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FOCUS: HONORING R. G. COOKS' ELECTION TO THE NATIONAL ACADEMY OF SCIENCES: RESEARCH ARTICLE

Assigning Peptide Disulfide Linkage Pattern Among Regio-Isomers via Methoxy Addition to Disulfide and Tandem Mass Spectrometry

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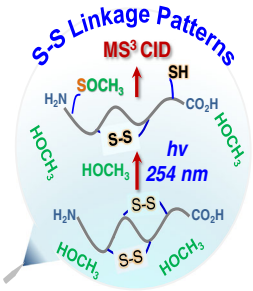

Abstract. Pinpointing disulfide linkage pattern is critical in the characterization of proteins and peptides consisting of multiple disulfide bonds. Herein, we report a method based on coupling online disulfide modification and tandem mass spectrometry (MS/MS) to distinguish peptide disulfide regio-isomers. Such a method relies on a new disulfide bond cleavage reaction in solution, involving methanol as a reactant and 254 nm ultraviolet (UV) irradiation. This reaction leads to selective cleavage of a disulfide bond and formation of sulfenic methyl ester (–$SOCH_3$) at one cysteine residue and a thiol (–SH) at the other. Under low energy collision-induced dissociation (CID), cysteine sulfenic methyl ester motif produces a signature methanol loss (–32 Da), allowing its identification from other possible isomeric structures such as S-hydroxylmethyl (–SCH_2OH) and methyl sulfoxide (–S(O)-CH_3). Since disulfide bond can be selectively cleaved and modified upon methoxy addition, subsequent MS^2 CID of the methoxy addition product provides enhanced sequence coverage as demonstrated by the analysis of bovine insulin. More importantly, this reaction does not induce disulfide scrambling, likely due to the fact that radical intermediates are not involved in the process. An approach based on methoxy addition followed by MS^3 CID has been developed for assigning disulfide linkage patterns in peptide disulfide regio-isomers. This methodology was successfully applied to characterizing peptide systems having two disulfide bonds and three disulfide linkage isomers: side-by-side, overlapped, and looped-within-a-loop configurations.

Keywords: Disulfide peptides, Collision-induced dissociation, Disulfide linkage, Sulfenic methyl ester, UV Photolysis

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Introduction

Formation of disulfide bond between two cysteine amino acid residues offers a possibility to covalently link segments within a polypeptide or among multiple peptides. It is one of the most common post-translational modifications in proteins, serving a vital role in defining and stabilizing three-dimensional structure in peptides and proteins [1, 2]. Peptides with multiple disulfide bonds are often encountered in biological systems, such as conotoxins (a group of neurotoxic peptides), defensins, and cyclotides [3–5]. Knotted disulfide bonds help them maintain a stable folded structure despite relatively small number of amino acid residues. Owing to different connection patterns of disulfide bonds, disulfide-rich peptides can have many disulfide regio-isomers, and usually only one specific isomer is biologically active [5].

Mass spectrometry (MS) has been demonstrated as a powerful tool for the identification and quantitation of peptides and proteins from complex mixtures. Characterization of disulfide-containing peptides requires information of both sequence and disulfide connectivity. The existence of disulfide bond, however, poses challenges in acquiring related information directly from mass spectrometry [6]. For instance, cleavage of disulfide bonds is not generally observed under low energy CID conditions for positively charged ions with mobile protons [6, 7]. This is explained by Lioe et al. from the aspect that higher activation energy is required for disulfide bond cleavage (40–70 kcal/mol) compared with that required for amide bond

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cleavages [8]. As a result, CID of the protonated peptide ions only produces sequence ions that fall outside of the disulfide covered region. To overcome this limitation, multiple methods have been developed with the aim of opening disulfide loops prior to MS analysis. The most commonly used approach is disulfide bond reduction followed by alkylation. This process, as well as electrolytic reduction of disulfide bonds, makes proteins more prone to subsequent enzymatic digestion and, therefore, renders increased sequence coverage in protein digests via MS/MS analysis [9, 10]. Consequently, disulfide bond linkage information is lost in this process. In order to preserve disulfide linkages, multi-enzyme digestion has been utilized to induce protein backbone cleavages within regions covered by one or multiple disulfide bonds. This method can release simple peptides connected by interchain disulfide bonds. Since there is no loop structure in these peptides, MS/MS via CID can be used to extract information on peptide sequences and disulfide bond linkages. It should be noted that delicate control of solution pH is key to avoiding disulfide bond scrambling, which can occur at pH over 8.3, the pKa of the cysteine side chain [11], while achieving relatively high enzyme activity. For highly knotted disulfide structures, partial disulfide bond reduction often needs to be coupled with multi-enzyme digestion. Dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) followed by alkylation have been used in disulfide bond partial reduction [12]. Achieving partial reduction rather than complete reduction, however, requires precise control of reaction conditions and can be a demanding task to accomplish.

