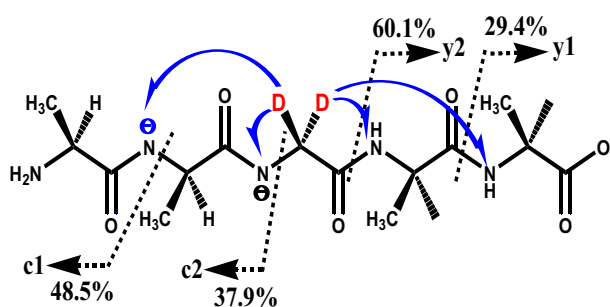


Intramolecular Hydrogen Transfer from the Alpha-Carbon (C α) and Backbone Amide Nitrogen (N $_b$) to Form c- and y-Ions in Negative-Ion CID of Peptides

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Abstract. The source of hydrogen in the formation of c- and y-ions produced by intramolecular hydrogen transfer in negative-ion CID experiments with peptides has been examined using C α -, C β -, and backbone amide (N $_b$)-deuterated peptides AAA(d3)AA, AAG(d2)AA, AAAG(d2)A, and AAAAA-d7, as well as five other peptides. The c- and y-ions produced by deuterium transfer from the deuterated residues were detected and identified by the exact *m/z* values obtained with a high-resolution

orbitrap mass spectrometer. The rate of deuterium transfer obtained indicates that over 50% of the hydrogen was originated from the backbone amide nitrogen, with the residual hydrogen coming from the backbone C α . It is clear that the hydrogen does not originate from the side chain C β . It is hypothesized that the intramolecular hydrogen transfer to form negative c- and y-ions takes place via 3-, 4-, 6-, 7-, 8-, and 9-membered ring transition states.

Keywords: Peptide, Negative ion, CID, c-ion, y-ion, Hydrogen transfer

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Introduction

Collision-induced dissociation (CID) combined with electrospray ionization (ESI) [1, 2] is a powerful tool for analyzing biological molecules such as peptides and proteins. Although there are many studies regarding the mechanistic and theoretical aspects of positive-ion low-energy (low-E) CID of peptides [3–6], negative-ion CID studies have been limited to reports by a small number of groups, namely those of Bowie [7], Harrison [8–10], Cassady [11–14], and Takayama [15, 16]. It is well-known that in positive-ion low-E CID of peptides, the formation of b- and y-ions can be explained by a mechanism involving a mobile proton [4, 5] and residue scrambling [6, 17–19]. Furthermore, the low-E CID results in residue-specific enhanced cleavage at the amide bond (C–N) of acidic Asp/Glu/Cys-Xxx residues and Xxx-Pro residues [5]. However, the residue-specific cleavage characteristics of negative-ion CID are quite different from those of positive-ion mode and are as follows (Table 1):

1. Specific cleavage at the N–C α bond of Xxx-Asp/Asn residues to form a c- and z-ion pair [7, 15]
2. Specific cleavage at the C–N bond of acidic Xxx-Asp/Glu/Cys residues to form b- and y-ions [16]
3. Specific cleavage at the N–C α bond of Xxx-Ser/Thr residues to form z-ions [20]
4. y1 ion formation due to the acidic carboxyl group at the carboxyl (C)-terminus [12, 15]

Here, we use the nomenclature of peptide fragments in as per the Biemann-Roepstorff notation [21, 22], i.e., amino (N)-terminal side a-, b-, and c-ions and C-terminal side x-, y-, and z-ions (Scheme 1), while the nomenclature with hydrogen addition (+H), protonation (+H⁺), and deprotonation (–H⁺) to describe c- and y-ions such as [y + 2H]⁺, [c + 2H]⁺, [c][–], and [y][–] is used according to the proposed nomenclature of Chu et al. [23].

With respect to the formation of c-ions in negative-ion CID of peptides, the Cassady group has reported that when using alanine heptamers (AAAAAAA) with or without an arginine (R) or lysine (K) residue, a dominant c4 ion is observed in the CID spectra independent of the position of the R and K residues [12].

Table 1. The Specific Cleavage Residues, Product Ions, and the Product Ions with Intramolecular Hydrogen Transfer in Positive- and Negative-Ion Low-Energy CID of Peptides

	Positive-ion	Negative-ion
Specific residue	Asp/Glu/Cys-Xxx, Xxx-Pro	Xxx-Asp/Glu/Cys, Xxx-Asp/Asn Xxx-Ser/Thr
Product ion	$[b]^+$, $[y+2H]^+$	$[b]^-$, $[y]^-$, $[y1]^-$, $[c]^-$, $[z]^-$
Hydrogen transfer	$[y+2H]^+$	$[y]^-$, $[c]^-$

