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Gas-Phase Oxidation via Ion/Ion Reactions: Pathways and Applications

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Abstract

Here, we provide an overview of pathways available upon the gas-phase oxidation of peptides and DNA via ion/ion reactions and explore potential applications of these chemistries. The oxidation of thioethers (i.e., methionine residues and S-alkyl cysteine residues), disulfide bonds, S-nitrosylated cysteine residues, and DNA to the $[M+H+O]^+$ derivative via ion/ion reactions with periodate and peroxymonosulfate anions is demonstrated. The oxidation of neutral basic sites to various oxidized structures, including the $[M+H+O]^+$, $[M-H]^+$, and $[M-H-NH_3]^+$ species, via ion/ion reactions is illustrated and the oxidation characteristics of two different oxidizing reagents, periodate and persulfate anions, are compared. Lastly, the highly efficient generation of molecular radical cations via ion/ion reactions with sulfate radical anion is summarized. Activation of the newly generated molecular radical peptide cations results in losses of various neutral side chains, several of which generate dehydroalanine residues that can be used to localize the amino acid from which the dehydroalanine was generated. The chemistries presented herein result in a diverse range of structures that can be used for a variety of applications including the identification and localization of S-alkyl cysteine residues, the oxidative cleavage of disulfide bonds, and the generation of molecular radical cations from even-electron doubly protonated peptides.

Graphical Abstract

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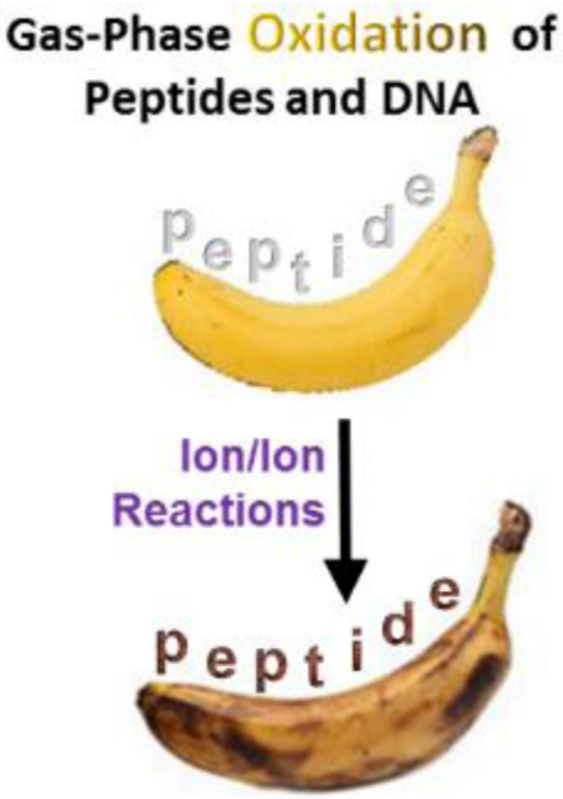

Keywords

Ion/ion reaction; oxidation; tandem mass spectrometry; radical cations

INTRODUCTION

The oxidation of peptides and proteins is a ubiquitous event that has been implicated in both normal and damaging cellular processes [1]. For example, protein oxidation has been implicated in signaling [2,3], gene regulation [4,5], aging [6,7], cancer [8,9], diabetes [10,11], atherosclerosis [12,13], and Parkinson's [14] and Alzheimer's diseases [15,16]. The oxidation of proteins results from interactions with reactive oxygen and nitrogen species that are either present natively or are generated as byproducts of oxygen metabolism [1,17]. Oxidative modifications can appear in a wide variety of forms ranging from the addition of an oxygen atom on amino acid side chains to protein cross-linking [1]. For example, proline, arginine, and lysine side chains can be oxidized to carbonyl derivatives [7,18], methionine is commonly oxidized to methionine sulfoxide, and less commonly to methionine sulfone [19], and cysteine oxidation can lead to intra- or inter-protein crosslinks [20,21,22]. Oxidative stress in red blood cells has been shown to induce hemoglobin crosslinking to the cytoskeleton, resulting in association of the heme center with the phospholipid membrane and allowing it to act as a local Fenton reagent [23]. Moreover, reactive oxygen and nitrogen species can also interact with and oxidize other bioanalytes, such as DNA, sugars, and lipids

[1,24,25,26]. For these and other reasons it is important to study the oxidation of bioanalytes.

Tandem mass spectrometry (MS^n) has been used for the analysis of various modifications [27,28,29] of peptides and proteins and is the most informative method currently used in the analysis of oxidative modifications [30]. As most types of oxidation result in a change in the overall mass of the peptide or protein of interest, MS is uniquely equipped to detect these changes and has been used to detect methionine oxidation [31], analyze glutathionylated hemoglobin [32], and determine the total load of oxidative modifications on a protein [33]. Mass spectrometric analysis of oxidation has furthermore been applied in the study of several diseases including Alzheimer's disease [16], diabetes [34], breast cancer [35], and influenza [36]. Some oxidative modifications can alter the fragmentation of peptides and proteins upon collision-induced dissociation (CID). For example, oxidation of the methionine side chain to the sulfoxide derivative results in highly efficient ejection of methanesulfenic acid (64 Da) [37,38,39,40,41], and cysteine residues oxidized to form intra- or intermolecular disulfide bonds can inhibit sequence information obtained for the regions protected within the disulfide linkage [42,43,44].

Recently, the oxidation of multiply protonated polypeptide cations to various forms, including $[M+H+O]^+$, $[M-H]^+$, and $M^{+\bullet}$ species, in the gas-phase via ion/ion reactions (i.e., the interaction of oppositely charged ions within a mass spectrometer) has been demonstrated [45,46]. Ion/ion reactions have several benefits over traditional solution-phase techniques for generating oxidized species including, for example, much faster reaction times, reduction of side-reactions via mass isolation of both reactants, and control over the extent of modification (i.e., the number of modifications that occur) [47]. Periodate anion (IO_4^-) selectively oxidizes non-modified peptides containing methionine residues, and to a lesser extent, tryptophan residues, to the oxygen transfer species $[M+H+O]^+$ [45]. Methionine-containing peptides then undergo efficient loss of 64 Da, corresponding to ejection of methanesulfenic acid, upon collision-induced dissociation (CID) of the $[M+H+O]^+$ species, in agreement with activation of methionine sulfoxide-containing peptides generated in solution [37–41,45]. Periodate anion is also capable of oxidizing neutral lysine and arginine residues, predominantly to the hydrogen deficient species $[M-H]^+$, in systems lacking other easily oxidized residues [48]. A suite of reagents generated upon negative nanoelectrospray ionization (nESI) of an aqueous solution of sodium persulfate, i.e., persulfate anion ($HS_2O_8^-$), peroxymonosulfate anion (HSO_5^-), and sulfate radical anion ($SO_4^{-\bullet}$), have also been shown to be oxidizing reagent anions for ion/ion reactions [46]. Peroxymonosulfate anion also generates oxygen transfer species upon activation of peroxymonosulfate-peptide complexes, and is a stronger oxidizing reagent anion than periodate as species both containing and lacking methionine/tryptophan residues undergo oxidation. Persulfate anion generates both oxygen transfer and hydrogen deficient species upon activation of the persulfate-peptide complex. Sulfate radical anion abstracts both a proton and a hydrogen atom upon activation of the ion/ion complex to generate a molecular radical cation.

Here, we summarize and illustrate the various chemistries that occur upon oxidation ion/ion reactions and present novel examples of the applications for which these chemistries can be

used. The oxygen transfer chemistry is demonstrated here using clusters of periodate anions (i.e., $H(IO_4)_2^-$), which results in oxidation of two methionine residues in the same peptide in a single ion/ion reaction step. Furthermore, the oxidation of maleimide-protected cysteine residues to the $[M+H+O]^+$ derivative is shown for the first time. The oxidation of a S-nitroso peptide is also demonstrated, though the NO• loss is too facile to detect the intact $[M+H+O]^+$ species. Rather, the $[M+H+O-NO•]^{+•}$ species is observed and undergoes radical-directed cleavages that confirm that the oxidation occurs on the S-nitroso cysteine sulfur atom. The selective oxidation of guanine nucleobases in a doubly protonated DNA oligomer is demonstrated here for the first time. The oxidation of neutral basic sites to $[M+H+O]^+$, $[M-H]^+$, and $[M-H-NH_3]^+$ species is demonstrated and the reactivities of periodate and persulfate anions are compared. Lastly, the generation of radical cations via ion/ion reactions with sulfate radical anion is briefly discussed. The oxidation chemistry described here results in the generation of a wide variety of species that can undergo fragmentation pathways dramatically distinct from their non-oxidized counterparts, thereby enabling strategies to facilitate the structural characterization of molecules with readily oxidizable sites.