In the gas phase different types of dissociation techniques have been developed to facilitate the analysis of disulfide-containing peptides in the last decade. These techniques include CID of deprotonated ions [13], peptide–metal complex (route 66 method) [14], and ultraviolet photodissociation (UVPD, at 157 nm or 266 nm) [15–17]. In electron-based dissociation, EXD, where X represents capture [18], transfer [19–22], or detachment [23], backbone cleavage as well as disulfide bond cleavage can be observed, allowing rich sequence information to be acquired. Radicals are known to be highly reactive towards disulfide bonds; a number of radical approaches have been used recently to cleave disulfide bonds and enhance disulfide peptide analysis [24]. These include distonic ion reaction approach [25], CID of TEMPO peptides [26], reactions with hydroxyl radical [27], sulfinyl radical [28], and hydroxyl methyl radical [29]. Because radical intermediates are produced in EXD processes and radical reactions, disulfide bond scrambling may happen in a millisecond (ms)-long detection/analysis window [24]. Consequently, the connectivity of multiple disulfide bonds in a peptide may not be accurately determined. For instance, Tan et al. studied ETD of peptide disulfide regio-isomers and found that the isomers all shared identical fragment ions resulting from radical cascades after initial electron transfer. Relative intensities of some fragment ions differed among isomers; however, the small differences could not be solely relied on for disulfide bond connectivity assignment [20].

The above discussed strategies, although have not fully addressed challenges in disulfide peptide/protein characterization, they clearly suggest a route based on either gas-phase or solution reaction to expand the capability of MS in their analysis, as long as the reaction is controllable and does not lead to disulfide rearrangement. Herein, we demonstrate an interesting disulfide bond cleavage pathway involving methanol upon 254 nm UV irradiation in solution. This new reaction results in the formation of sulfenic methyl ester (-S-O-CH_3) and free thiol (-SH) at the pair of separated cysteine residues. Although exact mechanism is not clear, experimental evidence suggests that this reaction does not involve radical intermediates; therefore, it should not lead to disulfide bond scrambling. Based on this new reaction, we have developed an online reaction-MS/MS (via low energy CID) approach to further explore its utility in the analysis of peptide systems containing one or more disulfide bonds.

Experimental

Materials

The sequence and disulfide bond connecting pattern of peptides studied in this work are listed in Table 1. Oxidized glutathione (**P1**), oxidized methionine sulfoxide, *N*-t-butyl phenylnitrone, and bovine insulin (**P5**) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DL-cystine 2, 2′, 3, 3, 3′, 3′-D_6 (**P2**) was purchased from CDN isotopes (Quebec, Canada). Fully reduced guanylin peptide (single letter sequence: PNTCEICAYAACTGC, precursor for **P3)** and fully reduced synthetic peptide (single letter sequence: CNKACGKLCLEVCR, the precursor for **P4**) were purchased from LifeTein LLC (Hillsborough, NJ, USA).

Synthesis of Disulfide Bonds

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 and Bailar [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): peptide. The reaction was allowed to proceed at room temperature for 1–3 hours and the reaction progress was monitored via MS. Following complete oxidation, peptide disulfide bond isomers were separated via reverse phase-high performance liquid chromatography (RP-HPLC). The separation condition is described in Supporting Information (SI), and LC separation chromatogram of **P3** regio-isomers is shown in SI Figure 1 as an example.

Methoxy Addition to Disulfide via 254 nm UV Irradiation and Mass Spectrometry

Reactions were initiated by UV irradiation from a low-pressure mercury (LP-Hg) lamp (20 mA, 2.54 cm lamp length, 0.64 cm diameter, double bore tubing with a synthetic quartz lamp casing) (model no 81-1057-5; BHK, Inc., Ontario, CA). The primary emission from the lamp is 254 nm. Peptides were

Table 1. List of Disulfide Linked Peptides Studied

Peptide	Structure	Peptide	Structure
P1	γECG γECG	P2	Cys-d_3 Cys-d_3
P3-I	PNTCEICAYAACTGC	P4-I	CNKACGKLCLEVCR
P3-II	PNTCEICAYAACTGC	P4-II	CNKACGKLCLEVCR
P3-III	PNTCEICAYAACTGC	P4-III	CNKACGKLCLEVCR
P5	GIVEQCCASVCSLYQLENYCN FVNQHLCGSHLVEALYLVCGERGFFYTPKA		

The peptides are indicated with single letter sequences. The connection line between two "C"s within a peptide represents a disulfide linkage

introduced via nanoelectrospray ionization (nanoESI) in methanol/water (v:v = 1:1) solution. A photo of the reaction setup is shown in SI Figure 2. A similar schematic and description of the experimental setup was published elsewhere [31]. A thin layer of aluminum foil was used to protect bulk part of the solution in tip from over-irradiation, with about 3 mm of taper exposed at the end. All experiments (except for insulin) were carried out on a 4000QTRAP tandem mass spectrometer (Applied Biosystems/SCIEX, Toronto, Canada). The triple quadrupole/linear ion trap configuration allowed 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. All the insulin data were collected using a LTQ-Orbitrap (Thermo Fisher Scientific, San Jose, CA, USA) with a resolution of 30,000 Da. The data shown were typically an average of 50 scans.

Nomenclature

In the context of this paper, peaks with a "SH" as a superscript indicate that the ion contains a free thiol group following disulfide cleavage. Labels containing "#" as a superscript suggests that the ion has been modified by the addition of the methoxy group to form the sulfenic methyl ester. Peaks labeled as "Ay_n" or "Bb_n" mean a sequence ion originating from that particular chain of a polypeptide, whereas in "ABb_n" or BA-y_n", the first letter indicates that the fragment contains either intact A or B chain, and the latter part ("Bb_n" or "Ay_n") refers to amide bond cleavage from either B or A chain. Fragment ions containing an "SSH" superscript represent C–S bond cleavage yielding disulfohydryl at the cleavage site. Its complimentary fragment ion has a dehydroalanine structure and is represented by a superscript of "CH_2".

