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Preferential Cleavage of N−N Hydrazone Bonds for Sequencing Bis-arylhydrazone Conjugated Peptides by Electron Transfer Dissociation

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Electron transfer dissociation (ETD) was used to sequence bis-arylhydrazone (BAH)-cross-linked peptides through preferential cleavage of the hydrazone bond. On average, 58% of the observed ETD product ion abundance was accounted for by fragment ions due to selective cleavage of the N12−N13 hydrazone bond. Dissociation of the N12−N13 hydrazone bond yielded the two constituent peptides, one an even-electron product ion termed Lα12, the other an odd-electron radical ion termed Lβ11$^{\bullet}$, which allowed each peptide to be individually sequenced by MS/MS methods and the site of cross-linking to be identified. The proposed pathway for the dissociation of the hydrazone bond involves transfer of the electron directly to the protonated hydrazone functionality and subsequent rearrangement to yield the Lα12 and Lβ11$^{\bullet}$ products. Collision induced dissociation (CID) of the even-electron Lα12 product yielded a series of b- and y-type ions; CID of the odd-electron Lβ11$^{\bullet}$ product resulted in a wide range of fragment ions including a-, b-, c-, y-, and z-type ions.

Tandem mass spectrometry continues to play an ever expanding role in the field of proteomics, particularly as a means to sequence and identify biological molecules.[1−5] Collision-induced dissociation (CID)[1,6,7] and electron transfer dissociation (ETD)[8,9] have found widespread use as these techniques typically yield high sequence coverage of peptides due to systematic cleavage of the peptide backbone. For peptide or protein sequencing applications, dissociation methods that are nonselective in terms of causing cleavages all along the backbone are preferred; however, in recent years, there has been a growing interest in designing selective gas-phase dissociation techniques to identify particular functional groups in peptides or proteins or to simplify interpretation of product ion mass spectra. For example, the Julian group has developed site-specific dissociation techniques to pinpoint tyrosine residues[10] and sites of phosphorylation[11] by radical directed dissociations methods utilizing ultraviolet photodissociation (UVPD) and CID. Mass spectrometric strategies that promote site-selective cleavages have the potential for high impact not only in traditional proteomics applications but also in the analysis of more complicated macromolecular systems, such as cross-linked protein complexes.

Chemical cross-linking generally involves covalently connecting pairs of amino acid residues that are spatially constrained within a single protein or a protein complex in order to facilitate the determination of the relative distances between residues or identify interacting interfaces in protein complexes.[12] In recent years, cross-linking with mass spectrometric analysis[12−14] has been used to determine the fold of a single protein[15] or the quaternary structure of protein−ligand complexes[16−19] and to elucidate conformational changes in protein structure upon activation[20] as well as ligand modulation of protein−protein interactions.[21] Bioconjugation of proteins and peptides to other biomolecules has also been an area of active interest as a means to couple independent functionalities of the constituent biomolecules in order to modulate the properties of the biomolecules or endow

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them with enhanced activities.[22] For example, therapeutic biomolecules are often coupled to proteins or cell penetrating peptides in order to traverse the cell membrane without degrading the "payload."[23,24] The coupling of an aldehyde-modified biomolecule to a second species with a hydrazine modification is often used for conjugation or cross-linking.[25,26] In order to identify the sites of cross-linking or conjugation, tandem mass spectrometric analyses are often performed.[27] Interpretation of product ion (MS/MS) spectra of cross-linked or conjugated peptides, which are produced upon the proteolytic digestion of cross-linked proteins or protein complexes, remains a great challenge as fragment ions may arise from bond cleavages along the backbone of either peptide, from cleavage of the cross-link moiety or from multiple cleavages. Moreover, differentiation of cross-linked products from other proteolytic species (e.g., peptides remote from any cross-linking site) remains a significant hurdle. Several analytical techniques have been developed to simplify the interpretation of the tandem spectra by designing cross-linkers that enhance selective cleavages or ones that allow facile distinction of true cross-linked products from noncross-linked ones. For example, an IR chromogenic cross-linker was designed to incorporate a phosphate chromophore that promoted selective infrared multiphoton dissociation (IRMPD) of cross-linked peptides, thus allowing ready differentiation of these species of interest from unmodified peptides.[28] In addition, IRMPD of these cross-linked peptides yielded a series of y-type product ions C-terminal to the cross-linked residues which allowed each constituent peptide to be sequenced without the need for MS^n methods.

Other groups have focused on developing cross-linkers that display characteristic fragmentation patterns upon collision-induced dissociation (CID) by including gas-phase labile bonds. The first method employed cleavable bonds to produce reporter ions upon low energy CID to readily identify and screen cross-linked peptides.[29,30] Chowdhury and co-workers designed a trifunctional cross-linker with an affinity group for enrichment and an NO_2 tag which was observed as a neutral loss upon CID.[31] In another strategy, labile bonds were incorporated into the cross-link itself such that the cross-link was cleaved selectively upon CID, yielding each constituent peptide with a characteristic modification.[30,32−37] For instance, Soderblom and Goshe incorporated an aspartyl-prolyl bond in the center of their cross-linker,[33] while the Reid group used a fixed-charged cross-linker containing a sulfonium ion which induced cleavage on either side of the sulfur atom.[35] More recently Dreiocker et al. developed a cross-linker which cleaved at a glycyl-prolyl bond upon CID through a nucleophilic attack by a thiourea moiety.[37] These approaches required an additional stage of CID to sequence either the cross-linked peptide for the reporter ion technique or the modified constituent peptides for the latter methods.

The cleavable cross-linking reagents developed to date all utilized IRMPD and CID methods in which selective dissociation may not be observed for all cross-linked peptides of interest. In fact, cross-linked peptides have been shown to exhibit dissociation trends similar to that of unmodified peptides in which fragmentation is dependent not only on amino acid composition and sequence but also on charge state.[38,39] For peptides with labile post-translational modifications (PTMs) such as phosphorylation, the losses of these modifications are often the preferred dissociation pathways that may be favored over cleavage of the labile bond located within the cross-linker.

