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Negative ion dissociation of peptides containing hydroxyl side chains

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The dissociation of deprotonated peptides containing hydroxyl side chains was studied by electrospray ionization coupled with Fourier transform ion cyclotron resonance (ESI-FTICR) via sustained off-resonance irradiation collision induced dissociation (SORI-CID). Dissociation under post-source decay (PSD) conditions was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). This work included hexapeptides with one residue of serine, threonine, or tyrosine and five inert alanine residues. During SORI-CID and PSD, dissociation of $[M–H]^-$ yielded c- and y-ions. Side-chain losses of formaldehyde (HCHO) from serine-containing peptides, acetaldehyde (CH_3CHO) from threonine-containing peptides, and 4-methylene-2,5-cycohexadienone (C_7H_6O) from tyrosine-containing peptides were generally observed in the negative ion PSD and SORI-CID spectra. Side-chain loss occurs much less from tyrosine-containing peptides than from serine- and threonine-containing peptides. This is probably due to the bulky side chain of tyrosine, resulting in steric hindrance and poor geometry for dissociation reactions. Additionally, a selective cleavage leading to the elimination of the C-terminal residue from $[M–H]^-$ was observed from the peptides with serine and threonine at the C-terminus. This cleavage does not occur in the dissociation of peptides with an amide group at the C-terminus or peptides with neutral or basic residues at the C-terminus. It also does not occur with tyrosine at the C-terminus. Both the C-terminal carboxylic acid group and the hydroxyl side chain of the C-terminal residue must play important roles in the mechanism of C-terminal residue loss. A mechanism involving both the C-terminal carboxylic acid group and a hydroxyl side chain of serine and threonine is proposed.

Historically, most efforts in sequencing peptides by mass spectrometry have been focused on the dissociation of protonated peptides.[1,2] Dissociation pathways of protonated peptides have been well established.[2] In contrast, much less information has been obtained concerning the fragmentation patterns of deprotonated peptides.[3–6] Fragmentation of deprotonated peptides has been shown to provide complementary structural information to their positive counterparts.[7,8] Therefore, sequencing unknown peptides can benefit from the interpretation of both positive and negative mass spectra.

Serine (S), threonine (T), and tyrosine (Y) residues have side chains with a hydroxyl group. The hydroxyl side chain is a potential deprotonation site in the negative mode and is expected to induce characteristic cleavages. Previous studies have shown that serine and threonine promote characteristic side-chain cleavages upon collisional activation in the negative mode; that is, loss of formaldehyde (HCHO) from serine and acetaldehyde (CH_3CHO) from threonine.[3,4,9–13] Dissociation of $[M–H]^-$ of tyrosine-containing peptides by Bowie and coworkers[3] showed no side-chain cleavage but γ and δ backbone cleavages (using their terminology), which result in the formation of c- and z-ions. The mechanism was proposed to result from the facile formation of a side-chain enolate anion. In the work by Beauchamp and co-workers,[9] a low-energy intermediate with intramolecular hydrogen bonding between the tyrosine side chain and the C-terminal carboxylic acid group was proposed to induce peptide dissociation. In addition, Lehmann and coworkers[14] and Bowie and coworkers[15] have shown that negatively charged phosphopeptides undergo efficient N–C_α bond cleavages at phosphorylated serine and threonine residues to give rise to abundant $[z_n–H_3PO_4]^-$ fragments under collision-induced dissociation (CID) conditions. Therefore, protein phosphorylation sites at serine or threonine can be located by negative CID.

In the positive mode, loss of the C-terminal residue with retention of the original carbonyl oxygen at the C-terminus, which is usually assigned as $[b_n+H_2O]^+$ or $[b_n+OH+$ alkali$]^+$, has been reported in the fragmentation of protonated peptides and metal-cationized peptides.[16–18] Several mechanisms have been proposed to explain this C-terminal residue loss.[16,19–23] It was reported that the position of a basic amino acid and its basicity had a significant influence on C-terminal residue loss.[24] C-terminal residue elimination is

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Table 1. SORI-CID fragmentations of deprotonated hexapeptides containing hydroxyl side chains

	SAAAAA					
	S, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-	w[a]	w	w	w		
$[c_n–H_2O]^-$		w				
$[c_n–30]^-$		w	m	w		
$[c_n–H_2O–30]^-$			w	w		
$y_{(7–n)}^-$	—[b]	w	w	w	w	
$[y_{(7–n)}–H_2O]^-$	w				w	
$[y_{(7–n)}–30]^-$	s		m			
	AASAAA					
	A, n = 1	A, n = 2	S, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-	w	w	w			
$[c_n–H_2O]^-$			w			
$[c_n–30]^-$			w			
$[c_n–H_2O–30]^-$			w			
$y_{(7–n)}^-$	—		w	w	w	w
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–30]^-$	s		w			
	AAAASA					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	S, n = 5	A, n = 6
c_n^-	w	w	m	w		
$[c_n–H_2O]^-$			w			
$y_{(7–n)}^-$	—		w	w	w	w
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–30]^-$	s	w	w	w	w	
	AAAAAS					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	S, n = 6
c_n^-		m	m	w		
$[c_n–H_2O]^-$		w				
$y_{(7–n)}^-$	—	w	w	w	w	m
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–30]^-$	s			w	w	w
Internal ions[c]	w, AAAAA					
$[M–H–S]^-$	s[d]					
$[S]^-$	w					
	TAAAAA					
	T, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-	w		w			
$[c_n–H_2O]^-$		w	w			
$[c_n–44]^-$	w	w	m	w		
$[c_n–H_2O–44]^-$			w	w		
$y_{(7–n)}^-$		w	w	w	w	
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–44]^-$	s					
	AATAAA					
	A, n = 1	A, n = 2	T, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-	w	w	w			
$[c_n–H_2O]^-$		w				
$[c_n–44]^-$			w	w		
$[c_n–H_2O–44]^-$			w	w		
$y_{(7–n)}^-$			w	w	w	w
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–44]^-$	s	w	w			