EXPERIMENTAL SECTION

Materials

Methanol and glacial acetic acid were purchased from Mallinckrodt (Phillipsburg, NJ). ARAMAKA, ARACAKA, HAHAHAA, and GRGMGRGMGRL were synthesized by Pepnome Limited (Shenzhen, China). KAKAKAA was synthesized by NeoBioSci (Cambridge, MA) and RARARAA was synthesized by CHI Scientific (Maynard, MA). KGAILCGIALK was synthesized by CPC Scientific (Sunnyvale, CA, USA). Sodium persulfate, sodium periodate, insulin, pepsin, S-nitrosoglutathione, and N-hydroxymaleimide were purchased from Sigma Aldrich (St. Louis, MO). ACCGAG was purchased from Integrated DNA Technologies (Coraville, IA, USA). All peptide and DNA stock solutions for positive nanoelectrospray 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 periodate solution was prepared in a 50/50 (vol/vol) solution of methanol/water at a concentration of ~1 mg/mL and diluted 10-fold prior to use. The proton bound dimer of periodate was observed upon negative nESI of a solution of sodium periodate in 50/50 vol/vol water/acetic acid. The persulfate solution was prepared in an aqueous solution at a concentration of ~1 mg/mL and diluted 10-fold prior to use.

Mass Spectrometry

All experiments were performed on a QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Concord, ON, Canada), previously modified for ion/ion reactions [49]. Multiply protonated peptides and singly charged anion reagent populations were sequentially injected into the instrument via alternately pulsed nano-electrospray (nESI) [50]. The peptide cations and oxidizing reagent anions were independently isolated in the Q1-mass filter prior to injection into the q2 reaction cell. Intact persulfate and sulfate radical anion were observed upon negative nESI of an aqueous solution of sodium persulfate. Peroxymonosulfate anion was also observed at a minor abundance upon negative

nESI of the sodium persulfate solution. However, a large nozzle-skimmer voltage difference increased the abundance of this ion species as it is a fragment generated from intact persulfate anion [46]. The ions of opposite polarity were allowed to react for a mutual storage reaction time of 20–1000 ms. Reactions with sulfate radical anion were optimized for time as the radical anion is extremely reactive and can react with neutrals in the trap. Mutual storage times for reactions with sulfate radical anion ranged from 20 – 200 ms. The ion/ion reaction products were then transferred to Q3, where the product ions were subjected to further characterization via MS^n and mass analysis using mass-selective axial ejection (MSAE) [51].

Syntheses

S-nitrosylated KGAILCGIALK was synthesized by combining 1 mg peptide with 1 mg S-nitrosoglutathione in 1 mL of water at 37 °C for 1 hr. The S-nitrosylation mixture was then diluted 100 fold in 49.5/49.5/1 vol/vol/vol methanol/water/acetic acid for electrospray. The peptic digest was performed by combining 0.1 mg insulin with 0.025 mg pepsin in 1% acetic acid at 37 °C for 6 hr [52]. The digestion mixture was diluted to 1–5 μM in 49/49/2 vol/vol/vol methanol/water/acetic acid and used without further purification. For the maleimide protection 0.1 mg N-hydroxymaleimide was added to a 1 mg/mL solution of the peptide ARACAKA in methanol. The solution was shaken at room temperature for 10 min, reconstituted in 49.5/49.5/1 vol/vol/vol methanol/water/acetic acid, and sprayed via positive nESI without any further purification.

RESULTS AND DISCUSSION

Generation of $[M+H+O]^+$ Species: Thioethers

The first demonstration of the gas-phase oxidation of peptides via ion/ion reactions involved methionine-containing peptides [45]. Doubly protonated peptides were subjected to ion/ion reactions with periodate anion. Upon reaction, all peptides underwent complex formation with periodate anion to generate the ion/ion complex $[M+2H+IO_4]^+$. However, only peptides containing methionine, and to a much lesser extent, tryptophan residues, generated an oxidized $[M+H+O]^+$ species upon activation of the ion/ion complex. All other peptides examined underwent loss of periodic acid (HIO_4) upon activation of the ion/ion complex to generate the charge-reduced $[M+H]^+$ species. Activation of the $[M+H+O]^+$ species generated in the gas-phase was compared to the $[M+H+O]^+$ species generated via solution-phase oxidation and the resulting spectra were identical suggesting that these two species were of the same primary structure. It was then found that the gas-phase ion/ion oxidation of thioethers was a general phenomenon as S-alkyl cysteine residues were also shown to undergo this oxygen transfer chemistry [53]. This was demonstrated with S-ethyl, S-farnesyl, and S-carbamidomethyl cysteine residues upon ion/ion reactions between doubly protonated peptides and both periodate and peroxymonosulfate anions. Furthermore, thioethers oxidized to the sulfoxide form undergo a facile rearrangement that leads to the loss of alkyl sulfenic acids (RSOH) [37–41,54]. For methionine residues this results in a loss of 64 Da corresponding to the ejection of methanesulfenic acid, a pathway that is well documented by groups investigating the solution-phase oxidation of methionine residues [37–41]. Interestingly, the loss of alkyl sulfenic acids from oxidized S-alkyl cysteine

residues results in the generation of a dehydroalanine residue (Dha) at the site of the original modified residue [53,54,55,56]. This is especially useful as Dha residues open a low energy pathway that leads to cleavage of the N-C_α bond of the Dha residue to generate c- and z-ions upon CID [56,57,58]. As c- and z-ions are not generally observed upon CID of protonated even-electron species, these ions can be used to identify the presence of Dha and localize the original S-alkyl cysteine within the peptide.

While we have reported on the oxidation of cysteine residues alkylated with common protecting agents (e.g., iodoacetamide and alkyl halides) [53], maleimide chemistry is another common method of protecting cysteine residues, the oxidation behavior of which is related here. The peptide ARACAKA was reacted with N-hydroxymaleimide to generate ARAC^{m}AKA (superscript m indicates that the cysteine has been protected with N-hydroxymaleimide) and the doubly protonated species was subjected to ion/ion reactions with an oxidizing reagent anion (Supplemental Figure S-1). Periodate anion (IO_4^-) and peroxymonosulfate anion (HSO_5^-) both generate the oxygen transfer $[M+H+O]^+$ species upon activation of ion/ion complexes with peptides containing thioethers, and are largely interchangeable for the examples shown here. A variety of examples using both oxidizing reagent anions are shown to demonstrate the versatility of the chemistry described herein. Activation of the ion/ion complex between doubly protonated ARAC^{m}AKA and HSO_5^- is shown in Figure 1(a) and predominantly generates the oxygen transfer species $[M+H+O]^+$. The structure of the oxidized species is shown in the insets of Figures 1(a) and (b). The $[M+H+O]^+$ species was then isolated and subjected to CID and the resulting spectrum is shown in Figure 1(b). Similar to other S-alkyl cysteine residues, oxidized ARAC^{m}AKA loses the maleimide protecting group as an alkyl sulfenic acid to generate a dehydroalanine-containing peptide (indicated by a red square, -RSOH in Figure 1). The mechanism for the loss of alkyl sulfenic acids upon CID of sulfoxide derivatives has been discussed previously [53,54,56]. Unique to this protecting group, however, is the dominant nominal loss of the maleimide group and a water (indicated by a blue square, -($R+H_2O$) in Figure 1). This loss occurs via abstraction of the β-hydrogen atom on the maleimide group rather than the peptide backbone and has been previously observed and discussed in detail for maleimide-protected cysteine residues oxidized in solution [59]. Activation of the -($R+H_2O$) species is shown in Figure 1(c) and generates a variety of b-ions, including the b_2, $b_4^{\blacksquare}$, and $b_6^{\blacksquare}$ species. The blue square superscript indicates fragments that have lost the ($R+H_2O$) group and can be used to localize the modification. Activation the dehydroalanine-containing species generated by alkyl sulfenic acid loss is shown in Figure 1(d) and generates an abundant c_3 ion in addition to the dominant $b_6^{\blacksquare}$ ion (red square superscripts indicate fragments containing a Dha residue whereas peaks shown in green are derived from the dehydroalanine effect). The peak 17 Da below the c_3 ion could either correspond to the b_3 ion or a sequential ammonia loss from the c_3 ion. Previous results have shown that c-ions generated via the dehydroalanine effect oftentimes readily lose ammonia indicating that this fragment is likely sequential in nature and is also derived from the dehydroalanine effect [53,55]. Since the dehydroalanine effect generates c- and z-ions N-terminal to the location of the original S-alkyl cysteine residue, this ion indicates that the modified cysteine residue is the fourth residue in the peptide, which could be useful in *de novo* sequencing applications. The control spectrum corresponding to CID of the protonated species is shown in

Supplemental Figure S-2 and predominantly yields the b_6 ion. Some loss of the protecting group is observed along with loss of the protecting group as a thiol to generate the dehydroalanine-containing species. A minor c_3 ion derived from sequential fragmentation of the Dha-containing species is also observed.

Generation of $[M+H+O]^+$ Species: Disulfide bonds

Similar to thioethers, disulfide bonds were also found to be susceptible to oxidation via ion/ion reactions [60]. Activation of the ion/ion complexes between intermolecularly disulfide-linked peptides and oxidizing reagent anions generates a combination of species resulting from (i) proton transfer from the peptide dication to the reagent anion to yield the charge-reduced $[M+H]^+$ species, (ii) oxygen transfer from the reagent to the peptide to generate the oxidized thiosulfinate $[M+H+O]^+$ derivative, and (iii) species corresponding to oxidative cleavage of the disulfide bond, likely due to secondary fragmentation of the fragile $[M+H+O]^+$ species. While disulfide bonds are important for the structure and function of proteins, their presence can complicate MS^n analysis, as CID of protonated peptides does not typically result in cleavage of the disulfide bonds [42]. Solution-phase oxidation is commonly done to cleave disulfide bonds, but it results in oxidation of the linked cysteine residues all the way to the cysteic acid derivative and may result in oxidation of other sites on the peptide/protein of interest [61].

