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ARTICLE *in* INTERNATIONAL JOURNAL OF MASS SPECTROMETRY · AUGUST 2014

Impact Factor: 1.97 · DOI: 10.1016/j.ijms.2014.08.027

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journal homepage: www.elsevier.com/locate/ijms

Intra-molecular reactions between cysteine sulfinyl radical and a disulfide bond within peptide ions

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ARTICLE INFO

Article history:
Received 30 April 2014
Received in revised form 11 August 2014
Accepted 12 August 2014
Available online xxx

Keywords:
Intra-molecular reactions
Disulfide bonds
Collision-induced dissociation
Cysteine sulfinyl radical
Radical migration
Disulfide bond scrambling

ABSTRACT

Cysteine sulfinyl radical (^{SO}•Cys) is a reactive intermediate discovered in the inactivation of enzymes utilizing the glycyl/thiyl radical in their catalytic functions upon exposure to air. ^{SO}•Cys has been recently formed and investigated in the gas phase via mass spectrometry (MS), with the aim being to acquire direct experimental evidence of the radical's intrinsic chemical reactivity. Ion/molecule reaction studies showed that ^{SO}•Cys was relatively chemically inert toward thiol (S—H) and disulfide (S—S) functional groups under the explored experimental conditions. Herein, we utilized intra-molecular reactions aided by collision-induced dissociation (CID) to overcome the limitations associated with the traditional bimolecular reactions and explore the reactivity of ^{SO}•Cys. Our results revealed a new reaction pathway in which the sulfinyl radical exchanged with an intrachain or interchain disulfide bond within a peptide ion, leading to the formation of a new disulfide bond and a sulfinyl radical. As a consequence, CID of peptide disulfide regio-isomers consisting of ^{SO}•Cys led to enhanced sequence information, however the disulfide bond linkage patterns could not be accurately assigned. This reaction pathway also has implications on disulfide bond scrambling in proteins initiated by a radical intermediate.

1. Introduction

Radicals or reactive oxygen species (ROS) play crucial roles in biological systems [1]. They are constantly generated in cells and their concentrations (in nM range) are rigorously regulated via multiple mechanisms. It was also revealed that many of the radical species are involved in cell signaling [2] and can be used by the immune system to fight invading organisms [3]. However, due to their highly reactive nature, radicals can induce irreversible modification of biomolecules and lead to cell death when their local concentrations are too high [4]. Cysteine, a sulfur-containing amino acid, is known to be very susceptible to radical attack [5]. Cysteine-containing peptides such as glutathione (in disulfide reduced or oxidized forms) function as redox buffers and antioxidants to maintain cell redox homeostasis [6,7].

Cysteine sulfinyl radical (^{SO}•Cys) has been detected as an intermediate during the inactivation of enzymes (i.e., pyruvate formate lyase) utilizing the glycyl/thiyl radical in their catalytic functions upon exposure to air [8]. Detailed knowledge of cysteine sulfinyl radical in protein systems such as their reactivity and structure is very limited from solution-phase studies partially owing to the transient nature and low concentration of bio-radicals under physiological conditions. Carefully examining the gas-phase chemistry of bio-radicals can provide experimental evidence of their intrinsic chemical properties; this information will also provide mechanistic insight of the fate of protein radical species including intra- or inter-molecular radical transfer after the initial formation. The formation of site-specific peptide sulfinyl radical ions in the gas phase has been reported by our group based on radical reactions in the nanoelectrospray ionization (nanoESI) plume and therefore allowed its fundamental chemical properties to be investigated in the gas phase in detail [9,10]. Gas-phase ion/molecule reactions have been used by many research groups as a means to study peptide radical reactivity, structure, and migration [11–17]. The reactivity of peptide cysteine sulfinyl radical ions with organic disulfides, thiols, and molecular oxygen in a linear ion trap mass spectrometer was investigated via ion/molecule reactions recently [18]. The sulfinyl radical appeared to be the least reactive relative to the thiyl and perthiyl radical, which agreed with studies in the condensed phase [19–21]. The low reactivity was in-part due to the delocalization of the spin between the sulfur and oxygen atom within ^{SO}•Cys as shown by theoretical calculations [22]. This

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chemical property of ^{SO}•Cys makes it difficult to further characterize its reaction products due to low reaction yields (typically <1%) and expand the scope of investigation to other reactions.

Note that the reaction yield of gas-phase ion/molecule reactions is affected by several factors including (1) collision rate constant ($k_{collision} \sim 10^{-9}$ cm^3/(molecule s)) [23–27], (2) the number density of neutral reagents, (3) inherent reaction efficiency (the fraction of collisions leading to reaction), and (4) reaction time (ms to s). For a reaction with inherent "low reactivity", increasing the partial pressure of the neutral reagent (to increase the number density) or reaction time will help, however, only to a limited degree. We recently utilized a new approach, "intra-molecular reactions", as an alternative means to study reactions having inherent low reactivity. The two functional groups intended for reaction are placed within the same peptide ion and collisional activation is used to overcome energy barriers associated with reactions and peptide conformation changes. In such a system, the chance of the two functional groups interacting is determined by the vibrational frequency of the peptide scaffold. This frequency is independent of the collisional rate constant in traditional ion/molecule reactions, and can be tuned to a much larger magnitude due to vibrational excitation of the ion from collisional activation. We have successfully applied this approach to probe the reaction between cysteine sulfinyl radical and free thiol. When sulfinyl radical was allowed to react with free thiol using the intra-molecular reaction approach, a new reaction channel, sulfinyl exchange with thiol was observed (Peptide-SO• + RS—H → Peptide-S—H + RSO•) [28]. This reaction channel required high activation energy (such as in beam-type CID) and was absent from ion/molecule reactions performed in a linear ion trap.

