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Gas-phase ion/ion interactions between peptides or proteins and iron ions in a quadrupole ion trap

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Abstract

The gas-phase ion/ion reactions of iron ions with oppositely charged peptide and protein ions were studied in a quadrupole ion trap. Both Fe^+ and $FeCO_2^-$ were investigated as possible reactant ions for gas-phase cleavage of peptide and protein ions. Several types of reaction products were observed. Charge exchange lowered the charge states of the proteins. Attachment resulted in a complex of the protein ion and the iron ion. In some cases bonds were broken in the protein ions, but it is unclear whether this is due to an insertion of the iron ion into a bond or due to the energetic reaction of oppositely charged species. Some preference was observed for bond cleavage near sulfur. Two disulfide bonds were broken in one case, and bonds adjacent to a cysteine residue were broken in another. (Int J Mass Spectrom 204 (2001) 47–54) © 2001 Elsevier Science B.V.

Keywords: Ion/ion reactions; Gas phase; Mass spectrometry; Proteins; Fragmentation

1. Introduction

Protein analysis by mass spectrometry offers tremendous advantages over traditional biochemical methods. Detection limits and analysis times have been drastically reduced with mass spectrometric techniques. Instead of days of analysis requiring picomoles of sample, proteins can now be analyzed in 5 min or less, and sample requirements are as little as 1 fmol of protein digested in solution or in the low femtomoles of protein digested in two-dimensional (2D) gels [1–6]. Mass spectrometry has been used for a variety of applications including de novo sequencing, protein identification, identifying microorganisms, accurate mass determination, analyzing carbohydrate attachments, monitoring protein folding, and locating modification sites and DNA sequencing error sites [3,7–15]. Many of these applications involve separation of a cell extract on a 2D gel and enzymatic digestion of the proteins in solution prior to analysis. This step can introduce a variety of problems. Digestion can require 6–24 h of reaction time. With potentially thousands of proteins separated on one gel, the digestion step is a bottleneck in the analysis of the separated proteins. Also, each additional solution phase step greatly increases both analysis time and potential for sample loss, and an in-gel digestion involves several steps of sample manipulation. The digestion can also complicate the ionization process. Ionization techniques, particularly electrospray ionization (ESI), are sensitive to many biochemical reagents. Detergents, surfactants, and buffers can all suppress an ESI signal significantly, even when

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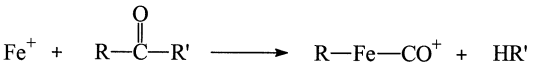
present in only small amounts. For example, sodium dodecyl sulfate (SDS) can almost completely suppress an ESI signal at a level of only 0.01% [16]. Enzymatic digestion requires the addition of some buffer salts and makes sample clean-up somewhat more challenging. Separation of contaminants from whole proteins is significantly easier than from digest fragments. Another source of contamination is the digestion procedure itself. The enzyme can undergo autolysis, and the resulting fragments can complicate the analysis of the sample digest fragments.

A larger problem is that the sample may contain more than one protein due to inadequate separation prior to digestion. In this case, digest fragments from both proteins are present in the sample. If the identity of these proteins is unknown, determining which fragments belong to each protein can be difficult. By moving the digestion to a different point in the analysis, many of these problems may be sidestepped. If digestion could be accomplished in the gas phase, the whole protein could be introduced into a trapping mass spectrometer, such as an ion cyclotron resonance instrument (ICR) or a quadrupole ion trap. In this case, it would be immediately apparent if more than one protein was present in the sample. Additionally, gas phase reactions require on the order of seconds as opposed to hours in solution. The analysis time would be greatly decreased for each sample, and another solution-phase step would be eliminated. An additional advantage is that the mass spectrum of the sample would be obtained without the need for a separate experiment. This would allow for simple and accurate determination of the molecular weight of the whole protein.

