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The dehydroalanine effect in the fragmentation of ions derived from polypeptides

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Abstract

The fragmentation of peptides and proteins upon collision-induced dissociation (CID) is highly dependent on sequence and ion type (e.g. protonated, deprotonated, sodiated, odd electron, etc.). Some amino acids, for example aspartic acid and proline, have been found to enhance certain cleavages along the backbone. Here, we show that peptides and proteins containing dehydroalanine, a non-proteinogenic amino acid with an unsaturated side-chain, undergo enhanced cleavage of the N—C α bond of the dehydroalanine residue to generate c- and z-ions. Because these fragment ion types are not commonly observed upon activation of positively charged even-electron species, they can be used to identify dehydroalanine residues and localize them within the peptide or protein chain. While dehydroalanine can be generated in solution, it can also be generated in the gas phase upon CID of various species. Oxidized S-alkyl cysteine residues generate dehydroalanine upon activation via highly efficient loss of the alkyl sulfenic acid. Asymmetric cleavage of disulfide bonds upon collisional activation of systems with limited proton mobility also generates dehydroalanine. Furthermore, we show that gas-phase ion/ion reactions can be used to facilitate the generation of dehydroalanine residues via, for example, oxidation of S-alkyl cysteine residues and conversion of multiply-protonated peptides to radical cations. In the latter case, loss of radical side-chains to generate dehydroalanine from some amino acids gives rise to the possibility for residue-specific backbone cleavage of polypeptide ions.

Keywords

dehydroalanine; selective cleavage; ion/ion reactions; low energy CID; c and z-ions

Introduction

Tandem mass spectrometry (MSⁿ) is a powerful approach for the analysis of peptides and proteins because of the primary structural information inherent in the observed products. The fragmentation of peptides and proteins depends heavily on the sequence and ion type of the species of interest. Certain amino acids can enhance cleavages at specific sites along the peptide or protein backbone under specific circumstances. Perhaps the two most well-known examples of enhanced tendencies for cleavage arise from the so-called aspartic acid^[1–3] and

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proline^[1–6] effects. The aspartic acid effect results in cleavage of the peptide bond C-terminal to aspartic acid residues to generate b- and y-ions. This process proceeds through the cyclization of the aspartic acid side-chain to cleave the peptide bond and is most prominent under proton mobility-limited environments (e.g. the number of arginine residues is greater than the number of excess protons).^[1–3] Conversely, the proline effect is most prominent when there is a mobile proton and results in cleavage of the peptide bond N-terminal to the pro-line residue. The proline amide group is a tertiary nitrogen and has a greater basicity than the surrounding amide bonds, thus facilitating the association of the mobile proton with the tertiary nitrogen and resulting in enhanced charge-directed cleavage N-terminal to the proline.^[1–6] Recently, the enhanced cleavage C-terminal to ornithine, a non-proteinogenic amino acid, has been described and dominated peptide fragmentation patterns, even when aspartic acid or proline residues were present.^[7]

Dehydroalanine (Dha) is another non-proteinogenic amino acid that can impact the fragmentation of peptides and proteins upon activation. Dehydroalanine is found in nature in peptide toxins (e.g. microcystins^[8]) and antibiotics (e.g. thiostrepton,^[9] nocathiacins,^[10] lantibiotics^[11–14]) as well as in proteins,^[15] and commonly undergoes reactions with various nucleophiles.^[11,12,16] Dehydroalanine is generated through the elimination of water^[11–13] and hydrogen sulfide^[17,18] from serine and cysteine, respectively. Additionally, conversion of tyrosine to Dha in the protein thyroglobulin is observed during thyroid hormone biosynthesis.^[19,20] There are also a variety of solution-phase synthetic routes to generate Dha, including oxidative elimination from S-alkyl cysteine and Se-phenylselenocysteine residues,^[21–26] the activation and elimination of serine derivatives,^[27] and enzymatic approaches.^[28] The synthesis of dehydroalanine is a widely investigated area of interest as Dha residues are readily transformed via conjugation to thiol^[21,22,29–31] and amine^[32] functionalities. For example, the conjugation of various thiol groups to Dha has been used to mimic various post-translational modifications (PTMs), including phosphorylation, glycosylation and lipidation.^[11,33–38]

Dehydroalanine residues can also be generated in the gas-phase. For example, asymmetric cleavage of a disulfide bond occurs upon collision-induced dissociation (CID) of disulfide-linked peptide/protein anions^[39,40] and proton mobility-limited cations^[41] and generates a Dha residue. The highly selective ejection of HOSR (R indicates an alkyl group) from oxidized S-alkyl cysteine residues upon CID has been reported to generate a Dha residue.^[42–44] Odd-electron species undergo neutral side-chain losses upon collisional activation, several of which generate dehydroalanine.^[45]

