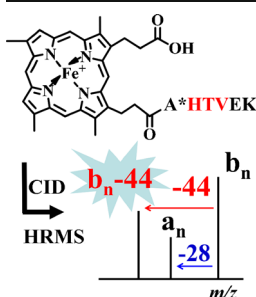


Investigation of b_n -44 Peptide Fragments Using High Resolution Mass Spectrometry and Isotope Labeling

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Abstract. An N-terminal deuterohemin-containing hexapeptide (DhHP-6) was designed as a short peptide cytochrome *c* (Cyt *c*) mimetic to study the effect of N-terminal charge on peptide fragmentation pathways. This peptide gave different dissociation patterns than normal tryptic peptides. Upon collision-induced dissociation (CID) with an ion trap mass spectrometer, the singly charged peptide ion containing no added proton generated abundant and characteristic b_n -44 ions instead of b_n -28 (a_n) ions. Studies by high resolution mass spectrometry (HRMS) and isotope labeling indicate that elimination of 44 Da fragments from b ions occurs via two different pathways: (1) loss of CH_3CHO (44.0262) from a Thr side chain; (2) loss of CO_2 (43.9898) from the oxazolone structure in the C-terminus. A series of analogues were designed and analyzed. The experimental results combined with Density Functional Theory (DFT) calculations on the proton affinity of the deuteroporphyrin demonstrate that the production of these novel b_n -44 ions is related to the N-terminal charge via a charge-remote rather than radical-directed fragmentation pathway.

Keywords: Deuterohemin, Charge-remote fragmentation, b_n -44 ions

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Introduction

A standard procedure for tandem mass spectrometry (MS/MS)-based peptide identification is matching experimental data obtained by collision-induced dissociation (CID) to theoretical spectra using bioinformatics software. The software is based on genomic information and assumes even cleavages occurring along the peptide backbone [1–3]. B and y ions generated by cleavage of amide bonds in protonated peptides, as well as a ions formed by loss of CO from b ions, are typical fragments. Many factors affect peptide fragmentation patterns: peptide sequence, presence and position of particular amino acids, charge, and even instrumental parameter settings [4–11]. In fact, some proteins in biological samples undergo post-translational modifications (PTMs), producing characteristic product ions [12–15].

Bing Wang and Jiayi Yu contributed equally to this work.

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Therefore, a clear understanding of the dissociation of peptide ions in gas phase is necessary; this will benefit de-novo sequencing and unambiguous protein identification in proteomics.

After great effort by the worldwide gas-phase ion chemistry community, the ‘mobile proton’ and ‘pathways in competition’ models have been established, which can explain the cleavages observed in protonated peptides [4–11]. Furthermore, fine details of structure, mechanism of formation, and activity of various fragment ions (such as b and a ions) have been extensively studied [7, 16, 17]. It is generally accepted that most common b ions are formed by nucleophilic attack of carbonyl oxygen on the adjacent amide carbon, generating an oxazolone structure in the C-terminus. However, amino acid side chains can competitively attack the same carbon to form isomeric b ions. For example, attack by the aspartic acid (Asp) side chain carboxylate forms a b ion with an anhydride structure; this ion loses CO_2 and CO (72 Da) upon further CID fragmentation [18, 19].

Many studies have focused on cleavage of tryptic peptides attributable to the numerous applications of trypsin in proteomics [2–5]. Tryptic peptides contain an arginine (R) or lysine (K) residue in their C-terminus. However, peptides with an R residue at the N-terminus can also be produced via non-tryptic

or tryptic cleavages from proteins containing KR, RR, and RP sequences [20, 21]. Compared with tryptic peptides, less attention is paid to the fragmentation pathways of these non-tryptic peptides. Recently, Paizs and co-workers demonstrated that the pathways leading to b_2 and $b_2 + H_2O$ fragments of the peptide RGD were different from those of common tryptic peptides, involving a salt-bridge stabilized configuration and an anhydride intermediate attributable to the R residue in the N-terminus of RGD [22].

In this work, an N-terminal deuterohemin-containing hexapeptide, DhHP-6, was chosen as a model N-terminal charged peptide. The structure is shown in Figure 1a. This peptide was initially designed as a short peptide mimetic of cytochrome *c* (Cyt *c*) and shows biological activities similar to microperoxidase-11, generated from hydrolytic cleavage of Cyt *c* [23]. The iron oxidation state has been determined unequivocally as Fe(III). This peptide can be singly charged through electrospray ionization (ESI) without protonation, consistent with previous reports and our results [24–26]. A series of novel b_n -44 ions (rather than b_n -28) were detected after CID using an ion trap mass spectrometer. High resolution mass spectra (HRMS) showed that the elimination of 44 Da could result from the loss of CO_2 (theoretical mass: 43.9898) or C_2H_4O (acetaldehyde; 44.0262). Because these fragment ions are seldom generated by common protonated peptides, the unusual fragmentation pathways implied piqued our interest.

Ternary metal complexes $[M^{n+}(L)^m-P]^{(n-m)+}$ (M: transition metal ion; L: suitable auxiliary ligand; P: neutral peptide or amino acid) have a similar structure to DhHP-6. These complexes produce radical cations upon CID [27–29]. In the MS/MS mass spectrum of Cyt *c*, along with a and y ions, c and z radical ions were detected [30]. Since these radical ions are commonly observed from electron capture dissociation (ECD), Breuker and McLafferty termed this unusual phenomenon

‘Native ECD’ and used these radical fragment ions to characterize the noncovalent interactions of Cyt *c* [30]. Our question is: Does the formation of b_n -44 ions from DhHP-6 involve a radical reaction in the gas phase? If not, which fragmentation mechanism generates these b_n -44 ions? To answer these questions, a series of peptides were designed, as shown in Figure 1b. In addition, we calculated the proton affinity of deuteroporphyrin with DFT and compared its PA value with that of Arg and Lys. Studies of these peptides and peptide-deuterohemin mixtures demonstrated that the gas-phase dissociation of DhHP-6 was greatly influenced by deuterohemin and that an N-terminal charge on the peptide rather than a radical reaction leads to formation of b_n -44 ions.

Experimental

Materials and Sample Preparation

All peptides, including isotope labeled peptides, used in this work (purity $\geq 90\%$) were synthesized using a conventional solid-phase method by Changchun BCHT Co., Ltd. Methanol (chromatographic grade) and formic acid (analytical grade) were purchased from Tedia Company Inc. (Cincinnati, OH, USA). Alanine-1- ^{13}C and Fmoc-chloride were purchased from Sigma Chemical (St. Louis MO, USA) to generate Fmoc-alanine-1- ^{13}C as described previously [31]. Each peptide was dissolved in Milli-Q water to make a stock solution of 2 mmol/L and diluted to a final concentration of 30 $\mu\text{mol/L}$ with methanol/water/formic acid at a volume ratio of 49.5:49.5:1, for direct ESI-MS analysis.

A noncovalent complex of [deuterohemin (Fe^{III}) (hexapeptide)]⁺ was prepared by mixing 100 μL of peptide stock solution and 100 μL of saturated deuterohemin solution (partially soluble in methanol, insoluble in water) and diluted with 800 μL methanol before ESI-MS analysis [28].