Previous ion/molecule reaction studies showed that peptide sulfinyl radical (Peptide-SO•) ions did react with a disulfide bond (RS—SR) although with a very low yield (<0.2%, reaction time: 3–10 s, trap pressure: 6 mTorr) [18]. The detected reaction proceeded via alkyl sulfide (RS—) abstraction by sulfinyl radical leading to the formation of thiyl radical and alkylthio-sulfinyl (S—O—SR) (Reaction (1)). An alternative reaction pathway, Reaction (2), was not observed which would involve peptide sulfinyl radical exchange with a disulfide bond, causing the formation of a new disulfide bond and alkyl sulfinyl radical. This reaction should be thermally neutral; however, it might require overcoming a significant energy barrier and was therefore not observed under conventional ion/molecule reaction conditions. If energetic collisions are applied to overcome the associated reaction energy barrier [29], this reaction could be observed.

$$\text{Peptide-SO•} + \text{RS—SR} \rightarrow \text{Peptide-SO—SR} + \text{RS•} \quad (1)$$

$$\text{Peptide-SO•} + \text{RS—SR} \rightarrow \text{Peptide-S—SR} + \text{RSO•} \quad (2)$$

In this study, intra-molecular reactions aided by collisional activation were further utilized to study the reactions between cysteine sulfinyl radical and a disulfide bond (both intra and interchain). Experimental results obtained from model peptide systems which contained one disulfide bond and one cysteine sulfinyl radical all showed that both Reactions (1) and (2) happened upon collisional activation of the peptide ions. These two types of intramolecular reactions of cysteine sulfinyl radicals induced disulfide bond opening or scrambling within a peptide ion system. As a consequence, collisional activation of peptide disulfide regio-isomers consisting of ^{SO}•Cys led to enhanced sequence information from backbone regions covered by a disulfide bond; however the disulfide bond linkage patterns could not be accurately assigned.

2. Experimental

2.1. Materials

The peptides studied herein are listed in Table 1 with their single letter sequences and corresponding disulfide bond connecting patterns also indicated. The precursor for the **P1** peptide (^{1}CAEK^{5}CIEK^{9}CLVR^{13}C, C1-C13 and C5–C9 disulfide bonds) was synthesized by SynBioSci (San Francisco, CA). Selectin binding peptide (^{1}CIELLQAR^{9}C, C1–C9 disulfide bond) was purchased from AnaSpec (Freemont, CA) and was used as the precursor for **P3** peptide. Fully reduced **P4** peptide (single letter sequence: CARICAKLCLEVCK) was purchased from CPC Scientific (Sunnyvale, CA). Thermolysin, dichlorobis(ethylenediamine)platinum ([Pt(en)$_2$Cl$_2$]) and *N*-acetyl-L-cysteine methyl ester (Cys**) were purchased from Sigma–Aldrich (St. Louis, MO). **P4-II** was used as the precursor for the **P2** peptide.

2.2. Synthesis of disulfide bonds within peptides

The oxidizing agent [Pt(en)$_2$(OH)$_2$Cl$_2$] was synthesized from [Pt(en)$_2$Cl$_2$] in-house following the procedure described by Heneghan et al. [30]. [Pt(en)$_2$(OH)$_2$Cl$_2$] was added to the dissolved peptide (1.0 mg/mL) in a molar ratio of 2:1–5:1 Pt(IV) to the peptide. The reaction was allowed to proceed at room temperature for 1–3 h and the reaction progress was monitored via MS. Following complete oxidation, the peptide disulfide bond isomers were separated via reverse phase-high performance liquid

Table 1
List of cysteinyl peptides studied herein.[a]

Peptide	Structure	Peptide	Structure
P1	Chain IEKC A CAEKC B LVRC C	P1N-SO•	Chain IEKC A (^{SO}•C)AEKC B
		P1C-SO•	Chain CAEK(^{SO}•C) B LVRC C
P2	Chain CAR A ICAKLCLEVCK B	P2C-SO•	Chain ICAKLCLEV(^{SO}•C)K B
P3	Chain CIELLQARC A Cys** B	P3N-SO•	Chain (^{SO}•C)IELLQARC A Cys** B
P4	CARICAKLCLEVCK	P4-I	CARICAKLCLEVCK
		P4-II	CARICAKLCLEVCK
		P4-III	CARICAKLCLEVCK

[a] The peptides are indicated with single letter sequences. The connection line between two "C"s within a peptide represents a disulfide linkage. The symbol, (^{SO}•C), stands for cysteine sulfinyl radical. *N*-acetyl-L-cysteine methyl ester is abbreviated as Cys**.

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chromatography (RP-HPLC). The **P3** peptide was synthesized by reacting reduced selectin binding peptide (0.5 mg/mL) with reduced *N*-acyl-L-cysteine methyl ester (0.5 mg/mL) in a 1:2 molar ratio. The reaction was air oxidized overnight and separated via RP-HPLC.

2.3. Enzymatic digestion

Peptic digests for the synthesis of **P1** (from **P1** precursor) and **P2** (from **P4-II**) peptides were obtained by reacting the oxidized peptides with thermolysin in a 10:1 molar ratio (peptide: thermolsyin) in a 100 mM Tris–HCl buffer (pH 6.5) at 65–70 °C for 30 min [31]. Peptic digests were separated via RP-HPLC as described below.

2.4. RP-HPLC

All peptides were separated using an Agilent 1200 series RP-HPLC System (Agilent Technologies, Santa Clara, CA) with a Zobrax C18 column. The **P4** peptide disulfide bond regio-isomers were separated at a flow rate of 0.65 mL/min with a linear gradient of 20–35% solvent B over 30 min. Solvent A was a mixture of 0.1% TFA in water and solvent B contained 0.085% TFA in 100% CH_3CN (acetonitrile). The **P3** peptide was separated via a stepwise gradient. An isocratic gradient (25–25% B) was used from 0–10 min followed by 25–27% B from 11–45 min at a flow rate of 0.5 mL/min. The eluent was detected at a wavelength of 206 nm. The collected eluent was vacuum dried overnight using a centrivap concentrator (Labconco, Kansas City, MO).