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Table 1. (Continued)

	AAAATA					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	T, n = 5	A, n = 6
c_n^-	w	w	w	w		
$[c_n–H_2O]^-$		w	w			
$y_{(7–n)}^-$					w	w
$[y_{(7–n)}–44]^-$	s	w	w	w	w	
	AAAAAT					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	T, n = 6
c_n^-	w	w	w	w		
$[c_n–H_2O]^-$		w	w			
$y_{(7–n)}^-$	—				w	w
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–44]^-$	s	w	w	w	w	w
Internal ions[c]	w, AAAAA					
$[M–H–T]^-$	m[d]					
	YAAAAA					
	Y, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-	s	w	s	w		
$[c_n–H_2O]^-$			w			
$[c_n–H_2O–106]^-$			w			
$y_{(7–n)}^-$	—	w	w	w	w	w
$[y_{(7–n)}–H_2O]^-$	w			w		
	AAYAAA					
	A, n = 1	A, n = 2	Y, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-	w	w	s	w		
$[c_n–H_2O]^-$			m	w		
$[c_n–106]^-$			w	w		
$[c_n–H_2O–106]^-$			w	w		
$y_{(7–n)}^-$		w	w	w	w	w
$[y_{(7–n)}–H_2O]^-$	w					
	AAAAYA					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	Y, n = 5	A, n = 6
c_n^-	w	w	s	w		
$[c_n–H_2O]^-$		w	w	w		
$y_{(7–n)}^-$	—	w	w	w	w	w
$[y_{(7–n)}–H_2O]^-$	w				w	
$[y_{(7–n)}–106]^-$			w	w	w	
	AAAAAY					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	Y, n = 6
c_n^-	w	w	s	w		
$[c_n–H_2O]^-$		w	w	w		
$y_{(7–n)}^-$	—	w	w	w	w	m
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–106]^-$						w
Internal ions[c]	w, AAAAA					

[a] Intensities where w is weak (<30% of the base peak), m is medium (30–65%), and s is strong (>65%).
[b] — indicates that this fragment has the *m/z* of the precursor ion.
[c] Internal ions are composed of the underlined residues.
[d] Corresponds to C-terminal residue loss.

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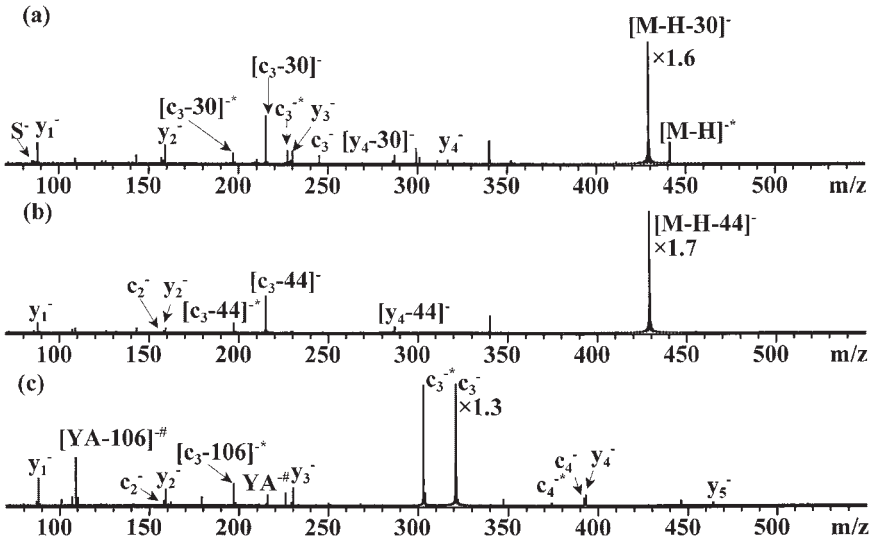

Figure 1. SORI-CID spectra of $[M–H]^-$ from (a) AASAAA, (b) AATAAA, and (c) AAYAAA. * and # correspond to loss of water and ammonia, respectively.

strongly promoted when arginine is the amino acid residue lost and also favored by the presence of an arginine residue at or near the N-terminus.[24–26] This is likely to be attributed to the interaction of a positively charged guanidino group of arginine with its own carbonyl group, resulting in facile attack of the C-terminal –OH on this carbonyl group.[24] Also, Padron and coworkers[24] found that the intensity of ions owing to the C-terminal residue loss increased with increasing gas-phase basicity (GB) of the residue at or near to the N-terminus.