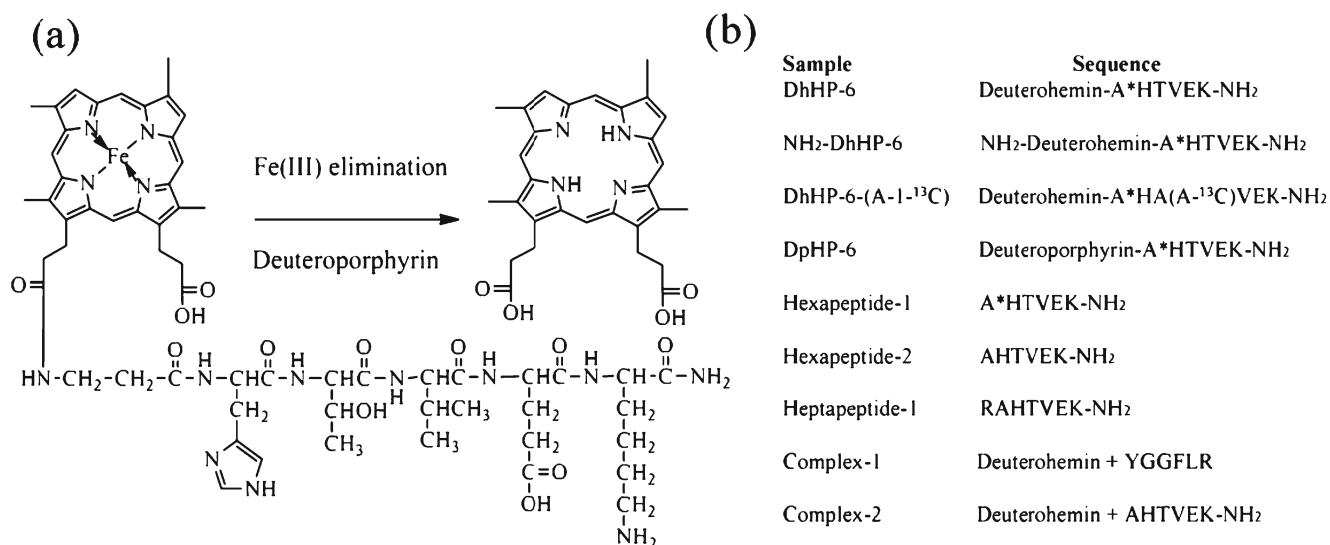


Figure 1. (a) Structures of N-terminal deuterohemin or deuteroporphyrin containing hexapeptides (DhHP-6 or DpHP-6). DhHP-6 molecular formula: $C_{59}H_{76}FeN_{14}O_{12}^+ Cl^-$; exact cation mass: 1228.5. (b) Sequences of DhHP-6 derived peptides and deuterohemin-peptide complexes (note: A* is β -Ala)

Mass Spectrometry

An Agilent G6300 ESI mass spectrometer (Agilent Technologies, Palo Alto, California, USA) in positive ion mode was used for MSⁿ experiments involving DhHP-6. Samples were infused at 180 μ L/h and determined in triplicate on 3 different days. The optimized instrumental conditions were as follows: spray voltage, 4000 V; capillary exit voltage, 150 V; skimmer voltage 50 V; dry gas, N₂, 325°C (5.00 L/min); nebulizer gas, N₂ (15 psi). For CID MS/MS experiments: isolation selection, standard; fragmentation cutoff, manually set; isolation width, 3.0 m/z ; fragmentation Ampl, 2.0 V for singly charged ions and 1.0 V for doubly and triply charged ions; 0.65 V for MS/MS/MS performance.

MicroTOF-Q II (Bruker Daltonics, Bremen, Germany) was used as a high resolution mass spectrometer to distinguish the loss of CO₂ (43.9898) from C₂H₄O (44.0262). Instrumental parameters were as follows: capillary voltage, 4000 V; dry gas, N₂, 200°C; nebulizer gas, N₂, 0.4 bar; isolation width 3.0 m/z (varied from 1.0 to 5.0 to examine potential isobaric contamination); ISCID energy, 10 eV; collision energy, 60 eV for singly charged precursor ions and 35 eV for doubly and triply charged ions. In addition, mass calibration was performed using sodium formate solution before sample injection into the ESI source.

Determination of Proton Affinity

The proton affinity (PA) of deuteroporphyrin was calculated at the same level as Lys and Arg using density functional theory (DFT), as reported by Paizs et al. [32]. Ab initio calculations were carried out to find the global minimum for deuteroporphyrin in neutral and protonated form. Molecular dynamics (MD) simulations were performed using Discovery Studio (DS) 2.5 [33] with a CHARMM force field [34]. The structures derived in this way were further optimized at the HF/3-21G, B3LYP/6-31G(d), and finally B3LYP/6-31 + G(d,p) levels. Total energies calculated at B3LYP/6-31 + G(d,p) level were corrected for zero-point vibration energies (ZPE) at the same level. The PA value of deuteroporphyrin was obtained as the difference between the ZPE-corrected DFT total energies of the protonated and neutral forms given in kcal mol⁻¹. All four imine nitrogen atoms in deuteroporphyrin were checked as protonation sites. All quantum chemistry calculations were performed with Gaussian 09 [35].

Results and Discussion

Fragmentation Patterns of DhHP-6 Ions are Affected by Labile Protons and Fixed Charge

The peptide DhHP-6 produces three precursor ions, m/z 1228.5(1+), 614.8(2+), and 410.2(3+), shown in Supplementary Figure S1 (Supporting Information). Figure 2 shows that each precursor ion gives different fragmentation patterns upon CID using an ion trap mass spectrometer.

The MS/MS spectrum of the singly charged ion presents a series of b ions as shown in Figure 2a. The peak at m/z 563.2 corresponds to positively charged deuterohemin. Note that the singly charged ion has no labile proton because the charge is carried by the N-terminal Fe(III). This phenomenon has been reported previously [24–26]. For peptide ions without a labile proton, it has been suggested that a proton can dissociate from the carboxylic acid group and attach to the amide nitrogen, initiating cleavage of the amide bond through a salt-bridge intermediate (Figure 2a, pathway A). This may explain the fragmentation of the singly charged ion [21, 22].

Our interest is in the detection of a series of abundant b_{n-44} ions (n : 2, 3, 4). A common b_n ion usually features a protonated oxazolone in its C-terminus that dissociates to produce a_n ions by loss of CO (28 Da) [7, 36, 37]. Some amino acid side chains can also be involved in competitive fragmentation reactions, resulting in b ions, as the “Asp effect” mentioned above [18, 19]. Therefore, it is conceivable that the generation of b_{n-44} ions involves either a specific b ion or a specific fragmentation pathway. Upon further dissociation of singly charged b_n ions (n : 2, 3, 4) only b_{n-44} ions were detected in the MS³ spectra (Supplementary Figure S2, Supporting Information), confirming that b_{n-44} ions were generated by dissociation of the corresponding b_n ions.

These b_{n-44} ions were also observed in the MS/MS spectrum of the doubly charged DhHP-6 ion. This extra proton can migrate to various sites upon excitation, resulting in cleavage of amide bonds and production of a series of complementary b/y sequencing ions (Figure 2b, pathway B) [5–7].

