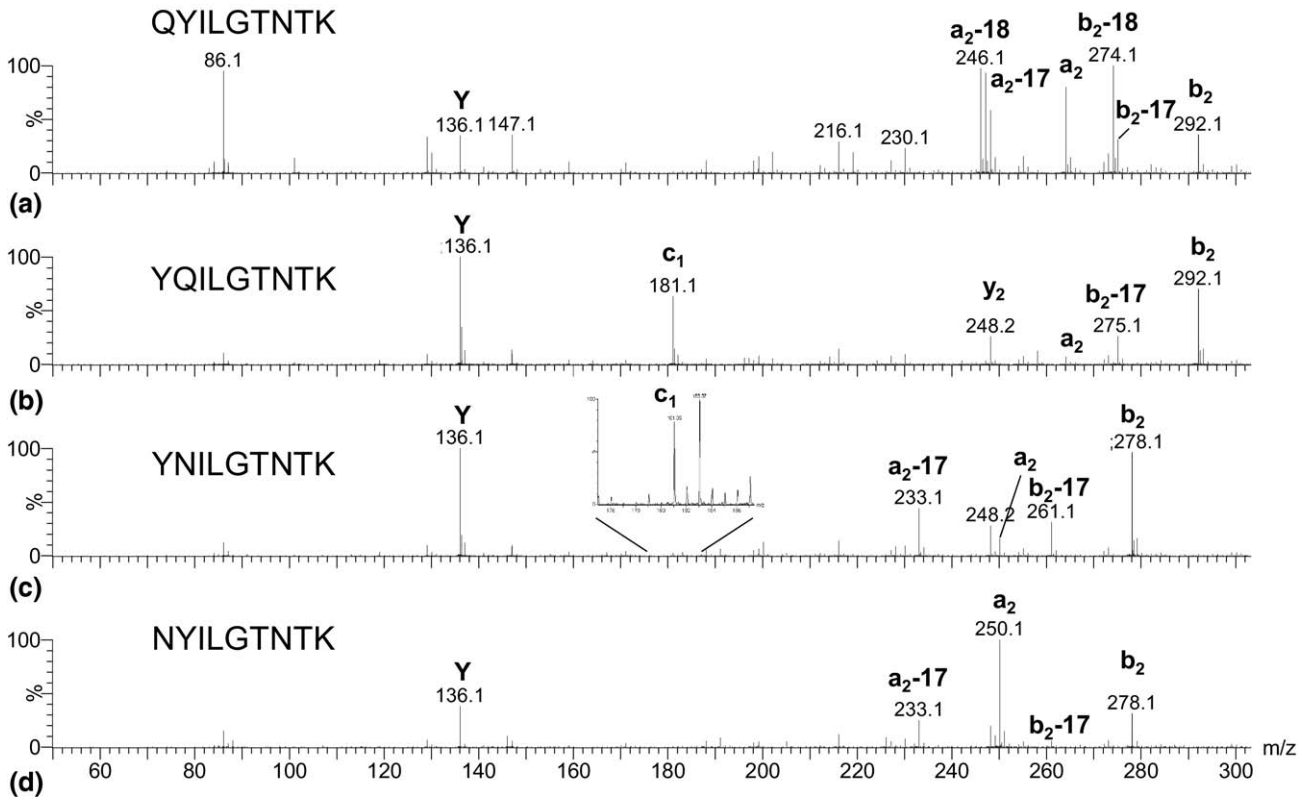

Figure 1. Low mass regions of the MS/MS-spectra of the synthetic peptides (**a**) QYILGTNTK, (**b**) YQILGTNTK, (**c**) YNILGTNTK, and (**d**) NYILGTNTK. The major ions originating from secondary fragmentations of the b_2 ion are indicated.

with N in position 2 shows a c_1 ion of low intensity. It is most likely that this ion is formed by direct fragmentation of the molecular ion without a b_2 ion intermediate. The difference in the signal intensity of the a_2 ion is presumably correlated with the more intense Y immonium ion which can be formed by secondary fragmentation of the a_2 ion [6]. To further investigate the differences in the fragmentation profiles of peptides containing N in their N-terminal dipeptide motif, the mass regions below the b_2 ions of our set of synthetic peptides were investigated. All N-terminal dipeptide motifs, except of those carrying R, showed a similar fragmentation behavior independent of the order of the amino acids. Only quantitative changes between N in position 1 or 2 were observed. A c_1 ion of low intensity was observed for peptides with N in position 2. These fragmentation patterns indicate that the structure of the b_2 ions containing N might be diketopiperazines, as an oxazolone structure would result in different fragmentation patterns for inverted sequence motifs [6, 7].

