Mobile Protons Versus Mobile Radicals: Gas-Phase Unimolecular Chemistry of Radical Cations of Cysteine-Containing Peptides^{*,†}

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A combination of electrospray ionization (ESI), multistage, and high-resolution mass spectrometry experiments are used to examine the gas-phase fragmentation reactions of radical cations of cysteine containing di- and tripeptides. Two different chemical methods were used to form initial populations of radical cations in which the radical sites were located at different positions: (1) sulfur-centered cysteinyl radicals via bond homolysis of protonated S-nitrosocysteine containing peptides; and (2) α -carbon backbone-centered radicals via Siu's sequence of reactions (*J. Am. Chem. Soc.* **2008**, *130*, 7862). Comparison of the fragmentation reactions of these regiospecifically generated radicals suggests that hydrogen atom transfer (HAT) between the α C–H of adjacent residues and the cysteinyl radical can occur. In addition, using accurate mass measurements, deuterium labeling, and comparison with an authentic sample, a novel loss of part of the N-terminal cysteine residue was shown to give rise to the protonated, truncated N-formyl peptide (an even-electron x_n ion). DFT calculations were performed on the radical cation $[\text{GCG}]^{+\bullet}$ to examine: the relative stabilities of isomers with different radical and protonation sites; the barriers associated with radical migration between four possible radical sites, $[\text{G}^{\bullet}\text{CG}]^+$, $[\text{GC}^{\bullet}\text{G}]^+$, $[\text{GCG}^{\bullet}]^+$, and $[\text{GC}(\text{S})\text{G}]^+$; and for dissociation from these sites to yield b_2 -type ions. (*J Am Soc Mass Spectrom* 2010, *21*, 1296–1312) © 2010 American Society for Mass Spectrometry

The mobile proton model, pioneered in large by the work of Gaskell and Wysocki, is an elegantly simple and well-established way of describing the low-energy fragmentation reactions of protonated even-electron peptides [1–5]. A key feature of this model is the competition between charge-directed fragmentation and charge-remote fragmentation. The former involves proton migration from the initial ground state of the protonated peptide to a higher energy isomer that can then facilitate heterolytic bond cleavage. When this site corresponds to a peptide bond, a neighboring nucleophile can induce peptide bond cleavage to form b- and y-type sequence ions [6]. In contrast, charge-remote fragmentation reactions are required when the proton is sequestered at a basic site. While there is still some debate on the structures of b-ions [7], the low-

energy fragmentation reactions of protonated peptides that result in the formation of both sequence and non-sequence ions are fairly well understood and have been the subject of several reviews [5–11]. This is due to the world-wide research efforts of many groups who have either conducted experimental and theoretical studies on small model peptides [5–10] or used statistical methods to interrogate large, curated libraries of MS/MS spectra of “real world” peptides, typically formed via tryptic digestion of proteins [12–22]. While more refined fragmentation models such as Paizs and Suhai's “pathways in competition (PIC)” have been developed [10], the “mobile proton” has remained center stage.

Over 50 years ago, Andersson performed the first MS-based study on peptides using electron ionization (EI) [23]. The key fragmentation reactions of the resultant radical cations were correctly described and the inherent problems of peptide volatility were recognized. The latter problem is the reason that gas-phase peptide radical cations largely remained a curiosity for several decades. Fortunately, this situation has been reversed, with a recent renaissance in the formation and reactions of radical ions of biomolecules. The motiva-

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* Dedicated to Vicki Wysocki and Simon Gaskell in honor of their winning the 2009 ASMS Distinguished Contribution in Mass Spectrometry award. Their pioneering work inspired a young lecturer (R.A.J.O.) over a decade ago to examine the mechanistic aspects of the fragmentation reactions of peptides.

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tion for these studies range from fundamental interest in species related to biological processes such as enzyme catalysis [24] and oxidative chemistry associated with damage to biomolecules such as proteins [25], through to the potential for developing novel mass spectrometry-based analytical applications, for example, in the area of proteomics [26]. There are two types of peptide radical cations that can be studied in the gas-phase: (1) hydrogen rich; and (2) canonical/hydrogen deficient. The former are formed via electron capture dissociation (ECD) [27] and electron-transfer dissociation (ETD) [28] and their mechanism of fragmentation has been the subject of intense scrutiny over the past decade [29–33]. The latter class of radical can be formed via a diverse range of methods, including EI; laser desorption coupled with photoionization [34–37]; oxidative dissociation of ternary metal peptide complexes [38–44]; UV-photodissociation of native peptides [45, 46]; homolytic photodissociation of the C–I bond in iodotyrosine-containing peptides and proteins [47, 48]; photodissociation of noncovalent complexes containing a photolabile precursor [49]; electron-induced dissociation [50, 51]; and through peptide derivatization to install a functional group with a weak bond that can undergo facile homolytic cleavage under collision induced dissociation (CID) [52–56].

The mechanistic aspects of the fragmentation reactions of canonical/hydrogen deficient radical cations are both interesting and challenging to unravel since they can potentially either undergo: (1) charge-directed or “spin-remote” [57] fragmentation reactions under mobile proton conditions in which the radical is a spectator; or (2) charge-remote, radical-driven fragmentation reactions [58]. Radical-driven fragmentation reactions can occur from a wide range of radical sites including from backbone radicals [55] and side-chain radicals [47, 49], and can result in cleavage of the peptide backbone to yield sequence ions [47, 49] or losses of small molecules or radicals from side chains to form non-sequence ions [49, 59, 60]. The latter fragmentation reactions allow differentiation between leucine and isoleucine residues [59].