Several studies have reported C-terminal residue loss for deprotonated peptides. In the work of Bowie and co-workers,[27] dissociation on $[M–H]^-$ of peptides from the bioactive amphibian peptide citropin 1 with $–CONH_2$ as the C-terminal group results in repeated C-terminal residue loss. This repeated cleavage was named β' cleavage. However, they found that the presence of serine, aspartic acid, or glutamic acid residues suppressed β' cleavage because facile side-chain loss was induced by these residues.[3] Harrison[28] reported C-terminal residue loss in the dissociation of dipeptides containing glutamic acid residues at the C-terminus. Recently, a study from our group reported that negative dissociation of peptides containing aspartic acid, glutamic acid, and serine at the C-terminus will eliminate the C-terminal residue.[29] In this study, three mechanisms were proposed to be involved to rationalize this process. These mechanisms involve nucleophilic attack of deprotonated carboxylic acid groups from either the C-terminus or an acidic side chain on the carbonyl carbon of the adjacent residue. The dissociation results of $[M–H]^-$ from AAAAAD, which was ^{18}O specifically labeled on the side-chain carboxylic group of the aspartic acid (D) residue, confirm that mechanisms involving the side chain and the C-terminal acidic groups occur in near equal probabilities.

In the present study, dissociation pathways of peptides containing hydroxyl side chains are investigated. Side-chain

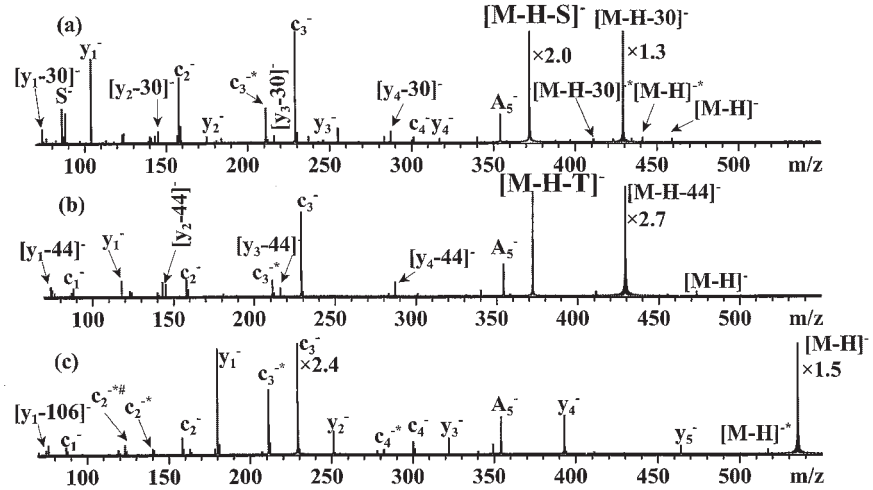

Figure 2. SORI-CID spectra of $[M–H]^-$ from (a) AAAAAS, (b) AAAAAT, and (c) AAAAAY. * and # correspond to loss of water and ammonia, respectively.

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Table 2. PSD fragmentations of deprotonated hexapeptides containing hydroxyl group side chains

	SAAAAA					
	S, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-		w	m	w		
$[c_n–H_2O]^-$				w		
$[c_n–30]^-$			s			
$y_{(7–n)}^-$	—[b]	w	m	w	s	s
$[y_{(7–n)}–30]^-$	w[a]					
	AASAAA					
	A, n = 1	A, n = 2	S, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-			w	w		
$[c_n–H_2O]^-$						
$[c_n–30]^-$			s			
$y_{(7–n)}^-$	—	w	w	w	m	w
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–30]^-$	m		m			
	AAAASA					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	S, n = 5	A, n = 6
c_n^-		w	s	w		
$[c_n–30]^-$					w	
$y_{(7–n)}^-$	—		w	m	w	w
$[y_{(7–n)}–H_2O]^-$	w	w				
$[y_{(7–n)}–30]^-$	w	w	w		m	
	AAAAAS					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	S, n = 6
c_n^-	w	w	s	w		
$y_{(7–n)}^-$	—	w	w	w	w	m
$[y_{(7–n)}–H_2O]^-$						
$[y_{(7–n)}–30]^-$	w	w	w	w	w	w
$[M–H–S]^-$	s[c]					
	TAAAAA					
	T, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-		w	w	w		
$[c_n–H_2O]^-$			w			
$[c_n–44]^-$		w	s	w		
$y_{(7–n)}^-$	—	w	w	w	w	w
$[y_{(7–n)}–44]^-$	m					
	AATAAA					
	A, n = 1	A, n = 2	T, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-			w	w		
$[c_n–44]^-$			m			
$y_{(7–n)}^-$	—		w	w	w	w
$[y_{(7–n)}–H_2O]^-$	w					
$[y_{(7–n)}–44]^-$	s	w	w			
	AAAATA					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	T, n = 5	A, n = 6
c_n^-		w	s	w		
$[c_n–H_2O]^-$						
$y_{(7–n)}^-$	—		w	w	w	w
$[y_{(7–n)}–44]^-$	s				w	