Results and Discussion

Reaction Phenomena

A simple disulfide-linked peptide containing two identical chains, viz. oxidized glutathione (**P1**), was used to demonstrate the typical reaction phenomena. The neutral peptide chain (γ$E^{S\bullet}$CG) after homolytic cleavage of the disulfide bond has a mass of 306.1 Da and is denoted with the letter A. To initiate the reaction, **P1** peptide solution (10 μM in CH_3OH/H_2O (v/v) = 1:1) was loaded into a pulled borosilicate nanospray tip and subjected to 254 nm UV irradiation from an LP-Hg lamp placed nearby (SI Figure 2) [29]. Beside the intact peptide ions at *m/z* 307.1 ($[M + 2H]^{2+}$), new peaks at *m/z* 308.1 and *m/z* 338.2 showed up as relatively abundant reaction products after irradiation for about 2 min (Figure 1a). This induction time suggests that it takes about 2 min for the reaction to reach a steady-state. Based on accurate mass measurement, the two main reaction products were identified as thiol reduced glutathione ($[^{SH}A + H]^+$) and CH_3O addition product ($[^{SOCH3}A + H]^+$), respectively. The thiol product had a slightly higher relative intensity than the CH_3O addition product (Figure 1a), likely due to its higher chemical stability. In order to determine the source of hydrogen atoms in the added CH_3O moiety, deuterated methanol [CD_3OH/H_2O (v/v) = 1:1)] was employed in nanoESI of **P1** and was subjected to 254 nm UV irradiation.

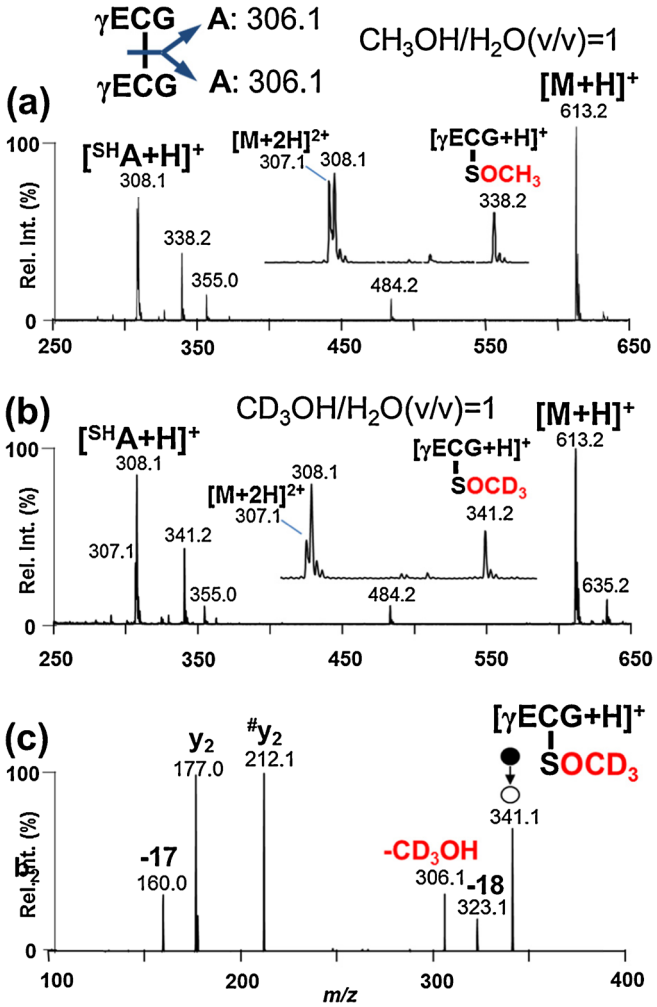


Figure 1. Methoxy addition to **P1** peptide upon 254 nm UV irradiation and product CID. MS^1 reaction spectra: (**a**) in presence of CH_3OH and (**b**) in presence of CD_3OH. (**c**) CID of methoxy addition product (*m/z* 341.2) observed in panel (**b**)

The reaction spectrum (Figure 1b) has two abundant product peaks at *m/z* 308.1 and 341.2. The increase of 3 Da for the peak at *m/z* 341.2 relative to product *m/z* 338.2 observed in CH_3OH experiment (Figure 1a) clearly suggests that (1) methanol is involved in the reaction and (2) the added moiety should be methoxy (OCH_3) rather than hydroxylmethyl (CH_2OH) since all methyl deuterium atoms are preserved. Examination of the isotopic distribution of product at *m/z* 341.2 showed that no H/D exchange happened in the process. MS^2 CID of the deuterated methoxy addition product (*m/z* 341.2) produced 35 Da neutral loss (CD_3OH) as well as y_2 fragments with or without the methoxy modification (Figure 1c). The above set of experiments demonstrates that disulfide bond is cleaved and modified in presence of methanol upon 254 nm UV irradiation, leading to the formation of thiol (-SH) on one cysteine and methoxy addition at the other cysteine residue.