A further interesting feature is the possibility of a “mobile radical,” whereby the initial radical site changes via the occurrence of intramolecular hydrogen atom transfer (HAT). The experimental evidence for such processes is often indirect, and typically involves examining the fragmentation chemistry of regiospecifically generated radical ions. There are three potential classes of HAT, which depend on the sites involved in the hydrogen atom migration: (1) backbone to backbone; (2) side-chain to backbone; (3) side-chain to side-chain. Removal of a backbone α C–H bond is a thermodynamically favored process [61], provided a captodative radical is formed [62]. Indirect evidence that HAT can occur from one α radical site to another (i.e., backbone-to-backbone HAT) comes from the observation that the CID spectra of different α radicals of the same peptide

are virtually identical [55]. Such reactions do not, however, always occur [63–65].

An example of side-chain-to-backbone HAT comes from the work of Ly and Julian [47], who suggested a mechanism whereby a tyrosine-based side-chain radical initiates backbone cleavage via H-atom abstraction of the adjacent N-terminal amide N–H bond. With regards to side-chain-to-side-chain HAT, Hao and Gross have noted [56] that peptides with regiospecifically generated cysteinyl radical sites give x-type sequence ions via radical type cleavages at sites, which are several residues away from the initial S radical. They suggest a HAT reaction from the β -carbon of threonine residues to the thiyl radical. Such a mechanism is consistent with the bond dissociation energies of S–H bonds (typically 81 kcal.mol^{−1}) [56] and the β -carbon of threonine (a theoretical estimate is 86.8 kcal.mol^{−1}) [66].

The side-chain thiolate radicals of cysteine residues represent an important class of radicals in peptide and protein chemistry that are involved in a number of enzyme reactions [24] and are implicated in protein damage [25]. Recent work by Schoeneich’s group has demonstrated that the side-chain thiolate radicals of cysteine residues can abstract hydrogen atoms from amino acids intermolecularly and also intramolecularly from adjacent amino acid residues [67–72]. Given the potential importance of these reactions in protein damage, as well as these being excellent model systems to examine side-chain-to-backbone HAT in gas-phase peptides, here we use a combination of multistage mass spectrometry experiments, collision-induced dissociation, deuterium labeling, and density functional theory (DFT) calculations to study the fragmentation reactions of regiospecifically generated radical cations of simple cysteine-containing di- and tripeptides. We also compare the fragmentation reactions of these radical cations with their even-electron counterparts, which have been previously published [73, 74].

Experimental

Chemicals

All chemicals and reagents obtained commercially were used without further purification. Cysteine, S-nitrosoglutathione, 2,2′:6′,2″-terpyridine and copper(II) nitrate were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Solvents of HPLC grade or higher were obtained from Burdick and Jackson (Muskegon, MI, USA). The peptides CG, GC, GCG, and GGC (90% purity) were obtained from Bachem, (Bubendorf, Switzerland). The peptides YCG, YGC, CYG, GYC, CGY, GCY, YC, CY, and CGG (<60% purity) were synthesized by Bio21 Peptide Technologies (Victoria, Australia). The labeled peptide cysteinyl(3,3-D₂)glycylglycine was synthesized using L-cysteine-N-Fmoc, S-Trityl (3,3-D₂, 98%) (Cambridge Isotope Laboratories, Andover, MA, USA) by Bio21 Peptide Technologies (Victoria, Australia).

Synthesis and Derivatization

The copper(II)-containing ternary complexes were prepared by reaction between equimolar amounts of copper(II) nitrate hemipentahydrate and 2,2':6',2''-terpyridine (Terpy) in EtOH following literature procedure [75] and purified via recrystallization. One milligram of this complex was dissolved in 140 μ L of methanol and 10 μ L aliquot of this solution was mixed with 10 μ L of a solution containing equimolar quantity of the corresponding tyrosine-containing tripeptides in 140 μ L of methanol. The mixture was allowed to sit at room temperature for 30 min, thereafter 140 μ L of methanol was added and the solution injected into the electrospray ionization (ESI) source of the mass spectrometer.

Trans-nitrosylation reactions in solution were carried out by mixing equimolar quantities of 1 mg/140 μ L solutions of S-Nitrosoglutathione (GSNO), and the corresponding cysteine-containing peptide in water, and the reaction was allowed to proceed at room temperature for 10 min. Before infusing the reaction mixture into the ESI source of the mass spectrometer, it was diluted 150-fold with methanol.

Methyl esterification of cysteine-containing peptides was performed by dissolving 10 mg of the lyophilized peptide in 1 mL of the methyl esterification reagent (prepared by the dropwise addition of 800 μ L of acetyl chloride to 5 mL of anhydrous methanol with stirring) and allowed to stand for 2 h at room temperature. The samples were lyophilized and used without further purification.

N-formylamino acids/peptides were prepared by modification of the procedure reported by Sheehan and Yang [76]. To 1.35×10^{-3} moles of the amino acid/small peptide dissolved in 3 mL of 88% formic acid, ca. 1.1 mL of acetic anhydride was added dropwise. The resulting solution was allowed to stir for one hour, after which time ca. 3 mL of ice-water was added. The solvent was evaporated by blowing nitrogen gas over the surface of the liquid. The resulting residue was recrystallized from aqueous ethanol to yield the desired products.

Mass Spectrometry Experiments

All mass spectrometric experiments were conducted on a Thermo Scientific (Bremen, Germany) LTQ FT hybrid mass spectrometer consisting of a linear ion trap (LTQ) coupled to a Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer. Methanolic samples were introduced into the ESI source of the mass spectrometer at a flow rate of 5 μ L/min. Sheath gas, capillary voltage, and temperature were adjusted to ca. 10 arbitrary units, 3.0 kV and 250 $^{\circ}$ C, respectively. Low-energy CID dissociation studies using helium as the collision gas were performed using the standard procedure of mass selec-

tion of the desired precursor ion and subsection of these ions to collisional activation. The activation time for these experiments was 30 ms with the percentage collisional energy optimized for each experiment.