In addition to providing advantages for conventional mass spectral analysis of proteins, gas phase digestion would allow novel types of experiments to be performed. For example, the gas phase three-dimensional structure of proteins could be investigated. The effect of the ionization process on the three-dimensional structure is disputed [17–19]. By first ionizing the whole protein and then performing a H/D exchange, the exposed parts of the protein ion will become deuterated, whereas the buried interior portions will retain their hydrogens [20–23]. The protein would be broken into fragments by means of gas-phase digestion at this point. Those fragments that increase in mass following the H/D exchange can be attributed to the exposed portions of the protein, whereas any that did not increase in mass can be assigned to interior sections. Comparison of these results with known solution phase structures could provide insight into the effect of the ionization process on protein structure. However, fragmentation using conventional techniques is problematic. Collision-induced dissociation (CID) often causes a scrambling of the H/D atoms, and, thus, positional information is lost [18]. A gas-phase digestion could break the protein into pieces without adding internal energy and eliminate scrambling of these isotopic labels. Unfortunately, proteolytic enzymes do not appear to function in the absence of solvent. Reagents that could be used as a chemical digestion have been investigated, and transition metal ions show promise.

Scheme 1.



Scheme 1.

Transition metal ions, such as Fe^+ and Cu^+, are well-known to cleave carbon–carbon bonds in gas phase reactions with neutral organics [24–29]. This reaction is especially prominent adjacent to carbonyls [30]. The metal ion inserts into the C-C bond, and the molecule is broken into two pieces at that point as shown in Scheme 1. This behavior has also been observed with neutral amino acids and small peptides [31,32]. The amino acids formed ions with Fe^+ and lost CO or H_2CO_2 as neutrals. The peptides formed fragments ionized by Fe^+, such as *a*, *b*, *c*, and *y*, according to conventional nomenclature [33].

Though metal ions show a propensity to cause cleavage in neutrals, a more useful reaction would involve protein ions. The generation of peptide neutrals is difficult and limited to very low molecular weight species. Large protein and peptide ions, however, can be very easily generated by ESI. These ions are either protonated or deprotonated versions of the

neutral peptides. The charge on the protein ions also provides a "handle" with which these species can be manipulated prior to reaction. Ion/ion reactions have been performed in the quadrupole ion trap and have demonstrated several useful properties [34]. Many of the advantages of a gas-phase digestion mentioned previously require ionized species, such as isolation and mass determination. Other advantages of using ions include building the signal intensity by trapping ions over long accumulation times, varying reaction times, and employing ion/molecule reactions prior to or following digestion. By performing these reactions in an ion trap, multiple stages of mass spectrometry (MSn) is readily available to identify the digest fragments within the same analysis.

Despite all the potential advantages, it is unknown whether protein ions will exhibit similar reactions to those with neutrals. This work describes a preliminary investigation of employing metal ion species to enact gas phase digestion of protein ions.

2. Experimental

All experiments were performed on a modified Finnigan ITMS controlled with ICMS software [35]. Peptide and protein ions were generated with a custom-built nanoelectrospray source. A buffer gas of helium at approximately 1 mTorr was added to the quadrupole ion trap. Tandem mass spectrometry (MS/MS) was achieved by means of CID using resonant excitation, as described previously [36].

Insulin and the peptide, RPPGFSPFR, were obtained from Sigma Chemical Company and used without purification. TIHDIILECV was synthesized in the Department of Biochemistry at the University of North Carolina at Chapel Hill. Electrospray solutions were prepared in two ways. For negative ESI, 100 μM protein solutions in 70:20:10 $CH_3OH/H_2O/NH_4OH$ were used. For positive ESI, 100 μM protein solutions were prepared in 75:20:5 $CH_3OH/H_2O/CH_3COOH$.

Protein ions were accumulated in the quadrupole ion trap prior to formation of iron ions. Iron ions (Fe^+ and $FeCO_2^-$) were generated using a frequency-doubled Nd:YAG laser (Continuum Surelight II). The laser was focused onto a stainless steel surface to produce the iron ions. A hole through the ring electrode allowed these ions to enter the trap. Isolation of one iron species was achieved using stored waveform inverse Fourier transform (SWIFT), as described previously [37]. SWIFT was employed to quickly eject all undesired laser-generated species before these ions could react with the simultaneously trapped protein ions [38–40]. The protein and iron ions were then allowed to react within the quadrupole ion trap for 10–2000 ms, most often for periods of 400–800 ms.