Furthermore, the same group reported that the c-ions can be produced by cleavage at the N-C α bond of the backbone region between the third and fourth residues from the C-terminus, i.e., C $_{m-3}$ and C $_{m-4}$ residues (m represents number of residues in the peptide), respectively, when C-terminus has a carboxyl group, while N-C α bond cleavage between the C $_{m-2}$ and C $_{m-3}$ residues (C $_{m-2,3}$) preferentially takes place when the C-terminus has an amide group [13]. The preferential cleavage at the N-C α bond to form c-ions may be explained by proton (H $^+$) abstraction from the backbone amide of the C $_{m-2,3}$ and C $_{m-3,4}$ residues via 8- and 11-membered ring transition states, respectively. The formation of negative c-ions by C $_{m-3,4}$ -H $^+$ abstraction via an 11-membered ring transition state is shown in Scheme 2, although Bokatzian-Johnson et al. have proposed the loss of 9-membered ring neutral with intramolecular proton transfer based on the DFT calculation [13]. According to the report of Bokatzian-Johnson et al. [14], it is likely that intramolecular proton abstraction from central amide backbone nitrogen (N $_b$) via 8- or 11-membered ring transition states excited with collisional activation takes place (Scheme 2). Another characteristic of the negative-ion CID spectra of peptides lacking Asp/Asn/Glu/Cys residues is the formation of y-ions [9, 12, 13]. Both c- and y-ions are produced by intramolecular hydrogen transfer to the backbone amide nitrogen (N $_b$). It is of interest to examine the source of hydrogen in the generation of c- and y-ions from the standpoints of appropriate and flexible conformations of gas-phase peptide molecules, which enable hydrogens to move to the amide nitrogen.

Here, we examine the source of hydrogen in the formation of c- and y-ions in higher energy collisional dissociation (HCD, corresponding to a conventional low-E CID) experiments with deprotonated peptides $[M-H]^-$ generated by ESI MS. To determine the source of hydrogen, peptides labeled with deuterium on the α -carbon, β -carbon, and backbone amide moieties were used. Peaks of c- and y-ions produced with and without intramolecular deuterium (D) transfer were detected and confirmed with a high-resolution mass spectrometer and exact m/z values.

Experimental

Materials

All peptides were purchased from the Peptide Institute (Minoh, Osaka, Japan). The samples used are an alanine pentamer (AAAAA), a lysine pentamer (KKKKK), and a phenylalanine pentamer (FFFFF). The deuterated peptides AAA(d3)AA, AAG(d2)AA, and AAAG(d2)A were supplied from the Peptide Institute. Acetic acid and acetonitrile (HPLC grade) were purchased from Wako Pure Chemicals (Osaka, Japan). Water

used in all the experiments was purified with a MilliQ water purification system from Millipore (Billerica, MA, USA). Deuterium oxide (D $_2$ O), acetonitrile-d $_3$ (CD $_3$ CN), and trifluoroacetic acid-d (TFA-d) were purchased from the Sigma-Aldrich (Steinheim, Germany).

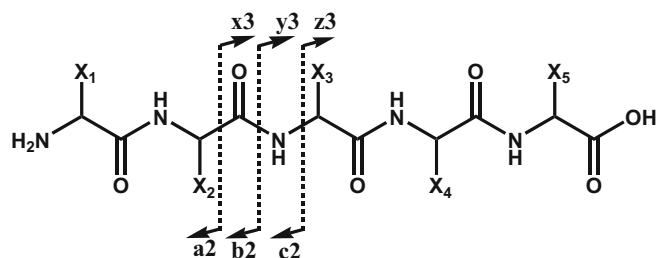
Mass Spectrometry and Sample Preparation

The HCD experiments were performed on a Q Exactive Focus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an ESI source. The mass resolving power for precursor ions was 70,000 at m/z 200 in FWHM. The width for selecting precursor ions was ± 0.4 Da. Target gas and collision energy were nitrogen and 10 eV, respectively. The sample was introduced into the ion source with an infusion inlet system at a flow rate of 30 μ L/min with nitrogen being used as both nebulizing and drying gas. The samples were prepared as 10 μ M solutions with a 1:1 (v/v) mixture of water/methanol with added 0.1% acetic acid. To obtain a deuterated peptide for alanine pentamer AAAAA, the peptide was dissolved in a mixture of D $_2$ O and CD $_3$ CN (1:1, v/v) and incubated for 24 h at room temperature.