High-resolution mass spectrometry spectra were acquired via transfer of ions from the linear ion trap into the FT-ICR cell. To increase mass accuracy, when experimental conditions permitted, the scanning window was narrowed to 15 Da centered around the ion of interest using the built-in selected reaction monitoring (SRM) and consecutive reaction monitoring (CRM) functionalities. Ions confirmed through accurate mass measurements have been denoted in the spectra by a superscripted double dagger symbol ($\ddagger$) next to its label, with measured and theoretical masses along with the error in ppm listed in Table T1 of the Supplementary Material, which can be found in the electronic version of this article. The positive mode calibration was performed via the automatic calibration function using the recommended LTQ calibration solution, consisting of caffeine, MRFA, and Ultramark 1621 solution.

Theoretical Methods

Geometry optimizations and electronic energy calculations were performed using the Gaussian 03 molecular modeling package [77]. Structures of minima and transition states were optimized at the B3-LYP level of theory [78, 79] with the 6-31++G(d,p) basis set. In the case of open-shell systems, spin-unrestricted calculations (UB3-LYP) were used. All optimized structures were subjected to vibrational frequency analysis to ensure they corresponded to either true minima (no imaginary frequencies) or transition states (one imaginary frequency). Intrinsic reaction coordinate (IRC) runs were performed on each transition state, followed by geometry optimizations to ensure that they connected to the appropriate reactant and product ion minima. The final energies used to calculate the potential energy surfaces were corrected with the (U)B3-LYP/6-31++G(d,p) zero-point vibrational energies, (ZPVE), ($E_{\text{reported}} = E_{\text{electronic}} + E_{\text{zpve}}$), with no scaling factors utilized. All energies are expressed as relative enthalpies, in ΔH , in kcal.mol $^{-1}$.

Results and Discussion

Formation of Radicals at Different Sites

Here we use two different chemical methods to regioselectively create radical sites within a peptide. The first involves the use of CID of protonated S-nitrosocysteine containing peptides to promote bond homolysis to yield sulfur-centered cysteinyl radicals (Scheme 1a), a method that has been successfully employed previously [56, 80]. The second method is one that has been recently utilized by Siu and involves two sequential stages of CID [64, 65]. In the first step, a ternary metal complex containing a peptide with a tyrosine residue is

Scheme 1. The use of two different chemical methods allowed the formation of initial populations of radical cations in which the radical sites were located at different positions: (a) bond homolysis of protonated S-nitrosocysteine-containing peptides yielded sulfur-centered cysteinyl radicals; (b) Siu's sequence of reactions [64, 65] in which a ternary metal complex containing a peptide with a tyrosine residue is subjected to collision-induced dissociation (CID) to yield a peptide radical cation, which, when isolated and subjected to a second stage of CID, forms an α -carbon backbone radical site via loss of a dehydroquinone from the tyrosine site.

subjected to CID to yield a peptide radical cation. This radical cation is isolated and subjected to a second stage of CID to form an α -carbon backbone radical site via loss of a dehydroquinone from the tyrosine residue (Scheme 1b). Generation of α -carbon-centered radical cations via this strategy was only successful for the N-terminal tyrosine peptides YC, YCG, and YGC, and readily proceeds via the spontaneous loss of the tyrosyl side chain. The corresponding tyrosine centered radical cations are unstable in the time span of our experiments and consequently are not observed in the CID spectra (Supplementary Material Figure S1A–C). A variety of ternary metal complexes consisting of various redox-active metals and auxiliary ligands were used in an attempt to generate α -carbon-centered radical cations at other sites without success. The peptides, M, studied included M = CY, CYG, and CGY, while their complexes that were subjected to CID included: $[\text{Cu}^{\text{II}}(\text{terpyBr})\text{M}]^{2+}$, $[\text{Cu}^{\text{II}}(\text{bipy})\text{M}]^{2+}$, $[\text{Cu}^{\text{II}}(12\text{-crown-4})\text{M}]^{2+}$, and $[\text{Fe}^{\text{III}}(\text{salen})\text{M}]^{+}$, $[\text{Fe}^{\text{III}}(\text{salenOCH}_3)\text{M}]^{+}$, $[\text{Mn}^{\text{III}}(\text{salen})\text{M}]^{+}$, where bipy = 2,2'-bipyridine and salen = *N,N'*-ethylenebis(salicylimine).

The fact that it is possible to regiospecifically generate radicals at different sites in these cysteine-containing peptides requires the use of a shorthand nomenclature that specifically identifies each unique radical site. Here we adopt the nomenclature of Chu and coworkers [64, 65] in which residues that contain an α -carbon radical have a superscripted solid circle to their right. As cysteine-centered radicals can be generated in two site-specific locations, we have used the notation C(S) to distinguish sulfur-centered sulfhydryl radicals from their α -carbon-centered counterparts, C. In cases where all possible radical sites for a system are being consid-

ered, the radical site is unspecified with the radical notation located after the square brackets. An analogous nomenclature is used to denote the location of the charge: in peptides in which the ionizing proton is sequestered by an arginine residue a charge symbol is placed next to the arginine one-letter code, whilst the charge notation is placed outside of the square brackets to describe systems in which the protonation site is unspecified. Examples of the nomenclature used in this text are summarized below.

- $[\text{G}^{\bullet}\text{CG}]^{+}$ = α -carbon radical located at the N-terminal glycine residue; site of proton is unspecified;
- $[\text{GC}(\text{S})\text{G}]^{+}$ = Sulfhydryl radical located at the internal cysteine residue; site of proton is unspecified;
- $[\text{GCG}]^{+\bullet}$ = Location of radical and proton sites are unspecified;
- $[\text{GC}(\text{S})\text{R}^{+}]$ = Sulfhydryl radical located at the internal cysteine residue; proton is sequestered by the arginine residue.