With respect to the ongoing interest in promoting specific bond cleavages in gas-phase ions, electron capture dissociation (ECD)[40] or ETD[8] typically result in cleavages of the $N-C_\alpha$ bonds of the peptide backbone which produces characteristic c- and $z^\bullet$-type ions with little preference as to the specific bond (i.e., any $N-C_\alpha$ bond between two specific amino acid residues). However, preferential cleavage of disulfide bonds was observed in early studies of proteins by ECD.[41] This high propensity for S−S bond dissociation was attributed to the high affinity of the disulfide bond for the hydrogen atom ($H^\bullet$). However, near complete sequence coverage of the protein was still obtained because a majority of the $N-C_\alpha$ backbone bones were also cleaved upon ECD.[41] The McLuckey group analyzed disulfide-bonded peptides by ETD, and for most of triply charged peptide cations studied, cleavage of the disulfide bond accounted for ∼70%−80% of the ETD product ions.[42] For quadruply charged peptide cations, lower yields (∼33%) of the product ions arose from selective disulfide bond cleavage.

In this work, electron transfer dissociation of bis-arylhydrazone-cross-linked peptides is explored in a linear ion trap mass spectrometer. Earlier studies showed that bis-arylhydrazone-(BAH)-cross-linked peptides could be selectively dissociated by ultraviolet photodissociation as a means to rapidly screen these species in complex mixtures based on selective photoabsorption

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of BAH-containing peptides.[43] In the present study, upon reaction with fluoranthene radical anions, the BAH-cross-linked peptides underwent ETD with preferential cleavage of the N−N hydrazone bond. Dissociation of the hydrazone bond yielded the two constituent peptides each with a unique mass tag at the cross-linking site, one an even-electron species and the other an odd-electron product. The propensity for cleavage of the hydrazone bond by ETD is compared to the preferential dissociation of the disulfide bond of peptides cross-linked with dithiobis (succinimidyl propionate) (DSP). A mechanism for the preferential cleavage of the hydrazone bond is proposed and discussed. Subsequent collision-induced dissociation of the primary product ions formed upon ETD-mediated cleavage of the hydrazone bond yields diagnostic sequence ions which allow the site of cross-linking to be unambiguously identified.

EXPERIMENTAL SECTION

Chemicals and Reagents. The peptides α-MSH (Ac-SYSMEHFRWKGPV-NH_2), neurotensin (Pyr-LYENKPRRPYIL), and Ac-RFMWMK-NH_2 were obtained from Bachem (Torrance, CA) and used without further purification. The peptides Ac-AAAKAAAAR, Ac-AAAKPAAAR, and Ac-AAAKAAAAR were synthesized at the Protein Microanalysis Facility at the University of Texas at Austin and purified by reversed-phase HPLC. Succinimidyl 4-formylbenzoate (SFB), succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH), and dithiobis (succinimidyl propionate) (DSP) were obtained from Pierce Biotechnology (Rockford, IL). All other reagents and chemicals were from Fisher Scientific (Fairlawn, NJ).

Peptide Chemical Conjugation and Cross-Linking. Peptides were cross-linked together as previously described as shown in Figure S-1 (Supporting Information).[43] Briefly, peptides were first modified by either SANH or SFB at a 1:10 molar ratio of peptide to derivatizing reagent (200 μM:2.0 mM) in a 20 mM HEPES, pH 8.0 buffer. After reacting overnight, the solutions were desalted via C_{18} solid-phase extraction using Waters tC_{18} SepPak cartridges (Milford, MA). The modified peptides were conjugated together in 99:1 MeOH/acetic acid (v/v) at an equimolar ratio (100 μM) at 55 °C overnight. Model peptides were also cross-linked with DSP. A fresh solution of DSP was prepared at 20 mM in dimethylsulfoxide (DMSO), and an aliquot was added to a solution containing the peptides to be cross-linked at a molar ratio of 3:2:2 (300 μM cross-linker/200 μM α-peptide/200 μM β-peptide) in 20 mM HEPES, pH 8.0 buffer. The percent volume of DMSO in the cross-linking solution was 5%. The peptides were cross-linked overnight and then desalted by C_{18} solid-phase extraction. All conjugated and cross-linked peptides were diluted to ~10 μM in 49.5:49.5:1 H_2O/MeOH/acetic acid (v/v/v) for ESI-MS/MS analysis. Samples were infused at a flow rate of 3.0 μL/min using an ESI voltage of 4.0 kV.

Mass Spectrometry. All mass spectrometry analyses were conducted on a Thermo Fisher Scientific LTQ XL linear quadrupole ion trap mass spectrometer (San Jose, CA) equipped with the standard ESI source operated at 4.0 kV and ETD capabilities, allowing for the chemical ionization and injection of fluoranthene radical anions. The mass spectrometer was controlled with the XCalibur 2.0.7 software package. Collision-induced dissociation experiments were performed by activating the precursor ions for 30 ms at a q-value of 0.25. Electron transfer dissociation was performed by injecting 1×10^5 reagent anions into the trap, and the reaction time was varied between 0.03 and 100 ms. The precursor ion population for CID and ETD was set to 1×10^4 ions. Time-resolved ETD experiments were performed by plotting the relative abundances of the observed product ions as a function of the ETD reaction time.