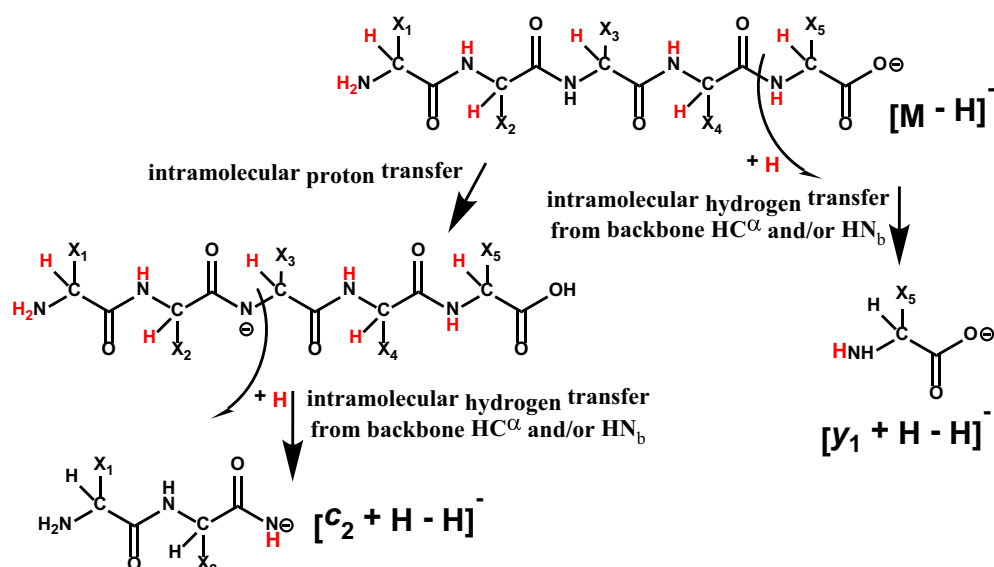
Results and Discussion

Preferential Formation of y1 and c2 Ions of Peptide Pentamers

Negative-ion HCD spectra of deprotonated molecules $[M-H]^-$ of three different pentamers AAAAA, KKKKK, and FFFFF are shown in Fig. 1. All the HCD spectra showed considerably intense peaks corresponding to y1 and c2 ions. The c2 ions originate from cleavage at the N-C α bond between the C $_{m-3}$ and C $_{m-4}$ residues (C $_{m-3,4}$) of the peptides [13], while the y1 ions originate from cleavage at the backbone amide C-N bond of the C-terminal residue [12, 14]. The CID spectra also



Scheme 1. Simplified Biemann-Roepstorff nomenclature for peptide fragments



Scheme 2. An adaptation of Cassidy's mechanism for the formation of c2 ions in negative-ion CID of pentapeptides [13]

showed the common product ions of c $_n$ ($n = 1-4$), y $_n$ ($n = 1-4$), c $_n$ -H $_2$ O, y $_n$ -H $_2$ O, and y $_n$ -CO $_2$. The intense c2 ions can be

explained by N-C $^{\alpha}$ bond cleavage through C $_{m-3,4}$ -H $^+$ abstraction via an 11-membered ring transition state [13] (Scheme 2).

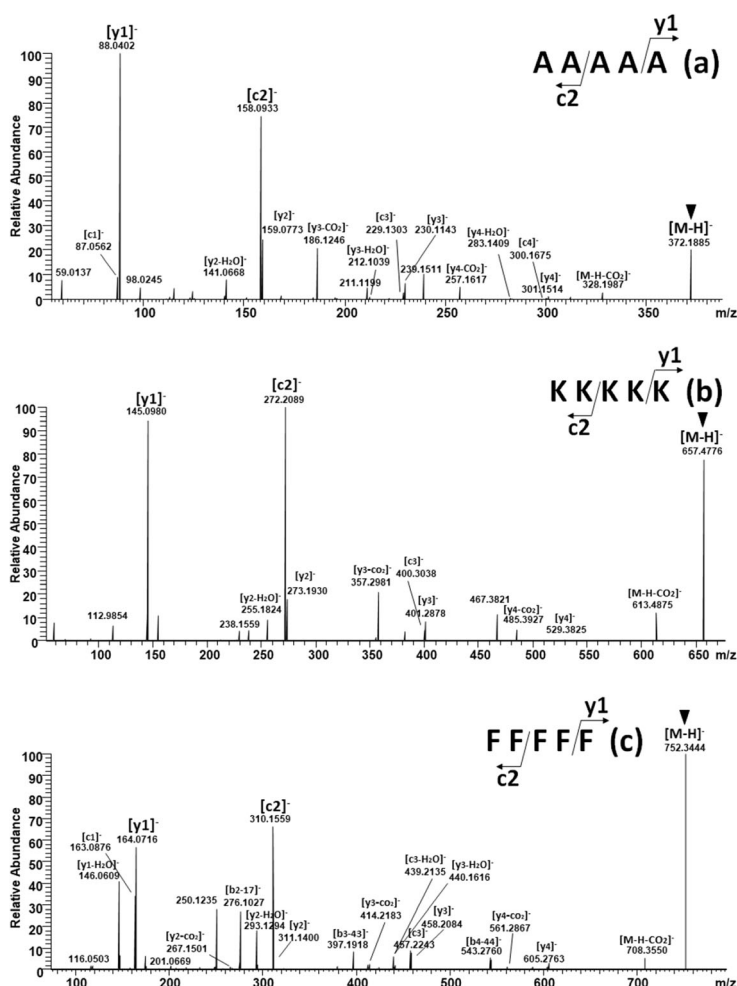


Figure 1. Negative-ion HCD spectra of deprotonated molecules [M-H] $^-$ of three different pentamers (a) AAAAA (M_r 373.2), (b) KKKKK (M_r 658.5), and (c) FFFFF (M_r 753.4)