Fragmentation Reactions of Radical Cations of the Dipeptides CG and GC

The sulfur-centered radical cations $[\text{C}(\text{S})\text{G}]^{+}$ and $[\text{GC}(\text{S})]^{+}$ were generated through CID of the corresponding S-nitrosylated peptide and subjected to a further stage of collision-induced dissociation. Figure 1a shows that CID of $[\text{C}(\text{S})\text{G}]^{+}$ predominantly gives rise to $[\text{H}_2\text{N} = \text{CHCH}_2\text{S}]^{+}$ at m/z 75, accompanied by a minor loss of ammonia (m/z 161). The low-energy CID of $[\text{GC}(\text{S})]^{+}$ (Figure 1b) yields a much richer spectrum, with abundant formation of the immonium ion $[\text{H}_2\text{N} = \text{CHCH}_2\text{SH}]^{+}$ (m/z 76), which requires hydrogen atom transfer (presumably, backbone to side chain) to occur before its formation; a series of minor fragmentation channels including loss of ammonia (m/z 161), loss of water and carbon monoxide (m/z 132), and formation of the $y_1\text{-H}_2\text{S}$ ion (m/z 88).

Collisional activation of the α -centered radical, $[\text{G}^{\bullet}\text{C}]^{+}$ (Figure 1c) results in a spectrum that is almost identical to that observed for the sulfhydryl centered radical cation $[\text{GC}(\text{S})]^{+}$, suggesting that interconversion occurs between $[\text{GC}(\text{S})]^{+}$ and $[\text{G}^{\bullet}\text{C}]^{+}$, presumably via HAT. Although further studies are required to determine whether $[\text{H}_2\text{N} = \text{CHCH}_2\text{SH}]^{+}$ (m/z 76) is formed via the radical-directed loss of COOH from the y_1 radical cation $[\text{C}(\text{S})]^{+}$, or via the charge-directed loss of CO , H_2O from the protonated y_1 ion, this result shows the role of side chains in facilitating radical migration.

The C-terminal methyl esters of these dipeptides were also investigated. Interestingly, the replacement of the alcohol with a methyl ester moiety does have an impact on the types of fragment ions formed. In the case of $[\text{C}(\text{S})\text{G-OCH}_3]^{+}$ (Figure 1d) a new ion at m/z 118 is observed. It corresponds to protonated N-formylglycine- OCH_3 , formed via the loss of $[\text{C}_2\text{H}_4\text{N}_2\text{S}]$. A related fragmentation reaction is observed for the

Figure 1. LTQ collision-induced dissociation of radical cations of cysteine-containing dipeptides formed via two methods: sulfur-centered radical cations generated via CID of S-nitrosylated derivatives: (a) $[\text{C}(\text{S})\text{G}]^+$; (b) $[\text{GC}(\text{S})]^+$; (d) $[\text{C}(\text{S})\text{G}-\text{OCH}_3]^+$; (e) $[\text{GC}(\text{S})-\text{OCH}_3]^+$; and the α -carbon-centered radical cations (c) $[\text{G}\cdot\text{C}]^+$, formed via oxidation dissociation of the ternary metal complex $[\text{Cu}^{\text{II}}(2,2':6',2''\text{-terpyridine})\text{YC}]^{2+}$. The double dagger symbol ($\ddagger$) indicates that the ion has been confirmed via HRMS. Note that the ion at m/z 150 in (b) and (c) arises from ion-molecule reactions of the product ion at m/z 132 with background water.

tripeptide $[\text{C}(\text{S})\text{GG}]^+$, which is discussed in detail below. In addition, fragment ions at m/z 75 ($[\text{H}_2\text{N}=\text{CHCH}_2\text{S}]^+$) and m/z 175 (loss of NH_3) are formed. Likewise, fragmentation of $[\text{GC}(\text{S})-\text{OCH}_3]^+$ (Figure 1e) resulted in a change to the types of fragment ions observed for the underivatized system, with major fragment ions corresponding to the loss of ammonia (m/z 175) and methanol (m/z 160). Finally, the presence of the y_1 ion at m/z 136 suggests an N-terminal backbone-to-side-chain hydrogen atom transfer.

Fragmentation Reactions of Radical Cations of the Tripeptides CGG, GCG, GG

The regiospecifically generated radical cations $[\text{C}(\text{S})\text{GG}]^+$, $[\text{GC}(\text{S})\text{G}]^+$, $[\text{GGC}(\text{S})]^+$, $[\text{G}\cdot\text{CG}]^+$, and $[\text{G}\cdot\text{GC}]^+$ were subjected to low-energy collision induced dissociation, resulting in the spectra shown in Figure 2a–f, respectively. Although we were unable to independently generate radicals at each of the α -carbon positions, general comparisons can be made with the previous studies performed on

Figure 2. LTQ collision-induced dissociation of radical cations of cysteine containing tripeptides: sulfur centered radical cations generated via CID of S-nitrosylated derivatives: (a) [C(S)GG]^{•‡}; (b) [GC(S)G]^{•‡}; (c) [GGC(S)]^{•‡}; (d) FT-MS spectrum of the [N-formyl-glycylglycine + H]⁺ ion from MS³ of [C(S)GG]^{•‡}; and α -carbon-centered radical cations formed via oxidative dissociation of various ternary metal complexes: (e) [G'CG]^{•‡} formed from MS/MS of [Cu^{II}(2,2':6',2''-terpyridine)YCG]²⁺; (f) [G'GC]^{•‡} formed from MS/MS of [Cu^{II}(2,2':6',2''-terpyridine)YGC]²⁺. The double dagger symbol (‡) indicates that the ion has been confirmed via HRMS. Note in (d) the dash lines correspond to the exact masses of the b₂ and [N-formyl-glycylglycine + H]⁺ ions calculated from their molecular formula.

the dissociation of [GGG]^{•‡} [64, 65]: (1) charge-directed sequence ions are not always present (Figure 2a); (2) due to the presence of the cysteinyl side-chain there is a greater number and variety of non-sequence ions, and unlike [GGG]^{•‡} many result from radical-directed processes (Fig-

ure 2a and c); and (3) depending on their initial location and the position of the side chain, migration of radical centers is facilitated by the presence of a cysteinyl side chain (Figure 2b, e, c, and f). The fragmentation chemistries of these cysteinyl radical cations are explored in more detail below.