3. Results

Two different charge states of insulin were isolated for the reaction of negatively charged insulin with Fe^+. The two reactions show slightly different product ions. With $[insulin-4H]^{4-}$, the reaction produced the 3^- and 2^- charge states, as well as both of these charge states with iron attachment (Fig. 1). However, in addition to lower charge states and attachments, reaction of $[insulin-5H]^{5-}$ with Fe^+ resulted in bond cleavage product ions (Fig. 2). Insulin is composed of two chains, A and B, linked by two disulfide bonds. The product ions observed correspond to A^- and B^{2-}, which would result from the cleavage of two disulfide bonds.

A smaller peptide was also used in a reaction with Fe^+. $[TIHDIILECV-2H]^{2-}$ reacted with Fe^+ similarly to insulin in that a lower charge state resulted, $[TIHDIILECV-2H]^{1-}$ (Fig. 3). Again, some fragmentation was also seen. The $[a_8+Fe]^-$ and $[b_8+Fe]^-$ product ions were observed as a result of this reaction. A third product ion was observed corresponding to the singly charged peptide minus H_2CO_2.

Many peptides gain protons more easily than lose them, so the use of positively charged peptides could be advantageous. Positively charged peptides were also investigated for use in these reactions. As would be expected, positively charged peptide ions did not react with the positively charged iron ion, Fe^+. However, many types of metal-containing ions, pos-

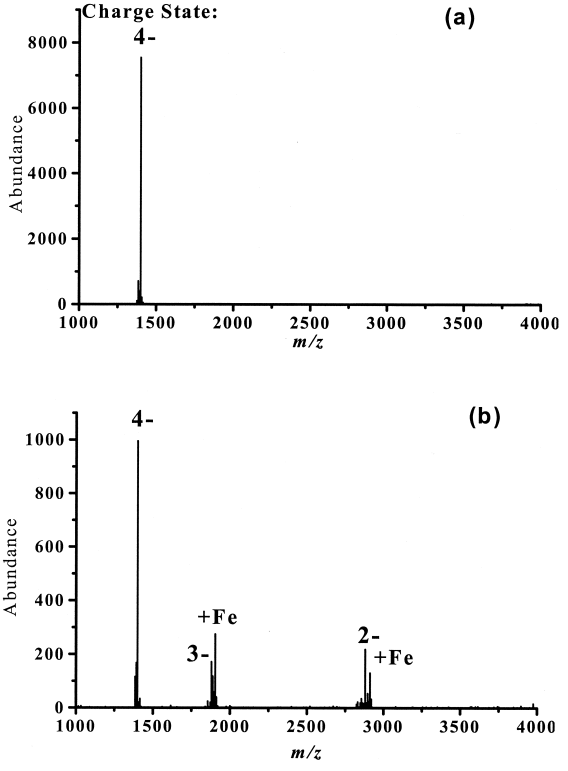


Fig. 1. Reaction of $[\text{insulin}-4\text{H}]^{4-}+\text{Fe}^{+}$. (a) Isolation of 4− charge state prior to reaction. (b) Following reaction with Fe^{+}.

itively and negatively charged, are formed from the laser pulse and trapped in the quadrupole ion trap. By altering the SWIFT isolation, $FeCO_2^-$ was chosen as the reacting species. Doubly charged bradykinin, $[\text{RPPGFSPFR}+2\text{H}]^{2+}$, was allowed to react with

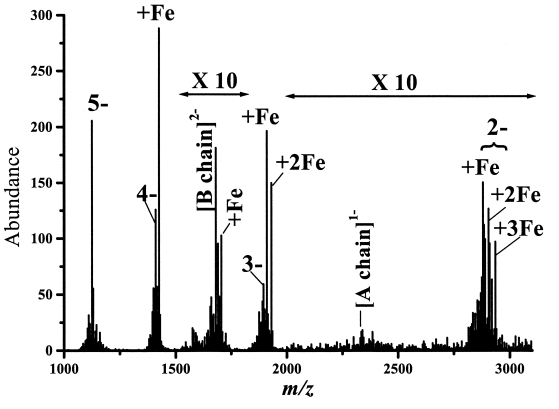


Fig. 2. Reaction of $[\text{insulin}-5\text{H}]^{5-}+\text{Fe}^{+}$ with Fe^{+}.