It has been shown that activation of dehydroalanine-containing species generated from asymmetric disulfide bond cleavage results in enhanced cleavage of the N—C α bond of the Dha residue to generate c- and z-type ions.^[39,40] We illustrate here that cleavage N-terminal to Dha residues at the relevant N—C α bond under low collision energy CID conditions is a general, and often dominant, effect, irrespective of the means for Dha generation. As c- and z-type ion types are not generally observed upon CID of positively charged even-electron species, they can be used to indicate the presence and location of Dha residues. We summarize several scenarios that arise from peptide ions generated directly from solution via electrospray ionization (ESI). These scenarios can give rise to the dehydroalanine effect

from species that can be encountered without special measures to promote Dha formation. Furthermore, we provide examples that involve measures to induce the formation of a Dha in the gas-phase. Hence, the gas-phase synthetic approaches provide selective and rapid means^[46] to convert particular residues to Dha residues for subsequent structural characterization by CID. The introduction of species that induce selective cleavages upon CID is analogous to the enzymatic digestions commonly done in solution and may provide complementary primary structural information to that obtained from the more common non-selective approaches.

Experimental section

Materials

Methanol, ammonium hydroxide and glacial acetic acid were purchased from Mallinckrodt (Phillipsburg, NJ, USA). Sodium periodate, sodium persulfate, 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octanol (PFO), bovine pancreas trypsin inhibitor (BPTI), melittin from honey bee venom, nisin from *Lactococcus lactis* and S-nitrosoglutathione were purchased from Sigma Aldrich (St. Louis, MO, USA). RAKGCKGR was synthesized by CHI Scientific (Maynard, MA, USA). The carbamidomethyl (cam)-modified BSA tryptic digest was purchased from ThermoFisher Scientific (Waltham, MA, USA). ARAMAKA and GRGMGRGMGRL were synthesized by Pepnome Limited (Shenzhen, China). RKRpSRAE was synthesized by AnaSpec (Fremont, CA, USA). All peptide and protein stock solutions for positive nanoelectrospray (nESI) were prepared in a 49.5/49.5/1 (vol/vol/vol) solution of methanol/water/acetic acid at an initial concentration of ~1 mg/ml and diluted 100-fold prior to use. The same solution was used for negative nESI, with ammonium hydroxide substituted for acetic acid. The solution of sodium periodate (50/50 vol/vol methanol/water) was prepared at a concentration of ~1 mg/ml and diluted 10-fold prior to use. Sulfate radical anion was observed upon negative nESI of an aqueous (~0.1 mg/ml) solution of sodium persulfate. PFO (~0.1 mg/ml) was sprayed from a 1% ammonium hydroxide methanol solution. S-nitroso-RAKGCKGR was prepared by combining 1 mg of RAKGCKGR with 1 mg of S-nitrosoglutathione in water for 1 h at 37 °C.

Mass spectrometry

All experiments were performed on either a QTRAP 4000 hybrid triple quadrupole/linear ion trap or a TripleTOF 5600 mass spectrometer (Sciex, Concord, ON, Canada), previously modified for ion/ion reactions.^[47] Analytes were generated via nESI and transmitted into Q3 (QTRAP 4000) or q2 (TripleTOF 5600) where they were subjected to MSⁿ. For ion/ion reactions, multiply protonated analytes and reagent anions were sequentially mass isolated in Q1 and injected into the q2 reaction cell via alternately pulsed nESI.^[48] The opposite polarity ions were allowed to react for 20–1000 ms. For experiments in the QTRAP 4000, the ion/ion reaction products were then transferred to Q3, where the complex was subjected to further characterization via MSⁿ and mass analysis using mass-selective axial ejection (MSAE).^[49] For experiments in the TripleTOF 5600, ions remained trapped in q2 for activation via ion trap CID. The experiments with BPTI and nisin were performed using the TripleTOF 5600 because of the higher resolution, mass accuracy and extended mass range needed for the larger systems.

Results and discussion

Several ways of generating dehydroalanine residues in solution and in the gas-phase via CID have been demonstrated. However, the effect of the Dha residue on the subsequent fragmentation of polypeptide ions has not been broadly investigated and is not widely appreciated. The presence of a Dha residue opens up a channel that leads to cleavage of the N—C α bond of the dehydroalanine residue^[39] to yield c- and z-type ions (see Scheme 1). We refer to this facile cleavage process as ‘the dehydroalanine effect’ in analogy with the term ‘proline effect’. Depending upon the relative facilities of other competing dissociation channels, the dehydroalanine effect can be prominent or even dominant (see below). Here we summarize a variety of scenarios in which dehydroalanine residues can arise in gas-phase polypeptide ions and describe their effect on subsequent backbone cleavage. In several scenarios, dehydroalanine residues can be generated upon CID of polypeptide ions generated directly via ESI. We also emphasize several approaches to modify polypeptide ions in the gas-phase so that they generate dehydroalanine residues upon CID.