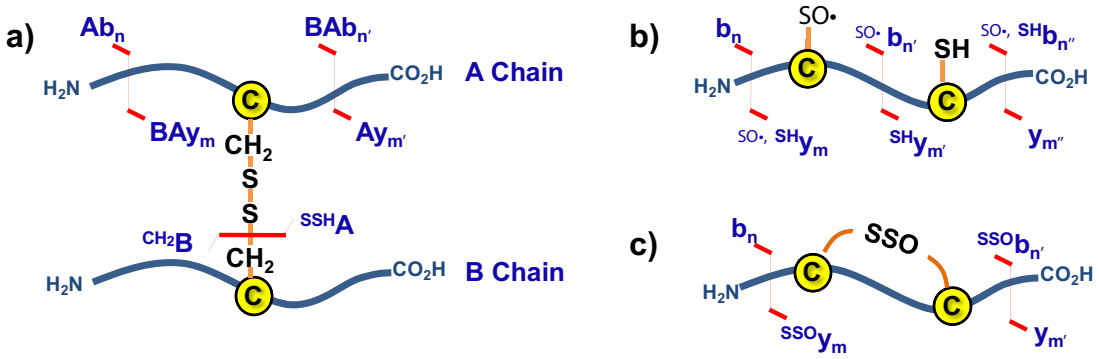
2.5. Radical reactions in nanoESI plume and mass spectrometry

A schematic of the experimental setup is shown in the Supporting Information (Scheme S1). The experimental setup has been described in detail before [32]. Briefly, peptide solutions for nanoelectrospray ionization (nanoESI) [33] were prepared at a concentration of 10 μM in 50/49/1.0 of water/methanol/acetic acid (v/v/v). The nanoESI tips were pulled from borosilicate glass capillaries (1.5 mm o.d. and 0.86 mm i.d.) using a Model P-1000 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA). The nanoESI emitter was inserted into the main arm of a T-shaped tube and was kept in line with the inlet of the mass spectrometer at a distance of 8–10 mm. A low temperature helium plasma was initiated in the side arm of the T-shape tube to form hydroxyl radicals as described previously [34]. The nanoESI plume of peptides was allowed to interact with hydroxyl radicals and the products formed in situ were analyzed on-line by mass spectrometry.

All experiments were carried out on a 4000 QTRAP tandem mass spectrometer (Applied Biosystems/SCIEX, Toronto, Canada). The triple quadrupole/linear ion trap configuration allows for two types of collisional activation methods, i.e., beam-type CID and ion trap CID. In beam-type CID, the precursor ions were isolated by Q1 and accelerated to q2 for collisional activation. The collision energy (CE), defined by the DC potential difference between Q0 and q2, was optimized and typically within the range of 5–10 V. Ion trap CID was carried out in Q3 linear ion trap, where a dipolar excitation was used for on-resonance collisional activation. The activation amplitudes were within the range of 20–60 mV and activation time of 200 ms was used. The characteristic parameters of the mass spectrometer during this study were set as follows: spray voltage, 1200–1800 V; curtain gas, 10 psi and declustering potential, 20 V. Mass analysis was achieved by using Q3 as a linear ion trap at a scan rate of 1000 Da/s. Data acquisition, processing, and instrument control were performed using Analyst 1.5 software.

2.6. Nomenclature

When disulfide linked peptide ions are subjected to collisional activation, cleavage can happen at the amide bond or the C—S bond within a disulfide linkage. The nomenclature of fragment ions resulting from the two types of cleavages are indicated in Scheme 1a using an interchain linked disulfide peptide as an example. An ion labeled as BAb_n or BAy_m indicates that the ion has an intact B chain disulfide linked to the fragment of A chain due to an amide cleavage. The C—S bond cleavage leads to the separation of the two peptide chains (A and B) and the formation of disulfohydryl and dehydroalanine at the cleavage site. These two complimentary ions are represented with superscripts "SSH" and "CH_2" to each chain, respectively, e.g., ^{SSH}A and $^{CH2}BCH_2$. The same rules apply for peptides consisting of intrachain disulfide bonds. However, for amide fragments containing an intact disulfide bond, labels such as $^{SS}y_n$ or $^{SS}b_n$ are used. When disulfide linked peptides are subjected to reactions with OH radicals, the OH radical cleaves the disulfide bond, forming a sulfinyl radical (—SO•) and a sulfhydryl (—SH) at the cleavage site (a representative structure is shown in Scheme 1b). Collisional activation of such peptide sulfinyl radical ions can lead to amide bond cleavages with the preservation of sulfinyl radical to the cysteine residue. Fragment ions containing the sulfinyl radical are symbolized with SO• in the superscript, e.g., $^{SO•}b_n$ or $^{SO•}y_m$, while $^{SH}y_m$ and $^{SH}b_n$ stand for the



Scheme 1. Nomenclature of fragments from cysteinyl peptide ions (charges are not indicated). (a) A peptide consisting of an interchain disulfide bond, (b) a peptide sulfinyl radical resulting from the reaction of an intrachain linked disulfide peptide with OH radical, and (c) a peptide consisting of thiosulfinyl (—SSO—) between two cysteine residues.

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complementary fragments which contain a free thiol (—SH) at the other cysteine residue. Fragments consisting of both —SO• and —SH are indicated as $^{SO\bullet,SH}b_n$ or $^{SO\bullet,SH}y_m$. Collisional activation of peptide sulfinyl radial ions can promote intramolecular reactions between the sulfinyl radical and a disulfide bond. Thiosulfinyl (—SSO—) can be formed between two cysteine residues from such process (a representative structure is shown in Scheme 1c). Amide bond cleavage outside the thiosulfinyl loop leads to the formation of $^{SSO}b_n$ or $^{SSO}y_m$ fragments.