No b_{n-44} ions were detected in the MS/MS spectrum of the triply charged DhHP-6 ion (Figure 2c). However, selective cleavage occurred at a His residue, leading to the generation of abundant $[b_2 + H]^{2+}$ ions. This histidine effect has been extensively studied because it results in the loss of sequence information [38]. It has been suggested that a proton moves from imidazole to the nitrogen of the C-terminal adjacent amide bond, promoting nucleophilic attack by the imidazole nitrogen atom on the carbon of the protonated amide. This process leads to the generation of a bicyclic ion as shown in Figure 2c, pathway C [38, 39]. Supplementary Figure S2b (Supporting Information) shows the MS³ spectrum of the $[b_2 + H]^{2+}$ ion. The $[a_2 + H]^{2+}$ ion was a unique product of $[b_2 + H]^{2+}$ through the loss of CO. This suggests differences between $[b_2 + H]^{2+}$ and singly charged b_2 ions in either structure or fragmentation pathways.

Comparing the product ions generated by DhHP-6 ions of different charge, we can conclude that the number of labile protons influences the fragmentation pathways of the precursor ions. In particular, a series of novel b_{n-44} fragments were detected in the MS/MS spectra of both singly and doubly charged ions. The questions remain, however: What is the cause of the neutral loss of 44 Da, and where does it come from? To address these questions, isotope labeling and high resolution mass spectrometry was employed to study a series of DhHP-6-derived peptides and deuterohemine-peptide complexes.

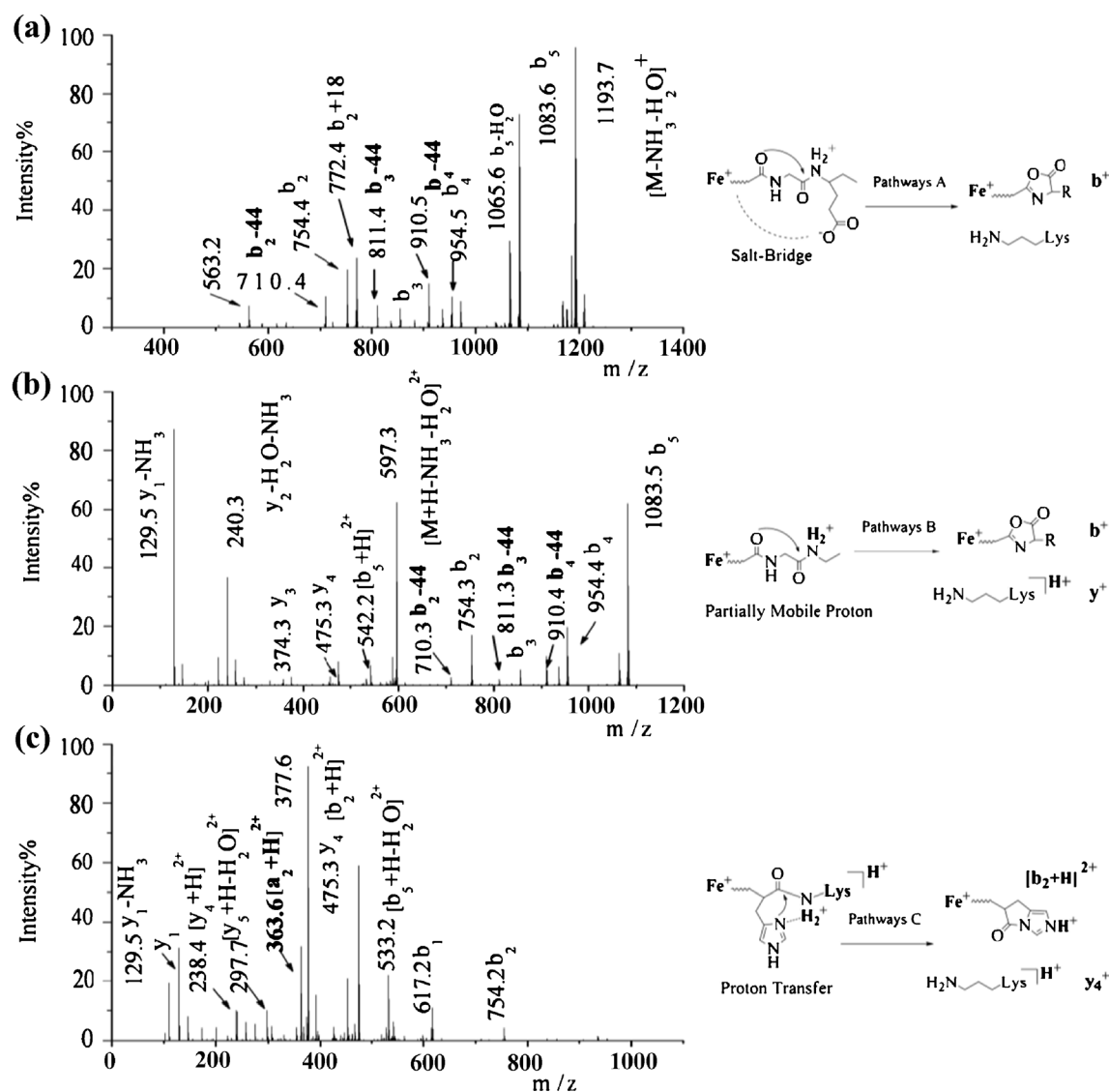


Figure 2. MS/MS spectra of each precursor ion (a) M^+ , (b) $[M^+ + H]^{2+}$, and (c) $[M^+ + 2H]^{3+}$ using the ion trap mass spectrometer (left) and corresponding fragmentation pathways of precursor ions to produce b and y ions (right)

Differentiation of C_2H_4O and CO_2 by High Resolution Mass Spectrometry

The ESI-Q-TOF instrument was used as a high resolution mass spectrometer to identify the neutral loss. Figure 3 gives the MS/MS spectrum of the singly charged ion generated by the ESI-Q-TOF instrument. The MS/MS spectra of the doubly and triply charged ions are presented in Supplementary Figure S3 (Supporting Information). To estimate potential isobaric contamination due to the 3.0 m/z isolation width, MS/MS spectra of the m/z 614.0 fragment with uni-resolution were performed (Supplementary Figure S4, Supporting Information).

Comparing Figures 2, 3, and Supplementary Figure S3, Supporting Information, different fragmentation patterns are observed for the same ions using different mass spectrometers [4, 5, 40]. In the ESI-Q/TOF mass spectra, abundant b_n - H_2O , b_n - CO , and b_n -44 ions

(n : 2, 3, 4) were obtained from the singly charged ion (Figure 3); the doubly charged DhHP-6 ion produced only b type ions (Supplementary Figure S3a, Supporting Information), in contrast to the b/y ions observed using the ion trap (Figure 2). These b ions showed different neutral losses: the singly charged b_n ions lost predominantly 44 Da (n : 2–4) fragments, whereas the doubly charged b ions lost CO (28 Da) and H_2O (18 Da). Note that two possible neutral losses of 44 Da can be unambiguously distinguished: CO_2 (43.9898) from the oxazolone or C_2H_4O (44.0262) from the Thr side chain. As shown in Figure 3a (right), the neutral loss of 43.993 Da from the b_2 ion could be assigned as CO_2 with a mass error of 0.0032 Da, and of 44.025 Da from the b_4 ions to C_2H_4O with a mass error of 0.0012 Da. However, the loss of 44.017 Da from the b_3 ion is intermediate between the masses