Naturally occurring dehydroalanine residues

Nisin, a polypeptide with antibiotic activity produced by *L. lactis*, is comprised of several unusual structural elements^[50] including two Dha residues, a dehydrobutyrine (Dhb) residue, a lanthionine ring generated by a thioether linkage between two alanine residues, and four β -methyl-lanthionine rings generated by thioether linkages between alanine and 2-aminobutyric acid (Abu) residues (see schematic in the insert to Fig. 1). This molecule has previously been subjected to study with fast-atom bombardment tandem mass spectrometry,^[51] plasma desorption mass spectrometry^[52] and ESI Fourier transform ion cyclotron resonance tandem mass spectrometry using sustained off-resonance irradiation (SORI) CID.^[53] The latter and most recent study provides detailed mass assignments for all product ions observed. When we examined this system, we noted that our sample was 2 Da higher in mass than that of native nisin, which suggests that one of the unsaturated side-chains or one of the lanthionine/ β -methyl-lanthionine rings was reduced. Nevertheless, the tandem mass spectrometry of the ions illustrates the dehydroalanine effect. We also observed species that corresponded to the addition of one water molecule, as was also noted in the SORI-CID study. Figure 1 shows the ion trap CID product ion spectrum of the singly protonated and reduced nisin. This ion was generated from multiply protonated species formed directly upon nESI via ion/ion proton transfer to deprotonated perfluorooctanol (PFO) anions.^[54] The base peak in the product ion spectrum corresponds to cleavage N-terminal to the Dha-33 residue to yield the c₃₂ product. Peaks corresponding to cleavages because of the dehydroalanine effect are indicated in green. We note that c-ions generated from the dehydroalanine effect readily lose ammonia upon subsequent CID (see below) to yield what is nominally the corresponding b-ion (b₃₂, in this case). It is likely that much of the b₃₂ signal noted in Fig. 1 actually arose from sequential cleavage of the c₃₂ ion. Hence, a dual label (i.e. b₃₂/c₃₂-NH₃) is provided in the figure. Other prominent products include the z₃₀ ion from the dehydroalanine effect from Dha-5 and the z₃₃ ion from the analogous process of Scheme 1 for the Dhb-2 residue. Products from the dehydroalanine effect are also prominent in the ion trap CID product ion spectra of the more highly charged precursor ions

examined in the SORI-CID studies. The ion trap CID product ion spectrum derived from the $[M + 4H]^{4+}$ precursor is provided in Supplemental Figure S-1.

Asymmetric cleavage of disulfide bonds

Asymmetric cleavage of disulfide bonds, namely, cleavage of one of the two C—S bonds, generates dehydroalanine residues. Typically, CID of multiply-protonated peptides does not result in cleavage of the disulfide bond, instead generating extensive backbone fragmentation.^[55] However, collisional activation of disulfide-linked anions and proton mobility-limited cations have both been shown to undergo asymmetric cleavage of disulfide bonds.^[39–41] The fragmentation patterns of disulfide-containing anions have been discussed in detail previously, and the mechanism of asymmetric disulfide cleavage has been theoretically modeled in a review by Bilusich and Bowie.^[40] In the same review, the cleavage of N—C α bonds N-terminal to other amino acids (Ser, Thr, Cys, Asp, Asn, Glu, Gln and sometimes Phe) in the negative mode was observed.^[40,56] Gln and Lys residues in the second position of peptide chains have also been observed to generate dominant c_1 ions under low-energy CID of protonated peptides,^[57,58] and calculations have shown that Ser, His and Arg may also be capable of generating these ions.^[59,60] Additionally, Kim and Beauchamp demonstrated the highly selective ejection of H_2S_2 , which involves cleavages at both —C—S— bonds of the disulfide linkage and generates two dehydroalanine residues, upon CID of positively charged sodiated and calciated peptides in a technique they have termed the ‘Route 66 Method’.^[61]

Figure 2 illustrates the dehydroalanine effect in the ion trap CID of a multiply charged anion of an intramolecularly disulfide-linked protein: namely, the $[M - 4H]^{4-}$ anion of bovine pancreatic trypsin inhibitor (BPTI).^[39] BPTI is a ~6.5-kDa protein containing three stapled disulfide bonds (stapled meaning that the first disulfide bond contains the other two disulfide bonds and the two smaller disulfide bonds overlap in the sequestered area). Some loss of H_2S as well as loss of H_2S_2 from the precursor ion takes place but the large majority of product ions arise from backbone cleavages. The major backbone cleavages are dominated by the dehydroalanine effect, as reflected by the abundant c- and z-type ions. Because of the presence of multiple disulfide linkages that may undergo disulfide bond scission before the backbone cleaves, some of the c- and z-type ions can be formed with more or less sulfur atoms than if only one disulfide linkage were present. In any case, this anion provides a clear case in which the dehydroalanine effect is dominant. We note that under beam-type CID conditions, losses of H_2S_2 were favored over formation of c- and z-type ions,^[39] which suggests that the dissociation kinetics for the successive cleavage of a —C—S— bond followed by N—C α bond cleavage differ significantly from successive cleavages of the two —C—S— bonds of a given disulfide linkage, which leads to H_2S_2 loss.