3. Results and discussion

3.1. Reactions of —SO• and S—S in an interchain disulfide peptide system

Peptides that vary in length, sequence and disulfide connectivity were tested (structures listed in Table 1) to investigate the reaction between the sulfinyl radical and a disulfide bond. The formation of site-specific cysteine sulfinyl radicals from disulfide linked peptides has been described elsewhere [9,10]. **P1** peptide is used as an example to illustrate such peptide sulfinyl radical ion formation and the subsequent CID in Fig. 1a. **P1** peptide (having three chains: A–B–C) was subjected to reactions with OH radicals in the nanoESI plume and either disulfide bond could be cleaved forming the sulfinyl radical at the cleavage site. Upon cleavage of the disulfide bond between chains B and C, a sulfinyl radical was formed at the N-terminal cysteine of B chain (**P1N-SO•**). The MS^1 spectra of **P1** peptide before and after radical reaction can be found in the Supporting Information (Fig. S1). This newly formed peptide sulfinyl radical ion (m/z 529.3, 2+) was mass selected and activated via beam-type CID (CE: 5 V, Fig. 1b). For clarity, all the peaks corresponding to the intra-molecular reaction products (Reaction (1) or (2)) are colored in red. Low energy collisional activation of this ion led to limited fragmentation channels corresponding to the separation of two chains (m/z 490.3, 507.3, 551.2, 567.2) and water loss (m/z 520.2). Fragment ion at m/z 567.2 has a +16 Da mass shift relative to the singly protonated B chain ($^{S\bullet}$CAEK $^{S\bullet}$C) and it is assigned as the structure containing an oxygen inserted into the intrachain disulfide bond. Its complementary fragment ion, A chain thiyl radical ion (IEK $^{S\bullet}$C), should appear at m/z 491.4. This ion is present with relative low intensity as it might undergo sequential loss of H atom (m/z 490.3) or other radical induced fragmentation channels. These two fragment ions are consistent with Reaction (1), in which the sulfinyl radical reacts with a disulfide bond by abstracting a sulfur atom.

The ion at m/z 551.2 has no mass shift relative to the singly protonated B chain ($[^{S\bullet}CAEK^{S\bullet}C+H]^+$, m/z 551.2), indicating formation of a disulfide bond within the peptide chain since it is unlikely to have two thiyl radicals within the structure. Indeed, the most abundant fragment from MS^3 CID of m/z 551.2 (Fig. 1c) is the internal loss of a lysine residue (-K), corroborating that the ion has a cyclic structure. The sequential C—S bond cleavage and peptide amide bond cleavage produce fragment ions at m/z 294.0, 336.1, 464.2 and thus further support the assignment of disulfide bond formation between the first and last cysteinyl residues. The

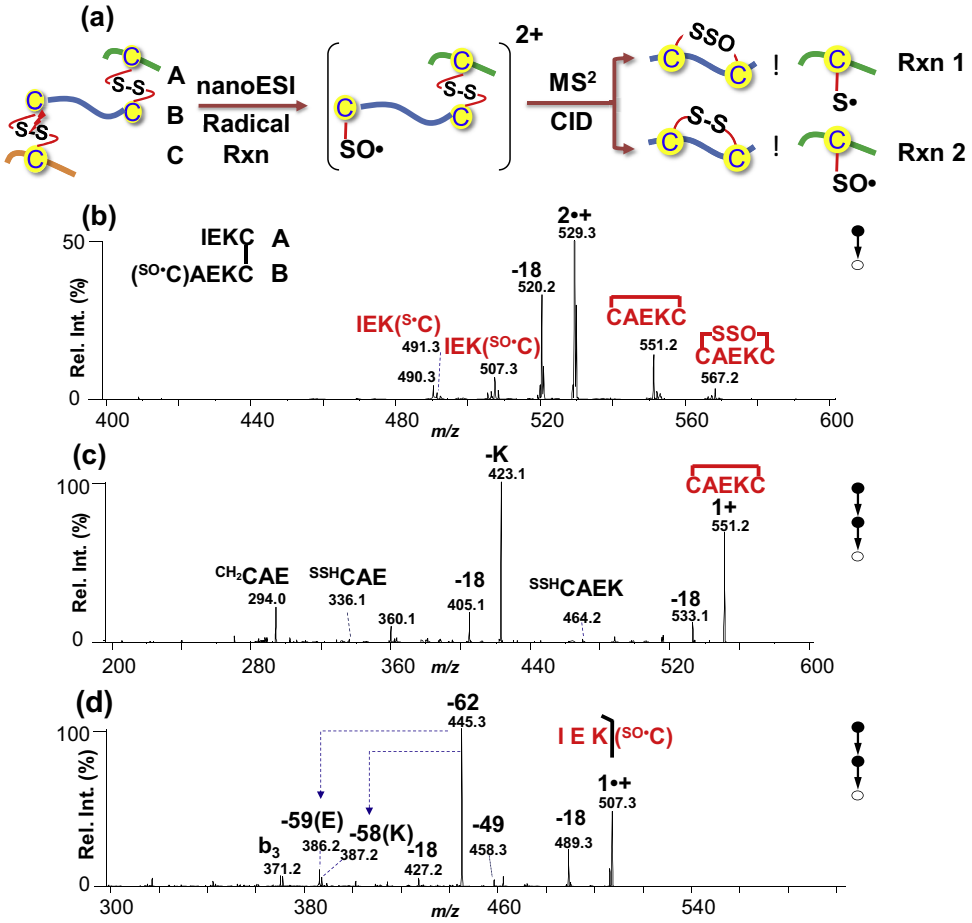

Fig. 1. (a) Method of generating **P1N-SO•** from radical reactions of **P1** and intramolecular reactions (Reactions (1) and (2)) resulting from subsequent collisional activation. (b) Beam-type CID of **P1N-SO•** (m/z 529.3, CE 5 V). (c) MS^3 CID of m/z 551.3 (activation energy 40 mV, activation time 200 ms). (d) MS^3 CID of m/z 507.3 (activation energy 40 mV, activation time 200 ms).