The gas-phase oxidation approach described here allows greater control over the extent of oxidation and enables the addition of exactly one oxygen atom and the cleavage of one disulfide bond. More oxygen atoms can be added via sequential ion/ion reactions with multiply charged species if needed. Other methods which selectively cleave disulfide bonds in the gas-phase, including irradiation at 266 nm [62], ion/ion reactions with transition metal-containing species [63,64,65], CID of peptides/proteins with limited proton mobility [66], and electron capture [67], electron transfer [68], and electron detachment dissociation [69], have also been described. The oxidation chemistry described here has the benefit of labeling disulfide bonds via the addition of an oxygen atom, which has been used to identify peaks of interest derived from enzymatic digestion mixtures of lysozyme and insulin without isolation or prior chromatographic separation [60]. All ions resulting from positive nESI of the digestion mixtures were subjected to ion/ion reaction experiments with an oxidizing reagent anion (IO_4^-) followed by a short period of dipolar DC excitation, a broad-band activation technique, to fragment the resulting ion/ion complexes. This experiment was then repeated using a proton transfer reagent anion (IO_3^-). The resulting proton transfer spectrum (generated via reaction with IO_3^-) was subtracted from the resulting oxidation spectrum (generated via reaction with IO_4^-), leaving only species that had undergone oxidation and resulted in a mass shift. This labeling procedure eliminates the need for reduction and alkylation in traditional disulfide-mapping experiments as well as pre-MS chromatographic separations, which are time-consuming.

The peptide NYCN/LVCGERGFF (slashes indicate two distinct peptides connected via intermolecular disulfide bond, the peptide on the left is referred to herein as ‘A’ while the peptide on the right is referred to as ‘B’) was generated from a peptic digest of insulin, a peptide hormone consisting of one intramolecular and two intermolecular disulfide bonds.

Activation of the protonated peptide is shown in Supplemental Figure S-3 and no fragments corresponding to cleavage of the disulfide bond are observed. Doubly protonated NYCN/LVCGERGFF was subjected to ion/ion reactions with IO_4^- and the resulting ion/ion complex was subjected to CID (Figure 2(a)). Generation of the proton transfer product, the oxygen transfer $[M+H+O]^+$ species, and fragments corresponding to oxidative cleavage of the disulfide bond are observed. Isolation and activation of the $[M+H+O]^+$ species is shown in Figure 2(b) and almost exclusively results in the generation of fragments corresponding to the oxidative cleavage of the disulfide bond (indicated in blue). The dominant peak observed is the $[B-H]^+$ species, indicating that the two chains have separated and the terminal carbon and sulfur atoms in the cysteine residue are connected by a double bond (similar in structure to the maleimide-protected cysteine after the loss of $R+H_2O$). The cluster of peaks centered around the $[B-H]^+$ ion (i.e., the $[B+H+O]^+$, $[B+H+S+O]^+$, and $[B-H-S]^+$ ions) are a signature pattern for the oxidative cleavage of intermolecular disulfide bonds and can be used to indicate one of the two originally disulfide-linked peptides [60]. This cluster of peaks is derived from abstraction of various hydrogen atoms by the oxygen or sulfur atoms as discussed previously [60]. There are also some smaller fragment ions corresponding to backbone cleavages along the B-chain (viz., By_4, By_6, Bb_6-H, and By_7-H). The Bb_6-H and By_7-H fragments are both secondary fragments derived from sequential cleavage of the disulfide bond and the peptide backbone. The By_6 and By_7-H ions localize the cysteine residue within the peptide chain. No fragments derived from the A-chain are observed, likely due to the lesser proton affinity of the A-chain as compared to the B-chain. The oxidative cleavage of disulfide bonds is useful in the analysis of peptides, proteins, and post-translational modifications, such as S-glutathionylation and S-cysteinylation, in which low molecular weight thiols are disulfide-linked to peptides/proteins [60].

Generation of $[M+H+O]^+$ Species: S-Nitroso Peptides

In addition to alkylation and the formation of disulfide bonds, the nitrosylation of sulfur atoms in cysteine residue is a common post-translational modification [70]. Activation of these S-nitroso compounds predominantly results in loss of NO• to generate a radical peptide cation [71]. As the modification is immediately lost upon CID it can be difficult to determine the site of original modification. S-nitroso cysteine residues are also susceptible to oxidation upon ion/ion reactions with oxidizing reagent anions, as is shown in Figure 3 for the reaction between doubly protonated S-NO KGAILCGIALK and IO_4^-. Activation of the ion/ion complex $[M+2H+IO_4]^+$ is shown in Figure 3(a) and generates a variety of peaks, the most prominent of which is the sequential loss of NO• from the oxidized species, i.e., $[M+H+O\text{-}NO•]^{+•}$. Interestingly, no intact $[M+H+O]^+$ is observed; presumably due to the facile nature of NO• loss. Furthermore, the proton transfer charge-reduced $[M+H]^+$ species is observed, along with the sequential NO• loss. Activation of the $[M+H+O\text{-}NO•]^{+•}$ species is shown in Figure 3(b) and results in a variety of neutral side chain losses and peptide backbone fragments. This spectrum is very similar to the control, viz., CID of the $[M+H\text{-}NO•]^{+•}$, shown in Figure 3(d), with the exception of the losses of SOH• and CH_2SOH• instead of SH• and CH_2SH• and the presence of the $y_{10}+O$ as opposed to the y_{10}. These peaks indicate that the oxidation has occurred on the sulfur atom of the original S-NO cysteine residue. An additional step of activation on the SOH• loss, which generates a Dha selectively at the site of the original S-nitrosylated cysteine, was done to localize the site of

the S-nitrosylation (Figure 3(c)). The c_5 ion is derived from the dehydroalanine effect and is the most dominant fragment observed in Figure 3(c). Since unmodified cysteine residues do not undergo oxidation the SOH• loss only occurs from, and selectively generates dehydroalanine at, modified (i.e., S-nitrosylated) cysteine residues. The enhanced generation of c- and z-ions N-terminal to Dha residues makes localization of the S-nitrosylation within the peptide straightforward.

Generation of $[M+H+O]^+$ Species Using Clusters

All gas-phase ion/ion oxidation experiments described to date have used a single oxidizing reagent anion to oxidize a peptide once. While sequential ion/ion reactions can be performed to add multiple oxidizing reagent anions and thus add multiple oxygen atoms to a peptide, this requires the use of a more highly charged peptide precursor to avoid neutralization, which is not always practical. One way to avoid this is the use of reagent clusters, as has been previously shown for the modification of multiple amine sites in peptides [72]. Here, we show the ion/ion reaction between a doubly protonated peptide containing two methionine residues, GRGMGRGMGRL, and a proton bound dimer of two periodate anions, $H(IO_4)_2^-$. Activation of the ion/ion complex, $[M+2H+H(IO_4)_2]^+$ is shown in Figure 4(a) and results in two dominant sequential losses of HIO_3 to generate $[M+2H+O+IO_4]^+$ and $[M+H+2(O)]^+$, respectively. Activation of the $[M+H+2(O)]^+$ species is shown in Figure 4(b) and predominantly results in loss of 64 Da corresponding to the ejection of methane sulfenic acid, as discussed previously (fragments that have lost one methanesulfenic acid moiety are denoted with a hollow square superscript). Further activation of the 64 Da loss from the $[M+H+2(O)]^+$ species is shown in Figure 4(c) and results in another dominant loss of 64 Da corresponding to ejection of another methanesulfenic acid (fragments that have lost two methanesulfenic acid moieties are indicated with a solid square superscript in Figure 4) from the second methionine residue, indicating that both methionine residue side chains have undergone oxidation. There is also a variety of backbone fragments in Figure 4 (b) and (c) that can be used to localize the oxidation sites to the two methionine side chains. The triply charged precursor of this peptide has previously been subjected to sequential ion/ion reactions with IO_4^- to generate the doubly adducted $[M+3H+2IO_4]^+$ species [45]. This species reacts almost identically to the one generated here using reagent cluster anions, however, it required a more highly charged precursor and multiple reagent anion adduction steps. The use of cluster reagent anions is a useful way to oxidize a peptide multiple times while minimizing the 'charge tax' associated with ion/ion reactions.

Generation of $[M+H+O]^+$ Species: DNA

All ion/ion oxidation experiments described to this point involved doubly protonated peptides and oxidizing reagent anions. Here, we show the oxidation of another type of bioanalyte of interest, DNA, via ion/ion reactions. The doubly protonated oligonucleotide ACCGAG was subjected to ion/ion reactions with HSO_5^-. The resulting ion/ion complex was subjected to CID and generated the spectrum shown in Figure 5(a). Activation of the $[M+2H+HSO_5]^+$ species resulted in proton transfer, oxygen transfer, and some secondary fragments, likely due to energetic transfer conditions between q2 and Q3. Further isolation and activation of the oxygen transfer species resulted in the spectrum shown in Figure 5(b), while activation of the proton transfer species generated the spectrum shown in Figure 5(c).