Figure 3, which compares ion trap CID of the $[M + 6H]^{6+}$ and $[M + H]^+$ ions of BPTI, is illustrative of the situation that often prevails with protonated disulfide linked proteins. The $[M + 6H]^{6+}$ precursor ions were generated directly via nano-ESI whereas the singly protonated precursor was generated via ion/ion proton transfer to deprotonated PFO.^[54,62] Ion trap CID of the $[M + 6H]^{6+}$ charge state is dominated by products from amide bond cleavage from residues that fall outside of all of the disulfide linkages (e.g. the b_3^+ , y_{55}^{5+}

and y_{56}^{5+} ions). There is a very minor signal that corresponds to the c_4^+ ion, which reflects a minor contribution from the dehydroalanine effect following asymmetric cleavage of the disulfide bond between Cys-5 and Cys-55. Nevertheless, as is often the case with multiply protonated polypeptides, evidence for disulfide bond cleavage is either absent or very minor. The main peaks observed upon CID of the $[M + H]^+$ are losses of H_2S and H_2S_2 from the various disulfide-linked cysteine residues. The z_{54} ion generated by the dehydroalanine effect and subsequent losses of H_2S and H_2S_2 from the z_{54} ion are also observed. The z_{45} ion is also observed at a significantly lower abundance. The z_{54} ion is generated from the dehydroalanine effect via asymmetric cleavage of the disulfide bond containing the largest number of amino acids (Cys-5 to Cys-55) and is the predominant ion formed because the Cys-5 to Cys-55 disulfide bond must be cleaved to observe any fragments within that region, including those from dehydroalanine residues generated upon asymmetric cleavage at either of the other disulfide bonds (Cys-14 to Cys-38 and Cys-30 to Cys-51). The z_{45} -ion is formed when both the Cys-5 to Cys-55 and the Cys-14 to Cys-38 di-sulfide bonds are cleaved and a dehydroalanine residue has been generated at Cys-14. This result is consistent with observations made with other disulfide-linked proteins ions under conditions of limited proton mobility. These situations prevail for protein anions,^[39] sodiated/calciated cations^[61] and when the number of excess protons is less than the number of arginine residues.^[41] In these ion-types, asymmetric disulfide bond cleavage, which generates dehydroalanine, can be highly competitive. Subsequent activation of these newly generated dehydroalanine residues results in cleavage of the N-terminal N—Ca bond to generate c- and z-ions that can be used to identify and localize cysteine residues involved in disulfide bonds.

Elimination of sulfenic acids from oxidized S-alkyl cysteine residues

Alkylated cysteine residues are generated from various PTMs, for example farnesylation and geranylgeranylation, and upon reduction and alkylation of disulfide bonds. Thus, the localization of these residues is important for disulfide and PTM mapping applications. S-alkyl cysteine residues oxidized in solution have been shown to undergo selective ejection of the alkyl sulfenic acid, namely, HOSR, where R is an alkyl group, upon CID.^[42–44] The loss of the HOSR moiety results in generation of a dehydroalanine residue at the site of the original S-alkyl cysteine residue. The analogous gas-phase oxidation of S-alkyl cysteine residues has recently been demonstrated via ion/ion reactions with the periodate anion, IO_4^- , and the resulting oxidized species lose alkyl sulfenic acids upon CID to generate dehydroalanine.^[63] Periodate anion has also been shown to oxidize disulfide bonds^[64] as well as methionine and tryptophan residues^[65] upon gas-phase ion/ion reactions, with methionine and S-alkyl cysteine residues being the most efficiently oxidized. The generation of dehydroalanine residues in an S-alkyl cysteine-containing peptide via gas-phase ion/ion reactions with periodate anion is shown in Fig. 4.