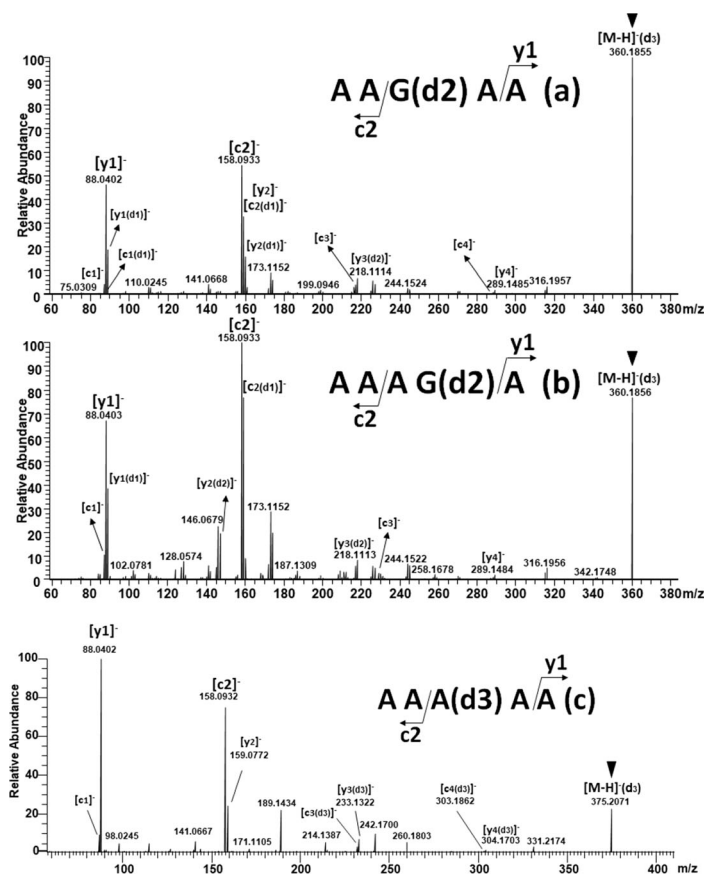


Figure 2. Negative-ion HCD spectra of deuterated glycine G(d2) and alanine A(d3) containing alanine clusters (a) AAG(d2)AA (*Mr* 361.3), (b) AAAG(d2)A (*Mr* 361.3), and (c) AAA(d3)AA (*Mr* 376.2)

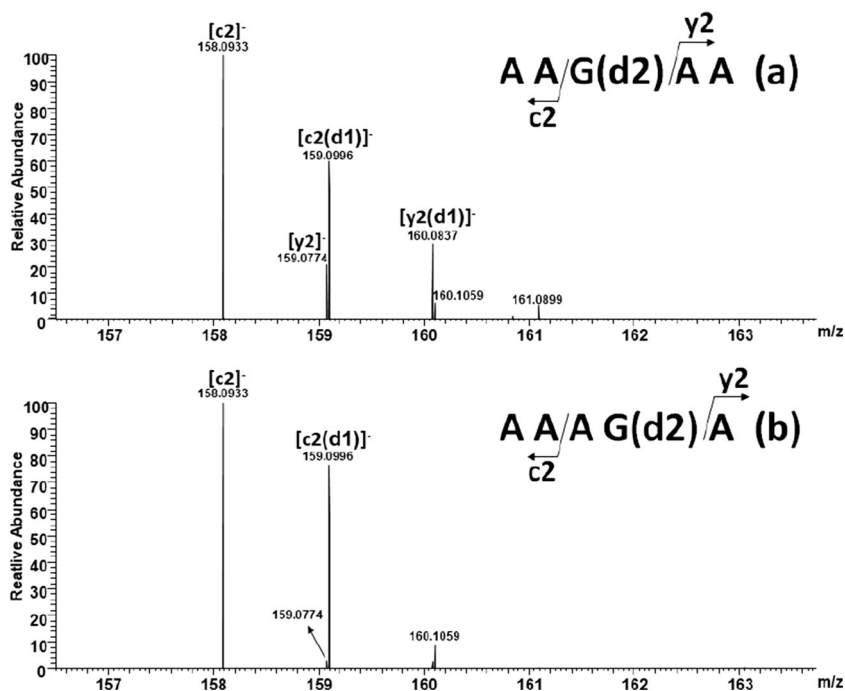


Figure 3. The c2 and y2 ion formation in negative-ion HCD spectra of deuterated glycine G(d2) containing alanine clusters (a) AAG(d2)AA (*Mr* 361.3) and (b) AAAG(d2)A (*Mr* 361.3)