Contrary to this observation, the fragmentation behavior of b_2 ions carrying Q is consistently position-dependent, which confirms the model introduced by Lee, Y. J. and Lee, Y. M. [8]. A feature left unanswered by these studies is the variable intensity of the c_1 ion in different types of synthetic peptides. To address this issue, the MS/MS spectra of the set of synthetic peptides with Q in position 2 were investigated. First, the signal intensities of the b_2 ions relative to the signal intensities of their corresponding c_1 ions were examined. Because of the low signal intensities of c_1 ions of peptides with N in position 2, only MS/MS spectra of peptides with Q in position 2 were taken into account at this point. The results are displayed in Figure 2.

From the data presented in Figure 2, two conclusions can be drawn: (1) a correlation between the length of the peptide and the intensity of the c_1 ion exists, (2) a correlation between the size of the residue of the amino acid in position 1 and the intensity of the c_1 ion exists. Despite of some exceptions, the relative intensity of the c_1 ion increases directly with molecular weight of the residue in position 1 and decreases with increasing peptide length. These correlations indicate that the formation of the c_1 ion is influenced by the remaining peptide sequence. One possible reason for this phenomenon is the occurrence of different b_2 ion structures, which result in different fragmentation behaviors and therefore variable intensities of the c_1 ions.

To test this hypothesis, pseudo MS^3 spectra of different b_2 ions from peptides with the same N-terminal dipeptide motif, which lead to varying c_1 ion intensities were performed. This was accomplished by fragmenting the intact peptide in the source of the mass spectrometer followed by fragmentation of the resulting b_2 ion in the collision cell by CID. A set of pure compound synthetic peptides (starting with YQ) was used for this

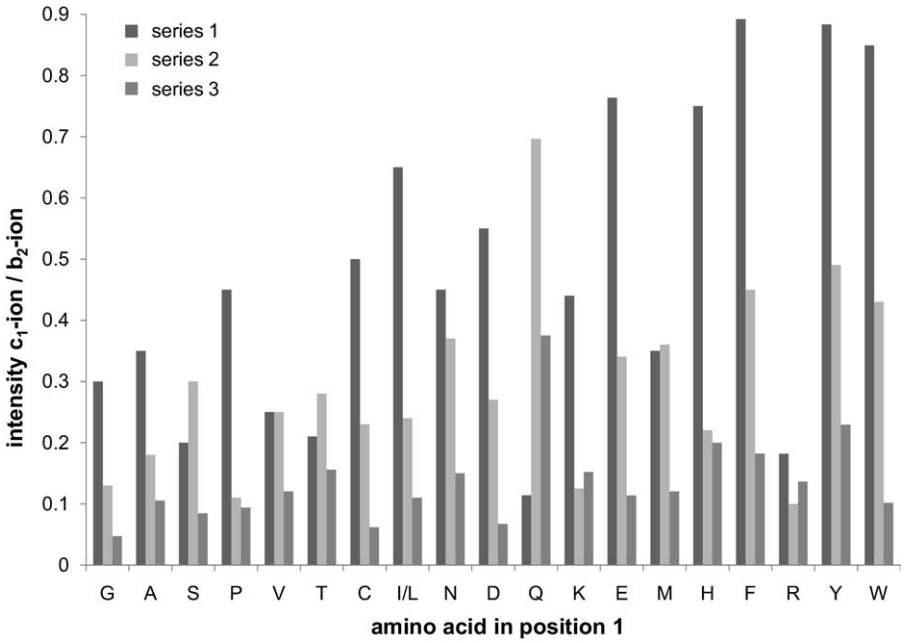

Figure 2. c_1 Ion signal intensities relative to those of corresponding b_2 ions of peptides with Q in position 2 of their N-terminal dipeptide motif. The values extracted from the MS/MS spectra of three different core sequences are displayed: series 1, X-QILGTNTK (9 mer, left, dark grey); series 2, X-QAEVNQLWTLR (12 mer, middle, light grey); series 3, X-QLIGHSQGGLTSR (14 mer, right, medium grey). X represents one of the 19 unmodified standard amino acids (L and I counted as one residue).

purpose. The MS^3 spectra of the b_2 ions and low mass regions of the MS/MS spectra of the corresponding precursor ions are shown in Figure 3.