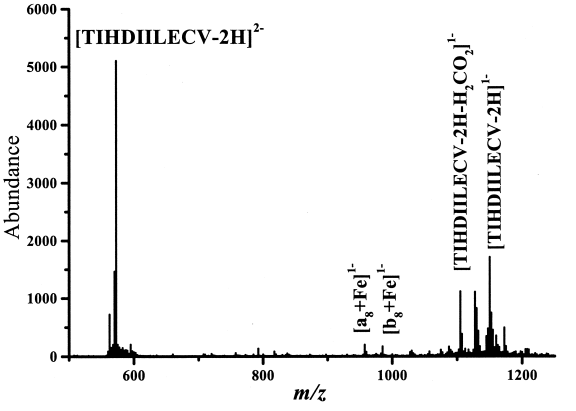


Fig. 3. Reaction of $[\text{TIHDIILECV}-2\text{H}]^{2-}$ with Fe^{+}.

$FeCO_2^-$. The results can be seen in Fig. 4, where several peptide product ions can be seen including the singly charged peptide. Though the attachment product, $[\text{RPPGFSPFR}+2\text{H}+FeCO_2]^{+}$, is not observed, several products incorporate the $FeCO_2^-$ ion. Some of these product ions involve the loss of small neutrals, including loss of ammonia, loss of both ammonia and water, and loss of ammonia and two waters. Additionally, the product ions $[a_6+FeCO_2]^{+}$ and $[y_5+FeCO_2]^{+}$ are observed.

4. Discussion

Several possible types of products can result from reactions of protein and iron ions. There are four types

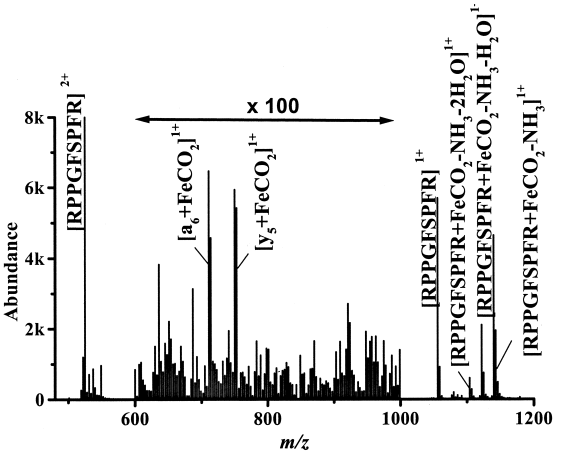


Fig. 4. Reaction of $[\text{RPPGFSPFR}+2\text{H}]^{2+}$ with $FeCO_2^-$.

of reactions that may be seen in the previous results: charge exchange, attachment, cleavage, and dissociation. The first two of these are not useful as a gas-phase digestion as bonds are not broken in the protein ion. The last two, cleavage and dissociation, both result in fragmentation of the protein ion, but the mechanisms of fragmentation differ. Some of these reactions may be useful for gas-phase digestion, while others may offer different advantages.

The first type of reaction, charge exchange, simply involves an electron transfer between the iron and protein ions. The result is that the iron ion is neutralized and the charge state of the protein is reduced. These products can be seen in each of the results described above, where the singly charged versions of RPPGFSPFR and TIHDIILECV are produced from the doubly charged, and the lower charge states are produced from the higher charge states of insulin. In fact, this type of reaction usually produces the most abundant product ion. This is not a surprising result taking into account the thermodynamics of charge exchange. In the case of a negatively charged protein reacting with Fe^+, the propensity for charge exchange depends upon the relative values of the recombination energy of Fe^+ [41] and the electron affinity of the protein ion. MOPAC calculations were used to estimate the electron affinity of a negatively charged peptide

$$Fe^+ + e^- \rightarrow Fe$$
$$\Delta H = -\text{recombination energy} = -7.8\ \text{eV} \quad (1)$$

$$[\text{peptide} - \text{H}]^- \rightarrow [\text{peptide} - \text{H}]^0 + e^-$$
$$\Delta H = \text{electron affinity} = 4\text{–}5\ \text{eV} \quad (2)$$

In this case, charge exchange is a favorable reaction, being exothermic by a few electron volts.

Attachment is a similar reaction in that the charge state of the protein is reduced. However, in this case, the iron remains attached to the protein ion, so the mass of the ion is also increased by 56 Da

$$[\text{peptide} - n\text{H}]^{n-} + Fe^+ \rightarrow$$
$$[\text{peptide} - n\text{H} + Fe]^{(n-1)-} \quad (3)$$

This reaction will also be exothermic as it involves the neutralization of a charge. Again, this is seen in the reaction with insulin (the peaks indicated with +Fe). However, the attachment product is not seen with either of the two peptides. This may be due to the smaller size and fewer degrees of freedom over which to partition the excess internal energy from the reaction.