The most striking difference between the spectra of Figures 5(b) and 5(c) is the presence of the prominent loss of the oxidized guanine base (-GOH) upon CID of the oxidized species. This species both indicates that one of the two guanine nucleobases undergoes oxidation upon reaction with peroxymonosulfate anion and demonstrates that the loss of an oxidized guanine is a more energetically favorable pathway than the loss of any other base. The small peak 16 Da above the GOH loss can correspond to either the loss of non-oxidized guanine, i.e., GH, or the loss of oxidized adenine, i.e., AOH. Ion/ion reactions were done between the poly-A 6-mer and peroxymonosulfate (data not shown) and the oxidized species was observed upon CID of the ion/ion complex, along with some loss of AOH upon CID of the oxidized species, indicating that this GH/AOH loss peak observed for ACCGAG likely consists of a combination of the two structures. However, the dominant loss of GOH from the precursor as well as smaller sequential losses of GOH from fragment ions indicate that the vast majority of the oxidation occurs selectively on the guanine. Also present in the spectrum corresponding to CID of the oxidized species are sequential base losses (i.e., -GOH-AH), and the oxidized w_5 ion, which also undergoes loss of GOH. This is almost identical to the control spectrum except for the addition of oxygen to the w_5 ion and the lack of the w_4 ion. Furthermore, guanine is the most readily oxidized nucleobase in condensed-phase experiments [73,74], supporting the observations from this gas-phase oxidation experiment. This is the first demonstration of the oxidation of DNA via ion/ion reactions.

Generation of $[M-H]^+$ and $[M-H-NH_3]^+$ species

Intact persulfate anion ($HS_2O_8^-$) is a stronger oxidizing reagent than periodate anion (IO_4^-) as it (i) oxidizes peptides both containing and lacking easily oxidized residues (i.e., Met and Trp) to the $[M+H+O]^+$ species, and (ii) generates a variety of oxidized products, i.e., $[M+H+O]^+$ and $[M-H]^+$, upon activation of the ion/ion complex regardless of the amino acid composition. Furthermore, the $[M+H+O]^+$ species generated upon CID of the persulfate-peptide ion/ion complex may be different in structure from that obtained for CID of the periodate-peptide ion/ion complex [46]. For example, activation of the ion/ion complex between doubly protonated ARAMAKA and periodate exclusively yielded the sulfoxide oxidation product whereas activation of the complex between the same species and persulfate resulted in a mixture of structures corresponding to the hydrogen deficient $[M-H]^+$ species, oxidation of the Met sulfur atom to the sulfoxide form, and oxidation of the terminal carbon atom in the methionine side chain.

The oxidation of neutral basic residues to $[M+H+O]^+$ and $[M-H]^+$ derivatives was first demonstrated with persulfate anion [46]. Activation of persulfate/peptide ion/ion complexes can undergo several pathways to generate oxidized or charge-reduced species. Loss of H_2SO_5 from the ion/ion complex, viz., $[M+2H+HS_2O_8]^+$, generates a species of the form $[M+H+SO_3]^+$. Further activation of this ion exclusively returns the charge-reduced species via loss of SO_3. Loss of SO_3 from the ion/ion complex generates a species of the form $[M+2H+HSO_5]^+$, which is identical to the ion/ion complex generated upon reaction between a doubly protonated peptide and peroxymonosulfate anion (HSO_5^-), which can yield either the oxygen transfer $[M+H+O]^+$ or the proton transfer $[M+H]^+$ species upon activation as discussed in the previous sections. Loss of H_2SO_4 from the ion/ion complex generates a species of the form $[M+H+SO_4]^+$. Activation of this species can either undergo loss of SO_3

to generate the oxygen transfer $[M+H+O]^+$ species (which is not necessarily the same structure as the $[M+H+O]^+$ species generated from CID of the SO_3 loss) or loss of H_2SO_4 to generate the $[M-H]^+$ species. Potential mechanisms and structures for these pathways have been discussed in detail previously [46]. Recently, we showed that periodate is also capable of oxidizing neutral basic sites in certain systems [48]. Peptides lacking easily oxidized residues (i.e., Met, Trp, disulfide bonds, etc.) and containing neutral basic sites can generate one or more of three species upon activation of periodate-peptide ion/ion complexes. The loss of iodic acid, HIO_3, to generate the oxygen transfer $[M+H+O]^+$ species can occur, though this pathway is always very minor in abundance. The loss of H_3IO_4 (while this may correspond to the loss of H_2O and HIO_3, these losses are not observed sequentially) to generate the $[M-H]^+$ species can occur, and is often dominant for arginine-containing systems. Lastly, the loss of $I(OH)_4NH_2$ to generate the $[M-H-NH_3]^+$ species has been observed from neutral lysine residues and is often dominant for lysine-containing peptides. While peaks corresponding to this mass are also observed for arginine-containing species they are derived from a sequential ammonia loss from the $[M-H]^+$ species instead of directly from the complex, in contrast with the case for lysine-containing peptides. (Comparison of the spectra derived from MS/MS of the $[M-H-NH_3]^+$ species directly from the complex and MS^3 of the ammonia loss from the $[M-H]^+$ species are identical for arginine-containing species but different for lysine-containing species, as discussed in detail previously [48].) Furthermore, while the oxidation of lysine residues to the $[M-H-NH_3]^+$ derivative was first noticed in ion/ion reactions with periodate anion, these species are also observed in reactions with persulfate anion where they are likely derived from a similar mechanism.

A direct comparison between the oxidation of various peptides containing neutral basic residues with periodate and persulfate anions is shown in Figure 6. A series of doubly protonated peptides of the form XAXAXAA, where X = Arg, Lys, or His, was subjected to ion/ion reactions with both persulfate (left of Figure 6) and periodate (right of Figure 6). The top panels show the CID spectra of the complex formed via ion/ion reactions between doubly protonated RARARAA and $HS_2O_8^-$ (Figure 6(a)) and IO_4^- (Figure 6(d)). A small amount of oxygen transfer product $[M+H+O]^+$ is observed upon activation of the periodate-RARARAA complex, though this pathway is extremely minor. This pathway was not observed for the persulfate-RARARAA complex. The $[M-H]^+$ product was observed upon activation of both ion/ion complexes, though it was observed at a much higher abundance for the periodate-RARARAA complex. The insets in Figure 6 show a zoom-in of the mass region around the $[M-H]^+$ and $[M+H]^+$ region. No proton transfer is observed for the persulfate oxidation reaction whereas a minor amount of proton transfer is observed for the periodate oxidation reaction indicating the high efficiency with which this peptide is oxidized with each of these reagents. Furthermore, the $[M-H-NH_3]^+$ species is the dominant peak observed upon persulfate oxidation and is present at a lower abundance upon periodate oxidation, but as mentioned previously, likely corresponds to a sequential loss of ammonia from the $[M-H]^+$ peak for this arginine-containing system.

The oxidation of doubly protonated KAKAKAA with persulfate and periodate is shown in Figure 6(b) and (e), respectively. Activation of the persulfate-peptide complex generated a small but significant amount of the oxygen transfer species $[M+H+O]^+$, whereas CID of the periodate-peptide complex generated an extremely minor amount of this species. The [M-

$H]^+$ species is generated at a similar ratio upon activation of the complexes between the peptide and both persulfate and periodate anion. The zoomed-in regions in the insets show that proton transfer is a dominant pathway upon activation of the complex with periodate whereas it is not observed for the complex with persulfate, reflecting the apparently stronger oxidation capability of persulfate. The $[M\text{-}H\text{-}NH_3]^+$ pathway, which is a unique oxidation pathway exclusively observed for lysine residues (i.e., while masses nominally corresponding to $[M\text{-}H\text{-}NH_3]^+$ species are observed for peptides lacking lysine residues, these species correspond to a sequential loss of ammonia from the $[M\text{-}H]^+$ species whereas it corresponds to a unique structure for lysine-containing peptides), is the dominant peak observed upon activation of the persulfate complex and is the dominant oxidation product observed for the periodate complex (secondary in abundance only to the proton transfer species).

Lastly, doubly protonated HAHAHAA was subjected to ion/ion reactions with persulfate and periodate anions and activation of the resulting ion/ion complexes is shown in Figures 6(c) and (f), respectively. Activation of the periodate-peptide complex exclusively results in loss of HIO_4 to generate the charge-reduced $[M+H]^+$ species. No oxidation is observed for this species, though previous reactions between periodate anion and other histidine-containing peptides have shown evidence for a very minor extent of oxygen transfer to the His side chain [48]. Activation of the HAHAHAA-persulfate complex, however, results in dominant formation of the oxygen transfer $[M+H+O]^+$ and hydrogen deficient $[M-H]^+$ species. Proton transfer is observed at a minor abundance. Ion trap CID of each of the oxidized HAHAHAA species is shown in Supplemental Figure S-4. Overall, persulfate anion is a stronger oxidizing reagent anion than periodate anion as it results in a higher degree of oxidation for all three of these systems (the proton transfer pathway is of moderate abundance upon activation of periodate-peptide complexes whereas it is of extremely minor abundance/not observed upon activation of persulfate-peptide complexes). Furthermore, additional steps of MS^n on the various oxidized species, with the exception of $[KAKAKAA+H+O]^+$, generates different spectra for peptides oxidized with periodate and persulfate (Supplemental Figures S-5 through S-8), indicating that the structures resulting from oxidation with the two reagents may be different. The oxidation of neutral basic sites is important in certain systems like proteins and cell-penetrating peptides, both of which contain a high density of unprotonated basic residues under traditional nESI conditions [48].