Identification of Cross-Linked Peptides and Product Ions. The ETD spectra of these cross-linked peptides were manually identified, as were the MS^3 CID spectra. Product ion spectra of cross-linked peptides were labeled according to the proposed nomenclature.[44] Briefly, the two constituent peptides are labeled as α and β, and for consistency, the α-peptide is designated as the one modified by SFB and the β-peptide is designated as the one modified by SANH; bis-arylhydrazone-cross-linked peptides are referred to as α + BAH + β (i.e., BAH = SFB/SANH). Dissociation of the peptide backbone of the α-peptide would yield $b_{n\alpha}$, $c_{n\alpha}$, $y_{n\alpha}$, and $z_{n\alpha}$ product ions. Similar notation is used for dissociation of the β-peptide. Lysine immonium ions cross-linked to either peptide are labeled as $K^L\alpha$ or $K^L\beta$. Product ions due to dissociation along the cross-linker are labeled based on the number of atoms away from the α-carbon of the cross-linked lysine of the peptide which retains the charge as shown in Scheme 1 with the numbering beginning from the α-peptide; similar numbering is used beginning from the β-peptide.

RESULTS AND DISCUSSION

While ECD and ETD have been used to preferentially cleave disulfide bonded peptides to identify natural disulfide linkages in proteins,[45−47] electron-based dissociation methods have not been utilized for the characterization of chemically cross-linked or conjugated peptides by exploiting selective bond cleavages. As shown in the present study, by introducing a hydrazone moiety into the cross-link functionality, the N−N bond is preferentially cleaved by ETD, yielding the two constituent peptides which can be sequenced individually by CID. We evaluate the ETD-selective cleavage of BAH-conjugated peptides, the preference for N−N versus S−S bond cleavage, and the ability to sequence the constituent peptides by CID after cleavage of the N−N cross-linker bond.

Electron Transfer Dissociation of Bis-arylhydrazone-Cross-Linked Peptides. Bis-arylhydrazone-cross-linked peptides were analyzed by CID versus ETD to assess the ability to sequence the conjugated peptides and locate the sites of the cross-links. For example, the peptide Ac-AAAKAAAAR (α-peptide) was cross-linked to neurotensin (PyrLYENKPRRPYIL, β-peptide) through SFB/SANH (BAH) conjugation. Upon ESI, the dominant products are [Ac-AAAKAAAAR + BAH + neurotensin +4H]$^{4+}$ and [Ac-

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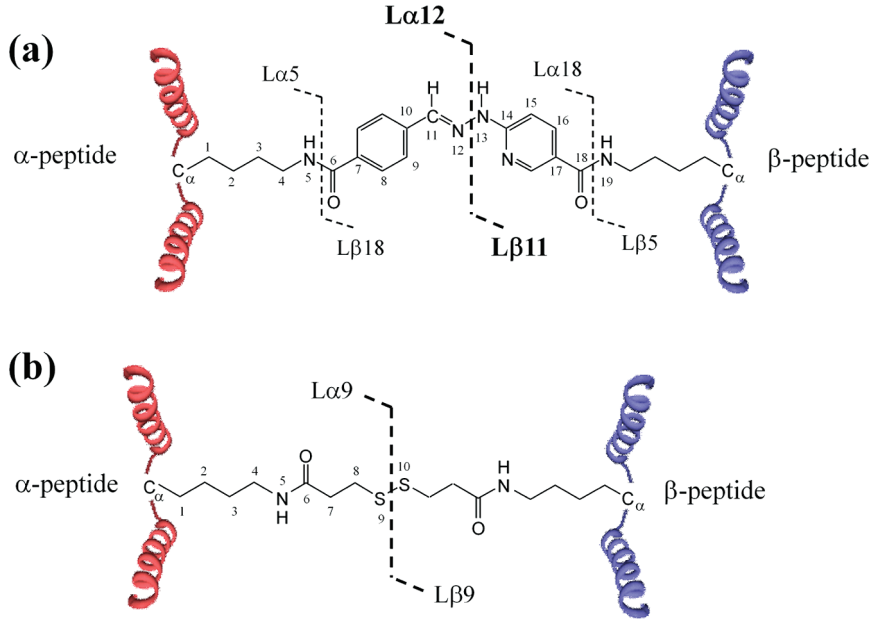
Scheme 1. Dissociation Nomenclature and Observed Bond Cleavages of (a) Bis-arylhydrazone and (b) DSP-Cross-Linked Peptides



AAAKAAAAR + BAH + neurotensin +3H]$^{3+}$. Collision-induced dissociation of the cross-linked peptide in the 4+ charge state, as shown in Figure 1a, predominantly resulted in cleavage of backbone amide bonds of the α-peptide (Ac-AAAKAAAAR), yielding a series of $y_{n\alpha}$ ($y_{2\alpha}$−$y_{5\alpha}$) and complementary cross-linked $b_{n\alpha}$ ions (b_n ion of the α-peptide cross-linked to intact neurotensin). One lysine immonium ion (from the α-peptide) cross-linked to neurotensin was also detected ($K^L\beta^{2+}$ ion). However, no product ions stemming from cleavage of the peptide backbone of neurotensin were observed in the CID mass spectrum, meaning that no diagnostic β-peptide ions were formed. In contrast, ETD of the same cross-linked peptide (4+) yielded three abundant product ions of *m/z* 896.9, 973.4, and 1793.7 (Figure 1b) as well as some lower abundance z$^\bullet$ and c ions of the type typically observed upon ETD of peptides. The former ions (*m/z* 896.9, 973.4, and 1793.7) arose from cleavage of the N12−N13 bond of the bis-arylhydrazone cross-link. (See atom labeling shown in Scheme 1a.) This yielded the two constituent peptides each with a unique mass tag at the cross-linked lysines, designated as the L*β*11 ion (*β*-peptide +119 Da, SANH tag, net *m/z* 896.9^{2+}) and the complementary Lα12 ion (α-peptide +131 Da, SFB tag, net *m/z* 973.4). The third most abundant product ion of *m/z* 1793.7 is the singly charged (i.e., charge reduced) analog of the L*β*11.