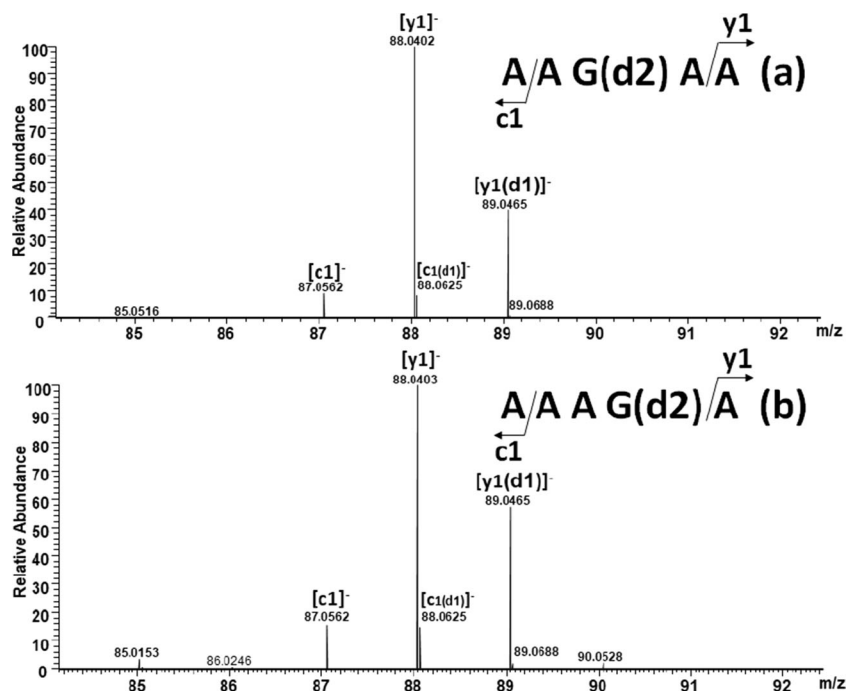


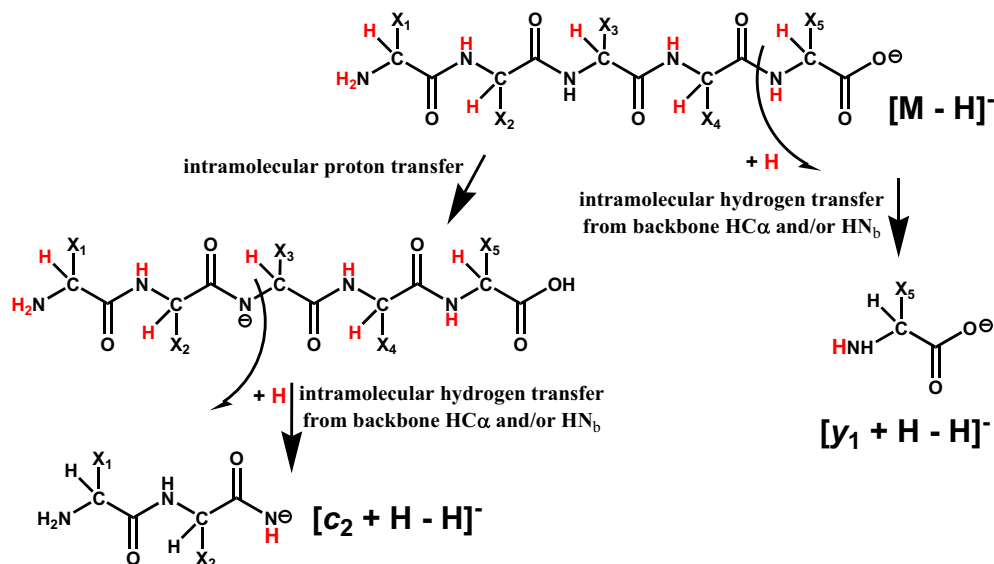
Figure 4. The c1 and y1 ion formation in negative-ion HCD spectra of deuterated glycine G(d2) containing alanine clusters (a) AAG(d2)AA (*Mr* 361.3) and (b) AAAG(d2)A (*Mr* 361.3)

The formation of the intense y1 ions observed in all the HCD spectra can be explained by specific cleavage at the C–N bond of the C-terminal residue due to the influence of the acidic carboxyl group [12, 15].

Formation of Deuterated c- and y-Ions Confirmed with Deuterated Peptides

In order to examine the possibility of hydrogen transfer from α -carbon ($C\alpha$) and β -carbon ($C\beta$) to form c and y ions, negative-ion HCD spectra of deuterated glycine containing peptides AAG(d2)AA and AAAG(d2)A and a deuterated alanine

pentamer AAA(d3)AA were obtained (Fig. 2). Figure 2a, b shows preferential c2 and y1 ion formation accompanied by deuterated products c2(d) and y1(d). Furthermore, c1 and y2 ions were also accompanied by deuterated products c1(d) and y2(d), respectively (Figs. 3 and 4). Although Fig. 2c also showed extraordinarily intense c2 and y1 ion peaks and c_n ($n=1-4$) and y_n ($n=1-4$) series ions, the c1, c2, y1, and y2 ions observed were not accompanied by any deuterated peaks of c1(d1), c2(d1), y1(d), and y2(d) due to deuterium transfer from the $C\beta$. This indicates that the hydrogen to form c1, c2, y1 and y2 ions does not originate from the $C\beta$ of the 3rd Ala(d3) residue. The results obtained above indicate that the hydrogen



Scheme 3. The formation of c- and y-ions due to intramolecular hydrogen transfer from the backbone $C\alpha$ and N_b sites

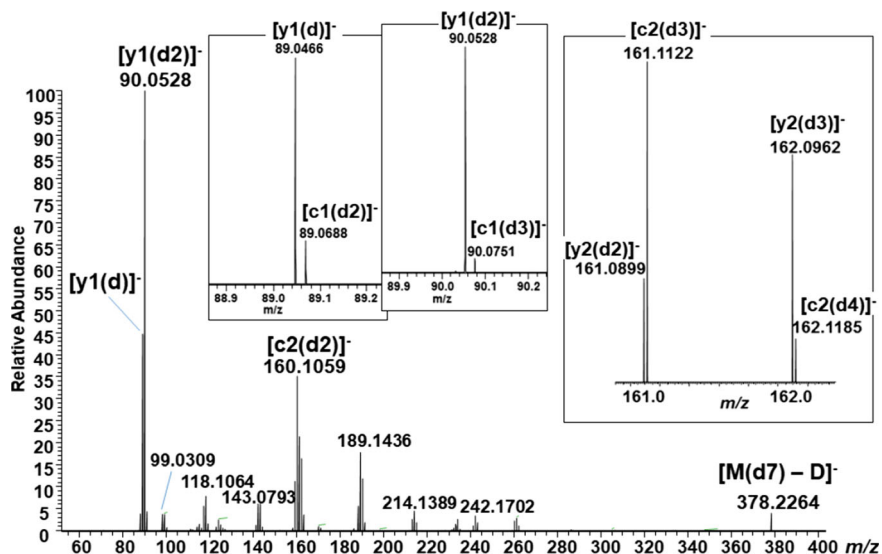


Figure 5. Negative-ion HCD spectrum of the precursor ion $[M(d7)-D]^-$ formed from deuterated alanine pentamer AAAAA-d7 (M_r 380.4). The insets represent enlarged spectra of c1, y1, c2, and y2 ions

to form c- and y-ions originates from the sites of the α -carbon (C_α) and/or amide nitrogen (N_b) of the peptide backbone (Scheme 3), and never come from the $C\beta$ sites.