Doubly protonated LFTFHADIC(cam)TLPDTEK, where 'cam' indicates an S-carbamidomethyl alkyl group (oxidized structure shown in Fig. 4(a)), was generated from a reduced and alkylated tryptic digest of BSA and was subjected to oxidation ion/ion reaction with IO_4^- . Activation of the $[M + H + O]^+$ species generated upon activation of the ion/ion peptide/periodate complex is shown in Fig. 4(a) and results in highly efficient ejection of the S-carbamidomethyl sulfenic acid, HOScam (107 Da), to generate the dehydroalanine residue

indicated by a red square. While this species was oxidized in the gas-phase via ion/ion reactions, $[M + H + O]^+$ species generated via solution- and gas-phase oxidation generate identical spectra upon activation,^[65] indicating that for this scenario, ion/ion reactions are not necessary to generate the Dha residue. Further activation of the dehydroalanine-containing protonated peptide (Fig. 4(b)) generates fragments along the peptide backbone, several of which are already observed in lower abundance upon CID of the $[M + H + O]^+$ species (Fig. 4(a)). Peptide fragments containing the dehydroalanine residue are indicated with a hollow square superscript. The most notable characteristic of the spectrum in Fig. 4(b) is the abundant formation of the c_8 ion resulting from cleavage of the N—C α bond N-terminal to the newly formed dehydroalanine residue. The peak 17 Da below the c_8 ion is indicated as a b_8 ion or a c_8 -NH₃ ion, with the latter assignment indicating the sequential nature of the mechanism for its formation. As CID of the c_8 ion pre-dominantly results in ammonia loss (data not shown), it is likely that the majority of this peak results from sequential loss of ammonia from the c_8 (viz. c_8 -NH₃). The peptide LFTFHADIC(cam)TLPDTEK also contains one proline residue and two aspartic acid residues, both of which can enhance cleavages adjacent to those residues upon CID. Fragments corresponding to cleavage N-terminal to proline residues are indicated in blue, while fragments corresponding to cleavage C-terminal to aspartic acid residues are indicated in red. While cleavages because of both the proline and aspartic acid effects are observed, they are in significantly lower abundance than the fragments generated because of the dehydroalanine effect for this system indicating that the dehydroalanine effect can compete with the proline and aspartic acid effects in at least some peptide/protein environments. The control spectrum showing CID of the unmodified $[M + H]^+$ species is shown in Supplemental Figure S-2 and is dominated by fragments derived from the aspartic acid and proline effects.

Loss of H₃PO₄ upon CID of phosphoserine-containing peptides

Collisional activation of phosphoserine-containing peptides frequently results in a dominant loss of phosphoric acid (H₃PO₄). A charge-remote β -elimination of phosphoric acid can generate a Dha residue. However, Palumbo, Tepe and Reid showed that phosphoric acid loss primarily occurs via a charge-directed S_N2 neighboring group participation reaction, which generates a cyclic oxazoline product. However, a minor fraction of the H₃PO₄ loss was determined to undergo the charge-remote reaction to generate the dehydroalanine product.^[66] The oxazoline product contains the N—C α bond of the original phosphoserine residue within a five-membered ring, making formation of the characteristic c- and z-ions unlikely. To look for evidence of the dehydroalanine effect in phosphoserine-containing peptides, the $[M + H]^+$ species of the peptide RKRpSRAE was subjected to CID; the resulting spectrum is shown in Fig. 5. As expected, the base peak corresponds to loss of phosphoric acid (Fig. 5(a)). Interestingly, c_3 and c_3 -NH₃ ions are also observed, indicating that at least a minor population of the ions may have generated dehydroalanine at the site of the phosphoserine residue upon CID. Further activation of the phosphoric acid loss yields a minor amount of the c_3 -ion, which is consistent with the finding of Palumbo, Tepe and Reid^[66] in that the majority of the H₃PO₄ loss likely generates the oxazoline product with only a minor population generating the dehydroalanine product. In Fig. 5, hollow squares are

used to indicate peptide fragments that have lost H_3PO_4 , which may either contain dehydroalanine or the oxazoline product.

Side-chain losses from radical peptide cations generated from (i) CID of S-nitroso peptides and (ii) ion/ion reactions with sulfate radical anion

Radical peptide cations undergo significantly different fragmentation pathways than their even-electron counterparts (e.g. protonated peptides) upon CID.^[67,68] For example, side-chain losses are commonly observed upon CID of odd-electron species whereas they are much less prominent upon CID of their even-electron analogs. The two main mechanisms by which side-chain loss occurs are abstraction of the α -hydrogen to generate dehydroalanine via loss of the radical side-chain and abstraction of the γ -hydrogen to generate a radical at the α -position via loss of the even-electron side-chain.^[69] In principle, several amino acids can generate a dehydroalanine residue via radical side-chain loss. Alanine, glycine and proline are the only amino acids that are unable to undergo this mechanism.^[69] However, valine and phenylalanine do not undergo significant side-chain losses by any mechanism and the generation of dehydroalanine from serine, threonine, tryptophan and tyrosine residues, while theoretically possible, is not observed.^[69] Nevertheless, 11 of the 20 common amino acids generate dehydroalanine upon activation of radical peptides. Several methods to generate radical cations have been developed, including CID of ternary metal–ligand–peptide complexes,^[70–76] photolysis of peptides with iodinated tyrosine residues^[77] or electrostatic complexes,^[69] and free radical-initiated reactions.^[78,79] Here, we demonstrate the generation of radical species from CID of S-nitroso peptides^[80,81] and ion/ion reactions with sulfate radical anion^[82] and the subsequent formation and dissociation of dehydroalanine-containing peptides.