A second BAH-cross-linked peptide contained α-MSH (Ac-SYSMEHFRWKGPV-NH_2) as the α-peptide and Ac-RFMWMK-NH_2 as the *β*-peptide. Upon ETD, the [α-MSH + BAH + Ac-RFMWMK-NH + 4H]$^{4+}$ ion of *m/z* 713.6 yielded three dominant product ions of *m/z* 898.4, 1059.3, and 1796.5, as

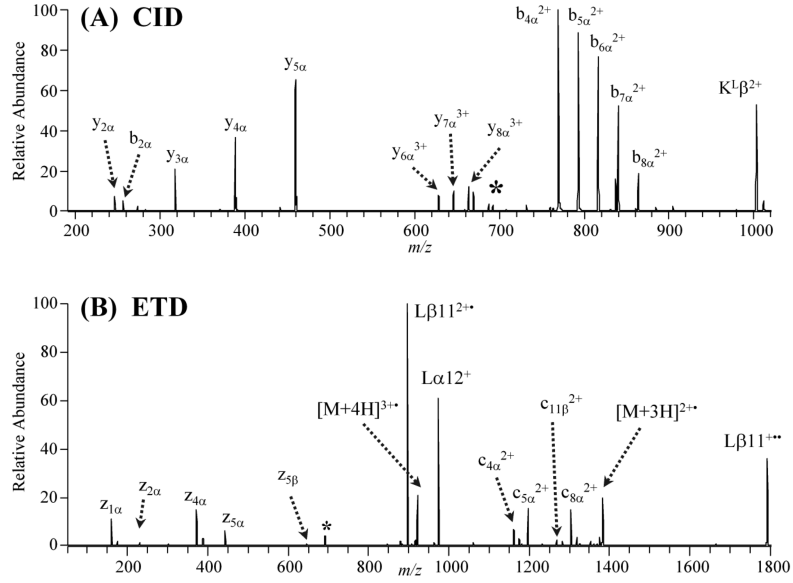


Figure 1. (A) CID and (B) ETD (100 ms ion−ion reaction time) product ion mass spectra of [Ac-AAAKAAAAR + BAH + PyrLYENKPRRPYIL + 4H]$^{4+}$ of *m/z* 692.3. The first peptide listed is referred to as α, and the second peptide is referred to as *β*. The precursor ion is represented by an asterisk (*).

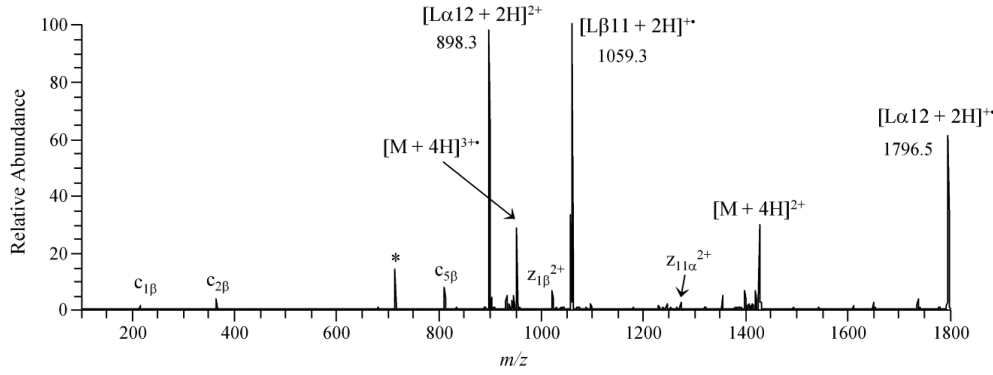
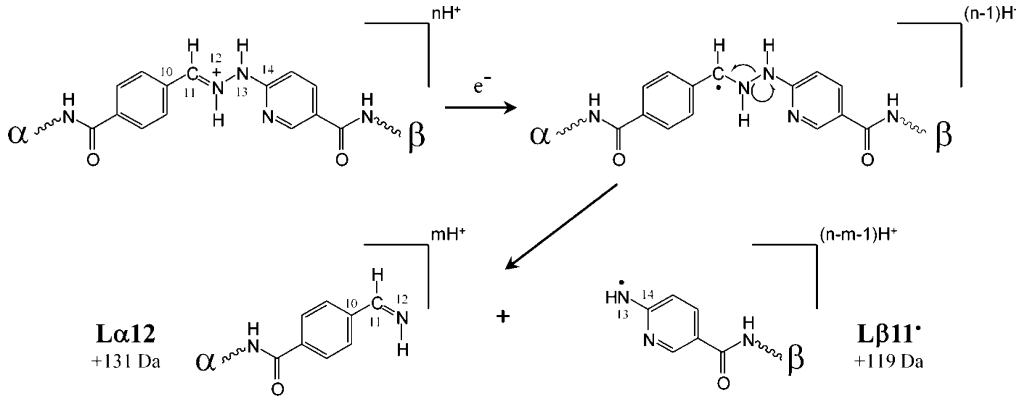


Figure 2. ETD mass spectra of [α-MSH + BAH + Ac-RFMWMK-NH_2 + 4H]$^{4+}$ of *m*/*z* 714.1 (40 ms reaction time). The first peptide listed is referred to as α, and the second peptide is referred to as *β*. The precursor ion is represented by an asterisk (*).

Scheme 2. Proposed Route for the Preferential Dissociation of the N12−N13 Bond by Electron Transfer Dissociation Yielding the Lα12 and Lβ11• Product Ions



shown in Figure 2. Cleavage of the N12−N13 hydrazone bond produces the Lα12 ion (α-peptide +131 Da, SFB tag, *m*/*z* 898.4^{2+}) and the complementary Lβ11 ion (*β*-peptide +119 Da, SANH tag, *m*/*z* 1059.3). The product ion of *m*/*z* 1796.7 is the charge reduced Lα12 species.