Addition of methoxy (CH_3O) to a sulfur atom can result in three possible isomeric structures: S-hydroxylmethyl (-S-CH_2OH), methyl sulfoxide (-S(O)-CH_3), and sulfenic methyl ester (S-O-CH_3). Our group has recently reported a method of synthesizing and characterizing S-hydroxylmethyl species from disulfide linked peptides at the ESI-MS interface. MS^2 CID of peptide S-hyroxylmethyl ions produces a distinct formaldehyde loss (30 Da), which can be utilized to identify S-hydroxylmethyl group [29]. Methionine oxidation leads to the formation of methyl sulfoxide at its side chain as a common product [32]. Neutral loss of methane sulfenic acid (CH_3SOH, 64 Da) is the dominant fragmentation pathway upon CID of protonated methionine sulfoxide containing peptide ions [33]. This neutral loss has been used to identify sulfoxide containing peptides and locate specific site of oxidized methionine [34, 35]. In terms of cysteine sulfenic methyl ester modification, there is no report on its gas-phase dissociation behavior, likely due to its chemical instability for synthesis and storage. Nevertheless, some insights into the structure of methoxy addition product should be gained by comparing tandem mass spectra of the methoxy addition product to the two known isomeric species (S-hydroxylmethyl and methyl sulfoxide). For the ease of structural identification, D6-cystine (**P2**), the simplest disulfide-linked peptide having deuterium atoms at all alpha- and beta-carbons, was used as a model system. As shown in Figure 2a, MS^2 CID of the methoxy addition product (*m/z* 155.0) resulting from 254 nm UV irradiation gives rise to a distinct neutral loss of 32 Da besides water and ammonia losses. Accurate mass measurement proves that the 32 Da loss corresponds to an elemental composition of CH_4O, viz. methanol. This methanol loss channel was consistently observed in

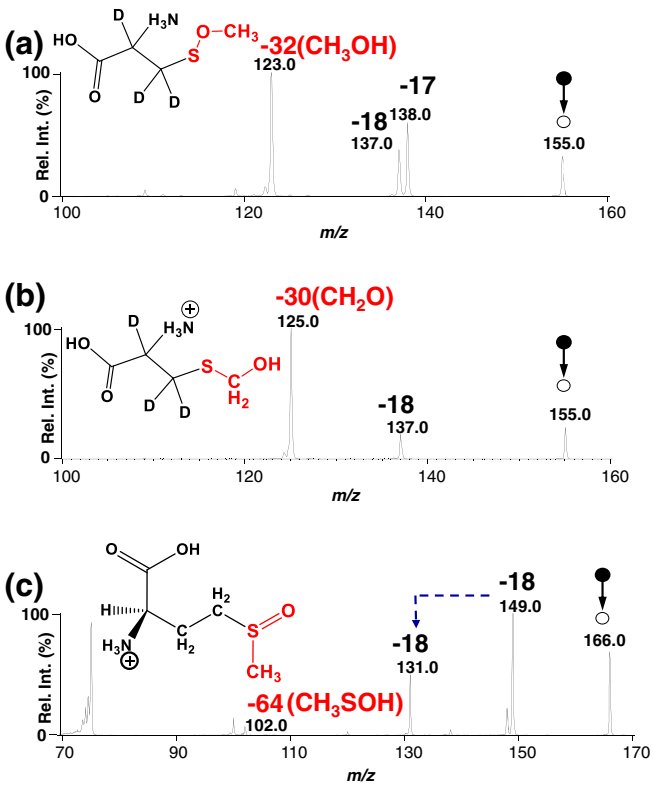
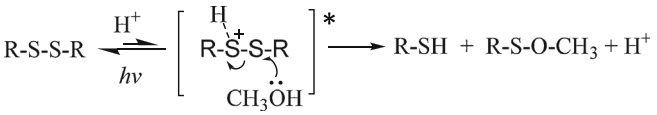


Figure 2. MS^2 CID of (**a**) protonated methoxy addition product (sulfenic methyl ester, *m/z* 155.0) of D_3-cysteine; (**b**) protonated D_3-cysteine S-hydroxylmethyl, *m/z* 155.0; (**c**) protonated methionine sulfoxide, m/z 166.0

peptide systems as well (i.e., glutathione data, Figure 1c). D_3-cysteine S-hydroxylmethyl, an isomer of the above methoxy addition product, was synthesized in situ based on procedures described before using D6-cystine as the precursor [29]. MS^2 CID of protonated D_3-cysteine S-hydroxylmethyl (*m/z* 155.0) produced a characteristic neutral loss of 30 Da (CH_2O, formaldehyde) as expected. On the other hand, CID of protonated methionine sulfoxide (*m/z* 166.0), a standard purchased commercially, produced sequential water loss peaks and a 64 Da (CH_3SOH) loss, signature of methionine sulfoxide side chain. Given that S-hydroxylmethyl and methyl sulfoxide structures both have fragmentation pathways different from the methoxy addition product, sulfenic methyl ester (S-O-CH_3) is left as the most probable structure for methoxy addition. Moreover, this structure is plausible for producing methanol loss under CID, where protonation of the sulfenic oxygen could readily trigger such a loss. Indeed, the data in Figure 2a point out that methanol loss requires mobile proton instead of alpha- or beta-carbon hydrogens in close proximity (all labeled with deuterium in the system).