The peptide S-nitroso RAKGCKGR was generated in solution by incubating 1 mg of S-nitrosoglutathione with 1 mg of the peptide in 1 ml of water for 1 h at 37 °C. The $[\text{M} + \text{H}]^+$ species of S-nitroso RAKGCKGR was isolated and dissociated via CID, and the resulting spectrum is shown in Fig. 6(a). As expected for S-nitroso peptides, the loss of $\text{NO}\bullet$ is the exclusive fragment observed. Further isolation and activation of the species generated by $\text{NO}\bullet$ loss are shown in Fig. 6(b) and results in several neutral side-chain losses as well as some backbone cleavages. The $\text{SH}\bullet$ loss generates a dehydroalanine in place of the cysteine residue, as indicated by a red square (Fig. 6(b)). The c_4 ion N-terminal to the original cysteine residue generated from the dehydroalanine effect is already observed. The $\text{C}_3\text{H}_8\text{N}_3\bullet$ loss from arginine also generates a dehydroalanine residue, although this pathway is observed at a minor abundance compared to the cysteine side-chain loss, likely because the radical was originally generated at the cysteine sulfur atom. The $\text{SH}\bullet$ loss was further isolated and activated, and the resulting spectrum is shown in Fig. 6(c). Several backbone fragments are observed, the most dominant of which is the c_4 ion from the dehydroalanine effect.

Several methods of generating radical cations via ion/ion reactions have been described,^[83] the most efficient method of which is ion/ion reactions with sulfate radical anion ($\text{SO}_4^{\bullet-}$).^[82] A proposed mechanism for the generation of a molecular radical cation and subsequent generation of dehydroalanine is outlined in Scheme 2. Upon formation of a stable ion/ion

complex between a doubly protonated peptide and sulfate radical anion, the sulfate radical anion abstracts a proton and a hydrogen atom from the peptide to yield the molecular radical cation, namely, $M^{+\bullet}$, via loss of H_2SO_4 . Subsequent radical rearrangement yields loss of a neutral radical side-chain to generate an even-electron, protonated, dehydroalanine-containing peptide. Doubly protonated ARAMAKA was subjected to ion/ion reactions with sulfate radical anion. Activation (both ion trap and beam-type collisional activation yielded essentially identical spectra) of the resulting ion/ion complex is shown in Fig. 7 and almost exclusively results in loss of H_2SO_4 to yield the molecular radical cation. Further activation of the $M^{+\bullet}$ predominantly results in loss of $C_2H_5S^\bullet$, which creates a dehydroalanine in place of the methionine residue, indicated with a red square in Fig. 7(b). One additional step of activation on the dehydroalanine-containing species is shown in Fig. 7(c) and results in backbone cleavages. The c_3 and b_6 ions are the dominant species formed, with the c_3 ion caused by the dehydroalanine effect. The b_6 ion is C-terminal to a lysine residue, which has been previously shown to undergo enhanced cleavages in certain environments.^[84]

Doubly protonated GRGMGRGMGRL was also subjected to ion/ion reactions with sulfate radical anion. As with ARAMAKA, activation of the ion/ion complex results in dominant formation of the molecular radical cation (data not shown). Collisional activation of the $M^{+\bullet}$ results in several side-chain losses along with some backbone cleavages (Fig. 8(a)). Of the neutral losses, the loss of $C_2H_5S^\bullet$ generates a dehydroalanine residue in place of one of the methionine residues (indicated by a red square with an 'M') while loss of $C_3H_8N_3^\bullet$ generates a dehydroalanine in place of one of the arginine residues (indicated by a red square with an 'R'). Further activation of the $C_2H_5S^\bullet$ loss is shown in Fig. 8(b) and predominantly yields the c_7 ion N-terminal to the second methionine residue, indicating that it has been converted to a dehydroalanine. The c_3 ion N-terminal to the first methionine residue is also observed, although at a significantly lesser abundance. Activation of the $C_3H_8N_3^\bullet$ loss is shown in Fig. 8(c) and yields various species from the dehydroalanine effect. The base peak in the spectrum corresponds to loss of $C(NH)_2$ (oftentimes observed from species containing neutral arginine residues) from the c_9 ion, which is also observed. Further CID of the c_9 - $C(NH)_2$ ion was performed to confirm the assignment and is shown in Supplemental Figure S-3. The c_5 ion is also observed at a significant abundance in Fig. 8(c). The c_5 ion corresponds to conversion of the second arginine to a dehydroalanine, while the c_9 ion corresponds to conversion of the third arginine to a dehydroalanine. The c_5 ion is isobaric with the y_4 ion so an additional step of CID was done to determine the identity of the peak (Supplemental Figure S-4). While evidence of both fragments is observed, the fragments derived from the c_5 ion (b_2 and b_4 and ammonia losses from each) are higher in abundance than the fragments derived from the y_4 ion (b_3 and $b_3 + H_2O$, assigned according to the sequence of the y_4 : MGRL), indicating that the c_5 ion generated from the dehydroalanine effect is a large contributor to the observed peak. The data of Fig. 8 illustrate a degree of selective control for polypeptide fragmentation via radical cation formation. For those residues that lose a radical side-chain to generate a dehydroalanine at the site of side-chain loss, selective backbone cleavage at those sites will likely occur upon subsequent fragmentation because of the dehydroalanine effect. The mass of the side-chain can be selected in an MS^3 experiment to enable a residue specific approach for inducing selective cleavages.