N-terminal Cysteine Residue: Fragmentation Reactions of [C(S')GG]⁺

The major CID product of [C(S')GG]⁺ (Figure 2a) corresponds to an ion at *m/z* 161. Accurate mass measurements on this ion suggests that it corresponds to an even-electron *x*₂ ion rather than the more commonly observed isobaric *b*₂ ion (Figure 2d). Other losses observed include NH₃ (*m/z* 218) and CH₂S (*m/z* 189). The latter loss was also observed in the low-energy CID spectrum of the cysteine radical cation [80]. Dissociation of the corresponding C-terminal methyl ester, [C(S')GG-OCH₃]⁺, shown in Supplementary Material Figure S2A, yields similar losses.

To provide structural evidence that the even-electron *x*₂ ion corresponds to protonated N-formyl-glycylglycine, the CID spectrum of protonated N-formyl-glycylglycine (Figure 3a) generated through collisional activation of [CGG]⁺ was compared with that of an independently synthesized sample of N-formyl-glycylglycine, which was subjected to ESI (Figure 3b). The two spectra appear indistinguishable, with identical product ions and relative abundances, confirming that the protonated N-formyl-glycylglycine is the major dissociative product of [C(S')GG]⁺.

How is this *x*₂ ion formed? DFT calculations (data not shown) on a range of isomeric structures of the [C₂H₄N,S] radical lost suggest that a likely candidate is the conjugated S-centered radical H₂NCHCHS. This in turn suggested a mechanism involving H atom attack to cleave the C–C bond (Scheme 2). To confirm the involvement of the β-H atom, a deuterium-labeled cysteinyl(3,3-D₂)glycylglycine was utilized, in which the two hydrogen atoms on the cysteine side chain were replaced with deuteriums. An examination of the CID spectrum of [C(3,3-D₂)(S')GG]⁺ shown in Figure 3c reveals that one deuterium is retained to yield an ion at *m/z* 162. Interestingly, this ion was found to be highly reactive towards adventitious background water within the ion trap, resulting in H/D exchange reactions to yield the corresponding unlabelled *x*₂ ion, protonated N-formyl-glycylglycine, at *m/z* 161, as confirmed by MS³ experiments (data not shown).

Puzzled by this H/D exchange, which is unexpected for the mechanism shown in Scheme 2, we carried out the following additional experiments. First, we subjected the *m/z* 162 ion from [C(3,3-D₂)(S')GG]⁺ to CID (Figure 3d) and observed a product corresponding to a deuterated *y*₁ ion at *m/z* 77, together with minor losses of CO (*m/z* 134), HDO (*m/z* 143), and H₂O (*m/z* 144). Next, we synthesized the expected deuterium-labeled product of Scheme 2, N-formyl(D)-glycylglycine and subjected it to ESI/MS. The resultant [M + H]⁺ was subjected to CID and gave the spectrum shown in Figure 3e. This spectrum is clearly different from that shown in Figure 3d, since it is dominated by the non-deuterated *y*₁ ion (*m/z* 76), while the losses of H₂O

(*m/z* 144), CO (*m/z* 134), and H₂O, CO (*m/z* 116) show no evidence for a mobile D⁺. Then we subjected the independently synthesized N-formyl-glycylglycine to ESI using a partially deuterium-labeled solvent system (1:1 MeOH: D₂O). The resultant [M + D]⁺, which is expected to have both mobile H⁺ and D⁺, gave the CID spectrum shown in Figure 3f, which closely resembles that of Figure 3d. Finally, we subjected the deuterium labeled ion [D₂NCH(CD₂S')C(O)NDCH₂C(O)NDCH₂CO₂D + D]⁺ to CID (Supplementary Figure S3) and discovered N-formyl-glycylglycine product ions with a range of deuterium labels at *m/z* 165, 166, and 167, which correspond to the losses of [C₂D₄N,S], [C₂H,D₃N,S], and [C₂H₂D₂N,S]. These various deuterium labeling experiments reveal that a more complicated mechanism is operating than that shown in Scheme 2.

C-terminal Cysteine Residue: Fragmentation Reactions of [GGC(S')]⁺ and [G'GC]⁺

The low-energy dissociation of [GGC(S')]⁺ shown in Figure 2c yields a more complex spectrum in comparison to those observed for [C(S')GG]⁺ and [GC(S')G]⁺ (Figure 2a and b). The spectrum is dominated by two ions: formation of [b₂-H]⁺ (*m/z* 114) and an ion corresponding to concomitant losses of HS' and CO₂ (*m/z* 158). Other notable losses corresponding to H₂O (*m/z* 217), CH₂S (*m/z* 189), [a₂-H]⁺ (*m/z* 86), the c₂ sequence ion (*m/z* 132), and the loss of HS' (*m/z* 202) can be observed. The combined loss of HS' and CO₂ uniquely identifies the C-terminal residue as cysteine, and Scheme 3 shows a potential concerted mechanism for this loss.