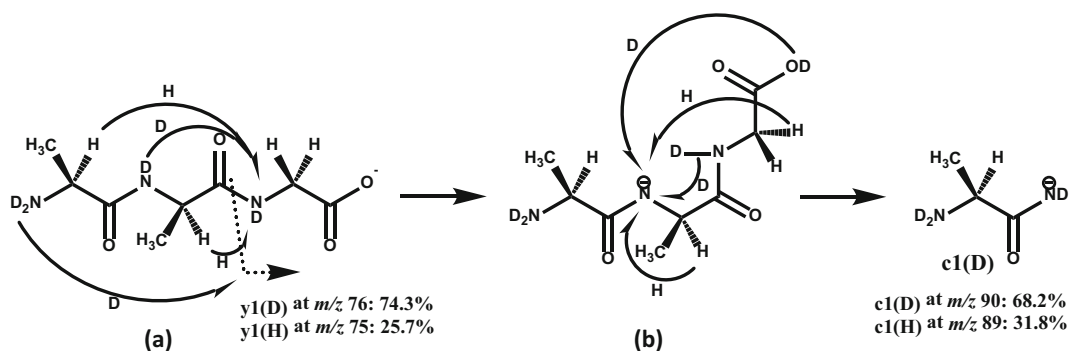
In order to confirm the intramolecular hydrogen transfer from the sites of the C_α and/or N_b of the peptide backbone, the negative-ion HCD spectrum of the de-deuterated analyte $[M(d7)-D]^-$ at m/z 378.2264 formed from a deuterated alanine pentamer AAAAA-d7 obtained by the incubation in D_2O/CD_3CN solution was obtained as shown in Fig. 5. The AAAAA having seven active hydrogens was deuterated with the rate of 72.7% (data not shown). The HCD spectrum preferentially showed y1(d), y1(d2), c2(d3), and y2(d3) ions, as shown in the insets of Fig. 5. The peaks at m/z 159.0996 and 160.1059 corresponding to c2(d1) and c2(d2) observed may be due to hydrogen and/or residue scrambling, although the mechanism is unclear.

Source of Hydrogen for the Formation of c- and Y-Ions

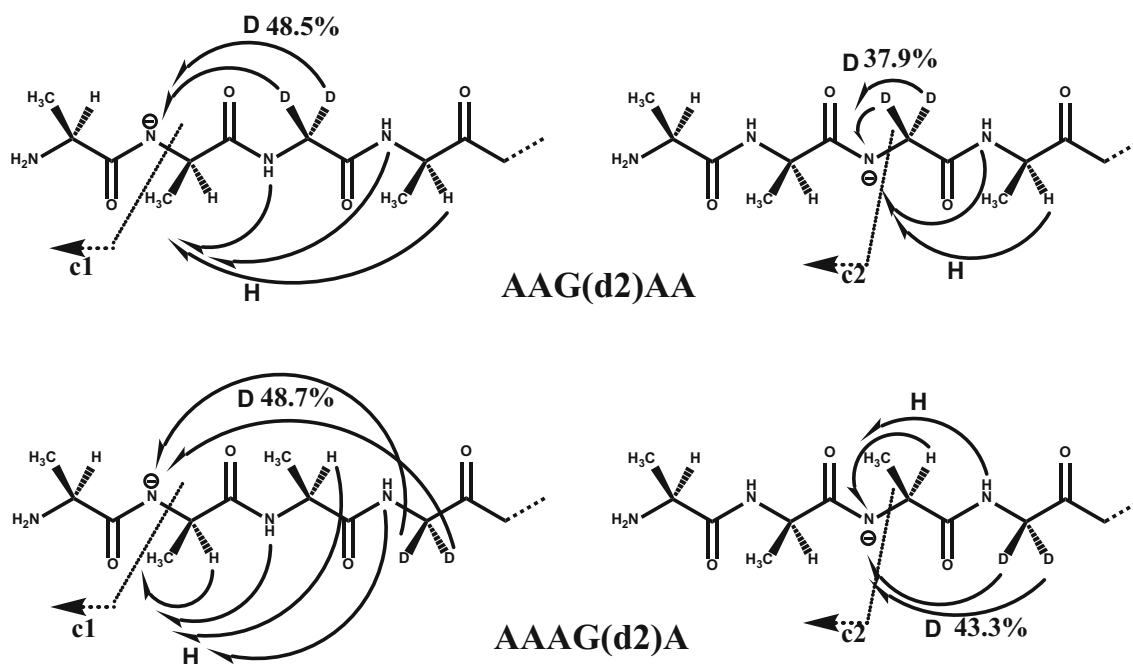
The intramolecular hydrogen transfer to form c- and y-ions in negative-ion CID spectra of peptides has been studied by