The concept of generating dehydroalanine residues for inducing selective cleavages in polypeptide ions is illustrated for a case with mixed cation sites (i.e. radical cation site and an excess proton). In such a case, radical-directed cleavages must compete with charge-directed cleavages, as discussed previously by Laskin *et al.*^[85] Melittin was subjected to nESI, and the $[M + 3H]^{3+}$ species was subjected to gas-phase ion/ion reactions with sulfate radical anion to generate the $[M + 3H + SO_4]^{2+\bullet}$ complex (m/z 1472.5). Gentle CID of the complex resulted in efficient and exclusive loss of H_2SO_4 to yield the $[M + H]^{2+\bullet}$ ion (m/z 1423.5). Activation of the $[M + H]^{2+\bullet}$ yielded a group of relatively abundant side-chain losses (Supplemental Figure S-5), the most dominant of which was the loss of $C_3H_7\bullet$ from the leucine residues (m/z 1402). Figure 9(a) shows the results from ion trap CID of the $[M + H-C_3H_7\bullet]^{2+}$, and Fig. 9(b) shows the ion trap CID of the $[M + 2H]^{2+}$ ion for comparison. The dehydroalanine effect clearly plays a major role in the observed backbone cleavages in Fig. 9(a) as reflected by the appearance of the $z_{21}^{2+\square}$ ion (Leu-6), the $z_{18}^{2+\square}$, $z_{18}^{+\square}$, c_8^+ and $c_8^+ - NH_3$ ions (Leu-9), the $z_{14}^{+\square}$ ion (Leu-13) and the $z_{11}^{+\square}$ and c_{15}^+ ions (Leu-16). No evidence for formation of z- or c-ions is apparent in the ion trap CID spectrum of the $[M + 2H]^{2+}$ ion. It is also noteworthy that no c- or z-ions from sites other than leucine residues, including those of the three isoleucine residues, appear in Fig. 9(a). In a radical cation, isoleucine is expected to lose $C_2H_5\bullet$ (29 Da) to generate a Dhb residue. Based on the nisin result described above, Dhb also appears to undergo the analogous process to yield c- and z-ions. This preliminary result for melittin points to the interesting possibility for developing a strategy for generating residue specific fragmentation in the structural characterization of polypeptide ions.

Conclusions

Dehydroalanine is a relatively rare amino acid in biology but can be encountered in a variety of scenarios in mass spectrometry, as illustrated here. They arise, for example, from asymmetric cleavage of disulfide bonds, loss of alkyl sulfenic acid from oxidized alkylated cysteine residues and loss of radical side-chains from radical cations. There is also evidence for dehydroalanine formation from phosphoric acid loss from phosphopeptides as a minor channel. Low energy collisional activation of a dehydroalanine-containing polypeptide ion results in enhanced cleavage of the N—C α bond of the dehydroalanine residue to yield N-terminal c- and z-ions. Such cleavages can be the dominant fragmentation processes observed upon ion trap CID, particularly in cases of relatively low proton mobility (e.g. anions and cations with less protons than the number of arginine residues). The c- and z-ions can be used to localize certain modifications (i.e. S-alkyl cysteines, disulfide-linked cysteines, phosphoserines, etc.) or various amino acids that generate dehydroalanine upon neutral side-chain losses from molecular radical cations. The latter observation is particularly significant in that polypeptide radical cations can be generated readily from multiply protonated polypeptides via gas-phase ion/ion reactions with the sulfate radical anion in an ion trap. By interrogating the ions generated by side-chain losses from amino acids that lose a radical species under conditions that favor the dehydroalanine effect, it may be possible to cleave polypeptide ions with the specificity normally associated with enzymes in solution. An illustration with melittin is provided here. This possibility will be further explored and developed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ion trap CID of the $[M + H]^+$ ion of a reduced (+2 Da) form of nisin. The singly protonated species was generated from multiply protonated ions via gas-phase proton transfer reactions with PFO anions. The lightning bolt indicates the species directly subjected to resonance excitation. Degree signs indicate water losses, and asterisks indicate ammonia losses. A peak corresponding to the attachment of a water molecule to the nisin was present in the original spectrum and after the final isolation step. This peak and the product ions generated from off-resonance power absorption of $[M + H + H_2O]^+$ were labeled with Δ signs.