(Continues)

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Table 2. (Continued)

	AAAAAT					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	T, n = 6
c_n^-		w	m	w	w	
$[c_n–H_2O]^-$						
$y_{(7–n)}^-$	—		s	w	w	s
$[y_{(7–n)}–44]^-$	m			w	w	
$[M–H–T]^-$	m[c]					

	YAAAAA					
	Y, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-	m	m	s	w		
$[c_n–H_2O]^-$						
$[c_n–106]^-$		w	w		w	
$y_{(7–n)}^-$	—	w	w	w	w	w

	AAYAAA					
	A, n = 1	A, n = 2	Y, n = 3	A, n = 4	A, n = 5	A, n = 6
c_n^-	w	w	s			
$[c_n–H_2O]^-$						
$[c_n–106]^-$			m			
$y_{(7–n)}^-$	—		w	w	m	w
$[y_{(7–n)}–106]^-$			w			

	AAAAYA					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	Y, n = 5	A, n = 6
c_n^-	w	m	s	w		
$[c_n–H_2O]^-$			w			
$y_{(7–n)}^-$	—	w	w	w	m	m
$[y_{(7–n)}–106]^-$			w		w	

	AAAAAY					
	A, n = 1	A, n = 2	A, n = 3	A, n = 4	A, n = 5	Y, n = 6
c_n^-	w	w	m	w		
$[c_n–H_2O]^-$						
$y_{(7–n)}^-$	—		w	w	m	s

[a] Intensities where w is weak (<30% of the base peak), m is medium (30–65%), and s is strong (>65%).
[b] — indicates that this fragment has the *m/z* of the precursor ion.
[c] Corresponds to C-terminal residue loss.

loss is observed throughout the dissociation of deprotonated peptides containing hydroxyl side chains. C-terminal residue loss is shown to occur from the dissociation of $[M–H]^-$ for peptides with serine and threonine at the C-terminus. A mechanism involving both the C-terminal carboxylic acid group and the hydroxyl side chains has been proposed to lead to this selective cleavage.

EXPERIMENTAL

All experiments were carried out on a BioApex 4.7e FT-ICR mass spectrometer from Bruker Daltonics (Billerica, MA, USA) equipped with a 4.7 T superconducting magnet. Ions were generated by an electrospray ionization (ESI) source from Analytica of Branford (Branford, CT, USA). The peptide solutions ranged in concentration from (1–9) × 10^{-5} M. A typical sample flow rate was 0.12 mL/h in the negative ion mode with a 50:50:1 mixture of methanol/water/ammonium hydroxide as the ESI solvent.

For low-energy CID experiments, precursor ions were isolated with resonance frequency ejection[30] and activated with sustained off-resonance irradiation (SORI).[31] SORI employed a 5–20 V_{pp} pulse at ca. 700 Hz off-resonance from the precursor ion (either higher or lower frequency) for a duration of 100–150 ms. The collision gas was argon, which reached a pulsed pressure of 10^{-5} Torr and was pumped away prior to detection. The collision energy was ca. 100 eV.

Post-source decay (PSD) experiments were carried out on a Reflex III MALDI/TOF mass spectrometer (Bruker Daltonics), which is equipped with a two-stage reflectron. The instrument has a flight path of 2.9 m. Sample ionization by matrix-assisted laser desorption/ionization (MALDI) involved irradiation with a model VSL-337ND-S nitrogen laser (Laser Science, Franklin, MA, USA) emitting at 337 nm.

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The peptides were dissolved in water to a concentration of 1 mg/mL ($(5–20) \times 10^{-4}$ M) and 1 μL aliquots were mixed with 3 μL of a commercial α-cyano-4-hydroxycinnamic acid matrix solution, which was obtained from Agilent Technologies (Palo Alto, CA, USA). A 1 μL aliquot of each final mixture was deposited on the sample plate and allowed to air dry prior to insertion into the source of the mass spectrometer. Ions were moved from the source to the flight tube via delayed extraction[32] with an extraction delay of 250 ns. For PSD experiments, a gated pulse was used to selectively allow the precursor ions to enter the fight tube. The mass window for precursor ion selection was ca. ±15 Da. For negative ion PSD, the reflectron voltage was stepped from −21 to −2.8 kV in nine stages, with fibrinopeptide B used for PSD calibration.[33] The mass spectrum at each PSD voltage step was the sum of 100–200 laser shots. Bruker XMASS software combined the spectra from the individual voltage steps to form a complete PSD mass spectrum.

Twelve hexapeptides, XAAAAA, AAXAAA, AAAAXA, AAAAAX (X = serine, threonine, and tyrosine), plus AAAAAS-NH_2 and AAAAAT-NH_2, were synthesized in our laboratory using a model 90 automated peptide synthesizer (Advanced ChemTech, Louisville, KY, USA). Standard Fmoc synthesis procedures were employed.[34] AAAAAS-NH_2 and AAAAAT-NH_2 have an amide group rather than a carboxylic acid group at the C-terminus. The synthesis of AAAAAS-NH_2 and AAAAAT-NH_2 starts from the C-terminus by using PAL resin, which includes –NH_2. Reagents used in synthesis were purchased from Advanced ChemTech.