Once the methoxy addition product was identified as sulfenic methyl ester, efforts were made to understand the reaction mechanism. It is worth noting that no methoxy addition was observed in the absence of 254 nm UV irradiation or methanol. In order to test if this reaction was specific to a disulfide bond, reduced glutathione was used. Very limited methoxy addition product was formed even after 10 min of 254 nm UV exposure. As a comparison, methoxyl addition to oxidized glutathione happened within 2 min of irradiation and the product ion intensity was about 20 times higher (*m/z* 341.2, SI Figure 3). Disulfide bond in cystine has an absorption shoulder around 254 nm with extinction coefficient about 300 M^{-1} cm^{-1}, which is two orders of magnitude higher than a thiol group in cysteine [36]. This band of UV absorption can lead to disulfide bond cleavage, forming thiyl radical at each sulfur atom. We subsequently monitored UV photolysis (254 nm) of model disulfide peptides in aqueous solutions without the addition of methanol via online MS detection. Disulfide reduction was observed as the only photolysis pathway, however, requiring at least 10 min of irradiation for the products to be detected. Given that methoxy addition product was observed at a much faster rate, it is unlikely that forming a thiyl radical is involved as an initial step for methoxy addition. In order to test if methoxy radical (CH_3O•) is generated when the solvent was subjected to UV exposure, we added *N-tert*-butyl-α-phenylnitrone, a known radical trap for methoxy radical, to the solution [37]. Stable isotope labeled experiments and accurate mass measurement data did not provide any evidence of the presence of methoxy radicals. Instead, a small amount of hydroxyl methyl radicals (•CH_2OH) were observed (SI, Figure 4). Thus, it is unlikely that methoxy radical is generated and responsible for the reaction. From the above experiments, we propose a reaction scheme where a disulfide bond is protonated or surrounded by positive charge; upon UV excitation, the disulfide bond can be cleaved via nucleophilic attack by methanol (Scheme 1). This cleavage pathway has been known as concomitant (electrophilic and nucleophilic) catalysis of disulfide bond [11]. Absorption by



Scheme 1. Proposed pathway for methoxy addition reaction with excited disulfide bond

disulfide bond at 254 nm would result in excitation of an electron from the sulfur lone-pair orbital to an antibonding level. Augenstine et al. suggested the possibility of excited disulfide bond extracting proton or hydrogen atom from a neighboring amino acid group, disrupting the hydrogen bonding network, which results in the inactivation of trypsin upon exposure to 254 nm UV light [38]. Upon photon excitation, disulfide bond is lengthened and it is more susceptible for nucleophilic attack [39]. In the absence of methoxy radical, we consider this hypothesis to be a probable reaction pathway.

Sequence Coverage in Insulin

Since methoxy addition can cleave disulfide bonds, we expect that this reaction can be used to enhance sequence coverage for peptides consisting of intramolecular disulfide bonds. Bovine insulin, (**P5**, a peptide consisting three disulfide bonds and two chains: A chain and B chain) was chosen for such a test. The yield of single methoxy addition product to +6 charge state of insulin is about 20% (MS^1 reaction spectrum shown in SI Figure 5). A single methoxy addition corresponds to single disulfide bond cleavage among one of three disulfide bonds. Due to limited reaction yield, obtaining high reaction yield for sequential methoxy addition could not be achieved under current reaction condition. Figure 3a shows the full MS^2 CID spectra of the single methoxy addition product (*m/z* 961.7, 6+); the zoomed-in region from *m/z* 700 to 1100 is shown in Figure 3b. Peaks labeled in black ($By_{3\text{-}6}$, $By_{11,19}^{2+}$, BAy_{19}^{5+}, BAb_{20}^{5+}, and BAb_{24}^{4+}) indicate sequence ions that fall outside of the disulfide loop region. Attention should be given to peaks labeled in red ($^{SH}By_{12\text{-}15}^{2+}$, $^{SH}Bb_{14\text{-}16}^{2+}$, $^{\#}BAy_{4\text{-}8}^{4+}$, and $ABb_{16\text{-}17}^{3+}$), which belong to a sequence originally covered within disulfide loops and are difficult to observe in conventional CID. These ions are now detectable because one of the two interchain disulfide bonds is cleaved upon methoxy addition and subsequent CID releases *b*/*y* type fragment ions within the previous loop region. It should be noted that detection of sequence ions within A-^{7}Cys and A-^{11}Cys region requires cleavages of two disulfide bonds. Their absence, therefore, did not indicate that the disulfide between A-^{7}Cys and A-^{11}Cys was not cleaved during the reaction, given that the single methoxy addition product was selected for CID. Fragmentation map of insulin 6+ methoxy reaction product is summarized in Figure 3c. This leads to 49% coverage of sequence (24 fragment sites/49 possible fragment sites), almost doubling that generated in the CID of the intact peptide (26%, 13 fragment sites/49 possible fragment sites, data not shown). The data in Figure 3 clearly demonstrate that methoxy addition reaction can be applied to peptide or proteins having multiple disulfide bonds for enhancing sequence coverage.