Cleavage is the reaction analogous to that seen with neutral organic compounds, where the iron ion inserts into a bond and cleaves it there. This is the reaction desired for gas-phase digestion. It is a specific reaction resulting from the chemical interaction of the iron ion with the protein. Dissociation also results in breaking bonds in the peptide, but the cause is not a specific cleavage reaction. Instead, this reaction is due to excess internal energy in the protein ion. Bond dissociation could result from this in a similar manner to that seen in collision-induced dissociation, where the increased internal energy causes bonds to be broken. This energy would be available from the exothermic reactions of charge exchange and attachment. For example, as shown above, charge exchange can have a few electron volts of excess energy, possibly more with larger proteins and higher charge states than with the peptide modeled. The product ions from both of these reactions will be fragments of the original peptide, possibly with iron ion attachment. As such, it is difficult to distinguish which process leads to the product ions.

Cleavage and/or dissociation product ions can be seen in most of the examples given. While the reaction of $[\text{insulin}-4\text{H}]^{4-}$ results only in charge exchange and attachment, product ions resulting from fragmentation are also observed following the reaction of $[\text{insulin}-5\text{H}]^{5-}$. There are some interesting features here. First, there are two disulfide bonds that are broken between the two chains. This indicates a propensity for cleavage of disulfides during the reaction with iron ions. Reactions of iron and cobalt ions, both positive and negative, have demonstrated an affinity for cleavage adjacent to sulfur and at disulfide bonds in neutral organic compounds [42]. The cleavage of two bonds could result from two iron ions reacting with the insulin, each responsible for cleav-

ing one disulfide bond. We do not yet have direct evidence of this, but the charge states of the product ions are consistent with this. The initial charge state of 5− combined with two positive iron ions could be expected to result in product ions with a combined charge of 3−, such as the A^- and B^{2-} ions observed. The 3− and 2− charge states also have multiple iron ions attached, which is further evidence of interaction with multiple iron ions. Differing iron incorporation is also seen in these product ions with both the B^{2-} chain as well as the $[B+Fe]^{2-}$ observed.

There are also peaks associated with the B^{2-} chain which may result from different sites of cleavage on the disulfide bond. If both C-S bonds and S-S bonds can be cleaved, the resulting product ions can contain one greater or one fewer S atoms. Small peaks are observed below both the B^{2-} and the $[B+Fe]^{2-}$ which could be these ions less one sulfur. A very small peak may also correspond to the $[B+Fe+S]^{2-}$. However, due to the low intensity signal and instrumental limitations in mass accuracy and resolution, these peaks cannot be definitively identified. Whether bond cleavage occurs solely at S-S bonds or also at C-S bonds will be an area for future investigation. Although these disulfide bond cleavages would not suffice for complete gas-phase digestion, it could prove useful for other types of protein analyses.

Another interesting feature is that these fragments were observed only with the 5− charge state of insulin. The 4− charge state attaches an iron ion and appears to react with two iron ions to get to the 2^- charge state, but no cleavage is observed nor is multiple iron attachment. It is possible that the two initial charge states simply have different chemistry with the iron ions, perhaps due to different three-dimensional structures. However, it may also be that the reactions of the oppositely charged ions by means of charge exchange or attachment are more exothermic with the higher charge state ion. This would result in greater internal energy available to cause dissociation of the protein ion in the charge exchange reaction. CID of these insulin ions has been performed for comparison with the products of the reaction with iron ions. The internal energy imparted by CID of insulin results in the formation of a wide variety of product ions, but with no apparent preference shown towards breaking the disulfide bonds. The Fe^+/ $[insulin-5H]^{5-}$ reaction is therefore interesting because it has a different result from a conventional dissociation method. However, whether this is due to a specific bond cleavage reaction or increased internal energy coupled with an association with the iron ion that makes the disulfide particularly susceptible is not known. Further study of other proteins containing disulfide bonds will be performed to evaluate the utility of iron ion reactions for this purpose.