RESULTS AND DISCUSSION

Dissociation by SORI-CID

The general fragmentation behavior for deprotonated peptides containing hydroxyl side chains is summarized in Table 1. Negative SORI-CID of a variety of hexapeptides containing one residue with an hydroxyl side chain (serine, threonine, and tyrosine) and five alanine (A) residues results in primarily backbone cleavage fragments, c_n^- and y_n^-. There is no evidence of enhanced backbone cleavage adjacent to the residues with hydroxyl side chains. Side-chain losses are observed abundantly along with the corresponding c_n^- and y_n^-. Formaldehyde (HCHO, 30 Da) is eliminated from serine-containing peptides and acetaldehyde (CH_3CHO, 44 Da) from threonine-containing peptides, irrespective of the position of the serine and threonine residues. In contrast, side-chain loss of 4-methylene-2,5-cyclohexadienone (C_7H_6O, 106 Da) is generated in much less abundance for tyrosine-containing peptides. Representative SORI-CID spectra for model hexapeptides with internal hydroxyl-containing residues are shown in Fig. 1.

A noteworthy feature of the spectra is that fragment ions which are produced from $[M–H]^-$ by loss of the C-terminal amino acid residue are generated abundantly from hexapeptides containing serine and threonine at the C-terminus (i.e.,

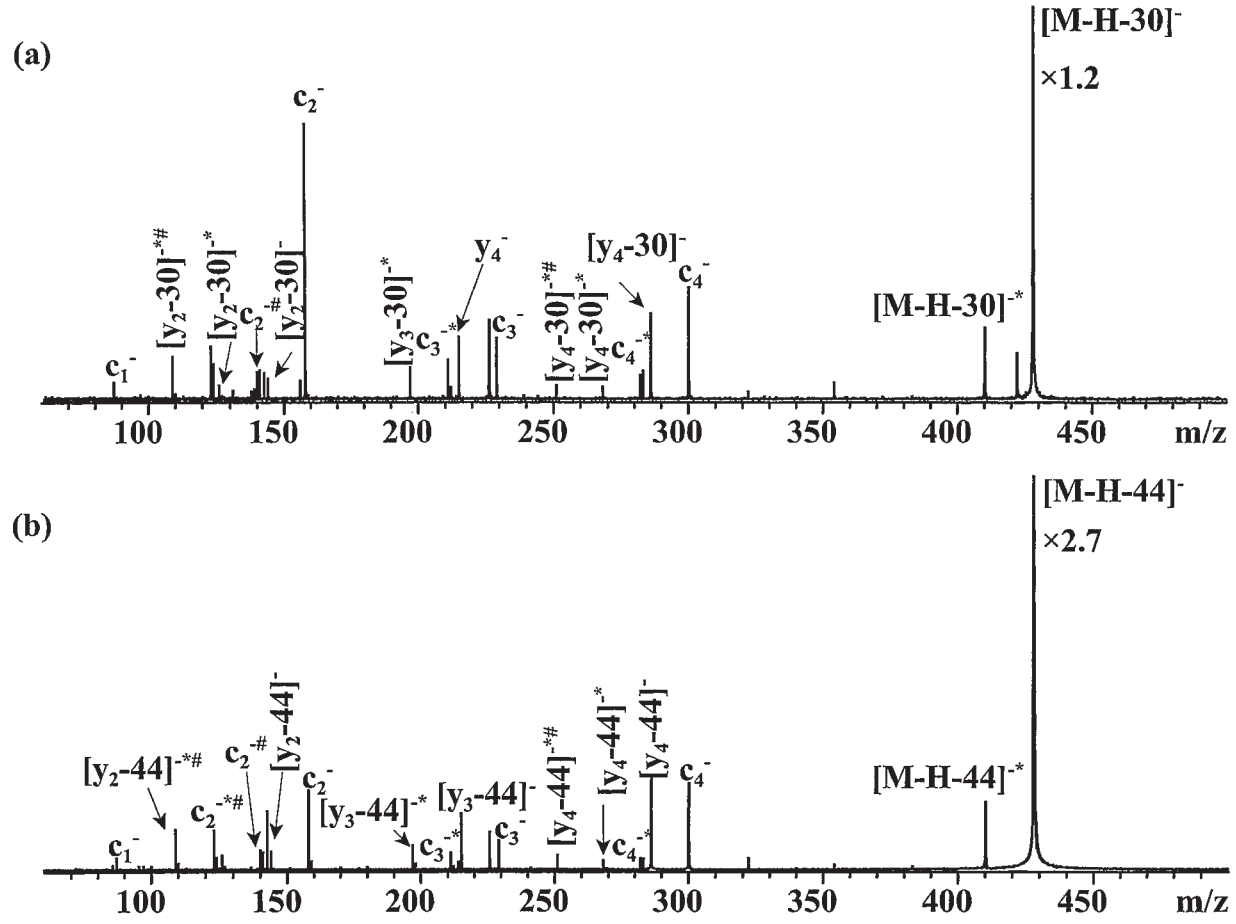

Figure 3. SORI-CID spectra of $[M–H]^-$ from (a) AAAAAS-NH_2 and (b) AAAAAT-NH_2. * and # correspond to loss of water and ammonia, respectively.