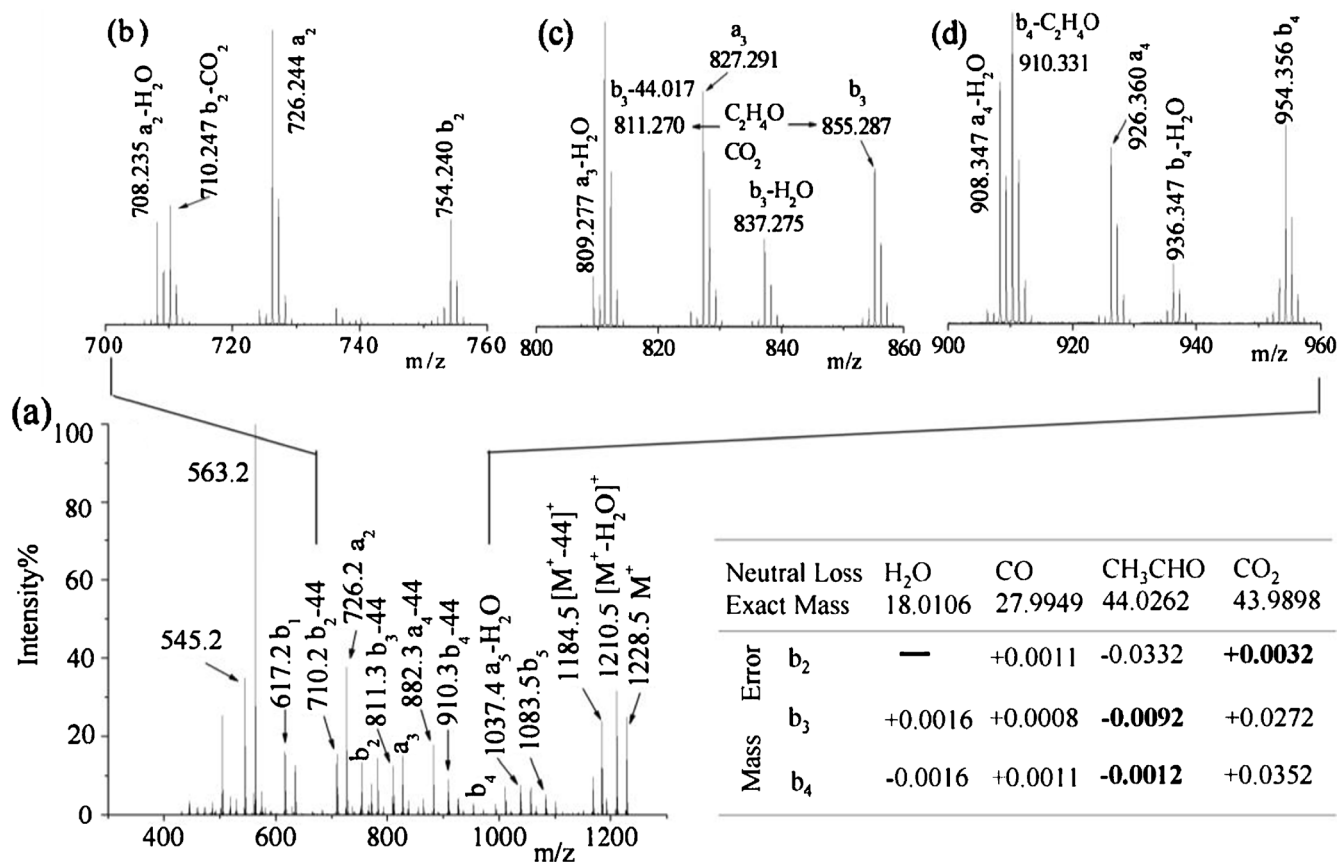


Figure 3. ESI-Q-TOF MS/MS spectra of the singly charged DhHP-6 ion. (a) Full scan spectrum (left) and differences between experimental and theoretical masses of the neutral loss fragments from b_n ; H₂O, CO, CH₃CHO, and CO₂ (right); extended MS/MS spectra of close to (b) the b_2^+ , (c) the b_3^+ , and (d) the b_4^+ ions

44.0262 (C₂H₄O) and 43.9898 (CO₂), which suggests a mixture of two molecules. This was investigated using isotope labeling.

Investigation of b_n -44 Ions Using Derivatives and Isotope Labeling

To further investigate the formation of b_n -44 ions, DhHP-6 was modified and isotopically labeled. First, considering that the N-terminal carboxylate of DhHP-6 may undergo decarboxylation, resulting in the loss of 44 Da (CO₂) [18, 27], NH₂-DhHP-6 was obtained by amidation of the N-terminal carboxylate of DhHP-6. Thus, there is one mass difference between DhHP-6 and NH₂-DhHP-6. However, decarboxylation is not apparent from the product ions generated from singly charged NH₂-DhHP-6, which differ by only 1 Da from the corresponding products of DhHP-6 (Supplementary Figure S5, Supporting Information).

To determine whether the 44 Da fragment was lost from the third Thr residue and to identify any effect of Thr on the formation of b_n -44 ions, the Thr residue in DhHP-6 was replaced with Ala. In addition, the carboxyl carbon was labeled with ¹³C to investigate whether ¹³CO₂ became apparent in the oxazolone structure of the b_3 ion. As expected, the b_2 -CO₂ ion was still abundant, in contrast with the less abundant b_3 -45

(¹³CO₂) ion; no b_n -C₂H₄O (n: 3, 4) or b_n -CO₂ (n: 4) ions were detected (Figure 4). Thus, it can be concluded that b_n ions (n: 2, 3) formed close to the N-terminus of DhHP-6 prefer CO₂ loss and that formation of b_n -C₂H₄O (n: 3, 4) may indeed be related to the Thr side chain. The absence of b_3 -C₂H₄O and the presence of the less abundant b_3 -45 (¹³CO₂) suggest that the loss of 44.017 Da from b_3 reflects both CO₂ and C₂H₄O.

Previous reports showed that protonated peptides containing Thr or Ser residues lose H₂O preferentially upon CID [7, 41–43]. CO₂ loss often involved a carboxylate, especially for peptide radical cations [18, 27]. The mechanism of the loss of the 44 Da in this case is investigated further as described below.

Factors Affecting the Formation of b_n -CO₂ and b_n -C₂H₄O

We have noted that DhHP-6 has similar composition to the [metal^{III}(salen)(peptide)] complex, except for a covalent connection instead of a noncovalent interaction between metal-ligand and peptide. The [Cu^{II}(ligand)(peptide)] complex gave various product ions, including both radical and neutral losses from amino acid side chains upon CID because both radical-driven redox reactions and charge-directed cleavages occurred [27–29, 44].