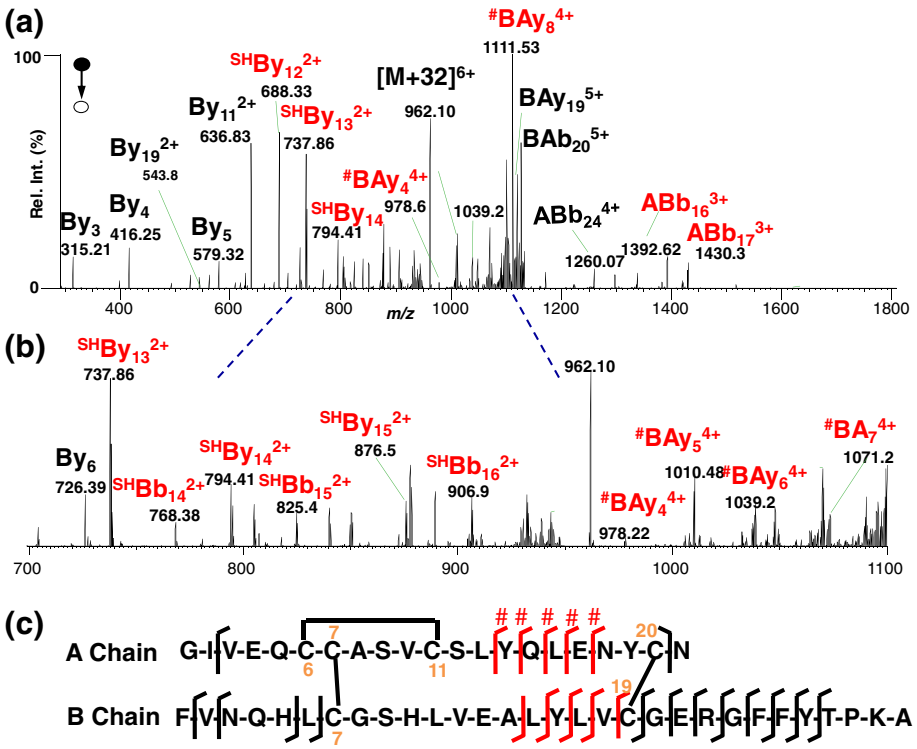
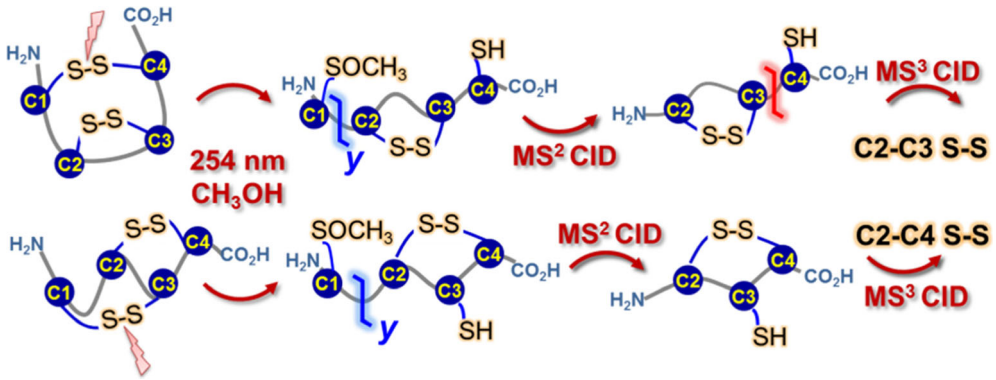


Figure 3. (**a**) MS^2 CID of methoxy reaction product of bovine insulin**, P5** (*m/z* 961.7, 6+); (**b**) zoomed-in region 700–1100 Da in panel (**a**); (**c**) bovine insulin sequence and fragmentation map, the ones indicated in black belong to exocyclic region of intact insulin whereas red sequence ions originate within the disulfide loops (#- sequence ion contains the methoxy modification)

Distinguish Disulfide Regio-Isomers

In order to fully characterize a biomolecule containing one or more disulfide bonds, one must know the sequence and disulfide connecting pattern. Knowing that the methoxy addition reaction can provide enhanced sequence information, the next logical challenge is distinguishing the disulfide bond connecting patterns in regio-isomers. Guanylin (**P3-III**), a biologically active human hormone peptide responsible for regulating water and electrolyte transport in renal and intestinal epithelia [5], was chosen as a model system for further studies. There are four cysteine residues (two disulfide bonds) in the sequence of guanylin peptide, and therefore three different disulfide connecting patterns are available (**P3-I**, **II**, **III** in Table 1). Following traditional MS^2 CID (*m/z* 758.3), the regio-isomer containing two side-by-side disulfide bonds (**P3-I**) can easily be distinguished by the appearance of *b* ($b_{8\text{-}11}$) and *y* ($y_{4\text{-}7}$) ions in the middle of the spectrum (SI Figure 6a). The other two regio-isomers (*m/z* 758.3) with loop-within-a-loop (**P3-II**, SI Figure 6b) and overlapped (**P3-III**, SI Figure 6c) disulfide connecting patterns produce the same sequence ions (b_3 and $y_{12\text{-}13}$) and those ions fall outside of the cyclic region, making it impossible to differentiate their disulfide-connecting patterns. There were also numerous internal fragment ions (neutral loss of A, I, Y, AY, AYA, AYAA) observed, once again proving that these two structures were mostly cyclic. In peptide systems containing two or more intramolecular disulfide bonds, low energy MS^2 CID cannot provide unique sequence ions that can be directly used to distinguish the disulfide bond connecting patterns between regio-isomers. Interestingly enough, the disulfide loop is opened upon formation of internal fragment ions, and we have demonstrated that further tandem MS of internal fragment ions can be used as a means to differentiate those regio-isomers in a previous study [40]. However, the generation of internal fragment is unpredictable, and an effective diagnostic precursor ion requires internal fragmentation at some specific position, which makes the method less reliable and spectra assignment quite challenging. Now with the use of methoxy reaction to cleave one disulfide bond, backbone sequence ions that are readily generated from MS^2 CID can be selected for MS^3 CID. In general, the approach to identify the connecting pattern in peptides containing two intrachain disulfide bonds is a three-step process (Scheme 2). In the first step, one disulfide loop is opened, while leaving the other disulfide bond(s) intact. The next step is to perform MS^2 CID of the reaction product to produce *b*/*y* ions containing at least one intact disulfide bond. Lastly, one of those *b* or *y* ions is subjected to MS^3 CID to produce unique sequence ions that fall outside of the disulfide loop, which allows for the differentiation between the regio-isomers.