Upon ETD of each of these two pairs of cross-linked peptides, the abundances of the characteristic Lα12 and Lβ11 product ions are greater than that of the charge reduced precursor ions (i.e., [M + 4H]$^{3+\bullet}$) or any conventional c- or z$^{\bullet}$-type sequence ions typically formed by ETD. The ability to preferentially cleave a specific bond within a cross-linker to yield two well-defined and predictable product ions offers great potential analytical utility as it streamlines data analysis and allows one to utilize well-established database search algorithms such as SEQUEST[48] and Mascot[49] to identify the cross-linked peptides. In our study, the formation of the complementary and highly diagnostic Lα12 and Lβ11 ions is observed upon ETD of all cross-linked peptides, a significant feature of the present strategy.

A proposed route for the preferential ETD-mediated cleavage of the hydrazone bond is shown in Scheme 2. Previous studies of hydrazone-containing molecules indicate the UV absorption profile shifts in both the gas-phase and solution-phase upon protonation of the hydrazone functionality, which is presumed to be due to its high gas-phase basicity.[43] Assuming that the N12 atom of the bis-arylhydrazone cross-link is protonated in the gas phase, the electron could likely be transferred to this functionality, resulting in a hypervalent species in which the extra electron would initially be localized at the C11 atom of the charge reduced species. Upon rearrangement, the N12−N13 bond is cleaved producing the complementary Lα12 and Lβ11$^{\bullet}$ product ions. While the radical is displayed as localized on the amine group of the final Lβ11$^{\bullet}$ product ion in Scheme 2, the radical can be resonance stabilized by the adjacent pyridinyl ring. The mass shifts observed for these two product ions are consistent with the proposed pathway in which the Lα12 species is an even-electron product (+131 Da, SFB tag) and Lβ11 (+119 Da, SANH tag) possesses the extra electron. There may be a high propensity for cleavage of the hydrazone bond by ETD because the mechanism does not require the migration of a hydrogen atom (H$^{\bullet}$) to initiate dissociation, as is necessary to produce conventional c- and z$^{\bullet}$-type ions.[40,50]

Preferential Cleavage of Hydrazone Bond by ETD. Preferential cleavage of the hydrazone bond was observed for the cross-linked peptides described above as well as for other model cross-linked peptides, including [Ac-AAAKAAAAR + BAH + Ac-AAAKAAAAR + 3H]$^{3+}$, [Ac-RFMWMK-NH_2 + BAH + Ac-RFMWMK-NH_2 + 3H]$^{3+}$, and [PyrLYENKPRRPYIL + BAH + Ac-SYSMEHFRWGKPV-NH_2 + 5H]$^{5+}$, as shown in Figure S-2 (Supporting Information). In addition, pairs of peptides were also reversed with respect to the orientation of conjugation (i.e., the α-peptide and *β*-peptide were reversed). For example, the α-MSH and Ac-RFMWMK-NH_2 peptides were conjugated to create [α-MSH + BAH + Ac-RFMWMK-NH_2] and [Ac-RFMWMK-

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NH_2 + BAH + α-MSH] products. Time-variable ETD experiments were performed to determine the appropriate ETD activation conditions to produce the greatest abundance of the diagnostic Lα12 and Lβ11$^\bullet$ ions. The relative abundances of the various types of product ions observed in the ETD mass spectra were plotted as a function of ETD reaction time for [α-MSH + BAH + Ac-RFMWMK-NH_2] as well as the same cross-linked peptides conjugated in the reverse orientation, [Ac-RFMWMK-NH_2 + BAH + α-MSH], in both the 3+ and 4+ charge states (Figure S-3, Supporting Information). For the cross-linked peptides in the 4+ charge state, the Lα12 and Lβ11$^\bullet$ product ions were observed to be the most abundant ions in the ETD mass spectra, in fact greater in abundance than the charge reduced precursor ions, when the ETD reaction time was greater than 25 ms (Figure S-3a,c, Supporting Information). In fact, greater than 70% of the total ion current was composed of these key Lα12 and Lβ11$^\bullet$ ions in the ETD mass spectra of the 4+ charged cross-linked peptides. In the ETD mass spectra of the same triply charged cross-linked peptides, the Lα12 and Lβ11$^\bullet$ ions are the most abundant fragment ions (i.e., products of the ETD reactions evolving from bond cleavage) but not the most abundant product ions. The most abundant product ions arise from electron transfer that yields the charge reduced precursor ($[M + 3H]^{2+\bullet}$) and proton transfer from the precursor to the reagent anion that yields $[M + 2H]^{2+}$ (Figure S-3b,d, Supporting Information). The summed relative abundance of the hydrazone cleavage products, Lα12 and Lβ11$^\bullet$, was ~25% upon ETD of the triply charged precursors. Charge reduction due to electron attachment without dissociation (ETnoD) accounted for almost 50% of the ion abundance in the ETD mass spectra of the 3+ charged precursors. Uninformative neutral loss products (e.g., side chain losses from Arg residues including loss of NH_3) and c/z$^\bullet$-type ions accounted for a maximum of ~6% of the total ion abundance in the ETD mass spectra of the 4+ charged cross-linked peptide ions and only 2%–3% each in the mass spectra of the 3+ charged precursors.

We speculate that the differences in the relative abundances of the hydrazone cleavage products (Lα12 and Lβ11$^\bullet$ ions) for the 4+ and 3+ charge states of the cross-linked peptides (i.e., such as for the α-MSH/AcRFMWMK-NH_2 pair described above) correlate with whether the hydrazone functionality is protonated. For the 4+ charge state, the hydrazone functionality is likely protonated, thus facilitating the pathway outlined in Scheme 2. In contrast, for the triply charged cross-linked peptides, it is likely that the two Arg residues are protonated, and then, the third proton is localized at either the hydrazone functionality or His-6 of α-MSH (Ac-SYSMEHFRWGKPV-NH_2), with the latter likely leading to c/z$^\bullet$-type ion production or charge reduction.