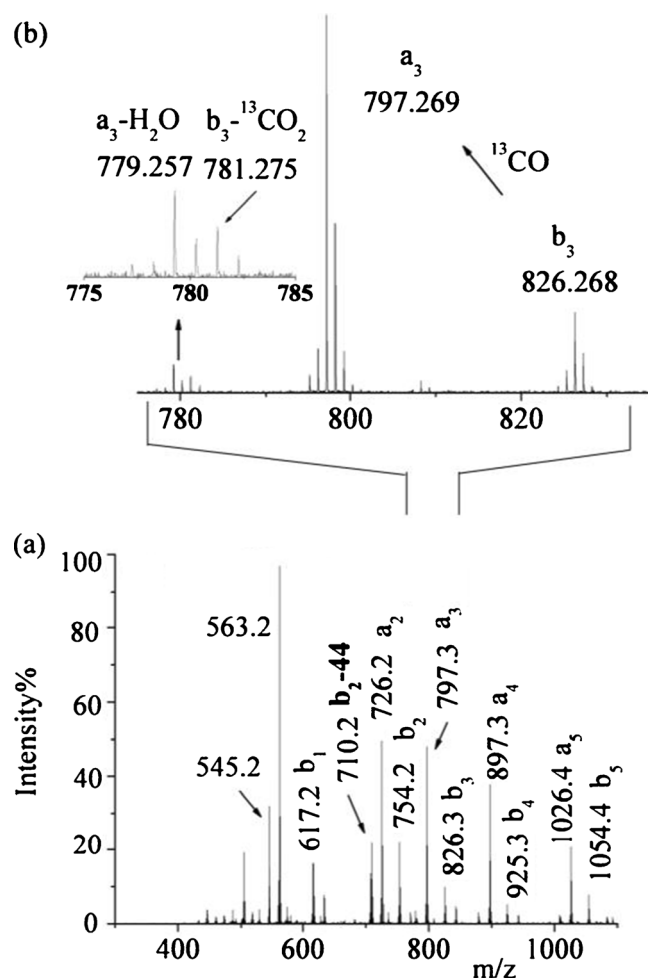


Figure 4. Q-TOF mass spectra: (a) full scan MS/MS spectrum of the singly charged DhHP-6-(A-1- ^{13}C) ion; (b) MS/MS spectrum from m/z 750 to 850

To investigate if the fragmentation of DhHP-6 ions involves radical reactions, deuterohemin(Fe^{III}) was mixed with the hexapeptide and subjected to CID. Because the hexapeptide YGGFLR has often been used to evaluate oxidation by metal-ligand complexes [27, 28], it was mixed with the deuterohemin. Figure 5a shows the MS/MS spectrum of the complex [deuterohemin(Fe^{III})(YGGFLR)] $^+$. The radical ion YGGFLR $^{\bullet+}$ (m/z : 711) was observed, as were [deuterohemin(Fe^{III})] $^+$ and [YGGFLR + H] $^+$ (m/z : 712). However, when YGGFLR was replaced with AHTVEK-NH $_2$, no radical cation P $^{\bullet+}$ was detected, as shown in Figure 5b. This suggests that a metalloporphyrin can serve as an alternative to the metal-ligand complex in the gas-phase redox reaction. However, it is evident that a great barrier exists for electron transfer from AHTVEK-NH $_2$ to Fe(III) in deuterohemin. Thus, radical-driven fragmentation of DhHP-6 is not feasible.

To investigate the effect of the N-terminal Fe(III) on fragmentation patterns of DhHP-6, DpHP-6 was obtained from DhHP-6 by removing Fe(III) from the N-terminal deuterohemin. Unlike DhHP-6, DpHP-6 was normally charged due to protonation. Similar product ions were observed in the

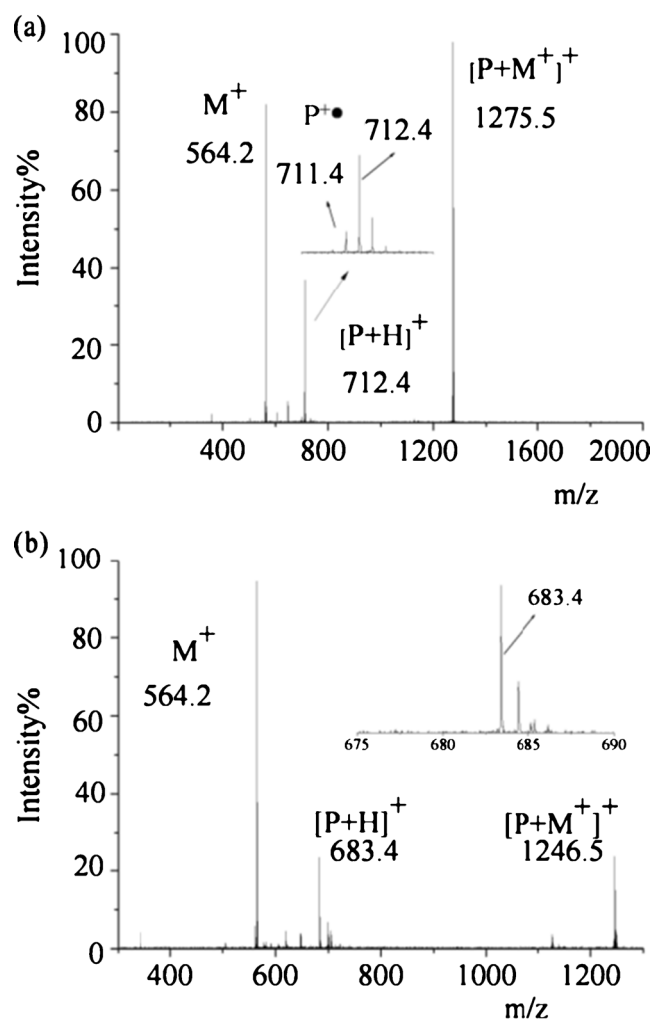


Figure 5. MS/MS spectra of (a) [deuterohemin(Fe^{III})(YGGFLR)] $^+$ and (b) [deuterohemin(Fe^{III})(AHTVEK-NH $_2$)] $^+$ using the Q-TOF mass spectrometer

MS/MS spectrum of DpHP-6 as for DhHP-6, except for a 53 Da shift (Supplementary Figure S6, Supporting Information). The singly charged DpHP-6 ion also produced a series of b-type fragments, and neutral losses from the b_n (n : 2, 3, 4) ions of DpHP-6 were the same as from DhHP-6. No y ions were detected, despite the fact that the basic residue Lys was located in the C-terminus of DpHP-6. “This suggests that the N-terminal deuteroporphyrin has a very strong proton affinity (PA), sequestering the added proton. This was confirmed by DFT calculations, which revealed a PA value for deuteroporphyrin of 248.2 kcal mol $^{-1}$, much higher than that of Lys (237.3 kcal mol $^{-1}$) and slightly lower than that of Arg (253.3 kcal mol $^{-1}$). These results suggest that the N-terminal charge plays a crucial role in the loss of the 44 Da neutral fragments through a charge-remote fragmentation pathway.