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cleavage at the C—S bond within a disulfide linkage leading to the formation of dehydroalanine at one cysteine and disulfohydryl at the other cysteine has been frequently observed for disulfide peptide ions under collisional activation conditions [35,36].

The fragment ion at m/z 507.3 is the A chain (IEK ($^{SO\bullet}$C)) containing the sulfinyl radical based on its MS^3 CID (Fig. 1d). The spectrum is dominated by the neutral loss of 62 Da (CH_2SO), a signature loss fragmentation channel for the sulfinyl radical ion, giving rise to a glycyl radical ion [9,22,32]. Radical-directed fragmentation of the thus formed glycyl radical ion leads to sequential losses of 59 Da (from glutamic acid side chain) and 58 Da (from lysine side chain) [9]. There is also a small degree of 49 Da (HSO) loss, which is another signature loss of sulfinyl radical. A small sequence ion, b_3, is also present. Its m/z 371.2 corresponds to the unmodified sequence (IEK) and thus suggests the sulfinyl radial is located at the C-terminus cysteine residue. It is worth noting that m/z 507.3 (IEK ($^{SO\bullet}$C)) and 551.2 ($\overline{\text{CAEKC}}$) product ions are complementary fragments from CID of **P1N-SO•**. A logical pathway of forming these products has to go through intra-molecular reaction between the —SO• and the S—S bond via Reaction (2), in which the exchange of —SO• with S—S leads to the formation of a new sulfinyl radical and a new disulfide bond. The MS^2 and MS^3 CID data of **P1N-SO•** (Fig. 1) clearly demonstrated the existence of two intra-molecular reaction pathways between sulfinyl radical and a disulfide bond. We also investigated CID of **P1C-SO•**; the same intra-molecular reaction phenomenon was observed with the aid of collisional activation. The data can be found in Fig. S2, Supporting Information. These results are of significance since sulfinyl radical exchange with a disulfide bond (Reaction (2)) is a new reaction channel only revealed by intra-molecular reactions with the assistance of collisional activation.

3.2. Reactions of —SO• and S—S in an intrachain disulfide peptide system

To determine whether the type of disulfide bond, i.e., interchain vs. intrachain, played a role in the intra-molecular reactions observed, the **P2** peptide was selected for studying reactions between —SO• and an intrachain disulfide bond. Fig. 2a outlines the method of forming **P2C-SO•** ions from radical reactions of **P2** peptide and subsequent CID experiments. **P2C-SO•** has a sequence of $I^2CAKL^6CLEV^{10}CK$, in which a disulfide bond is formed between C2 and C6 and a sulfinyl radical is formed at C10. In order to detect intra-molecular reactions, **P2C-SO•** (m/z 618.4, 2+) was subjected to collisional activation (Fig. 2b, ion trap CID, activation energy 60 mV for 200 ms). Several fragments belonging to the original structure of **P2C-SO•** (i.e., no sulfinyl radical transfer) are present such as sequence ions b_{6-9} and the internal loss of both lysine and leucine (-KL, m/z 994.4). We also observed b/y-types of fragment ions indicating the change of the original ion structure due to reactions of the C10 sulfinyl radical and the C2–C6 disulfide bond. For instance, y-type fragment ions (m/z 692.3, 805.4, 933.4) containing an intrachain disulfide bond between C6 and C10 were detected. Fragments due to mass shift of +16 Da relative to the intact b ions from the original structure were observed at m/z 646.2 and 759.4.

MS^3 CID was used to further verify the identities of these product ions. Fig. 2c shows the MS^3 CID of m/z 692.3, which has a