Generation of molecular radical cations

The generation of radical peptide cations is an area of ongoing research as odd-electron peptides yield fragments, such as those derived from side chain losses, upon CID that are complementary to those generated from their even-electron counterparts [75,76]. Several methods of generating radical peptide cations for tandem MS analysis have been demonstrated. Collisional activation of ternary metal-ligand peptide complexes [77,78,79,80], free radical-initiated reactions [81,82], CID of nitroso peptides [71,83], and photolysis of peptides with iodinated tyrosine residues or electrostatic complexes [84] have all been shown to generate radical molecular cations. Furthermore, several methods of generating radical cations via ion/ion reactions have recently been reviewed [85]. The most efficient means of generating a peptide radical cation via ion/ion reaction is through ion/ion

reactions between a multiply protonated peptide of interest and sulfate radical anion [46]. Activation of the resulting ion/ion complex results in generation of the molecular radical cation via highly efficient loss of sulfuric acid. Similar to the loss of alkyl sulfenic acids from oxidized S-alkyl cysteine side chains, some odd-electron neutral losses from various amino acids upon CID of peptide radical cations generate dehydroalanine residues [86]. This chemistry has recently been used to selectively cleave and localize various amino acids within peptides via fragments derived from the dehydroalanine effect [55].

Doubly protonated KAKAKAA was subjected to ion/ion reactions with sulfate radical anion ($SO_4^{-\bullet}$). Activation of the resulting ion/ion complex $[M+2H+SO_4]^{+\bullet}$ is shown in Figure 7(a) and results exclusively in loss of H_2SO_4 to generate the molecular radical cation, viz., $M^{+\bullet}$. Activation of the complex is a highly efficient method for generating the molecular radical cation. However, it is often already observed upon ion/ion reaction, without the need for an additional activation step for the complex. Results from activation of the molecular radical cation are shown in Figure 7(b), which show a variety of peaks corresponding to backbone fragments and neutral side chain losses characteristic of CID of odd-electron peptide cations. Of particular interest is the loss of $C_3H_8N\bullet$ (58 Da, indicated with a red square in Figure 7(b) and (c)), which generates a dehydroalanine residue in the place of one of the three lysine residues. Further isolation and activation of this species is shown in Figure 7(c). Notably, the c_2 ion is the most dominant peak in the spectrum. This ion is generated via the cleavage of the N-C_α bond of the second lysine residue (indicating conversion to Dha upon loss of $C_3H_8N\bullet$) and is derived from the dehydroalanine effect as well. Also observed are the $z_5^{\square}$, c_4, c_4-NH_3, and c_2-NH_3 ions, which are all derived from the dehydroalanine effect. The hollow square indicates the z-type ion generated by the dehydroalanie effect. (While the c_4-NH_3 and c_2-NH_3 species could theoretically correspond to the b_4 and b_2 species, respectively, previous experiments indicate that these peaks are likely sequential losses from the c_4 and c_2 ions [53,55].) Furthermore, the loss of ammonia from the parent ion may also be derived from the dehydroalanine effect when the lysine at the N-terminus has been converted into Dha (i.e., an ammonia loss can also be considered a $z_7^{\square}$ ion). Other fragments both containing ($b_3^{\blacksquare}$, $b_4^{\blacksquare}$, $b_5^{\blacksquare}$, $b_6^{\blacksquare}$, $y_5^{\blacksquare}$, and $y_6^{\blacksquare}$) and lacking (b_3) modified lysine residues are also observed. Ions derived from the dehydroalanine effect are observed next to all three of the lysine residues (though the ammonia loss from the precursor is somewhat ambiguous as peptides commonly lose ammonia upon CID), and can be used to localize the lysine residues within the peptide. This approach has also been used to localize methionine residues in the peptide ARAMAKA, both methionine and arginine residues in the peptide GRGMGRGMGRL, and leucine residues in a melittin system with mixed cation sites (sequence: GIGAVLKVLTTGLPALISWIKRKRQQ) [55]. Furthermore, the loss of SH• from the cysteine side chain observed upon CID of the molecular radical cation generated upon NO• loss from an S-NO peptide was used to localize cysteine residues [55].

CONCLUSIONS

Here, we have drawn together a variety of gas-phase oxidation reactions recently discovered via ion/ion chemistry and described some possible applications for these reactions that are already apparent. We have discussed the oxidation of thioethers in methionine and S-alkyl cysteine residues and demonstrated a new example illustrating the oxidation of a cysteine

residue protected with N-hydroxymaleimide. Similar to previous examples demonstrating the oxidation of S-alkyl cysteine residues, this system underwent loss of the alkyl sulfenic acid to generate the dehydroalanine species, which was then used to localize the modified cysteine residue via the generation of c- and z-ions derived from the dehydroalanine effect. Unique to the hydroxymaleimide protecting group, however, was the dominant loss of the maleimide plus the elements of water, which was previously observed for maleimide-protected cysteine residues oxidized in solution. Furthermore, we demonstrated the simultaneous oxidation of multiple methionine residues in one peptide via ion/ion reactions with reagent clusters of the form $H(IO_4)_2^-$. We demonstrated the oxidation of an S-nitrosylated peptide, which could aid in the localization of the nitrosylation to a specific cysteine side chain, and an intermolecularly disulfide-linked peptide, which underwent highly efficient oxidative cleavage of the disulfide bond upon oxidation. We also demonstrated the oxidation of DNA to the $[M+H+O]^+$ derivative. This species predominantly lost the oxidized guanine base, indicating that the majority of the oxidation chemistry likely occurred on the guanine nucleobase, in analogy with solution-phase observations. We also compared the oxidation of neutral basic residues to $[M+H+O]^+$, $[M-H]^+$, and $[M-H-NH_3]^+$ species via ion/ion reactions with persulfate and periodate anions. Lastly, we demonstrated the generation of molecular radical cations via ion/ion reactions with sulfate radical anion, a reagent derived upon negative nESI of an aqueous solution of sodium persulfate. This is especially useful as molecular radical cations undergo fragmentation distinct from their even-electron counterparts upon CID, some of which generate Dha residues that can be used to selectively localize amino acids within the peptide of interest. Overall, we have demonstrated a variety of oxidation ion/ion reactions and identified applications in which they may be useful. The applications of these chemistries are diverse and range from mapping disulfide bonds to obtaining complementary sequence information from molecular radical cations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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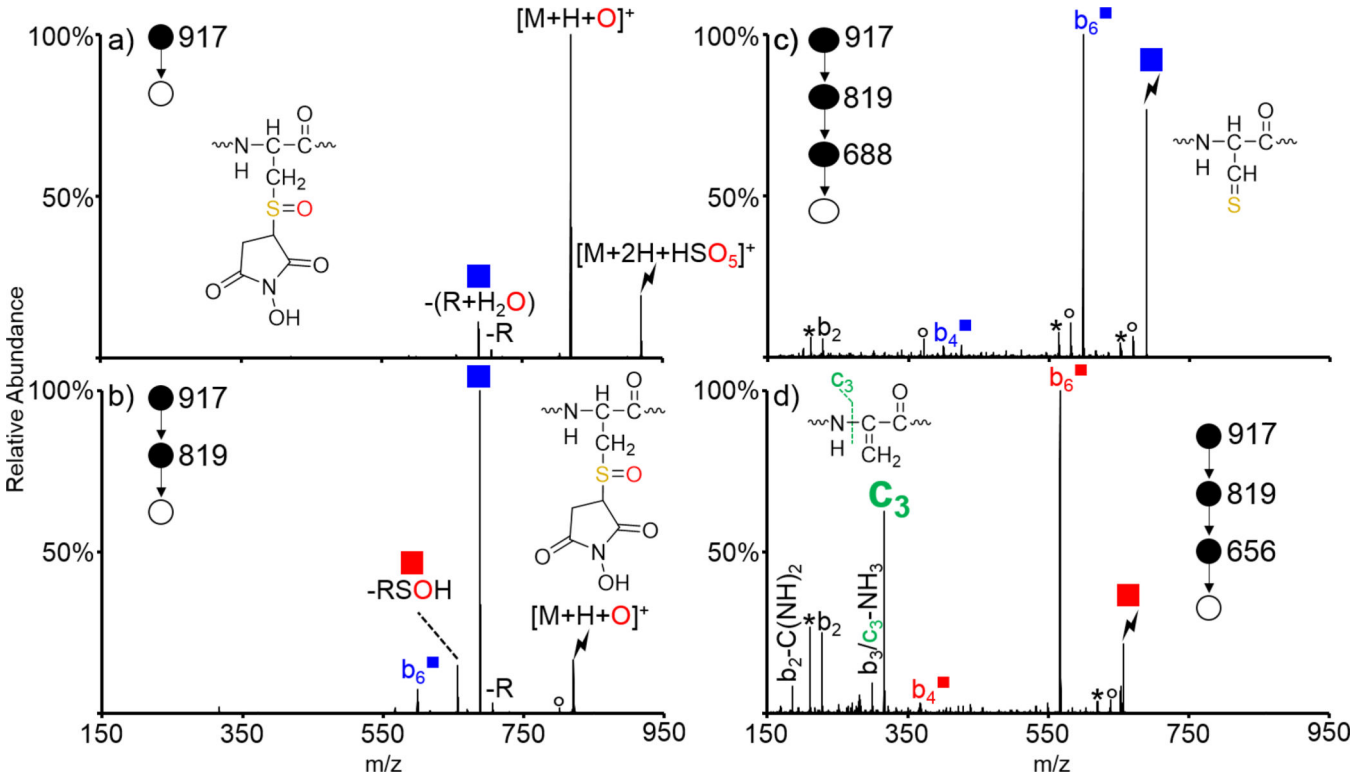

Figure 1.