Figure 3b, d, and f confirm the strong dependence of the c_1 ion intensity of the rest of the peptide sequence. For all sequences used, different intensities of the c_1 ion relative to the b_2 ion were observed. But as Figure 3a, c, and e show, the fragmentation of the b_2 ion itself led to similar intensities for the c_1 ion in all cases. These data reveal that the structure of the b_2 ion is similar in all

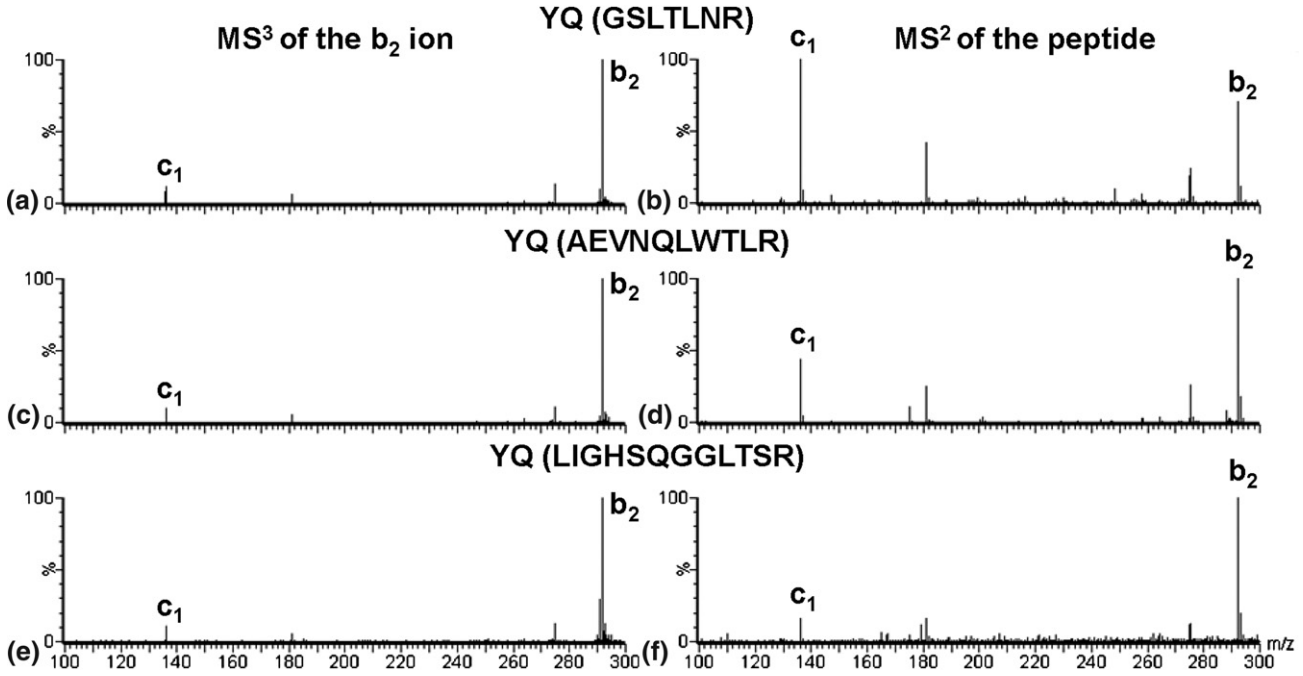

Figure 3. Low mass regions of the MS/MS spectra of the molecular ions (**b**), (**d**), (**f**) and pseudo MS^3 spectra of the b_2 ions (**a**), (**c**), (**e**) of three different synthetic peptides. The sequences used were: (**a/b**) YQGSLTLNR (series 1), (**c/d**) YQAEVNQLWTLR (series 2), (**e/f**) YQLIGHSQGGLTSR (series 3).

three cases, and that the differences in the intensities of the c_1 ion are not related to differing b_2 ion structures, but must have their origin in another process. Such a result indicates a significant impact of a second mechanism of c_1 ion formation as was deduced from the presence of z_{max-1} ions in an earlier study [6].