Following such a strategy, MS^2 CID of methoxy addition product of guanylin regio-isomers was first conducted and the data are shown in Figure 4. Neutral loss of 32 Da was observed in all three regio-isomers, from both intact precursor ions and fragment ions, confirming the formation of sulfenic methyl ester. MS^2 CID (*m/z* 774.3, 2+) of the regio-isomer containing



Scheme 2. Three steps in identifying disulfide linkage pattern of peptides with two intrachain disulfide bonds

side-by-side disulfide bonds (**P3-I**) produced numerous unique *b* ($b_{8\text{-}11}$) and *y* ($y_{4\text{-}7}$) ions that fall in the middle of the sequence, making this structure distinctive (Figure 4a). There are also ions indicating that one of the two disulfide bonds was cleaved, with addition of a hydrogen (-SH) or methoxy (-$SOCH_3$) ($^{\#}y_{4\text{-}6, 9\text{-}10}$, $^{SH}b_{5\text{-}6}$, $^{SH}y_{9\text{-}10}$, and $^{\#}b_{10\text{-}11}$). The sequence ions observed in-between the two disulfide bonds are sufficient to distinguish the disulfide bond connecting patterns so that no further analysis is necessary. For the other two disulfide regio-isomers (*m/z* 774.3, 2+), loop-within-a-loop (**P3-II**, Figure 4b) and overlapped (**P3-III**, Figure 4c), there is clear evidence that one disulfide bond has been cleaved ($^{SH}y_{9\text{-}11}$) and modified ($^{\#}b_{5\text{-}6}$) by the methoxy group. These ions are absent in the CID of the intact regio-isomers (SI Figure 6). The fragmentation patterns are very similar for **P3-II** and **P3-III** and it is difficult to distinguish between the two based only on the MS^2 CID.

Note that although $^{SH}y_{9\text{-}11}$ ions resulting from CID of **P3-II** and **P3-III** isomers share the same sequence, they contain an intrachain disulfide bond at different locations. This difference may lead to different fragmentation patterns upon CID that can be used to distinguish disulfide bond connecting patterns. Figure 5 shows MS^3 CID of $^{SH}y_9$ (*m/z* 860.3) ions originating from the latter two regio-isomers. The predicted structure for $^{SH}y_9$ ion originating from the loop-within-a-loop structure (**P3-II**) contains a disulfide bond between Cys 7 and Cys 12. In Figure 5a, the appearance of $b_{6\text{-}8}$ suggests that it should be exocyclic from Cys 12 to Cys 15. Moreover, internal fragment ions (b_6-A, *b*6-AA, and b_8-Y) also indicate there is a disulfide

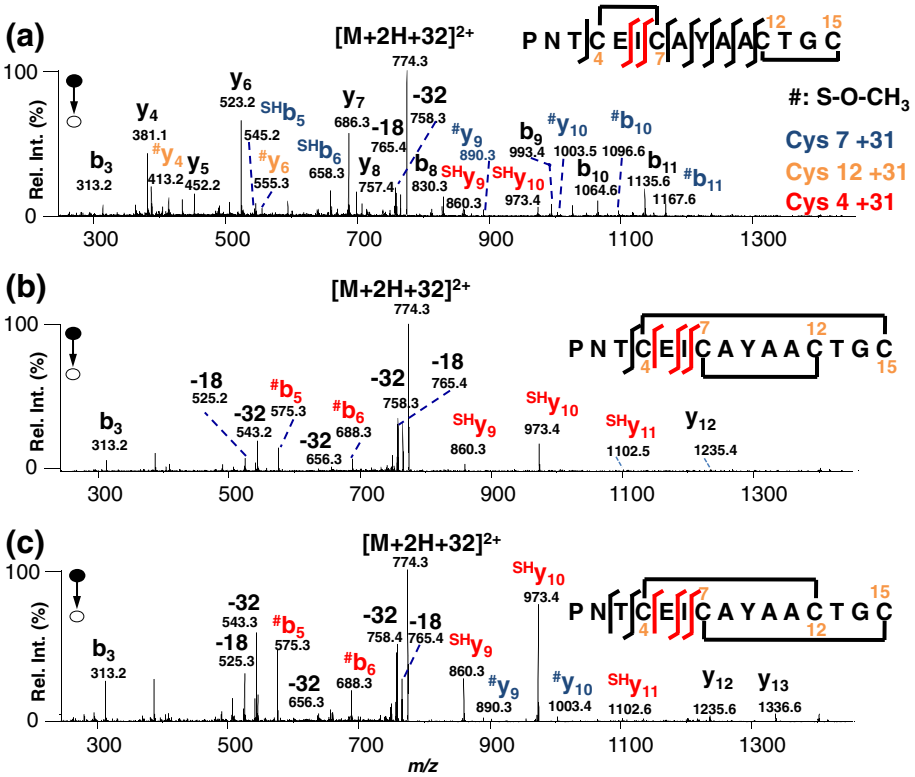


Figure 4. MS^2 CID of the doubly protonated methoxy addition product of guanylin isomers (**a**) **P3-I,** side-by-side disulfide bonds; (**b**) **P3-II,** loop-within-a-loop disulfide bonds; (**c**) **P3-III,** overlapped disulfide bonds