The CID spectrum of [G'GC]⁺ (Figure 2f) is simpler than that of [GGC(S')]⁺ since it only exhibits ions corresponding to [b₂-H]⁺ (*m/z* 114), loss of H₂O (*m/z* 217) and [a₂-H]⁺ (*m/z* 86). Since the relative abundances of these fragment ions are very similar to those observed in the CID spectrum of [GGC(S')]⁺, this suggests that [GGC(S')]⁺ rearranges to [G'GC]⁺ in competition with other fragmentation channels (e.g., HS', CO₂, and CH₂S). In contrast, the absence of losses of HS', CO₂, and CH₂S from the CID spectrum of [G'GC]⁺ suggests that [G'GC]⁺ does not rearrange to [GGC(S')]⁺.

Internal Cysteine Residue: Fragmentation Reactions of [GC(S')G]⁺ and [G'CG]⁺

CID of [GC(S')G]⁺ and [G'CG]⁺ resulted in the spectra shown in Figure 2b and e, which look very similar and are dominated by the formation of the radical cation [b₂-H]⁺ (*m/z* 160). This same ion can be observed following dissociation of [GC(S')G-OCH₃]⁺ (Supplementary Material Figure S2B). The formation of the [b₂-H]⁺ ion can occur via a charge-directed process in which the radical is immobile, and [b₂-H]⁺ is produced

Figure 3. LTQ collision-induced dissociation of: (a) protonated N-formyl-glycylglycine synthesized in the gas-phase via MS⁴ of [C(S)GG]⁺; (b) an “authentic” structure of protonated N-formyl-glycylglycine formed via ESI on a sample of N-formyl-glycylglycine synthesized in the condensed phase; [76] (c) [C(3,3-D₂)(S)GG]⁺; (d) deuterated N-formyl-glycylglycine synthesized in the gas-phase via MS⁴ of [C(3,3-D₂)(S)GG]⁺; (e) an “authentic” structure of protonated N-formyl(D)-glycylglycine formed via ESI on a sample of N-formyl(D)-glycylglycine synthesized in the condensed phase; [76] (f) an “authentic” structure of deuterated N-formyl-glycylglycine formed via ESI on a sample (1:1 MeOH:D₂O) of N-formyl-glycylglycine synthesized in the condensed phase [76]. The double dagger symbol (‡) indicates that the ion has been confirmed via HRMS.

directly from either the sulfur-centered radical cation [GC(S)G]⁺ or the α -carbon-centered radical cation [G[•]CG]⁺. Conversely, its formation may operate via a “mobile radical” process in which the radical migrates to a site away from its initial site of generation to one of three intermediates [GC(S)G]⁺, [G[•]CG]⁺, or [GC[•]G]⁺, followed by dissociation of the peptide via charge-

directed processes. Unfortunately, MS⁴ studies on each of the two [b₂-H]⁺ ions were unable to shed light on the origin of these ions, since they both fragment via the loss of CO (Supplementary Material Figures S4). Thus, in the next section we use DFT calculations to elucidate the structures and possible fragmentation reactions of radical cations of GCG.

Scheme 2. Mechanism for the formation of N-formyl-peptides.

DFT Calculations on Mobile Radicals Versus Mobile Protons in the Radical Cations of the Tripeptide Containing an “Interior” Cysteine Residue: [GCG]⁺

Due to the considerable theoretical complexities involved in studying radical cations of tripeptides (i.e., site of radical, site of proton), the DFT calculations were restricted to the systems which gave the simplest CID spectra: [GC(S)G]⁺, and [G[•]CG]⁺. As discussed above, the MS/MS spectra of these two regiospecifically generated radicals resulted in similar-looking spectra. Thus a key objective of these studies was to establish whether interconversion between the radical sites occurs before fragmentation.

Since there are numerous permutations for intramolecular hydrogen-atom and proton transfers, a comprehensive examination of all possible pathways and their corresponding potential energy surfaces is beyond the scope of this work. Instead, we have adopted a similar methodology to that used to examine the interconversion of α -carbon-centered triglycine radical cations [64], whereby only hydrogen atom migrations between α -carbons were examined. In addition, as suggested by the gas-phase experiments above, the presence of the cysteinyl side chain can facilitate radical migrations and consequently HAT from the sulfhydryl site to each of the three α -carbons was also explored. Investigations into nitrogen and oxygen centered radical migrations were also conducted, but as the enthalpic barriers obtained for these systems are higher than the corresponding carbon- and sulfhydryl-centers, these results are not presented here. A further challenge is the presence of multiple potential protonation sites, and with the exception of protonation on the thiol site, every possible heteroatom protonation site was examined. Only those that are directly involved in HAT/dissociation are, however, presented here. Although these results are not meant to represent a comprehensive study of all the possible pathways for hydrogen-atom and proton transfer reactions, the aim of these DFT calculations was to offer some insights into acces-

sible fragmentation pathways of [GCG]⁺ under conditions of low-energy CID (Figure 2b and e).

Low-Energy Structures of the Hydrogen-Deficient [GCG]⁺ Radical Cation

First, a survey of the low-energy structures of the various tautomers of hydrogen-deficient GCG radical cation was conducted. All possible combinations of the radical sites at the sulfhydryl and α -carbon positions together with the protonation sites at the amino nitrogen and the carbonyl oxygen were considered, giving rise to the sixteen tautomeric structures shown in Table 1, with the energy of each structure in kcal.mol⁻¹ and relative to the lowest energy tautomer, **02a**. Due to the large number of degrees of freedom of this tripeptide the entire conformational space for each of these tautomers was not examined. Instead, the structures shown in Table 1 represent the most stable structures found through a combination of geometry optimizations based on input guesses that maximize the number of internal hydrogen-bonds and those discovered from IRC runs that connected local minima to the various transition states for HAT and fragmentation discussed further below.