Oxidation of ARAC^{m}AKA via ion/ion reactions with HSO_5^-, where C^{m} indicates a cysteine residue protected with N-hydroxymaleimide. CID of (a) the ion/ion complex between doubly protonated ARAC^{m}AKA and HSO_5^-, (b) the $[M+H+O]^+$ species, (c) loss of (R $+H_2O$) from the oxidized species, and (d) the dehydroalanine species generated upon loss of the alkyl sulfenic acid from the oxidized species. Lightning bolts indicate the species subjected to CID. Asterisks indicate ammonia losses, degree signs indicate water losses, red and blue squares indicate loss of RSOH and ($R+H_2O$) from the oxidized species, respectively. Red/blue fragments with square superscripts indicate fragment ions that have lost RSOH/($R+H_2O$).

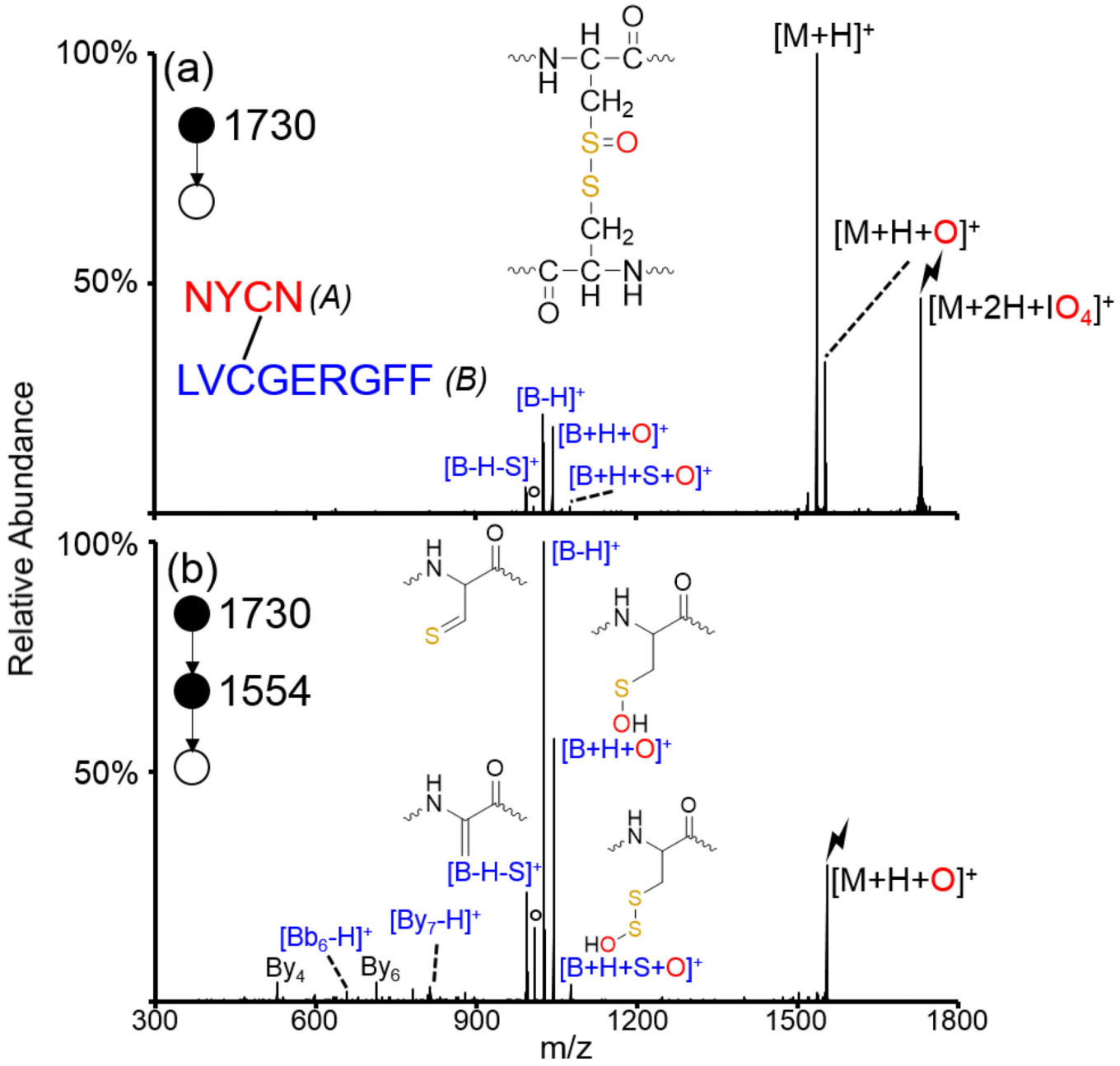

Figure 2.

Oxidation of an intermolecularly disulfide-linked peptide generated from a peptic digest of insulin. CID of (a) the ion/ion complex between doubly protonated NYCN/LVCGERGFF and IO_4^-, and (b) CID of the oxidized species. The sequence of the peptide is shown inset in (a) along with the structure of the $[M+H+O]^+$ species. Structures of the $[B+H+S+O]^+$, $[B+H+O]^+$, $[B-H]^+$, and $[B-H-S]^+$ species are shown in (b). Fragments derived from cleavage of the disulfide bond are indicated in blue as all fragments observed correspond to fragments from the B-chain (indicated in blue in inset). Lightning bolts indicate species subjected to CID and degree signs indicate water losses.

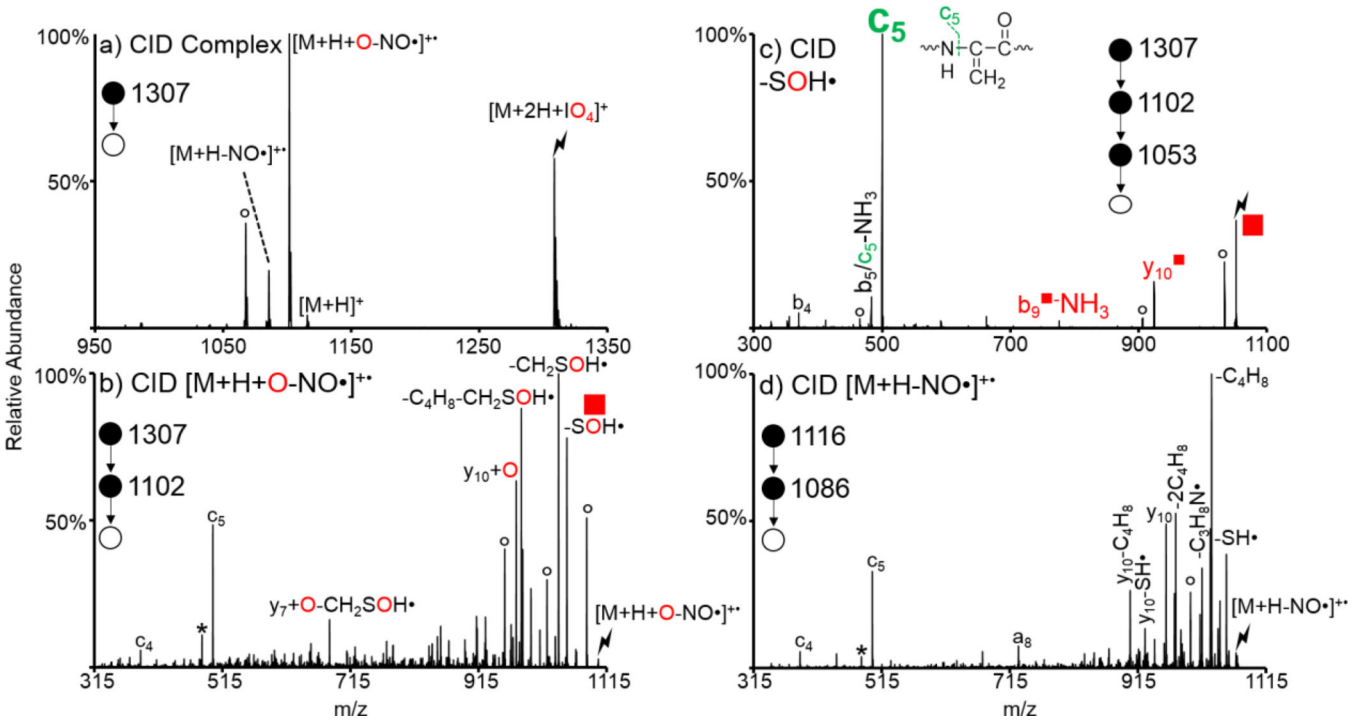

Figure 3.