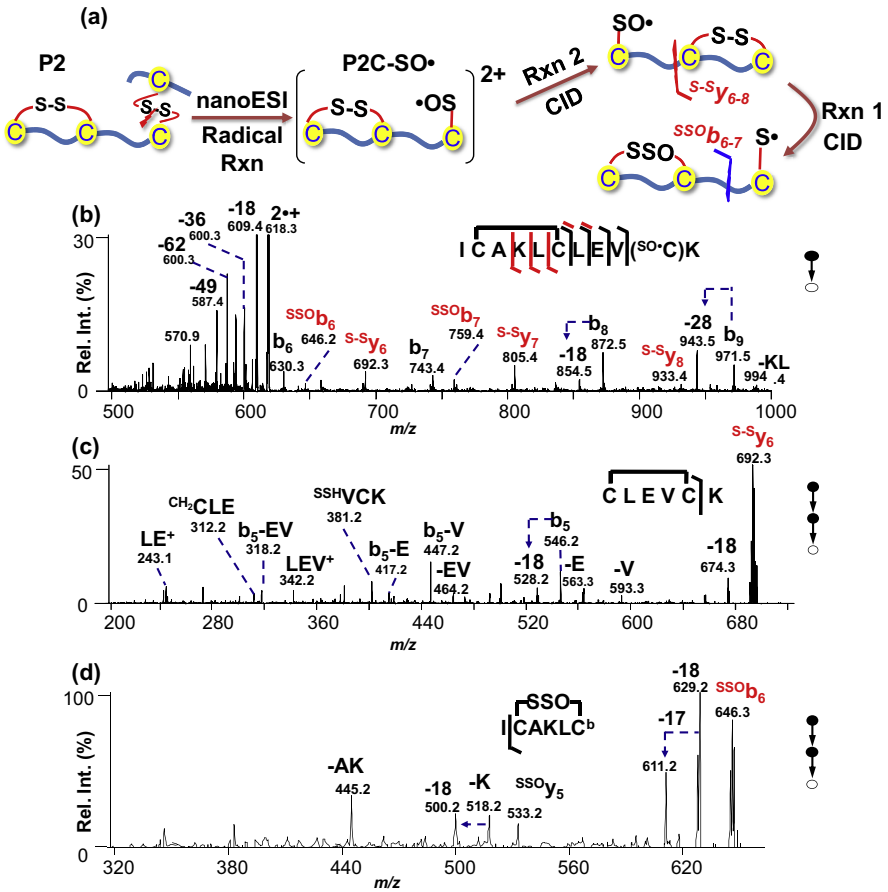

Fig. 2. (a) Method of generating **P2C-SO•** from radical reactions of **P2** and intramolecular reactions (Reactions (1) and (2)) resulting from subsequent collisional activation. (b) Ion trap CID of the site-specific sulfinyl radical **P2C-SO•** peptide (m/z 618.4, 2+, activation energy 60 mV and 200 ms activation time). (c) MS^3 CID of $^{S-S}y_6$ (m/z 692.3, activation energy 40 mV and 200 ms activation time). (d) MS^3 CID of $^{SSO}b_6$ (m/z 646.3, activation energy 33 mV and 200 ms activation time.

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mass match to y_6 consisting of a disulfide bond (^{1}CLEV^{5}CK, C1–C5 disulfide bond), denoted as $^{SS}y_6$. The loss of internal amino acid residue(s) (-V, E,-EV) is consistent with the presence of a cyclic structure. The only detected sequence ion, b_5, also has a cyclic structure evidenced by its internal loss of valine (b_5-V, *m/z* 447.2) and glutamic acid (b_5-E, *m/z* 417.2). Those ions all support the structural assignment of $^{SS}y_6$. Similar methods were applied to *m/z* 805.4 and 933.4, and those ions were assigned as $^{SS}y_7$ and $^{SS}y_8$, respectively. The ion at *m/z* 646.3 (labeled as $^{SSO}b_6$ in Fig. 2b) has a +16 Da mass shift relative to b_6 (sequence: I^{2}CAKL^{6}C, C2–C6 disulfide bond). MS^3 CID of this ion is shown in Fig. 2d. The internal losses (-K, -AK) indicate that the ion still has a cyclic structure. The only sequence ion observed is $^{SSO}y_5$, which occurs outside of the cyclic backbone region and contains the +16 Da mass shift. Since no fragment ions corresponding to sulfinyl radical (i.e., loss of 62 or 49 Da) are observed, this ion should be an even electron species and the addition of +16 Da likely occurs at the disulfide bridge.

The possible pathways of intramolecular reactions and subsequent fragmentation leading to $^{SS}y_{6-8}$ and $^{SSO}b_{6,7}$ ions are suggested in Fig. 2a. Reaction (2) leads to the formation of a new disulfide bond between C6 and C10, transferring sulfinyl radical to C2 residue. Amide bond cleavages outside the C6–C10 disulfide loop produce $^{SS}y_{6-8}$ ions. This surviving Reaction (2) product can further undergo Reaction (1), transforming the original disulfide bond within **P2C-SO•** to the thiosulfinyl structure. The amide bond cleavages outside the thiosulfinyl loop give rise to $^{SSO}b_{6,7}$ ions. As evidenced by the data in Fig. 2, both types of intramolecular reactions are detected between sulfinyl radical and an intrachain disulfide bond.

3.3. Effect of ion charge state on reactions of —SO• and S—S

The **P3** peptide was used for formation of peptide sulfinyl radical ions (**P3C-SO•**, structure shown in Fig. 3a) with different charge states so that the effect of the charge state on intra-molecular reactions could be investigated. The CID for the doubly charged **P3C-SO•** is shown in Fig. 3a. In this charge state where a mobile proton is present, b/y-type sequence ions dominate the spectrum. The presence of sequence ions $BAy_{1-5,7}$ and $^{SO•}b_{4-8}$ is consistent with the original ion structure of **P3N-SO•**, i.e., sulfinyl radical is located at the C-terminal cysteine of A chain. However, the fragment ions labeled in red, i.e., $^{SO•}y_{2-4,6,7}$ and $BAb_{2-5,7,8}$, could only have resulted from sulfinyl radical exchange with a disulfide bond (Reaction (2)), leading to a new structure with B chain disulfide linked to the N-terminal cysteine of A chain and sulfinyl radical located at the C-terminal cysteine of A chain (**P3C-SO•**, structure indicated in Fig. 3a inset). Depending on the relative orientation of sulfinyl radical and a disulfide bond, Reaction (2) can also lead to sulfinyl radical transfer to B chain and a new disulfide bond being formed within A chain (^{1}CIELLQAR^{9}C, C1–C9 disulfide bond). Indeed, the peak at *m/z* 1046.6 corresponds to A chain consisting of a disulfide bond, although at relatively low abundance. Its complementary reaction product, B chain sulfinyl radical ion, was not detected likely due to the low mass cutoff in ion trap CID.

MS^2 CID of the singly charged **P3N-SO•** (Fig. 3b) shows a very different fragmentation pattern as compared to the 2+ charge stage. Since no mobile proton is present in this ion, the peptide amide bond cleavages are largely suppressed and other reaction/fragmentation channels become more competitive. For instance, the peak at *m/z* 1046.6 (A chain with an intrachain disulfide bond), resulting from sulfinyl radical exchange with a disulfide bond, is a prominent channel as compared to that observed from CID of 2+ ions. There is no direct evidence of forming **P3C-SO•** from Reaction (2) since the new structure is isomeric to the parent ion (**P3N-SO•**) and no sequence ions are generated from 1+ charge state. Other sulfinyl radical associated fragmentation channels are observed, including losses of 49 Da (SOH) and 62 Da (CH_2SO). The 62 Da loss leaves a carbon centered glycyl radical at the original $^{SO•}$Cys site, which can further attack the interchain disulfide bond leading to the formation of *m/z* 1032.6. The neutral loss of 15 Da peak might come from chemical interferences having close *m/z* to **P3N-SO•** upon radical ion formation. Since sulfinyl radical exchange with the disulfide bond was observed from the singly charged as well as