From the structures shown in Table 1, apparently the energy of the various tautomers is dictated by the extent of stabilization of both the ionizing proton and the radical. The lowest-energy structure of all 16 tautomers, **02a**, contains a seven-membered ring in which the charge of the ionizing proton is delocalized onto the internal carbonyl oxygen. In addition, the N-terminal α -carbon radical center is captodatively stabilized by both the strongly electron-donating properties of the amino group, in combination with the strongly electron-withdrawing properties of the protonated first carbonyl oxygen. The effect of these two stabilization energies are evident in the Structures **03** and **01** in which the site of the proton is located on the second carbonyl oxygen and the amino group respectively. Although for Structure **03** the charge remains delocalized over a seven-membered ring, the extent of captodative stabilization of the radical center is lowered due to the weaker electron-withdrawing ability of the -CONH- group; consequently, **03** is higher in enthalpy than **02a** by 16.5 kcal.mol⁻¹. Protonation of the amino moiety to give Structure **01a**, changes the strongly electron-donating NH₂⁻ into an electron-withdrawing N⁺H₃⁺ group, destabilizing the radical center to give a structure that is ca. 35 kcal.mol⁻¹ higher in energy than **02a**. This type of destabilization has been previously

Scheme 3. Potential mechanism for the loss of HS[•] and CO₂ from [GGC(S)]⁺.

Table 1. Sixteen tautomeric low-energy structures of the hydrogen-deficient $[\text{GCG}]^+$ radical cation

Radical site	Protonation site			
	Amino	Carbonyl oxygen 1	Carbonyl oxygen 2	Carbonyl oxygen 3
G [•] CG	 01 <u>35.1</u>	 02a <u>0.0</u>	 03 <u>16.5</u>	 04 <u>29.5</u>
GC [•] G	 05 <u>20.7</u>	 06a <u>24.5</u>	 07a <u>6.5</u>	 08 <u>27.8</u>
GCG [•]	 09 <u>15.5</u>	 10a <u>15.4</u>	 11 <u>17.5</u>	 12 <u>35.8</u>
GC(S [•])G	 13 <u>16.0</u>	 14a <u>14.4</u>	 15 <u>20.4</u>	 16 <u>36.9</u>

The structures were generated by examining all possible combinations of the radical sites at the α -carbon and sulfur positions together with protonation at the amino group and the carbonyl oxygen. The optimized structures and their corresponding energies were calculated using (U)B3-LYP/6-31++G(d,p). The lower underlined number corresponds to the energy of the structure (in kcal.mol⁻¹) relative to **02a**. For structures and cartesian coordinates of each species, refer to Figure S5 of the Supplementary Material.

noted by Yu et al., who found a much higher BDE for the C–H bond in protonated glycine relative to neutral glycine [81]. Another way of thinking about this phenomenon is that installation of a radical site adjacent to the protonated N-terminus enhances its acidity and thus makes other sites in the peptide more attractive to locate the proton on. Thus, a radical site on the α carbon directly next to the protonated N-terminus facilitates mobilization of the proton.

Due to the weaker electron-donating properties of the amide nitrogens, the radical centers located on the α -carbon of the second and third residues, $[\text{GC}^{\bullet}\text{G}]^+$ and $[\text{GCG}^{\bullet}]^+$, are less stabilized and, consequently, higher in energy than their N-terminal α -carbon radical counterparts. The most stable tautomer of $[\text{GC}^{\bullet}\text{G}]^+$ is **07a**, in which the α -centered radical is located on the internal residue and is captodatively stabilized by the first amide nitrogen, and the strongly electron-withdrawing $-\text{C}(\text{OH}^+)\text{NH}-$ group. Protonation on the other sites does not contribute towards captodative stabilization,

thus making them higher in energy. Protonation on the second carbonyl oxygen for $[\text{GCG}^{\bullet}]^+$ (**11**), yields a structure that is higher in energy (17.5 kcal.mol⁻¹) than the corresponding structures protonated on the amino (**09**) and N-terminal carbonyl oxygen (**10a**) (15.5 and 15.4 kcal.mol⁻¹, respectively).

Finally, the two lowest energy structures of the sulfur-centered $[\text{GC}(\text{S}^{\bullet})\text{G}]^+$ are the amino and carbonyl oxygen 1 species (**13** and **14**) possibly due to the delocalization of the charge through hydrogen bonding to form five- and seven-membered rings, respectively.

Potential Mechanisms for the Dissociation of the $[\text{GCG}]^+$ Radical Cation

Since the CID spectra of $[\text{GCG}]^+$ essentially only give a single product, three possible dissociative pathways for the generation of the $[\text{b}_2\text{-H}]^+$ ion were considered, together with a fourth pathway in which the radical

center may migrate to the α -carbon of the C-terminal glycine residue, resulting in the formation of an even-electron b_2 ion (not observed experimentally). The potential energy surfaces (PES) are given in Figure 4a–d, and each of these are considered in separate sections

below. As the radical cation generated from the dissociation of $[\text{GC}(\text{SNO})\text{G}]^+$ would be expected to dissociate to yield the sulfur-centered radical cation $[\text{GC}(\text{S})\text{G}]^+$, the various pathways originate from the lowest energy tautomer of $[\text{GC}(\text{S})\text{G}]^+$, **14a**.

Figure 4. (U)B3-LYP/6-31++G(d,p) calculated reaction pathways and associated structures for key structures involved in the loss of glycine from $[\text{GC}(\text{S})\text{G}]^+$ via: (a) $[\text{GC}(\text{S})\text{G}]^+$; (b) $[\text{G}^+\text{CG}]^+$; (c) $[\text{GC}^+\text{G}]^+$; and the loss of $[\text{Gly-H}]^+$ from (d) $[\text{GCG}]^+$. For structures and cartesian coordinates of each species, refer to Figure S5 of the Supplementary Material. All numbers (in kcal.mol⁻¹) are relative to **02a**.