Oxidation of S-NO KGAILCGIALK via ion/ion reactions with IO_4^-. Ion trap CID of (a) the ion/ion complex between doubly protonated S-NO KGAILCGIALK and IO_4^-, (b) the oxidized $[M+H+O\text{-}NO\bullet]^{+\bullet}$ species, (c) the SOH• loss species (■) from (b) to generate the Dha species, and (d) the NO• loss from the protonated peptide (control spectrum). Lightning bolts indicate species subjected to CID, degree signs indicate water losses, and asterisks indicate ammonia losses.

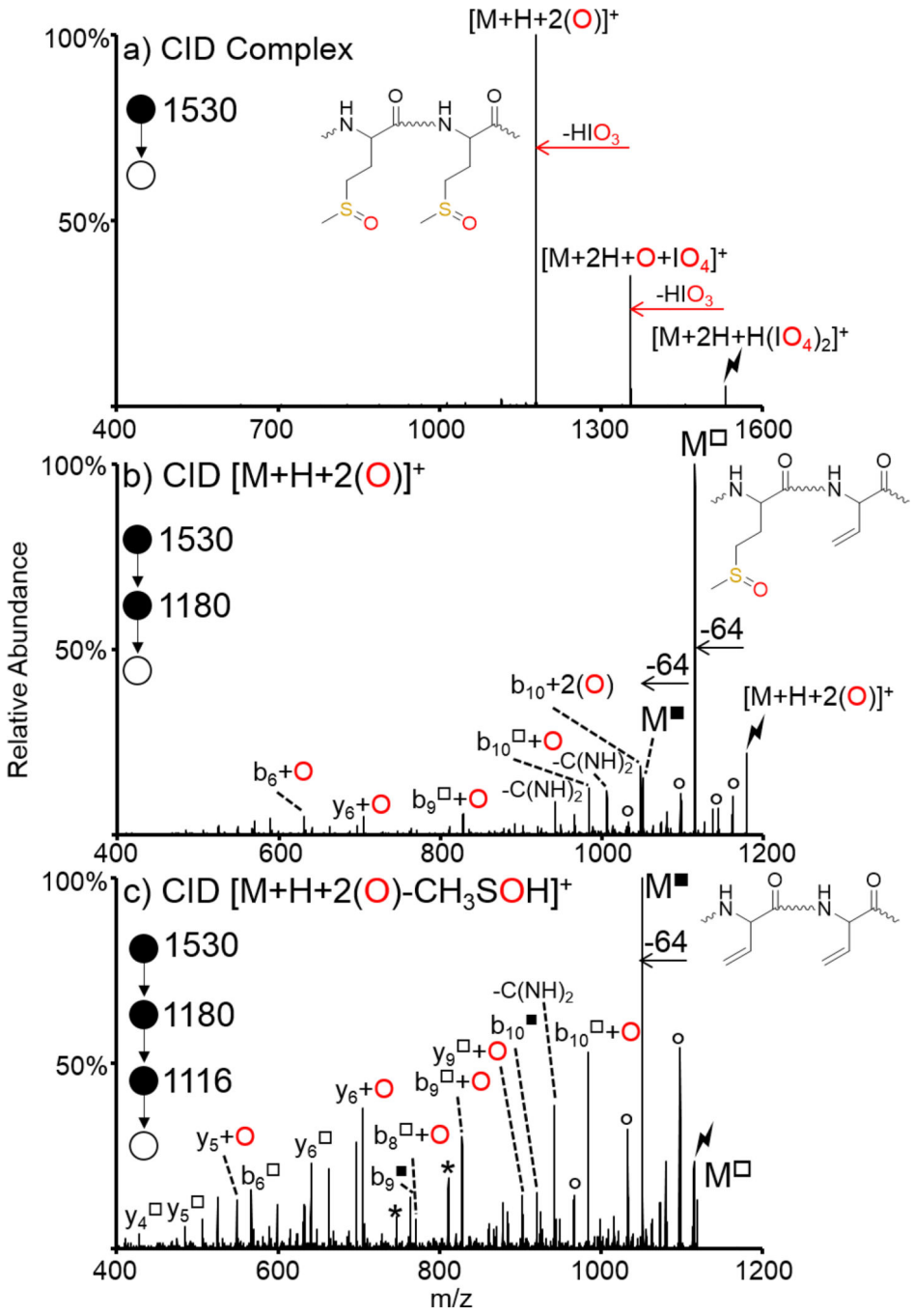

Figure 4.

Oxidation of both methionine residues in GRGMGRGMGRL via a single ion/ion reaction with a periodate cluster. CID of (a) ion/ion complex between doubly protonated GRGMGRGMGRL and $H(IO_4)_2^-$, (b) the doubly oxidized $[M+H+2(O)]^+$ species, and (c) the 64 Da loss (CH_3SOH) from the doubly oxidized species. Insets show methionine residues in GRGMGRGMGRL where (a) both Met residues are oxidized, (b) one of the Met residues has undergone loss of CH_3SOH (64 Da), and (c) both Met residues have lost CH_3SOH. Lightning bolts indicate species subjected to CID, asterisks indicate ammonia

losses, and degree signs indicate water losses. Hollow square superscripts indicate species that have lost one CH_3SOH group while solid square superscripts indicate species that have lost two CH_3SOH groups.

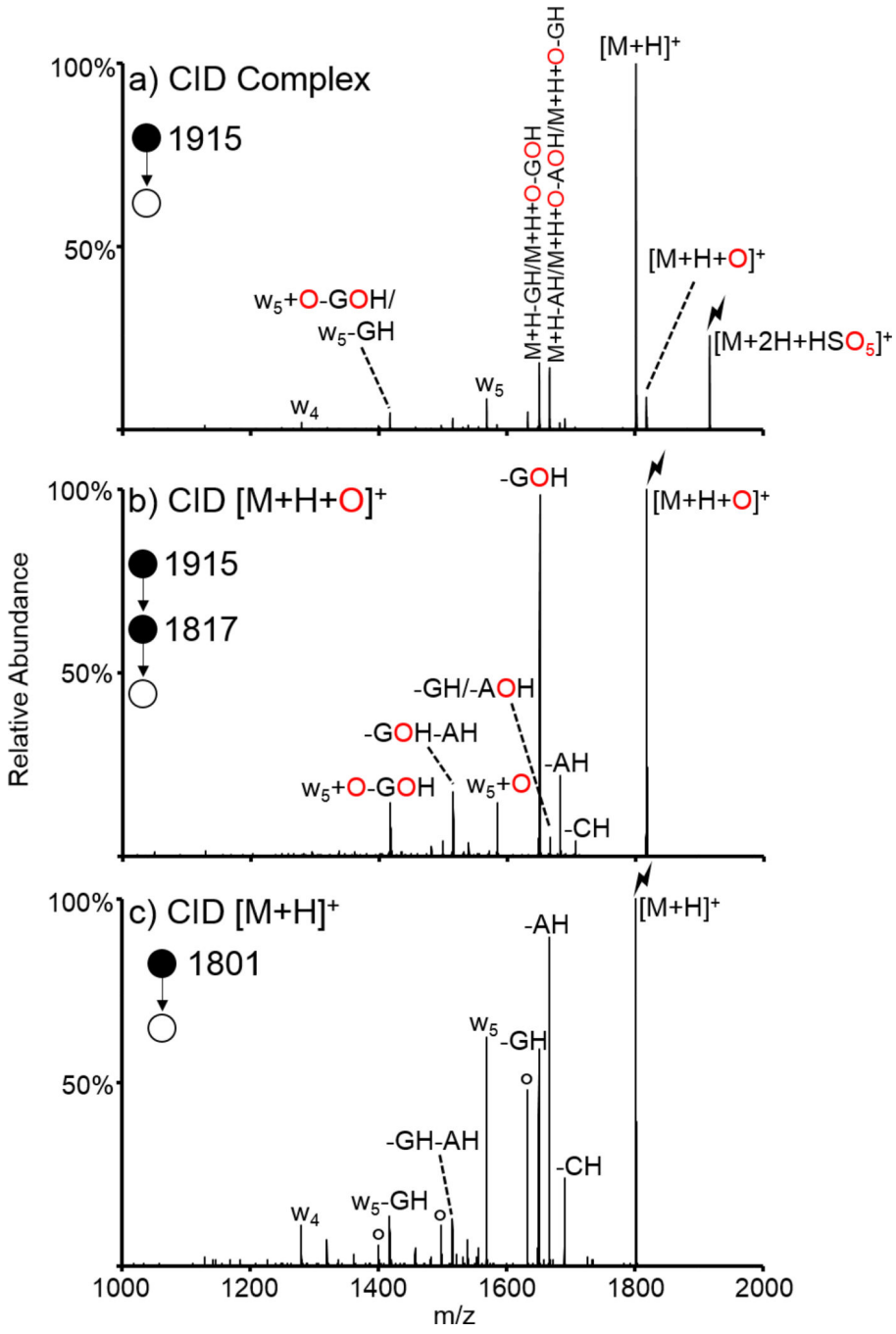

Figure 5.