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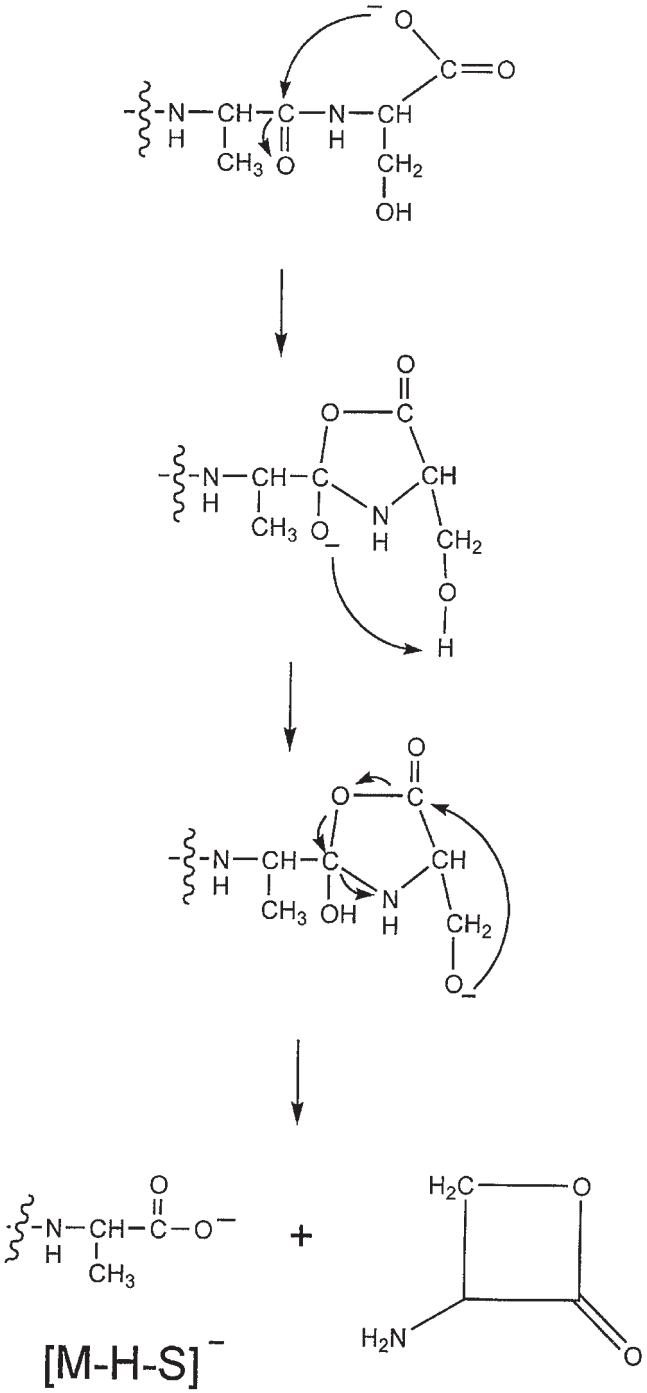

Figure 4. Proposed mechanism for formation of $[M–H–S]^-$ from $[M–H]^-$ of AAAAAS.

AAAAAS and AAAAAT). This can be seen in Fig. 2. In contrast, C-terminal residue loss is not generated from peptides with tyrosine at the C-terminus.

Dissociation by PSD

Negative PSD of serine-, threonine-, and tyrosine-containing peptides produces mainly backbone cleavages, c_n^- and y_n^-, along with side-chain losses. Table 2 lists the fragment ions produced by PSD. Again, $[M–H–S]^-$ and $[M–H–T]^-$ are formed from the dissociation of AAAAAS and AAAAAT in large abundance, while $[M–H–Y]^-$ is not generated from the dissociation of AAAAAY. The SORI-CID and PSD spectra generally contain the same ions, although sometimes in different relative abundances. PSD does, however, show some evidence of possible x-series ions, especially for AAAAAY.

MS/MS on $[M–H]^-$ of AAAAAS-NH_2 and AAAAAT-NH_2

In order to obtain a better understanding of the cleavage mechanism leading to the C-terminal residue loss from $[M–H]^-$, the C-terminal carboxylic acid groups of AAAAAS and AAAAAT were replaced with an amide group. That is, –OH was replaced with $–NH_2$ at the C-terminus. The negative SORI-CID spectra of AAAAS-NH_2 and AAAAAT-NH_2 are shown in Fig. 3. Backbone cleavage fragment ions, c_n^- and y_n^-, along with side-chain loss are observed. Interestingly, fragment ions due to loss of the C-terminal residue from $[M–H]^-$ were not produced. This is different from the experimental results obtained by Bowie and coworkers.[27] In their studies, repeated C-terminal residue loss was observed in negative dissociation of the bioactive amphibian peptide citropin 1 with $–CONH_2$ as the C-terminal group. A possible reason is that high-energy CID was employed in their studies. Their collision energy is on the order of kiloelectron volts; thus, more energy is imparted into precursor ions and processes that require higher energy can occur. In contrast, our studies use PSD and SORI-CID, which are low-energy techniques.