Harrison [8]. From negative-ion CID spectra of tripeptides AAG and AAG-d₅ (Fig. 3 in the report of Harrison [8]), the rate of intramolecular hydrogen (deuterium) transfer to form c1 and y1 ions can be estimated as shown in Scheme 4. The rate of H (D) transfer estimated from the report of Harrison [8] indicates that 68.2% of deuterium in the c1 ion originates from the backbone amide nitrogen (N_b) between Ala2 and Gly3 or C-terminal carboxyl group (Scheme 4b), while 31.8% of the hydrogen originates from the C_α sites of Ala2 and Gly3. On the other hand, 74.3% of the deuterium in the y1 ion originates from N_b between Ala1 and Ala2 and/or the N-terminal amino group (Scheme 4a), while 25.7% originates from the C_α sites of Ala1 and Ala2. The intramolecular deuterium transfer from N_b to another N_b site to form c1(D) in Scheme 4a may be explained by a 5- or 8-membered ring transition states (Scheme 4b).

In a similar manner, the rate of intramolecular hydrogen (deuterium) transfer in the HCD spectra of Figs. 2, 3, and 4 can be estimated from the peak intensity. The rate of intramolecular deuterium transfer from the C_α of Gly3(d2) and Gly4(d2) residue to form c1 and c2 ions is represented in Scheme 5. It



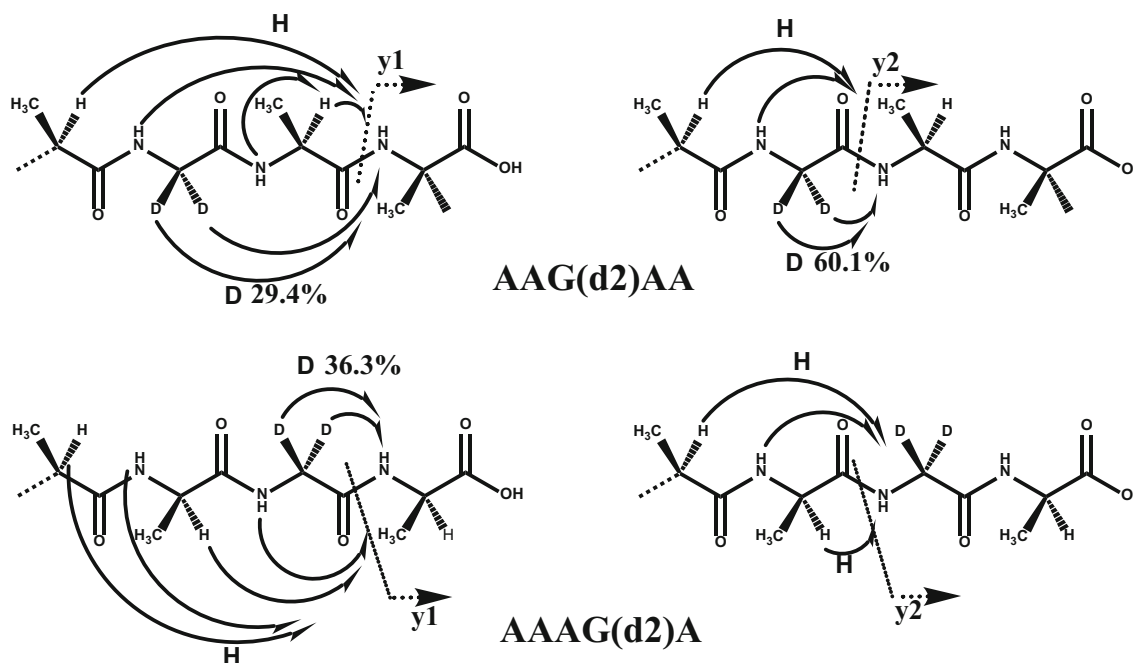
Scheme 4. (a) The rate of hydrogen (deuterium) transfer for negative c1 and y1 ions in the paper of Harrison [8] and proposed mechanisms of (b) c1 ion formation via 5- or 8-membered ring transition states



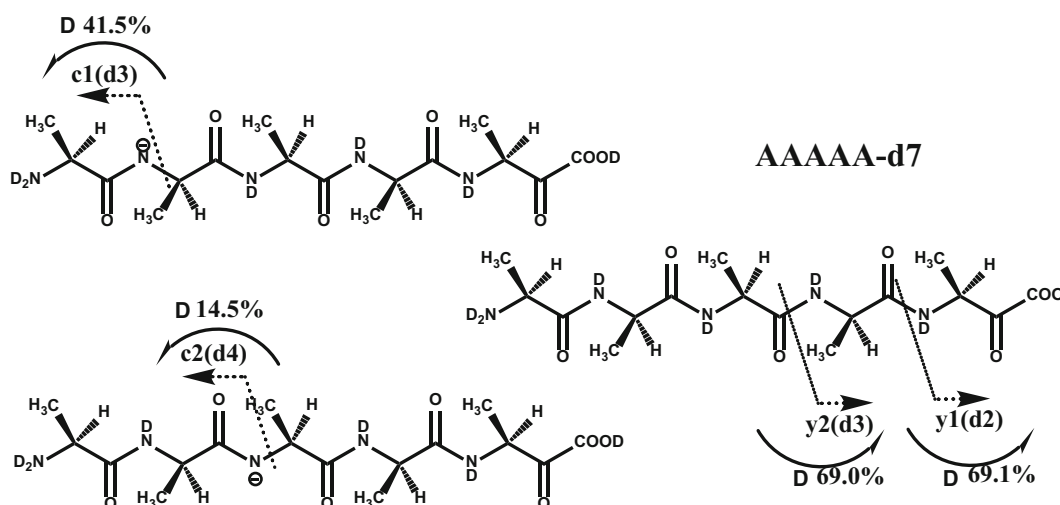
Scheme 5. The rate of deuterium transfer from the C α of Gly3(d2) and Gly4(d2) residues of deuterated peptides AAG(d2)AA and AAAG(d2)A in the formation of c1 and c2 ions