Oxidation of DNA via ion/ion reactions with HSO_5^-. CID of (a) the ion/ion complex between $ACCGAG^{2+}$ and HSO_5^-, (b) the oxidized species, and (c) the protonated (control) species. Lightning bolts indicate species subjected to CID, degree signs indicate water losses.

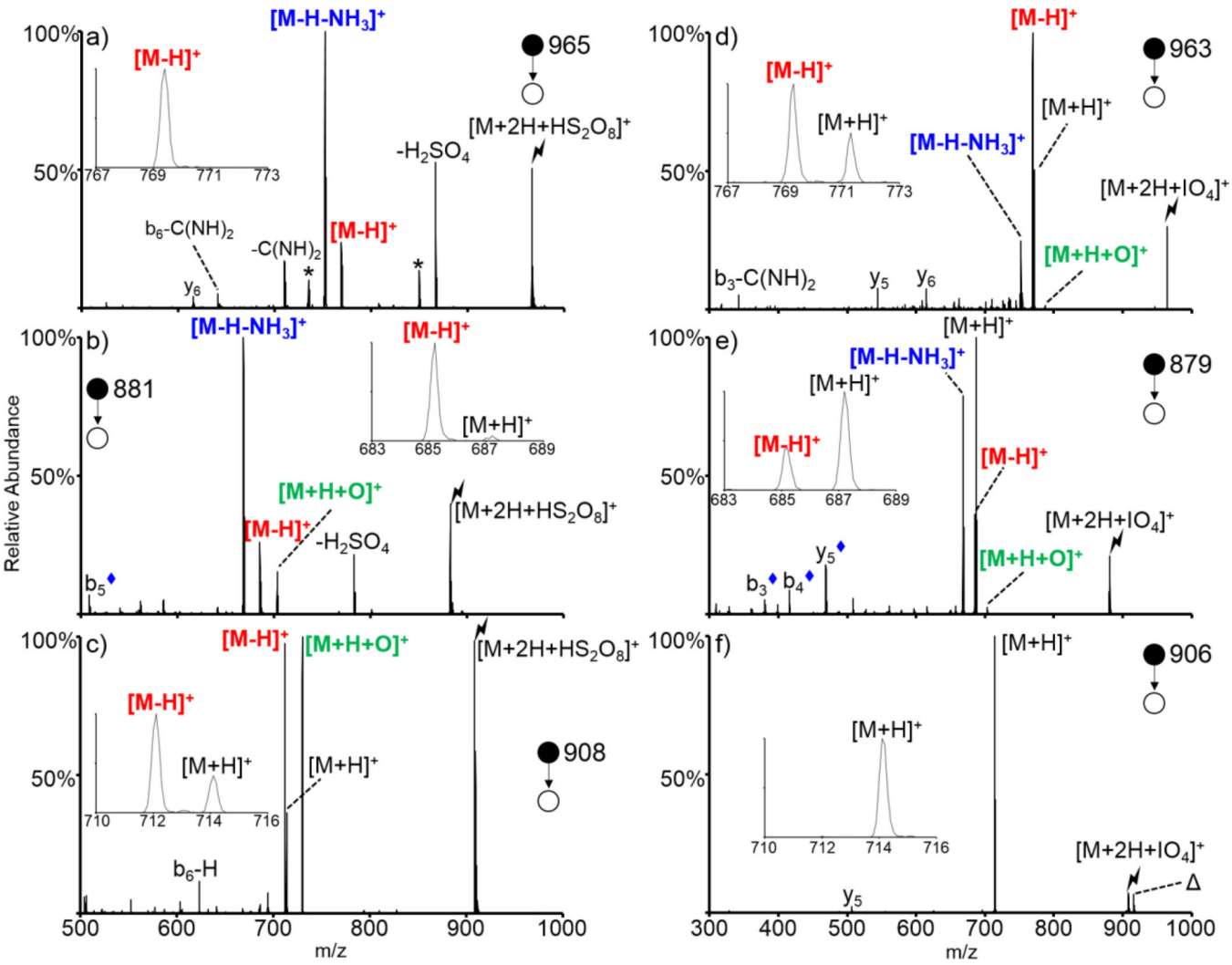

Figure 6.

Comparison of the oxidation of neutral basic sites via ion/ion reactions with $HS_2O_8^-$(left) and IO_4^- (right). CID of the ion/ion complex between doubly protonated RARARAA and (a) $HS_2O_8^-$, (d) IO_4^-, KAKAKAA and (b) $HS_2O_8^-$, (e) IO_4^-, and HAHAHAA and (c) $HS_2O_8^-$, (f) IO_4^-. Lightning bolts indicate species subjected to CID, asterisks indicate loss of ammonia, delta signs indicate contaminants present in the isolation, and blue diamonds indicate $[b/y\text{-}H\text{-}NH_3]^+$ fragments. Insets show the zoomed in mass region around the $[M\text{-}H]^+$ and $[M+H]^+$ species.

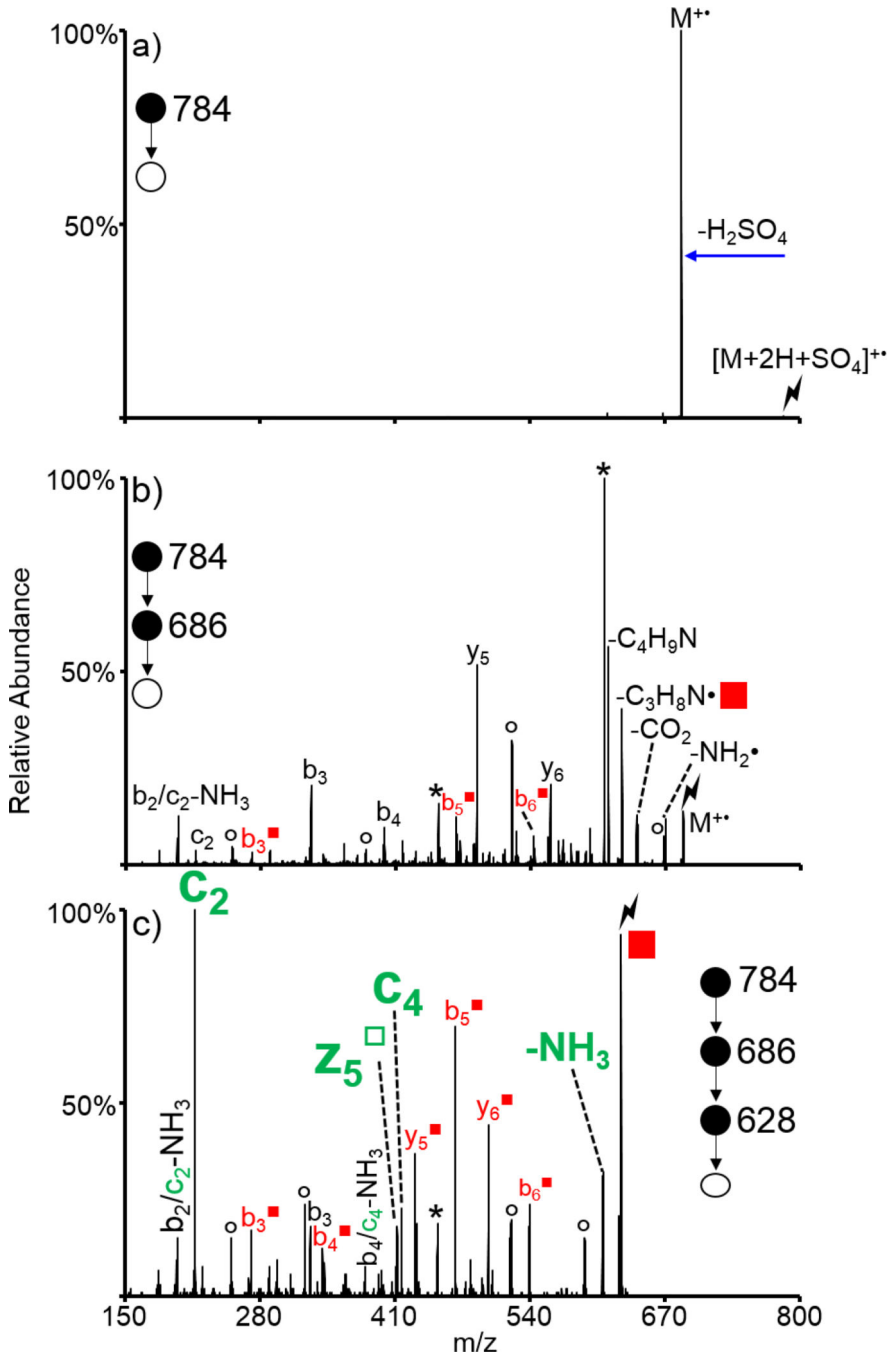

Figure 7.

Generation of a molecular radical cation of KAKAKAA via ion/ion reactions with sulfate radical anion. CID of (a) the ion/ion complex between doubly protonated KAKAKAA and $SO_4^{-\bullet}$, (b) the molecular radical cation of KAKAKAA, and (c) the loss of $C_3H_8N\bullet$ from (b) to generate a Dha residue out of one of the lysine residues. Lightning bolts indicate species subjected to CID, asterisks indicate loss of ammonia, degree signs indicate loss of water, red

squares indicate Dha-containing species, and fragments derived from the dehydroalanine effect are indicated in green.