To confirm the effect of a fixed charge on the formation of b_n -44 ions, an Arg residue was used in place of the N-terminal deuterohemin of DhHP-6 [10, 18]. The MS/MS spectrum of singly charged RAHTVEK-NH $_2$ was compared with that of singly charged AHTVEK-NH $_2$. The b_n -44 ions (n : 4–5),

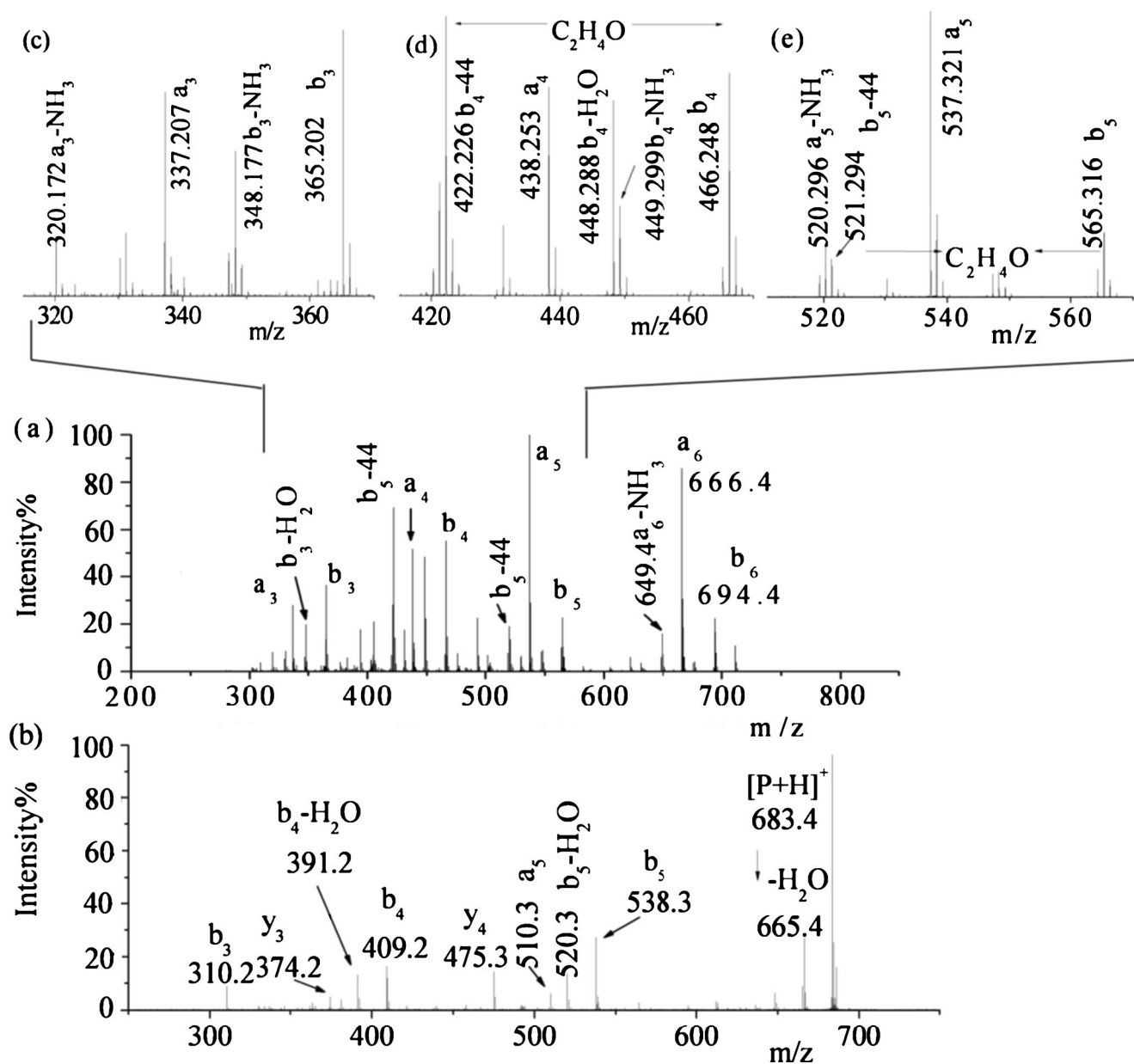


Figure 6. MS/MS spectra of the singly charged (a) RAHTVEK-NH₂ and (b) AHTVEK-NH₂. Inset: extended MS/MS spectra of RAHTVEK-NH₂ in m/z range of (c) 315–370; (d) 415–470; and (e) 510–570

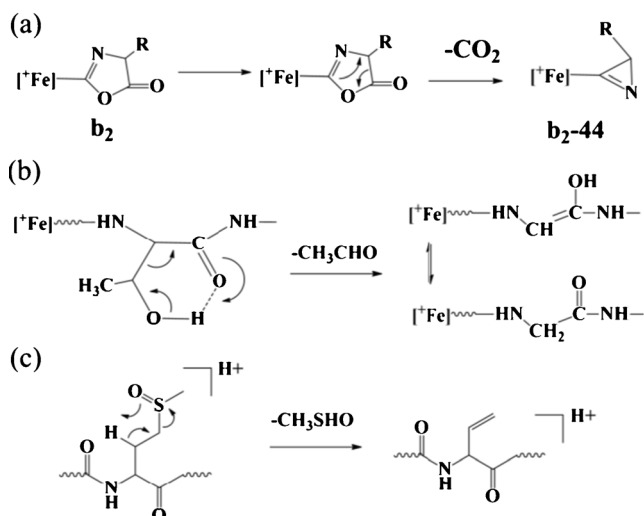
corresponding to b_n -C₂H₄O ions, were generated exclusively by RAHTVEK-NH₂; no b_n -CO₂ ions were detected among accurate m/z values (Figure 6). This suggests that deuterohemin plays an important role in the formation of the b_n -CO₂ ions and that the formation of b_n -C₂H₄O ions (n : 3–5) is directly related to the Thr residue and N-terminal fixed charge.

Proposed Mechanism for the Elimination of CO₂ and CH₃CHO

Dehydration of Thr side chains initiated by a labile proton has been widely reported [7, 41–43]. However, there is no labile proton in the singly charged b_n ions of DhHP-6, DpHP-6, and RAHTVEK-NH₂: the proton is sequestered in the N-terminus.

In addition, it is evident that CH₃CHO is eliminated from the Thr side chain without the involvement of a labile proton. A charge-remote elimination reaction involving a six-membered ring and H rearrangement is suggested, corresponding to the formation of b_n -CH₃CHO as shown in Scheme 1b. This charge-remote fragmentation pathway, resulting in CH₃CHO loss, is similar to the loss of methane sulfenic acid (CH₃SHO, 64 Da) from methionine sulfoxide-containing peptide ions in the absence of labile protons (Scheme 1c) [15].

CO₂ loss from the non-protonated oxazolone ring of DhHP-6 b_2 ion may involve a 2*H*-azirine structure (Scheme 1a). This structure has been widely reported [45]. It was proposed by Van Stipdonk et al. to explain fragmentation of metal complexes with positively charged peptides upon CID using an ion-



Scheme 1. Proposed mechanism for the generation of (a) b_n - CO_2 and (b) b_n - CH_3CHO ions, and (c) the loss of CH_3SHO

trap mass spectrometer [46]. Aromatic groups (phenyl, chlorophenyl, etc.) have been reported to promote the synthesis of 2*H*-azirine compounds [45, 47]. We speculate that porphyrin or imidazole may play a similar role in the stabilization of this structure because of the similar π -system.