Figure 2.
Ion trap collision-induced dissociation of the $[M - 4H]^{4-}$ anion of bovine pancreatic trypsin inhibitor.

Figure 3. Ion trap CID product ion spectra of the (a) $[M + 6H]^{6+}$ ion and (b) $[M + H]^+$ ion of bovine pancreatic trypsin inhibitor.

Figure 4.

CID of (a) the LFTFHADIC(cam)TLPDTEK $[M + H + O]^+$ species generated via gas-phase oxidation with the periodate anion, and (b) the dehydroalanine species generated in (a). Degree signs indicate water losses, asterisks indicate ammonia losses and lightning bolts indicate species subjected to CID. Red squares indicate formation of the dehydroalanine product while hollow square superscripts indicate fragment ions that contain a dehydroalanine residue. Green is used to indicate fragments generated from the dehydroalanine effect. Red and blue are used to indicate fragments generated from aspartic acid and proline effects, respectively.

Figure 5. CID of (a) RKRpSRAE $[M + H]^+$, and (b) the H_3PO_4 loss from (a). Degree signs indicate water losses, asterisks indicate ammonia losses and lightning bolts indicate species subjected to CID. Green is used to indicate fragments generated from the dehydroalanine effect. Hollow squares indicate fragments that have lost H_3PO_4 .

Figure 6.

CID of (a) S-nitroso RAKGCKGR $[M + H]^+$, (b) the NO• loss species and (c) the loss of SH• from (b). Degree signs indicate water losses, and lightning bolts indicate species subjected to CID. Red squares indicate formation of the dehydroalanine product, while hollow square superscripts indicate fragment ions that contain a dehydroalanine residue. Green is used to indicate fragments generated from the dehydroalanine effect.

Figure 7.

CID of (a) the ion/ion complex between doubly protonated ARAMAKA and sulfate radical anion, (b) the molecular radical cation generated in (a), and (c) the $\text{C}_2\text{H}_5\text{S}^\bullet$ loss from (b) which generates a dehydroalanine from the methionine residue. Degree signs indicate water losses, asterisks indicate ammonia losses and lightning bolts indicate species subjected to CID. Red squares indicate formation of the dehydroalanine product, while hollow square superscripts indicate fragment ions that contain a dehydroalanine residue. Green is used to indicate fragments generated from the dehydroalanine effect.

Figure 8.

CID of (a) the molecular radical cation of the peptide GRGMGRGMGRL generated via ion/ion reaction with sulfate radical anion, (b) the $C_2H_5S^{\bullet}$ loss from (a) which generates a dehydroalanine residue from one of the methionine residues and (c) the $C_3H_8N_3^{\bullet}$ loss from (a) which generates a dehydroalanine residue from one of the arginine residues. Degree signs indicate water losses, asterisks indicate ammonia losses and lightning bolts indicate species subjected to CID. Red squares indicate formation of the dehydroalanine product, with the letter inside indicating the amino acid that has been transformed into dehydroalanine. Hollow square superscripts indicate fragment ions that contain a dehydroalanine residue. Green is used to indicate fragments generated from the dehydroalanine effect.

Figure 9.

Ion trap CID of (a) the $[M + H-C_3H_7\bullet]^{2+}$ and (b) the $[M + 2H]^{2+}$ ions derived from melittin. Degree signs indicate water losses, asterisks indicate ammonia losses and lightning bolts indicate species subjected to CID. Red squares indicate formation of the dehydroalanine product, with the letter inside indicating the amino acid that has been transformed into dehydroalanine. Hollow square superscripts indicate fragment ions that contain a dehydroalanine residue. Green is used to indicate fragments generated from the dehydroalanine effect.

Scheme 1.

Proposed mechanism for generation of c- and z-ions N-terminal to dehydroalanine residues upon CID.^[39]

Scheme 2.

Proposed mechanism for the ion/ion reaction between sulfate radical anion and a doubly protonated peptide and subsequent loss of the neutral radical side-chain to generate dehydroalanine.