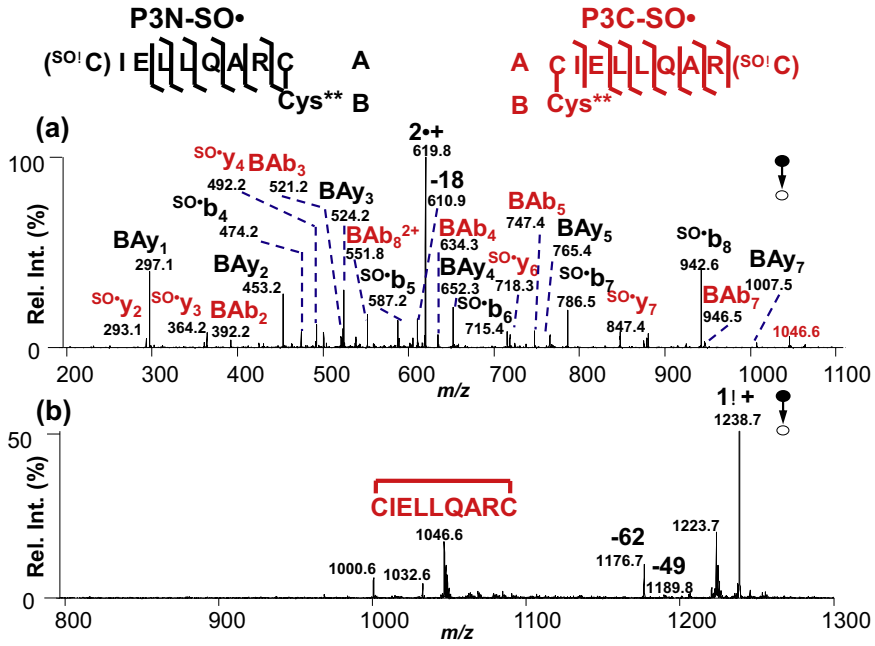

Fig. 3. (a) Ion trap CID of doubly charged **P3N-SO•** peptide (*m/z* 619.8, activation energy 20 mV and 200 ms activation time). (b) Ion trap CID of the singly charged **P3N-SO•** (*m/z* 1238.7 activation energy 40 mV and 200 ms activation time).

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the doubly charged ion, it is safe to assume that ion charge state did not play a significant role in affecting the sulfinyl radical reactivity.

3.4. Implications in the analysis of disulfide linked peptide regio-isomers

Formation of disulfide bonds is one of the most common post translation modifications and plays a vital role in stabilizing the three-dimensional structure and function of many proteins and naturally occurring peptides [37,38]. In many naturally occurring peptides, only one specific disulfide bond bridging pattern makes the peptide biologically active, although theoretically there are many ways of connecting disulfide bonds [39]. Pinpointing the disulfide bond connectivity in systems containing multiple disulfide bonds is crucial in understanding the structure and function of those peptides. We therefore purposely introduced $^{SO\bullet}$Cys to peptide disulfide regio-isomer systems to test how radical reactivity would affect the extent of structural information that could be obtained from CID conditions. Three disulfide bond regio-isomers were obtained from forming two disulfide bonds within the **P4** peptide (CARICAKLCLEVCK) due to the existence of four cysteine residues. The specific disulfide bond connecting patterns are shown in Table 1. The oxidized **P4** peptide contains two disulfide bonds; therefore, products resulting from OH radical reaction at one or two disulfide bonds can be both observed. The reaction product (m/z 522.7, 3+), corresponding to the cleavage of one disulfide bond can be readily isolated for CID since it has a distinct mass shift of +17 Da (OH) relative to the intact peptide ion (m/z 517.0, 3+). It is worth noting that the radical ions formed this way are a mixture of sulfinyl radicals at different cysteine residue locations due to the lack of selectivity of the radical reaction toward a specific disulfide bond. The CID data of the cysteine sulfinyl radical ions formed from **P4** disulfide bond regio-isomers are summarized in Fig. 4. Ion trap CID of the sulfinyl radical ions (m/z 522.7, 3+, Fig. 4a) from **P4-I**, which has a C1–C5 and C9–C13 disulfide bond connecting pattern, produces fragments b_{5-8}, y_{6-7} and b_{13}^{3+} from backbone region outside of the disulfide loops. These ions do not contain any mass shift due to radical reactions (adding an oxygen and a hydrogen to a disulfide bond) and are also present in the CID of protonated disulfide intact **P4-I** ions as discussed in detail elsewhere [35]. The signature neutral loss of 62 Da (CH_2SO) from sulfinyl radical is also present, which can undergo another neutral loss of 46 Da (SCH_2) to give rise to the 108 Da loss [32]. Peptide backbone fragments consisting of the radical reaction modified cysteinyl residue(s) are also observed, including $^{SH}y_{3,5}$, $^{SO\bullet,SH}b_{6,7}$, $^{SO\bullet,SH}y_{6,7}$. The superscripts of "SH" and "SO•" indicate the mass increase due to the addition of an H (+1 Da) or both O and H (+17 Da) relative to the original structure of **P4-I**. The observation of $^{SH}y_{3,5}$ from the backbone region which is originally covered by a disulfide bond (C9–C13) proves that this disulfide bond is cleaved from the radical reactions. The presence of $^{SO\bullet,SH}b_{6,7}$ and $^{SO\bullet,SH}y_{6,7}$ ions indicates the addition of OH to either disulfide bond (C1–C5 or C9–C13).