To investigate if this pathway may lead to the discrepancies in signal intensities for the c_1 ions of the differing peptides, the intensities of the z_{max-1} ions and the c_1 ions were compared in the MS/MS spectra of peptides with Q in position 2. For example, the signal intensities of the c_1 and z_{max-1} ions relative to the intensity of the b_2 ion are depicted for two sequence motifs. For this purpose, peptides with A and W in position 1 were chosen, as both residues are nonpolar amino acids and the b_2 ions containing these two residues show similar fragmentation behaviors [7], which should result in similar fragmentation mechanisms. In Figure 4, the signal intensities of the c_1 ions and the z_{max-1} ions relative to the b_2 ion are shown for both dipeptide motifs and the different peptide sequences.

The comparison of the relative signal intensities of the c_1 ions and the z_{max-1} ions shows a clear correlation between the intensities of the two species. Peptides with W in position 1 show more intense c_1 and z_{max-1} ions than peptides with A in position 1. Additionally, both species show a decrease in signal intensity of the c_1 ion with increasing peptide length. We conclude from these data that the size of the amino acid in position 1 has an effect on the formation of the c_1 ion, which seems to be particularly true for the direct formation of the c_1 ion without a b_2 ion intermediate, as peptides with higher c_1 ion intensities also show higher z_{max-1} ion intensities. A similar phenomenon is observed in Figure 2 as the relative signal intensities of the c_1 ions increase directly with the molecular weight of the first amino acid residue. In addition, the differences between the three peptide core sequences increase as well with the molecular weight of the first residue. As the relative c_1 ion intensity originating from the further fragmentation of the b_2 ion is constant (see Figure 3), these differences must be due to the c_1 ion generated by direct formation from the molecular ion. This seems to be more common for larger residues in position 1. To investigate further the formation of the c_1 ion from the molecular ion, its complementary ion, the z_{max-1} ion, of the synthetic peptide AQPIASTK was fragmented in a pseudo MS^3 experiment. The resulting spectrum is displayed in Figure 5.

The fragment ion spectrum of the z_{max-1} ion is composed of y ions and the complementary ions, named *b ions. The whole series of fragment ions is present down to the $*b_1/y_6$ ion pair. These data indicate that only the first two residues of the peptide are involved in the formation of the c_1/z_{max-1} ion pair. Supporting for this fragmentation behavior is the difference in signal intensity between peptides with N and Q in position 2. If the fragmentation into a c_1 and z_{max-1} ion would proceed via a previous cyclization of the peptide in the gas phase [11], one would expect minor differences in the fragmentation behavior as the difference between N and Q is only one CH_2 group. Additionally, the fragmentation of the peptide bond between Q and P, as shown in Figure 5, proceeds in a normal fashion because the $*b_1$ ion is observed. This implies that neither the carboxy nor the amino group of the peptide bond Q–P is involved in the formation of the c_1 ion.

A factor likely to be involved in these mechanisms is the mobile proton [3], which would explain the decreasing intensity of the c_1 ion with increasing peptide length. With increasing length, the probability of the mobile proton being located at a specific residue of a peptide decreases. This influence would denote that the formation of the c_1 ion is dependent on the presence of a proton at the amide nitrogen of the peptide bond allowing the cyclization of the amino acid residue and resulting in the formation of c_1/z_{max-1} ions. Building on these assumptions, a mechanism for the formation of the c_1/z_{max-1} ions from peptides with Q and N in N-terminal position 2 is proposed in Figure 6.

As this mechanism does not involve the carbonyl group as suggested [8], the fragmentation of peptides with ornithine (O) in N-terminal position 2 should lead to the formation of a c_1 ion as well. To investigate this

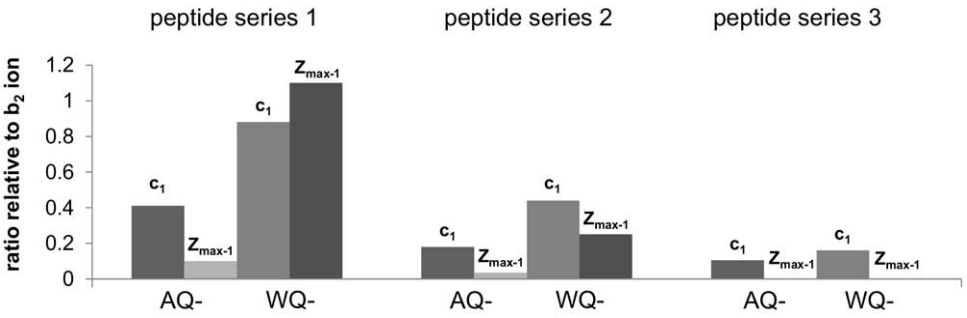

Figure 4. Signal intensities of the c_1 and z_{max-1} ions relative to the b_2 ions of peptides with Q in position 2 of their N-terminal dipeptide motif. Values of three series of synthetic peptides are displayed. Series 1, A/W-QILGTNTK (9 mer); series 2, A/W-QAEVNQLWTLR (12 mer); series 3, A/W-QLIGHSQGGLTSR (14 mer).