The only noticeable difference upon ETD of the reverse orientation cross-linked peptides, e.g., [α-MSH + BAH + Ac-RFMWMK-NH_2] and [Ac-RFMWMK-NH_2 + BAH + α-MSH], is that the abundance of the hydrazone cleavage product containing the α-MSH peptide (Lα12 in Figure S-3a,b; Lβ11$^\bullet$ in Figure S-3c,d; Supporting Information) was always 1.5 to 2 times greater than the complementary product containing the Ac-RFMWMK-NH_2 peptide (Lβ11$^\bullet$ in Figure S-3a,b; Lα12 in Figure S-3c,d; Supporting Information). This phenomenon was further investigated by measuring the ratio of Lα12 to Lβ11$^\bullet$ product ions for other charge states of the cross-linked α-MSH/Ac-RFMWMK-NH_2 and Ac-RFMWMK-NH_2/α-MSH peptides as well as other BAH-cross-linked peptides, and the results are summarized in Table S-1 (Supporting Information). The product ion containing the larger or more basic peptide (e.g., the Lα12 ion of PyrLYENKPRRPYIL + BAH + Ac-RFMWMK-NH_2) in general is observed at a 12–15 times greater abundance than the complementary product ion (e.g., Lβ11$^\bullet$) upon ETD of the lower charge state precursor. For the intermolecularly cross-linked peptides in which the α and β peptides are identical, the Lα12/Lβ11$^\bullet$ abundance ratios ranged between 0.8 and 2.3.

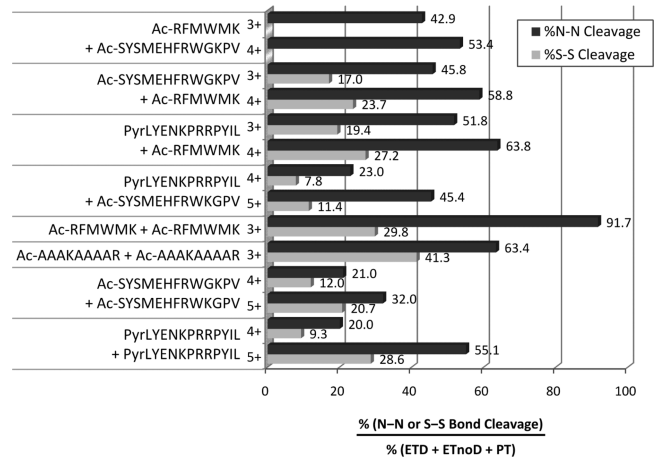

Figure 3. Percent abundance of product ions due to cleavage of the N–N hydrazone bond of BAH-cross-linked peptides or of the S–S bond of DSP-cross-linked peptides relative to the total abundance of ETD, ETnoD (electron transfer without dissociation), and PT (proton transfer) product ions in the mass spectra. Values are the average of four ETD ion–ion reaction times (25, 50, 75, and 100 ms).

Preferential Dissociation of Hydrazone Bonds vs Disulfide Bonds by ETD. The preferential cleavage of the hydrazone bond was compared to the propensity for disulfide bond cleavage for peptides cross-linked through disulfide bonds. For these comparative experiments, peptides were cross-linked by DSP which contains a disulfide bond at the center of the cross-linking reagent (Scheme 1b). ETD mass spectra of [α-MSH + DSP + Ac-RFMWMK-NH_2] in the 4+ and 3+ charge states are shown in Figure S-4 (Supporting Information). ETD of the cross-linked peptide in the 4+ charge state predominantly resulted in cleavage of the disulfide bond, producing the complementary Lα9 and Lβ9$^\bullet$ (or Lα9$^\bullet$ and Lβ9) ions. (See ETD mass spectrum in Figure S-4a (Supporting Information) and Lα9 and Lβ9 nomenclature in Scheme 1b.) A high abundance of the charge reduced precursor was also observed upon ETD of the DSP-cross-linked peptides (4+). Upon ETD of the triply charged cross-linked peptide, charge reduction via electron attachment without subsequent dissociation was the dominant pathway, with only minor production of the Lα9 product ion stemming from cleavage of the disulfide bond (Figure S-4b, Supporting Information). The complementary Lβ9 fragment ion was not detected.

The propensity for cleavage of the N12–N13 bond of BAH-cross-linked peptides is compared to the preferential dissociation of the S–S bond of DSP-cross-linked peptides in Figure 3. Upon ETD of the BAH-cross-linked peptides which possess a mobile