Conclusions

This work provides significant information on the effect of the deuterohemin group in the N-terminus on the dissociation patterns of DhHP-6 upon CID process. These gas-phase reactions are also influenced by charge states, the identity and position of the amino acids, and even the instruments used. Our conclusion is based mainly on results obtained using a Q-TOF instrument. A number of b_n -44 ions were detected. Two different neutral fragments [CH_3CHO (44.0262 Da) and CO_2 (43.9898 Da)] have been unambiguously identified by accurate mass measurements, and the formation pathways have been proposed. The experimental and theoretical results gave evidence that both deuterohemin and deuteroporphyrin can function as a charge carrier, similar to the N-terminal R residue, leading to the loss of CH_3CHO from Thr through a charge-remote reaction. This novel fragmentation of Thr in the absence of labile protons provides featured additional information, which could be used for promoting the identification of peptide sequence. The porphyrin may show a neighbor effect, resulting in loss of CO_2 from the non-protonated oxazolone nearby. This could lead to the generation of a novel 2*H*-azirine structure. This is consistent with prior results in the synthesis of 2*H*-azirines: aromatic groups are known to increase the stability of aziridine and azirine rings. Because one-third of natural proteins are metalloproteins, the present work using a model peptide presents important fragmentation information. In addition, we demonstrate that the deuterohemin group can induce formation of radical peptide ions by gas-phase redox reactions

when mixed with a suitable peptide. This result suggests that it may be possible to develop and apply deuterohemin to produce radical fragment ions in CID without the use of ECD and ETD, providing structural information and, therefore, facilitating protein identification. Much more research on dissociation of metalloproteins is required; this research may be of value in manual de-novo sequencing for identification of metalloproteins.

Acknowledgment

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References

1. Aebersold, R., Goodlett, D.R.: Mass spectrometry in proteomics. *Chem. Rev.* **101**, 269–296 (2001)
2. Seidler, J., Zinn, N., Boehm, M.E., Lehmann, W.D.: De novo sequencing of peptides by MS/MS. *Proteomics* **10**, 634–649 (2010)
3. Medzihradsky, K.F., Chalkley, R.J.: Lessons in *de novo* peptide sequencing by tandem mass spectrometry. *Mass Spectrom. Rev.* 1–21 (2013). doi:10.1002/mas.21406
4. Escobar, H., Reyes-Vargas, E., Jensen, P.E., Delgado, J.C., Crockett, D.K.: Utility of characteristic QTOF MS/MS fragmentation for MHC class I peptides. *J. Proteome Res.* **10**, 2494–2507 (2011)
5. Mouls, L., Aubagnac, J.L., Martinez, J., Enjalbal, C.: Low energy peptide fragmentations in an ESI-Q-ToF type mass spectrometer. *J. Proteome Res.* **6**, 1378–1391 (2007)
6. Dongre, A.R., Jones, J.L., Somogyi, Á., Wysocki, V.H.: Influence of peptide composition, gas-phase basicity, and chemical modification on fragmentation efficiency: evidence for the mobile proton model. *J. Am. Chem. Soc.* **118**, 8365–8374 (1996)
7. Paizs, B., Suhai, S.: Fragmentation pathways of protonated peptides. *Mass Spectrom. Rev.* **24**, 508–548 (2005)
8. Tabb, D.L., Huang, Y., Wysocki, V.H., Yates, J.R.: Influence of basic residue content on fragment ion peak intensities in low-energy collision-induced dissociation spectra of peptides. *Anal. Chem.* **76**, 1243–1248 (2004)
9. Huang, Y., Triscari, J.M., Tseng, G.C., Pasa-Tolic, L., Lipton, M.S., Smith, R.D., Wysocki, V.H.: Statistical characterization of the charge state and residue dependence of low-energy CID peptide dissociation patterns. *Anal. Chem.* **77**, 5800–5813 (2005)
10. Laskin, J., Yang, Z., Song, T., Lam, C., Chu, I.K.: Effect of the basic residue on the energetics, dynamics, and mechanisms of gas-phase fragmentation of protonated peptides. *J. Am. Chem. Soc.* **132**, 16006–16016 (2010)
11. Tsapralis, G., Nair, H., Somogyi, Á., Wysocki, V.H., Zhong, W., Futrell, J.H., Summerfield, S.G., Gaskell, S.J.: Influence of secondary structure on the fragmentation of protonated peptides. *J. Am. Chem. Soc.* **121**, 5142–5154 (1999)
12. Schweppe, R.E., Haydon, C.E., Lewis, T.S., Resing, K.A., Ahn, N.G.: The characterization of protein post-translational modifications by mass spectrometry. *Acc. Chem. Res.* **36**, 453–461 (2003)
13. Sprung, R., Chen, Y., Zhang, K., Cheng, D., Zhang, T., Peng, J., Zhao, Y.: Identification and validation of eukaryotic aspartate and glutamate methylation in proteins. *J. Proteome Res.* **7**, 1001–1006 (2008)
14. Palumbo, A.M., Tepe, J.J., Reid, G.E.: Mechanistic insights into the multistage gas-phase fragmentation behavior of phosphoserine- and phosphothreonine-containing peptides. *J. Proteome Res.* **7**, 771–779 (2008)
15. Reid, G.E., Roberts, K.D., Kapp, E.A., Simpson, R.J.: Statistical and mechanistic approaches to understanding the gas-phase fragmentation behavior of methionine sulfoxide containing peptides. *J. Proteome Res.* **3**, 751–759 (2004)
16. Harrison, A.G.: To b or not to b: the ongoing saga of peptide b ions. *Mass Spectrom. Rev.* **28**, 640–654 (2009)