The peptide $[TIHDIILECV-2H]^{2-}$ also fragments upon reaction with Fe^+. Two of the product ions, $[a_8+Fe]^-$ and $[b_8+Fe]^-$, incorporate the iron reactant ion. This incorporation could result from attachment of the Fe^+ ion to the peptide followed by dissociation. However, incorporation would also result from cleavage. The mechanism requires the iron ion to insert into a bond, inducing the reaction. When the bond is cleaved, the iron remains attached to one piece of the protein ion. The *a* and *b* ions are similar to the types of ions seen following CID. This may lend credence to the argument that this is dissociation due to excess internal energy as a result of attachment. However, it should also be noted that the bonds cleaved are adjacent to a cysteine residue. It has been observed that in peptides cationized with Fe^{2+}, the iron interacts strongly with the thiol group, and bonds adjacent to cysteine residues dissociate preferentially upon CID [43]. This may be the same effect we observe here and may also explain the cleavage of the disulfide bonds in insulin. Again, the iron interaction may have some affinity for sulfur-containing residues and make adjacent bonds more susceptible to cleavage. Another product ion seen from this reaction is the loss of H_2CO_2 from the charge exchange product. Although this could again be a dissociation, it is interesting to note that the loss of H_2CO_2 was also seen by Amster's group in the reaction of neutral amino acids. Therefore, this loss may be characteristic of cleavage.

The reaction of $[RPPGFSPFR+2H]^{2+}$ shows several product ions, all of which incorporate the $FeCO_2$ reactant. The attachment product, $[RPPGFSPFR+2H+FeCO_2]^+$, is not observed, but all of the product ions incorporate $FeCO_2$. The

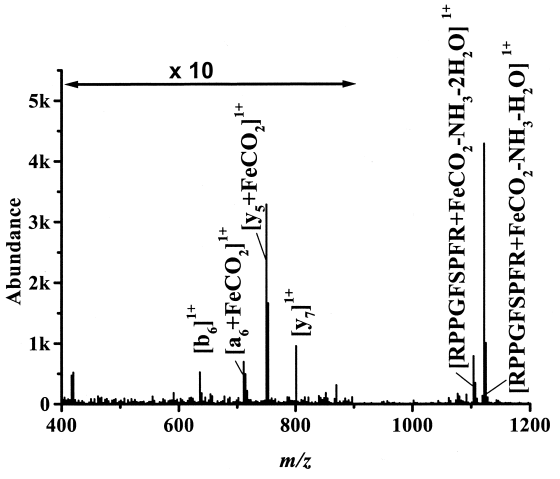


Fig. 5. MS/MS of [RPPGFSPFR+$FeCO_2^-$−NH3].

$[RPPGFSPFR+2H+FeCO_2-NH3]^{2+}$ product ion was isolated and dissociated by means of CID. This produced the same water loss, a_6, and y_5 product ions seen in the ion/ion reaction spectrum (Fig. 5). The process of CID simply increases the internal energy of the ion through collisions with a buffer gas. Because an increase in internal energy caused exactly the same dissociation pattern as that seen from the reaction with $FeCO_2^-$, it is likely that excess internal energy is the cause of dissociation in both cases. Therefore, cleavage reactions were likely not observed in the reaction of $[RPPGFSPFR+2H]^{2+}$ with $FeCO_2^-$, but rather dissociation following attachment.

5. Conclusions

Iron ions are well known to cleave carbon-carbon bonds in gas-phase reactions with neutral species. For this reason, these ions were explored as possible reactant species for use in the gas-phase digestion of protein ions. However, the metal ions and protein ions do not appear to react in the same manner as neutrals. Although some bonds are broken in the protein ions, it is unclear whether this is due to cleavage or dissociation. In the case of a positive peptide reacting with $FeCO_2^-$, dissociation is almost certainly the cause. Another type of dissociation, electron capture dissociation, has been investigated recently and has demonstrated some advantages over CID [44]. These ion/ion reactions will be investigated further to determine if they offer similar advantages. With negative peptides and proteins, reaction with Fe^+ appears to cause fragmentation close to cysteine residues, either adjacent to this residue or a disulfide bond between two cysteines. Future experiments will explore other disulfide and cysteine-containing peptides and proteins. Also, positive ions containing cysteine will be explored to determine whether $FeCO_2^-$ shows the same propensity for fragmentation close to cysteine.

Acknowledgements

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