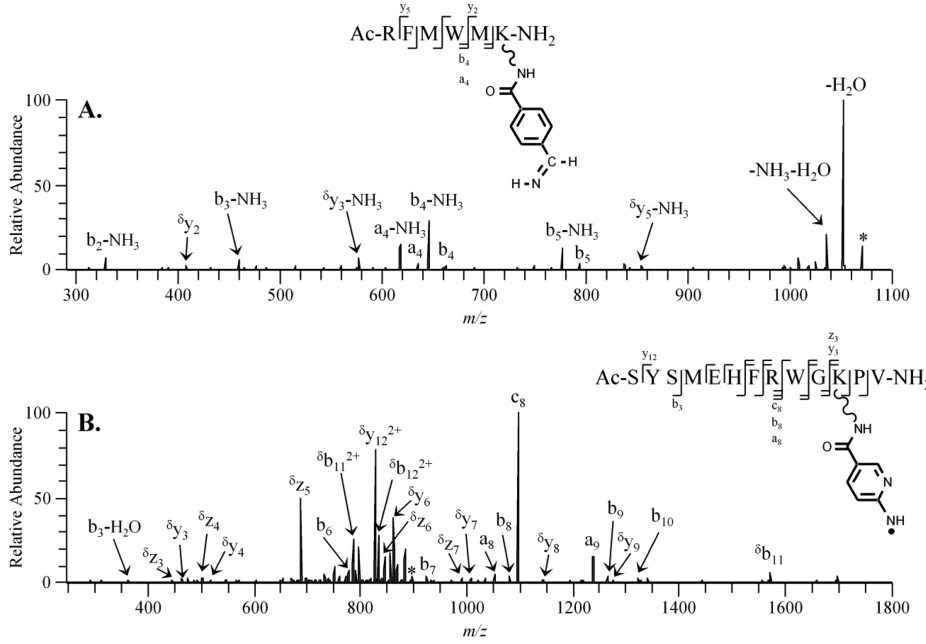

Figure 4. CID mass spectra (MS^3) of product ions from ETD of $[Ac\text{-}RFMWMK\text{-}NH_2 + BAH + \alpha\text{-}MSH + 4H]^{4+}$ of *m/z* 714.1 (30 ms reaction time). The first peptide listed is referred to as α, and the second peptide is referred to as *β*. (A) MS^3 CID mass spectrum of $[L\alpha 12 + H]^+$ of *m/z* 1070.2 (17% NCE). (B) MS^3 CID mass spectrum of $[L\beta 11 + 2H]^{2+\bullet}$ of *m/z* 892.6 (15% NCE). Product ions which retain the modification are labeled with *δ*. The precursor ion is represented by an asterisk (*).

proton (i.e., in which the hydrazone functionality is presumed to be protonated), on average 58 ± 17% of the product ions were due to cleavage of the N12−N13 bond, leading to the diagnostic $L\alpha 12/L\beta 11^{\bullet}$ products. Upon ETD of the DSP-cross-linked peptides in the higher charge states, only 26 ± 9% of the product ions were due to disulfide bond cleavage. In the lower charge states, the percent of product ions due to preferential cleavage dropped to approximately 34 ± 14% for the BAH-cross-linked peptides and 13 ± 5% for the DSP-cross-linked peptides. ETD of BAH-cross-linked peptides also yielded charge reduced (e.g., electron transfer without dissociation or proton transfer) products at 5−20% lower abundance than the DSP-analogs. As compared to previously identified bonds which are preferentially cleaved by electron transfer,[41,42] the present results indicate that there is a greater preference for the specific cleavage of the hydrazone bond by ETD, making the hydrazone functionality an attractive platform for ETD-selective conjugation strategies.

Sequencing of Cross-Linked Peptides by ETD-CID MS^n Methods. The high propensity for the hydrazone bond cleavage upon ETD provides a means to individually sequence the two constituent peptides of intermolecularly BAH-cross-linked peptides through an MS^3 strategy. After conjugation of the two peptides of interest, ETD of the cross-linked species yields the two peptides, each containing a unique mass tag based on its original conjugation reagent: 131 Da for the peptide covalently modified by SFB (the Lα12 product) or 119 Da for the peptide modified by SANH (the $L\beta 11^{\bullet}$ product). These key primary product ions, Lα12 and $L\beta 11^{\bullet}$, formed upon ETD are excellent candidates for subsequent CID in order to sequence each constituent peptide individually. As illustrated below, subsequent CID (MS^3) of each modified peptide produces diagnostic fragment ions that allow sequencing of each peptide and location of the site of cross-linking.

The sequence coverage obtained by the ETD-CID method was compared to CID alone for the cross-linked complex containing α-MSH (Ac-SYSMEHFRWGKPV-NH_2) and Ac-RFMWMK-NH_2, first with α-MSH as the α-peptide and Ac-RFMWMK-NH_2 as the *β*-peptide, then reversed. The CID mass spectra of these cross-linked peptides are shown in Figure S-5, Supporting Information. CID predominantly results in cleavage of amide bonds near the cross-linked lysines, yielding cross-linker-containing fragment ions such as lysine immonium ions linked to the other peptide (e.g., $K^L\alpha$ or $K^L\beta$) or ions due to cleavage of the cross-link amide bond (e.g., Lα18 or Lβ18; see Scheme 1a). Only a single abundant peptide sequence ion was observed, the b_5 ion of Ac-RFMWMK-NH_2 ($b_{5\beta}$), and no fragment ions of α-MSH were detected. The CID results did not provide sufficient diagnostic sequence information to identify the peptides nor locate the sites of the cross-links.

The corresponding ETD-CID results are shown in Figure 4. In this case, the $[L\alpha 12 + H]^+$ and $[L\beta 11 + 2H]^{2+\bullet}$ produced upon ETD of the (Ac-RFMWMK-NH_2 + BAH + α-MSH) complexes were selected for CID. CID of the even-electron Lα12 ion (Ac-RFMWMK-NH_2 + 131 Da) yielded a variety of a-, b-, and y-ions, the types of product ions which are typically observed upon CID of peptides (Figure 4a). Full sequence coverage (as defined as number of observed backbone cleavages divided by the number of backbone bonds) of the Ac-RFMWMK-NH_2 peptide was obtained, and the cross-link site was confirmed at Lys-6 through the observation of the unmodified b_5 ion and the y_2 ion which contains the 131 Da mass tag. In fact, all of the y-ions contained the cross-linker mass tag. Likewise, there were no ions arising from cleavage of the amide bond connecting the SFB moiety and the lysine side chain. CID of the complementary odd-electron $L\beta 11^{\bullet}$ ion (α-MSH + 119 Da)

yielded a-, b-, c-, y- and z-type ions, a mixture of product ions which are generally observed upon CID (a-, b-, and y-ions) and ETD (c/z$^{\bullet}$-ions) of conventional peptides (Figure 4b). The c- and z-type ions are most likely produced by radical-driven backbone cleavages in which the radical is mobilized upon collisional activation. Since the radical can also be stabilized by delocalization within the pyridinyl ring, dissociation of backbone amide bonds also occurs, yielding the b- and y-ions which are conventional charge-directed fragment ions. While spectral interpretation can be more difficult when a greater array of types of fragment ion are produced, in this case, near complete sequence coverage (91.7%) of the β-peptide is obtained. The 119 Da (SANH) mass tag was retained upon CID of this Lβ11$^{\bullet}$ peptide, thus allowing the cross-linking site to be pinpointed to the Lys-11 site based on the detection of the unmodified b_{10} and modified b_{11} ions. CID of the Lβ11$^{\bullet}$ and Lα12 ions formed upon ETD of the same peptide cross-linked in the opposite orientation, (α-MSH + BAH + Ac-RFMWMK-NH_2), also provided near complete sequence coverage of the two peptides (Figure S-6, Supporting Information).