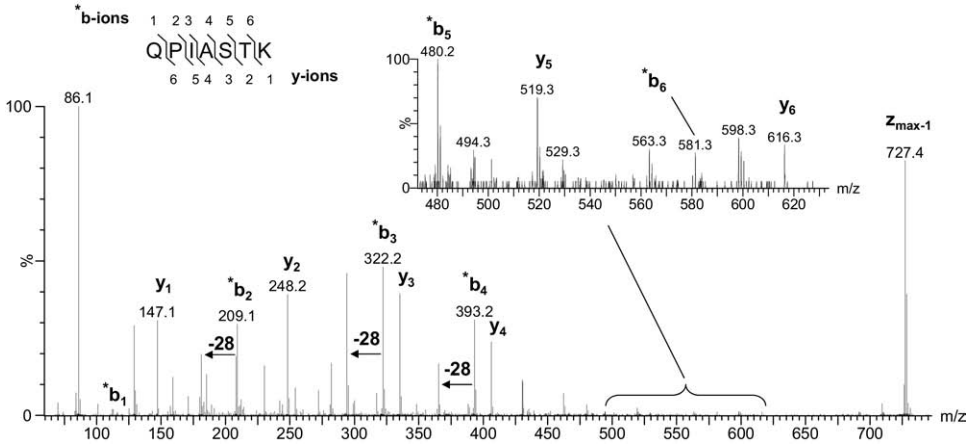

Figure 5. Pseudo MS^3 spectrum of the z_{max-1} ion of the synthetic peptide AQPIASTK. The peptide was fragmented in the ion source of the mass spectrometer by an elevated cone voltage. The z_{max-1} ion was further fragmented in the collision cell by CID. All major fragment ions are indicated.

further, three peptides were synthesized composed of the N-terminal dipeptide motif LO and the three core sequences. These peptides were then fragmented using static nanoESI-MS/MS. For the three sequences investigated, a prominent c_1 ion was observed. The intensity of the c_1 ion relative to the b_2 ion showed the same dependency on the remaining peptide sequence as observed for the c_1 ions formed from peptides with Q in position 2. As an example, the low mass region of the peptide LOILGTNTK and the c_1 ion intensities relative to the corresponding b_2 ion intensities are displayed in Figure 7.

The fragmentation of the ornithine-containing peptides led to the formation of c_1 ions, with intensities lower compared to peptides containing Q, but higher compared to peptides with N in position 2. The further fragmentation of the b_2 ion by pseudo MS^3 does not result in a c_1 ion (see Figure 7a bottom), which indicates that the formation of the c_1 ion proceeds exclusively by fragmentation of the molecular ion.

Conclusion

The data presented in this study reveal that there are two major pathways for the formation of c_1 ions during CID. The first pathway involves a b_2 ion intermediate which then further fragments to the c_1 ion [8]. The second pathway, as described herein, involves the side chain nitrogen of either Q, N, or O, leading to a direct cyclization of the amino group with the α C-atom of the second peptide residue from the N-terminus leading to the formation of the c_1 ion from the molecular ion without b_2 ion intermediate.