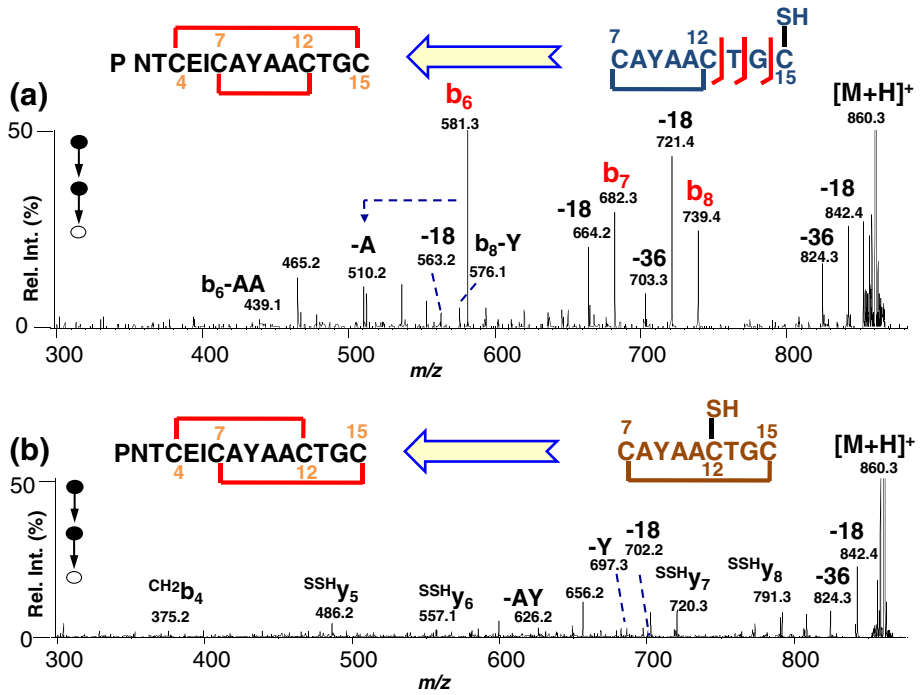


Figure 5. MS^3 CID of $^{SH}y_9$ (*m/z* 860.3) ion generated after dissociation of methoxy addition product from guanlyin disulfide region-isomers (**a**) **P3-II**, loop-within-a-loop disulfide bonds; (**b**) **P3-III**, overlapped disulfide bonds

bond between Cys 7 and Cys 12. These ions serve as definitive evidence for the assignment of disulfide linkage in **P3-II**. On the other hand, the MS^3 CID spectrum for $^{SH}y_9$ ion derived from **P3-III** (Figure 5b) is dominated by internal fragment ions (losses of Y and AY) with no conventional *b* or *y* ions. Some modified *b*/*y* ions are formed due to C–S bond cleavage leading to the formation of dehydroalanine at one cysteine ($^{CH2}b_4$) and disulfohydryl at the other cysteine ($^{SSH}y_{5\text{-}8}$). This suggests that most of the sequence is covered by a disulfide bond, which is in agreement with the predicted structure for the $^{SH}y_9$ ion, having a disulfide bridge between Cys 7 and Cys 15 (**P3-III**). Using this information, one could readily work backwards to decipher disulfide linkage pattern. Another synthetic system (**P4** peptides) containing four cysteines, two disulfide bonds, and three regio-isomers was also studied using the methoxy reactions followed by CID (SI Figure 7). MS^3 CID of the $^{SH}y_{12}$ (*m/z* 660.8) ion produced distinctive sequence ions that allowed for the differentiation of the loop-within-a-loop and overlapped isomers (SI Figure 8). In summary, MS^3 CID of sequence ions containing one intact disulfide bond can produce unique fragment ions that allow for quick and accurate assignment of disulfide bond connecting patterns in regio-isomers.

There are several MS/MS techniques relying on radical mechanism (i.e., ETD) to analyze peptides or proteins with disulfide bond. Although they succeed in giving enhanced sequence coverage compared with CID, many fail to provide disulfide connectivity due to radical cascades that result in disulfide scrambling or consecutive disulfide cleavage [24, 41]. As our mechanistic studies have shown, methoxy addition reaction does not involve radical reactants or intermediates. Moreover, upon cleavage of a disulfide bond, methoxy and hydrogen each add on to cysteine sulfur atoms forming an even electron species. As a result, disulfide bond connectivity is well retained for subsequent analysis and disulfide regio-isomers can be unambiguously identified.

Conclusions

UV photolysis at 254 nm of disulfide linked peptides in the presence of methanol leads to cleavage of disulfide bond and formation of free thiol and sulfenic methyl ester at the pair of cysteine residues. MS^2 CID of methoxy addition product produces a distinct neutral loss of 32 Da (CH_3OH), allowing the use of tandem mass spectrometry to distinguish this product from other possible isomeric structures (S-hydroxyl methyl and methyl sulfoxide). Although the exact mechanism for methoxy addition is not clear, experimental evidences suggest that cleavage of disulfide bond is likely to go through nucleophilic attack by methanol under 254 nm UV excitation instead of radical pathways. Given its capability of cleaving disulfide bond, this reaction was coupled online with MS^2 CID to provide enhanced sequence information for peptides and small proteins containing multiple disulfide bonds. For instance, CID of the methoxy addition product of bovine insulin almost doubled the detection of sequence ions compared with CID of the intact insulin ions. A strategy based on online methoxy addition – MS^3 CID was developed and applied to the analysis of peptide disulfide regio-isomers containing two disulfide bonds. No disulfide bond scrambling was observed and distinctive fragment ions were obtained that allowed for the assignment of disulfide bond connecting patterns with high confidence.

Beside the above advantages, relatively low reaction yield of methoxy addition reaction limits its immediate application to the analysis of more complicated disulfide peptide/protein systems. Our future studies will focus on reaction mechanism elucidation and reaction yield improvement.

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