For both the Lβ11$^{\bullet}$ and Lα12 ions created upon ETD of the cross-linked peptides, the SFB and SANH mass tags were retained upon collisional activation, allowing conclusive identification of the site of the cross-link. It is critical that the cross-linker mass tags are retained upon ion activation; if the cross-link amide bonds are cleaved upon CID, then all tracking information about the unique location of the site of cross-linking is lost. These key amide bonds are in fact cleaved upon CID of the cross-linked peptides, and thus, the sites of cross-linking cannot be determined for the constituent peptides by direct CID. Another advantage of the ETD-CID strategy as opposed to direct CID is that double-cleavage cross-linked product ions are not observed. CID of cross-linked peptides often yields product ions which arise from the cleavage of multiple backbone amide bonds, such as combined cleavage of an amide bond of the α-peptide and an amide bond of the β-peptide, producing fragment ions of the form $b_{n\alpha}{}^{L}y_{n\beta}$.[39] While these types of product ions can be informative, they are difficult to identify and typically require manual interpretation. In contrast, the ETD-CID technique allows the two constituent peptides to be sequenced individually and simplifies spectral interpretation.

Collision-induced dissociation of the charge-reduced products arising from electron transfer (but without dissociation) of the cross-linked peptides was also performed to determine if preferential cleavage of the hydrazone bond could be observed from the nondissociative electron transfer product (Figure S-7, Supporting Information). An example of this type of charge-reduced ion (m/z 951.6) was noted in Figure 2 upon ETD of [Ac-RFMWMK-NH_2 + BAH + α-MSH]$^{4+}$. Typically, supplemental activation in the form of resonant collisional excitation[51] or IR irradiation[52,53] of charge-reduced peptides yields c- and z$^{\bullet}$-type fragment ions. It is thought that the c and z$^{\bullet}$ ions are initially held together by noncovalent interactions in the charge-reduced products, and supplemental activation releases them as free fragment ions. In the present study, CID of the charge-reduced [Ac-RFMWMK-NH_2 + BAH + α-MSH]$^{3+\bullet}$ ion resulted in a broad array of c- and z$^{\bullet}$-type ions, in addition to even-electron b- and y-type fragment ions (Figure S-7, Supporting Information). The abundances of Lα12 and Lβ11$^{\bullet}$ ions was low. These results suggest that only a small fraction of the charge-reduced cross-linked peptides are composed of the Lα12 and Lβ11$^{\bullet}$ ions held together by noncovalent bonds.

The sequence information obtained upon CID of the Lα9 and Lβ9 product ions formed upon ETD of the DSP-cross-linked peptides was compared to the ETD-CID results for the analogous BAH-cross-linked peptides. CID of the Lα9 product ion formed upon ETD of α-MSH + DSP + Ac-RFMWMK-NH_2 produced many unidentifiable product ions in addition to neutral losses stemming from the CO$-CH_2-CH_2-$SH modification at Lys-11 of α-MSH (data not shown). The Lβ9 product ion (i.e., [Ac-RFMWMK-NH_2 + CO$-CH_2-CH_2-$SH]) yielded a complex array of a-, b-, c-, y-, and z-type ions. The amide bond between the cross-linked Lys residue and the CO$-CH_2-CH_2-$SH tag was also cleaved upon CID of the complementary Lα9 and Lβ9 ions, and thus, the tracking information to identify the site of cross-link was lost. In general, significantly lower sequence coverage was obtained using the ETD-CID technique for the DSP-cross-linked peptides compared to the BAH-cross-linked peptides. In addition, upon ETD of these cross-linked peptides, greater preferential cleavage of the hydrazone bond compared to disulfide bond cleavage was observed, thus showcasing the compelling advantages of ETD for the analysis of hydrazone-conjugated peptides.

CONCLUSIONS

Electron transfer dissociation of intermolecularly bis-arylhydrazone-cross-linked peptides resulted in preferential dissociation of the N12−N13 hydrazone bond. Cleavage of the hydrazone bond yields the two constituent peptides: one an even-electron product with a 131 Da mass tag (Lα12) and the other an odd-electron product with a 119 Da mass tag (Lβ11$^{\bullet}$). Greater than 65% of the ion current is composed of these two key products for BAH-cross-linked peptides that possess a mobile proton which is presumed to be at least partially localized at the hydrazone functionality. The hydrazone bond cleaves more efficiently than the disulfide bond in analogous DSP-cross-linked peptides. The high propensity for dissociation of the N12−N13 hydrazone bond is presumed to be due to capture of the electron directly at the protonated hydrazone functionality and subsequent rearrangement. This pathway does not require the migration of a hydrogen atom to initiate dissociation as is proposed for the conventional production of c- and z$^{\bullet}$-type fragment ions upon ETD of peptides. Upon subsequent CID, these primary ETD product ions, Lα12 and Lβ11$^{\bullet}$, afford near complete sequence coverage of each constituent peptide. CID of the Lβ11$^{\bullet}$ ion yielded a-, b-, c-, y-, and z-type ions, and CID of the complementary Lα12 ion produced predominantly a-, b-, and y-type ions, with conclusive identification of the cross-linking site. CID of the intact cross-linked peptides did not afford full sequence coverage of each peptide nor identification of the cross-link site. The ETD-CID technique decouples the two constituent peptides and treats the cross-linked peptide instead

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as two modified peptides which can be sequenced individually, thus allowing the potential use of database search algorithms for peptide sequencing and identification.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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