C-terminal residue loss from $[M–H]^-$

C-terminal residue loss has been observed in the dissociation of deprotonated peptides with aspartic acid and glutamic acid residues at the C-terminus.[9,15,28,29] The C-terminal residue loss from peptides with C-terminal serine has been also reported by a recent study in our group by Li *et al*.[29] Li *et al*. suggested that C-terminal residue loss from peptides with the serine residue at the C-terminus occurs through a mechanism involving the nucleophilic attack of $–COO^-$ on the adjacent backbone carbonyl group. In the present work, the C-terminal $–COO^-$ is definitively shown to be involved in the mechanism of C-terminal residue loss because $[M–H–S]^-$ and $[M–H–T]^-$ are not generated from negative mode dissociation of AAAAAS-NH_2 and AAAAAT-NH_2, which lack –COOH groups.

The hydroxyl side chains of serine and threonine in AAAAAS and AAAAAT should be involved in the mechanism because C-terminal residue loss is not observed in the dissociation of deprotonated peptides containing neutral or basic residues at the C-terminus. Also, C-terminal residue loss does not occur in AAAAAY where the hydroxyl side chain of the tyrosine residue is far removed from the C-terminal carboxylate. This suggests that C-terminal residue loss requires that the hydroxyl side chains are in proximity to the C-terminal carboxylic acid group. Taking these factors into consideration, a mechanism is proposed to rationalize the process of C-terminal residue loss in AAAAAS and AAAAAT. This mechanism, which is shown in Fig. 4, is modified from the previously proposed mechanism[29] to include hydroxyl side chain involvement.

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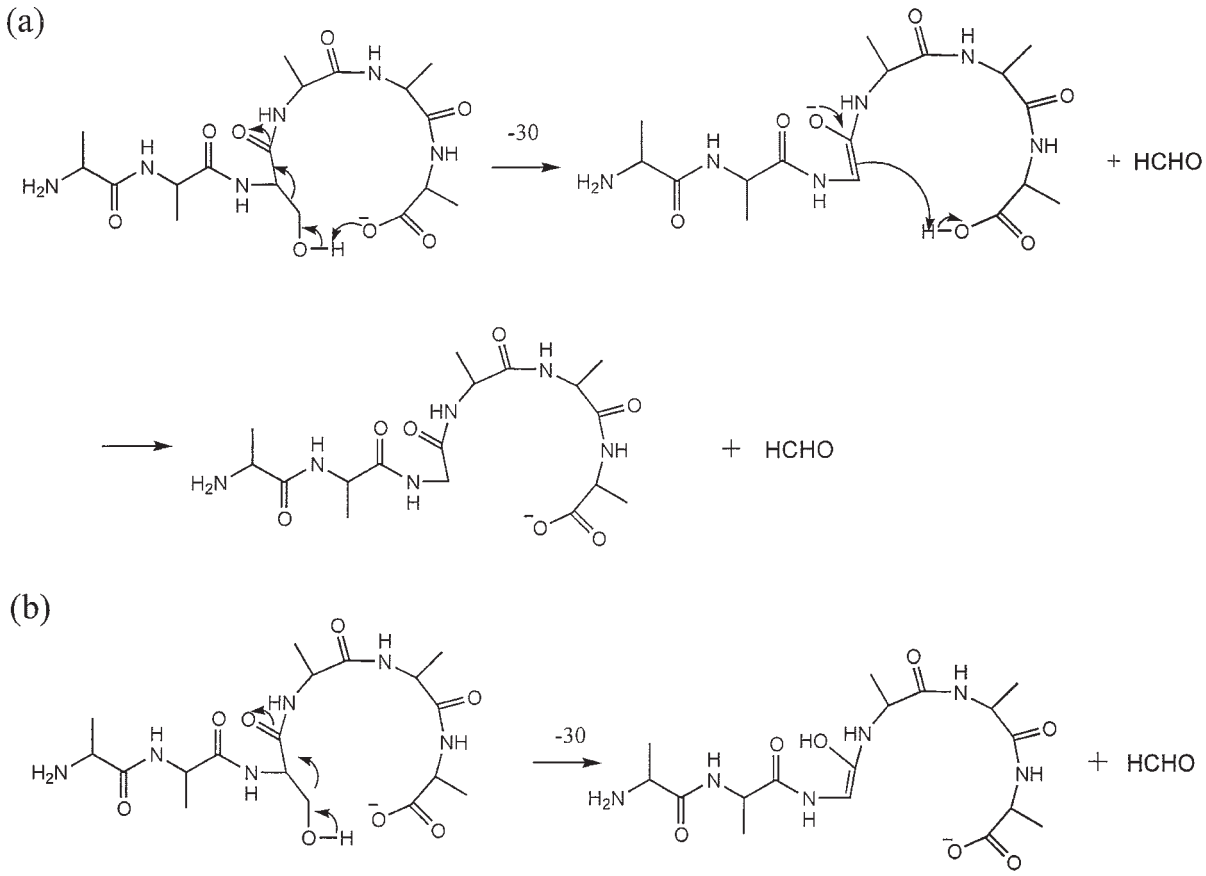

Figure 5. Side-chain loss from dissociation of $[M–H]^-$ of AASAAA, as adapted from Reiter *et al*,[12] by (a) a charge-induced mechanism and (b) a charge-remote mechanism.