17. Polfer, N.C., Oomens, J., Suhai, S., Paizs, B.: Spectroscopic and theoretical evidence for oxazolone ring formation in collision-induced dissociation of peptides. *J. Am. Chem. Soc.* **127**, 17154–17155 (2005)
18. Gu, C., Tsapralis, G., Brecci, L., Wysocki, V.H.: Selective gas-phase cleavage at the peptide bond C-terminal to aspartic acid in fixed-charge derivatives of Asp-containing peptides. *Anal. Chem.* **72**, 5804–5813 (2000)
19. Wang, B., Shang, J.Z., Qin, Y.J., Yan, B.N., Guo, X.H.: Differentiation of α - or β -aspartic isomers in the heptapeptides by the fragments of [M+Na]⁺ using ion trap tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **22**, 1453–1462 (2011)
20. Switzar, L., Giera, M., Niessen, W.M.: Protein digestion: an overview of the available techniques and recent developments. *J. Proteome Res.* **12**, 1067–1077 (2013)
21. Bythell, B.J., Csonka, I.P., Suhai, S., Barofsky, D.F., Paizs, B.: Gas-phase structure and fragmentation pathways of singly protonated peptides with N-terminal arginine. *J. Phys. Chem. B* **114**, 15092–15105 (2010)
22. Bythell, B.J., Suhai, S., Somogyi, Á., Paizs, B.: Proton-driven amide bond-cleavage pathways of gas-phase peptide ions lacking mobile protons. *J. Am. Chem. Soc.* **131**, 14057–14065 (2009)
23. Guan, S., Li, P., Luo, J., Li, Y., Huang, L., Wang, G., Zhu, L., Fan, H., Li, W., Wang, L.: A deuterohemin peptide extends lifespan and increases stress resistance in *Caenorhabditis elegans*. *Free Radic. Res.* **44**, 813–820 (2010)
24. He, F., Hendrickson, C.L., Marshall, A.G.: Unequivocal determination of metal atom oxidation state in naked heme proteins: Fe (III) myoglobin, Fe (III) cytochrome *c*, Fe (III) cytochrome *b5*, and Fe (III) cytochrome *b5* L47R. *J. Am. Soc. Mass Spectrom.* **11**, 120–126 (2000)
25. Yang, F., Bogdanov, B., Strittmatter, E.F., Vilkov, A.N., Gritsenko, M., Shi, L., Elias, D.A., Ni, S., Romine, M., Paša-Tolic, L.: Characterization of purified c-type heme-containing peptides and identification of c-type heme-attachment sites in *Shewanella oneidensis* cytochromes using mass spectrometry. *J. Proteome Res.* **4**, 846–854 (2005)
26. Pashynska, V.A., Van den Heuvel, H., Claeys, M., Kosevich, M.V.: Characterization of noncovalent complexes of antimalarial agents of the artemisinin-type and FE (III)-heme by electrospray mass spectrometry and collisional activation tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **15**, 1181–1190 (2004)
27. Tureček, F.: Copper-biomolecule complexes in the gas phase. The ternary way. *Mass Spectrom. Rev.* **26**, 563–582 (2007)
28. Barlow, C.K., McFadyen, W.D., O'Hair, R.A.: Formation of cationic peptide radicals by gas-phase redox reactions with trivalent chromium, manganese, iron, and cobalt complexes. *J. Am. Chem. Soc.* **127**, 6109–6115 (2005)
29. Chu, I.K., Rodriguez, C.F., Lau, T.C., Hopkinson, A.C., Siu, K.M.: Molecular radical cations of oligopeptides. *J. Phys. Chem. B* **104**, 3393–3397 (2000)
30. Breuker, K., McLafferty, F.W.: Native electron capture dissociation for the structural characterization of noncovalent interactions in native cytochrome *c*^{**}. *Angew. Chem.* **115**, 5048–5052 (2003)
31. Chan, W.C., White, P.D. (eds.): Fmoc solid phase peptide synthesis—a practical approach. Oxford University Press Inc, New York (2000)
32. Bleiholder, C., Paizs, B., Suhai, S.: Revising the proton affinity scale of the naturally occurring α -amino acids. *J. Am. Soc. Mass Spectrom.* **17**, 1275–1281 (2006)
33. Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Scalmani, G., Barone, V., Mennucci, B., Petersson, G.A., Nakatsuji, H., Caricato, M., Li, X., Hratchian, H.P., Izmaylov, A.F., Bloino, J., Zheng, G., Sonnenberg, J.L., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Vreven, T., Montgomery Jr., J.A., Peralta, J.E., Ogliaro, F., Bearpark, M., Heyd, J.J., Brothers, E., Kudin, K.N., Staroverov, V.N., Kobayashi, R., Normand, J., Raghavachari, K., Rendell, A., Burant, J.C., Iyengar, S.S., Tomasi, J., Cossi, M., Rega, N., Millam, N.J., Klene, M., Knox, J.E., Cross, J.B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R.E., Yazyev, O., Austin, A.J., Cammi, R., Pomelli, C., Ochterski, J.W., Martin, R.L., Morokuma, K., Zakrzewski, V.G., Voth, G.A., Salvador, P., Dannenberg, J.J., Dapprich, S., Daniels, A.D., Farkas, Ö., Foresman, J.B., Ortiz, J.V., Cioslowski, J., Fox, D.: Gaussian 09, revision A.01. Gaussian, Inc, Wallingford (2009)
34. MacKerell, A.D., Bashford, D., Bellott, M., Dunbrack, R.L., Evanseck, J.D., Field, M.J., Fischer, S., Gao, J., Guo, H., Ha, S., Joseph-McCarthy, D., Kuchnir, L., Kuczera, K., Lau, F.T.K., Mattos, C., Michnick, S., Ngo, T., Nguyen, D.T., Prodhom, B., Reiher, W.E., Roux, B., Schlenkrich, M., Smith, J.C., Stote, R., Straub, J., Watanabe, M., Wiorkiewicz-Kuczera, J., Yin, D., Karplus, M.: All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J. Phys. Chem. B* **102**, 3586–3616 (1998)
35. Discovery Studio 2.5; Accelrys Inc.: San Diego (2009)
36. Allen, J.M., Racine, A.H., Berman, A.M., Johnson, J.S., Bythell, B.J., Paizs, B., Glush, G.L.: Why are a3 ions rarely observed? *J. Am. Soc. Mass Spectrom.* **19**, 1764–1770 (2008)
37. Sinha, R.K., Erlekam, U., Bythell, B.J., Paizs, B., Maitre, P.: Diagnosing the protonation site of b2 peptide fragment ions using IRMPD in the X–H (X = O, N, and C) stretching region. *J. Am. Soc. Mass Spectrom.* **22**, 1645–1650 (2011)
38. Tsapralis, G., Nair, H., Zhong, W., Kuppanan, K., Futrell, J.H., Wysocki, V.H.: A mechanistic investigation of the enhanced cleavage at histidine in the gas-phase dissociation of protonated peptides. *Anal. Chem.* **76**, 2083–2094 (2004)
39. Farrugia, J.M., Tavemer, T., O'Hair, R.A.: Side-chain involvement in the fragmentation reactions of the protonated methyl esters of histidine and its peptides. *Int. J. Mass Spectrom.* **209**, 99–112 (2001)
40. Barton, S.J., Whittaker, J.C.: Review of factors that influence the abundance of ions produced in a tandem mass spectrometer and statistical methods for discovering these factors. *Mass Spectrom. Rev.* **28**, 177–187 (2009)
41. Reid, G.E., Simpson, R.J., O'Hair, R.A.: Leaving group and gas phase neighboring group effects in the side chain losses from protonated serine and its derivatives. *J. Am. Soc. Mass Spectrom.* **11**, 1047–1060 (2000)
42. Neta, P., Pu, Q.L., Yang, X., Stein, S.E.: Consecutive neutral losses of H₂O and C₂H₄O from N-terminal Thr–Thr and Thr–Ser in collision-induced dissociation of protonated peptides Position dependent water loss from single Thr or Ser. *Int. J. Mass Spectrom.* **267**, 295–301 (2007)
43. Serafin, S.V., Zhang, K., Aurelio, L., Hughes, A.B., Morton, T.H.: Decomposition of protonated threonine, its stereoisomers, and its homologues in the gas phase: evidence for internal backside displacement. *Org. Lett.* **6**, 1561–1564 (2004)
44. Laskin, J., Yang, Z., Ng, C.M., Chu, I.K.: Fragmentation of α -radical cations of arginine-containing peptides. *J. Am. Soc. Mass Spectrom.* **21**, 511–521 (2010)
45. Singh, G.S., D'hooghe, M., Kimpe, N.D.: Synthesis and reactivity of C-heteroatom-substituted aziridines. *Chem. Rev.* **107**, 2080–2135 (2007)
46. Cooper, T.J., Talaty, E.R., Van Stipdonk, M.J.: Novel fragmentation pathway for CID of (b_n-1+Cat)⁺ ions from model, metal cationized peptides. *J. Am. Soc. Mass Spectrom.* **16**, 1305–1310 (2005)
47. Katritzky, A.R., Wang, M., Wilkerson, C.R., Yang, H.: A novel approach to substituted 2H-azirines. *J. Org. Chem.* **68**, 9105–9108 (2003)