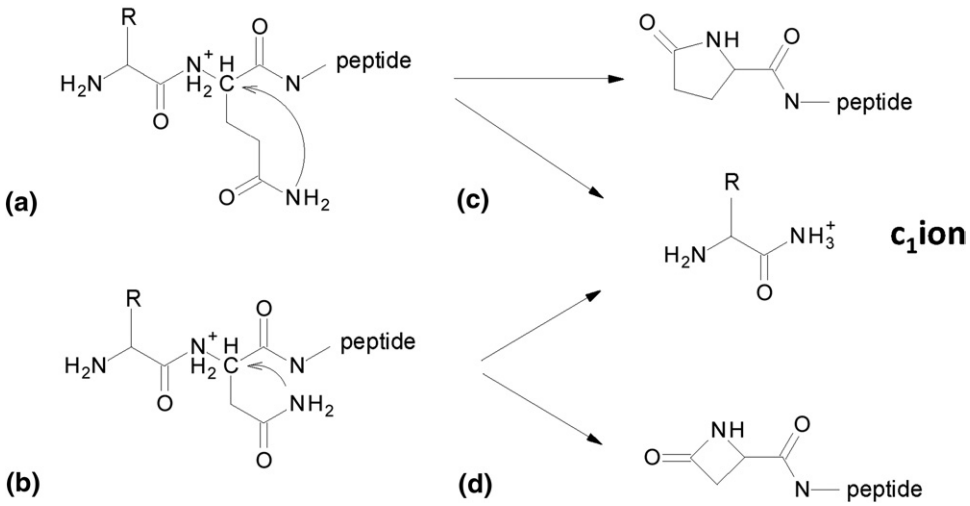

Figure 6. Proposed mechanism for the formation of c_1/z_{max-1} ions from peptides with Q or N in N-terminal position 2. Peptides with Q in position 2 (**a**) fragment to a c_1 ion under formation of a five-ring at the peptide N-terminus (**c**). Peptides with N in position 2 (**b**) lead to the formation of a c_1 ion and a peptide with a four-ring structure at the N-terminal residue (**d**).

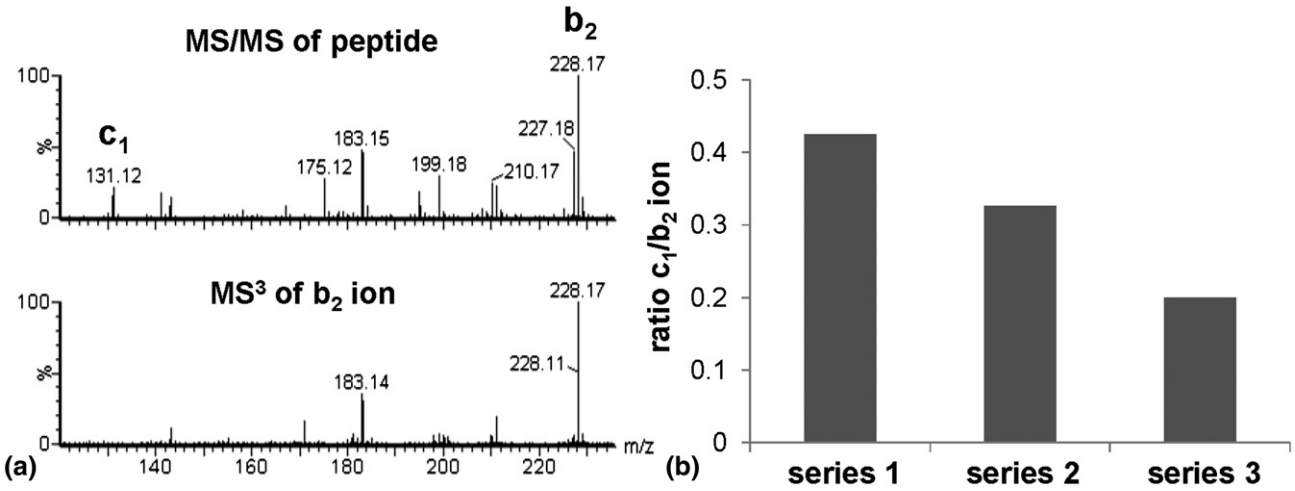

Figure 7. (**a**) Low mass region of the MS/MS spectrum of the synthetic peptide LOILGTNTK (top) and pseudo MS^3 spectrum of the b_2 ion (bottom); (**b**) ratio of the c_1 ion relative to the b_2 ion for the three different core sequences: series 1, LO-ILGTNTK (9 mer); series 2, LO-AEVNQLWTLR (12 mer); series 3, LO-LIGHSQGGLTSR (14 mer).

This allows further insight in the fragmentation mechanisms during CID of protonated peptides. The knowledge of these mechanisms and the presence of the resulting ions may serve as valuable information for de novo sequencing of peptides or for algorithm supported interpretation of fragment ion spectra. For these purposes, the appearance of the c_1/z_{max-1} ions is of great value because they serve as marker ion pair and allow for unambiguous identification of the N-terminal dipeptide motif, which is of great value for the interpretation of fragment ion spectra.

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