As illustrated in Fig. 4, a five-membered ring intermediate is formed as a result of nucleophilic attack of the negatively charged C-terminal carboxylic acid group on the adjacent backbone carbonyl group. Then hydrogen transfer occurs followed by nucleophilic attack of a deprotonated hydroxyl side chain on the ring carbonyl carbon. The five-membered ring breaks apart, leading to the formation of $[M–H–X]^-$ (X = serine or threonine). The mechanism shown in Fig. 4 is not only capable of explaining the disappearance of the C-terminal residue loss when –COOH is replaced with $–CONH_2$, but is also able to explain why the C-terminal loss occurs only in peptides with serine and threonine at the C-terminus. As predicted from the proposed mechanism, the absence of C-terminal residue loss from the dissociation of $[M–H]^-$ of AAAAAY occurs because the rigid side chain of tyrosine cannot attack the hydroxyl side chain on the carbonyl group of the ring.

Characteristic side-chain loss

Side-chain losses of formaldehyde (HCHO) and acetaldehyde (CH_3CHO) from serine- and threonine-containing peptides, respectively, have been reported in the negative mode.[3,4,9–13,35] In addition, neutral loss of HCHO (as well as backbone cleavages) have also been recently reported for singly charged dilithiated peptides, $[M–H+2Li]^+$, which indicates that the deprotonation site can induce these reactions irrespective of the total precursor ion charge.[36] For these side-chain eliminations from deprotonated peptides, charge-directed and charge-remote mechanisms have been proposed, as shown in Fig. 5.[12] Charge-remote cleavages, which require more energy than charge-directed cleavages, generally occur in high-energy dissociation techniques.[37,38] SORI-CID is well known as a low-energy CID method[31] and cleavages via low-energy processes are most common.[39] In contrast, PSD is generally a higher energy process than SORI-CID.[39–41] Excess energy can be imparted to the precursor ions by laser irradiation during the MALDI process. Studies conducted by Gross and coworkers[42] have shown that a charge-remote mechanism is feasible for the dissociation of fatty acids under PSD conditions. Therefore, side-chain loss observed in our PSD studies could occur via a charge-induced mechanism (Fig. 5(a)) or a charge-remote rearrangement (Fig. 5(b)). In contrast, a charge-induced mechanism that involves lower energy is probably the dominant process in our SORI-CID studies. The mechanism of Fig. 5(a) contains proton movement between the C-terminal carboxylic acid group and the hydroxyl side chain. Deprotonation of the hydroxyl side chains of serine and threonine requires ca. 40 kcal/mol more energy than deprotonation of the C-terminal carboxylic acid group.[9] In addition, computations conducted by Adams and coworkers[12] have shown that a stable structure can exist with hydrogen bonding formed between hydroxyl side chains and the C-terminal carboxylic acid group. Thus, an endothermic process involving hydrogen transfer from the side chains of serine or threonine residues to the C-terminal

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RCM

carboxylic acid group should be possible upon low-energy collisional activation, such as our SORI-CID and PSD experiments.

Our experimental results show that characteristic side-chain loss of 4-methylene-2,5-cyclohexadienone (C_7H_6O) occurs in low abundance from the dissociation of deprotonated tyrosine-containing peptides (see, e.g., Fig. 1). The acidity of the side chain of tyrosine is comparable to the acidicity of the C-terminal carboxylic acid group (342.9 kcal/mol for deprotonated p-ethylphenol, which can represent the side chain of tyrosine, versus 341.6 kcal/mol for deprotonated glycine, which can represent the C-terminal carboxylic acid group)[43] and deprotonation at the hydroxyl side chain of the tyrosine residue can occur upon collisional activation. Because of geometric constraints owing to the rigid, bulky side chain of tyrosine, side-chain loss is hindered and is not abundant in the MS/MS spectra of deprotonated tyrosine-containing peptides.

CONCLUSIONS

Negative SORI-CID and PSD yielded comparable fragmentations for peptides containing serine, threonine, and tyrosine. In both PSD and SORI-CID studies, backbone cleavages resulting in c_n^- and y_n^- were primarily observed. The presence of residues with hydroxyl side chains leads to two characteristic cleavages. One is abundant side-chain elimination; that is, loss of formaldehyde (HCHO) from serine-containing peptides, acetaldehyde (CH_3CHO) from threonine-containing peptides, and 4-methylene-2,5-cyclohexadienone (C_7H_6O) from tyrosine-containing peptides. Side-chain loss occurs much less from tyrosine-containing peptides than from serine- and threonine-containing peptides. This is probably due to the rigid, bulky side chain of tyrosine, resulting in poor geometry for dissociation reactions. The other characteristic cleavage was the C-terminal residue loss from peptides containing serine and threonine at the C-terminus. This selective cleavage does not occur in the dissociation of peptides with an amide group at the C-terminus. Therefore, the C-terminal carboxylic acid group plays an important part in the mechanism of C-terminal residue loss. Hydroxyl side chains should also be involved because C-terminal residue loss does not occur in the dissociation of peptides with neutral or basic residues at the C-terminus.

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