is hypothesized that the c1 and c2 ions for AAG(d2)AA are formed by intramolecular deuterium transfer from the C α via 6- and 3-membered ring transition states, respectively (Scheme 5 upper). The c1(d) and c2(d) ions for AAAG(d2)A are formed by deuterium transfer via 9- and 6-membered ring transition states, respectively (Scheme 5 lower). This indicates that about 39–49% of hydrogens which form c1 and c2 ions originate from the C α of the Gly residue, while the residual hydrogens

(51–61%) come from the C α of Ala and the backbone amide nitrogen (N $_b$). Considering the result of Harrison [8] and our results of Schemes 4 and 5, it may be concluded that the major source of hydrogen in the formation of c-ions is both the N $_b$ and the Ala(C α) sites of peptides. In contrast, the formation of y1(d) and y2(d) ions can be explained by intramolecular deuterium transfer from the C α of the Gly(d2) residue via 7- and 4-membered ring transition states with rates of 29–60%



Scheme 6. The rate of deuterium transfer from the C α of Gly3(d2) and Gly4(d2) residues of deuterated peptides AAG(d2)AA and AAAG(d2)A in the formation of y1 and y2 ions



Scheme 7. The rate of deuterium transfer from the amide nitrogen (N_b) and N-terminal amino group of deuterated peptides AAG(d2)AA, AAAG(d2)A in the formation of y1 and y2 ions

(Scheme 6). The major source of hydrogens in the formation of y-ions may also originate from the backbone amide (N_b), as shown in Schemes 4a and 6.

In the negative-ion HCD spectrum of $[M(d7)-D]^-$ showed in the insets of Fig. 5, the peak intensities of the y1(d) and y1(d2) indicate that 69.1% of deuterium in the y1(d2) ion originates from the amide nitrogens (N_b) and/or the N-terminal amino group (Scheme 7), while 30.9% of the hydrogen originates from the $C\alpha$ sites. The peak intensities of the c1(d2) and c1(d3) indicate that 41.5% of deuterium in the c1(d3) ion originates from the N_b sites and/or the C-terminal carboxyl group, while 58.5% of the hydrogen originates from the $C\alpha$ sites. In a similar manner, the rates of intramolecular D (H) transfer to form y2(d3) and c2(d4) ions can be estimated from the insets of Fig. 5, as shown in Scheme 7.

The rates of intramolecular deuterium transfer from the $C\alpha$ sites of Gly3(d2) for AAG(d2)AA and Gly4(d2) for AAAG(d2)A and from the N_b sites of AAAAA-d7 residues to form c1, c2, c3, y1, and y2 ions in negative-ion HCD spectra (Figs. 2, 3, 4, and 5) are summarized in Table 2.

Conclusions

The characteristics of residue-specific cleavage and product ions in negative-ion CID of peptides are quite different from

those of positive-ion CID, as summarized in Table 1. However, intramolecular hydrogen transfer to the amide nitrogen (N_b) for the formation of c- and y-ions is common to both positive- and negative-ion CID, and it is of interest from the standpoints of the conformation and flexibility of gas-phase peptide ions suitable for hydrogen transfer. The use of the deuterated peptides AAA(d3)AA, AAG(d2)AA, AAAG(d2)A and AAAAA-d7 in negative-ion HCD experiments gave information about the source of hydrogen in the formation of c- and y-ions. The results obtained indicated that the major source of hydrogen, over 50% in the rate of intramolecular hydrogen transfer to form c- and y-ions in the peptides AAG(d2)AA and AAAG(d2)A, is the backbone amide nitrogen (N_b), while another source is the backbone $C\alpha$ sites. The hydrogen did not originate from the $C\beta$ sites. In the case of the peptide AAAAA-d7, the hydrogen to form y-ions comes from the N_b sites with the rate of 69%, while the hydrogen to form c1 and c2 ions comes from the N_b sites with the rate of 41.5 and 14.4%, respectively. For the intramolecular hydrogen transfer to form negative c- and y-ions, it is suggested that deprotonated peptides $[M-H]^-$ transiently form at least 3-, 4-, 6-, 7-, 8-, and 9-membered ring transition states, indicating the flexibility of gas-phase peptides.

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Table 2. Rate (%) of the Intramolecular Deuterium Transfer from the $C\alpha$ Sites of Gly3(d2) for AAG(d2)AA and Gly4(d2) for AAAG(d2)A and from the N_b Sites of AAAAA-d7 Residues to Form c1, c2, c3, y1, and/or y2 Ions in Negative-Ion HCD Spectra

Product ion	AAG(d2)AA	AAAG(d2)A	AAAAA-d7
c1	48.5	48.7	41.5
c2	37.9	43.3	14.5
c3	–	46.3	–
y1	29.4	36.3	69.1
y2	60.1	–	69.0

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