Collisional activation of sulfinyl radical ions with the other two disulfide regio-isomers, **P4-II** (an overlapped disulfide connecting pattern, C1–C13 and C5–C9) and **P4-III** (an intertwined disulfide connecting pattern, C1–C9 and C5–C13) are shown in Fig. 4b and c, respectively. The two CID spectra are almost identical to each other and only show a small difference to Fig. 4a in terms of the ion identity and ion relative intensity. Peptide amide bond fragments from one disulfide bond covered region are observed including $^{SO\bullet}b_3$, $^{SH}y_3$, $^{SH}y_5$ and $^{SO\bullet,SH}y_6$. These ions are consistent from cleavage of one disulfide bond from radical reactions. However, it is interesting that backbone fragments $^{SS}b_{5-8}$ and $^{SS}y_{6-7}$ which are derived from the backbone region originally covered by two disulfide bonds (from C5 to C9 in **P4-II** and **III**) are also present.

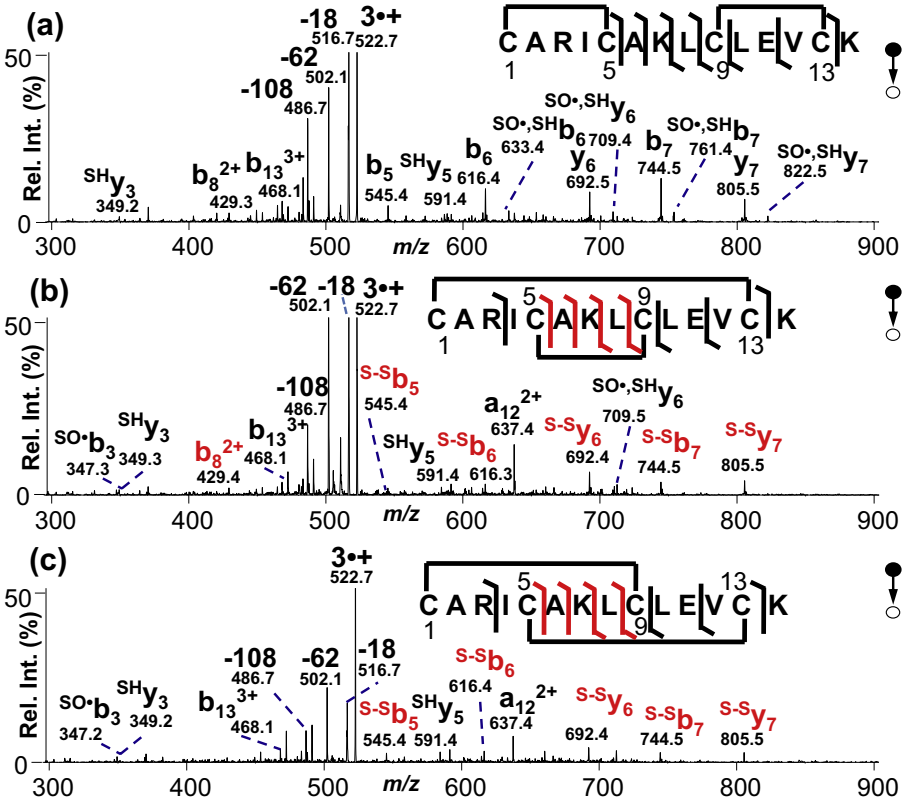

Fig. 4. Ion trap CID of triply protonated sulfinyl radical ions (m/z 522.7, 3+, activation energy 40 mV and 200 ms activation time) derived from radical reactions of peptide disulfide regio-isomers (a) **P4-I**, (b) **P4-II** and (c) **P4-III**.

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Note that $^{SS}b_{5-8}$ and $^{SS}y_{6-7}$ ions have the same mass as those detected from **P4-I** and they have one disulfide bond. The only possible way of forming these fragment ions is through intra-molecular sulfinyl radical exchange with a disulfide bond as discussed earlier. It is also because of intra-molecular radical transfer reaction that the fragmentation patterns of the three disulfide regio-isomers are very similar to each other. On one side, this results in the difficulty in distinguishing disulfide connecting patterns directly from CID of these sulfinyl radical ions. On the other side, the radical transfer reactions allow more sequence information to be obtained due to the fact that sulfinyl radical reactions open up peptide backbone regions to produce the traditional b- and y-type sequence ions. A similar radical induced disulfide bond opening phenomenon has been observed for the same peptide system (**P4** peptides) using ETD as the dissociation technique [40], indicating the common nature of the disulfide bond related radical chemistry independent of how the peptide radical ions are formed initially.

4. Conclusions

Intra-molecular reactions assisted by collisional activation proved to be an effective means to investigate sulfinyl radical reactivity. A new reaction channel was discovered, involving sulfinyl radical exchange with a disulfide bond, leading to the formation of a new disulfide bond and a new sulfinyl radical within polypeptide ion systems. The identities of the reaction products were confirmed using MS^n CID. This reaction phenomenon was consistently observed in different peptide systems that varied in lengths, amino acid compositions, and disulfide bond connecting properties. Peptide ion charge state did not show significant effect on sulfinyl radical reactivity. When $^{SO\bullet}$Cys was introduced to peptide disulfide regio-isomer systems, the exchange of sulfinyl radical with disulfide bond allowed more sequence information to be obtained; however, the disulfide bond connecting pattern could not be determined due to the radical induced disulfide bond scrambling. The reactivity study of sulfinyl radical might shed light on how a reactive protein radical intermediate attacks neighboring disulfide bonds causing disulfide bond scrambling and ultimately loss of function for a protein. Although we only studied the reactions between sulfinyl radical and a disulfide bond in this report, the intra-molecular reaction approach could be modified to study the reaction between any two functional groups that can be successfully synthesized into the same molecule.

Acknowledgements

Financial support from NSFCHE-1308114 is greatly appreciated. Y.X acknowledges ASMS research awards for supporting research on radical ion chemistry studies. K.L.D would like to thank the Midwest Crossroads Alliance for Graduate Education and the Professoriate (AGEP) and the Purdue doctoral fellowship for support during the course of this research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijms.2014.08.027.

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