Mechanism for Formation of the $[b_2-H]^+$ Radical Cation from $[GC(S')G]^+$

The loss of neutral glycine to yield the oxazolone radical cation $[b_2-H]^+$ is predicted to occur via **14b**, which is 9.9 kcal.mol⁻¹ higher in energy than **14a** (Figure 4a). The dissociation occurs via a concerted mechanism in which the ionizing proton is transferred from the first carbonyl oxygen onto the terminal amide nitrogen, thereby allowing for a neighboring group attack by this first carbonyl oxygen onto the second carbonyl carbon. This process occurs via transition-state **TS14b-17**, which has a barrier of 39.0 kcal.mol⁻¹ above **02a** to yield the ion-molecule complex **17**, which upon separation yields the oxazolone radical cation, **18**, with an overall endothermicity of 48.7 kcal.mol⁻¹.

Mechanism for Formation of the $[b_2-H]^+$ Radical Cation from $[G'CG]^+$

Figure 4b shows the PES for the interconversion of $[GC(S')G]^+$ to $[G'CG]^+$, followed by dissociation to yield the b_2 -type ion **20**. Thus the key first step in the fragmentation pathway must involve H-atom migration from the N-terminal α -carbon to the sulfur in the precursor **14c**, to give **02b**, which has been calculated to have a barrier of 41.5 kcal.mol⁻¹. The subsequent dissociation from conformer **02c** begins through protonation of the C-terminal amide from the first carbonyl oxygen, followed immediately by nucleophilic attack of the N-terminal carbonyl oxygen to yield the ion-molecule complex **19**. The resulting $[b_2-H]^+$ ion **20** is 17.7 kcal.mol⁻¹ more stable than the isomeric **18**.

Mechanism for Formation of the $[b_2-H]^+$ Radical Cation from $[GCG]^+$

Figure 4c shows that the migration of the radical center from $[GC(S')G]^+$ to $[GCG]^+$ occurs via a 1,3-H atom transfer from the low-energy conformer **14a** via **TS14a-07a** to yield **07a**. The barrier for this interconversion is 38.1 kcal.mol⁻¹. The subsequent dissociation of this ion occurs stepwise: formation of the intermediate **21b** through proton migration from the second carbonyl oxygen onto the nearby N-terminal amide via **TS07b-21b** that lies 65.3 kcal.mol⁻¹ higher in energy than **02a**, followed by dissociation via **TS21a-22** to finally give the $[b_2-H]^+$ isomer **23** which has an endothermicity of 42.1 kcal.mol⁻¹. The substantial barrier associated with **TS07b-21b** suggests this pathway may not occur¹.

¹ A reviewer has pointed out that the barrier against proton migration seems too high. Unfortunately we were unable to locate any transition states involving proton transfer between the first two carbonyl oxygen atoms. In addition attempts were made to locate transition states involving protonation of the thiol moiety as suggested by the reviewer, but these were also unsuccessful.

Mechanism for Formation of the b_2 Cation from $[GCG]^+$

The 1,6-H atom transfer required for the interconversion from $[GC(S')G]^+$ to $[GCG]^+$ occurs via **TS14d-10b** resulting in **10b** (Figure 4d). This transition-state has a barrier 16.7 kcal.mol⁻¹ higher than the preceding ion, **14d**. In a mechanism similar to that observed for $[GC(S')G]^+$ and $[G'CG]^+$, this isomer dissociates via a concerted mechanism. The proton is transferred from the first carbonyl oxygen onto the N-terminal peptide bond, followed by neighboring group attack by the same carbonyl oxygen to result in the b_2 ion solvated by glycine, **24**. The barrier for this dissociation is 47.7 kcal.mol⁻¹ (**TS10e-24**) relatively to **02a**. Removal of the neutral glycine from the ion-molecule complex **24** gives the b_2 ion which is 37.2 kcal.mol⁻¹ above **02a**. As the barrier required for this reaction is much higher than the barriers for dissociation of $[GC(S')G]^+$ and $[G'CG]^+$, this loss is not observed in Figure 2b.

Potential Energy Surfaces for the Interconversions Among the α -Carbon Centered $[G'CG]^+$, $[GC'G]^+$, and $[GCG]^+$

Conversion from $[G'CG]^+$ to $[GC'G]^+$. Figure 5a shows the potential energy surface for the interconversion of $[G'CG]^+$, **02d**, to $[GC'G]^+$, **21c**, via a 1,4-H atom transfer from the α -carbon of the cysteine residue to the α -carbon of the N-terminal glycine residue. The barrier is 33.3 kcal.mol⁻¹ relative to **02d** and 27.7 kcal.mol⁻¹ relative to **06a**. To link **06a** to the structure required for the dissociation, **21a**, the transition-state **TS06b-21c** was calculated, corresponding to the barrier involved in the migration of the proton from the N-terminal carbonyl to the amide bond located on the C-terminus. The barrier for this proton transfer is 26.8 kcal.mol⁻¹ relative to the lowest energy Structure **02a**.

Conversion from $[GC'G]^+$ to $[GCG]^+$. The potential energy surface for the interconversion of $[GC'G]^+$ to $[GCG]^+$ is shown in Figure 5b. This involves a 1,4-H atom transfer between the α -carbons of the second and third residues, and proceeds via **TS07b-10c**, which is predicted lie 47.1 kcal.mol⁻¹ higher in energy than **02a**.

Conversion from $[GCG]^+$ to $[G'CG]^+$. Figure 5c shows the potential energy surface for the 1,7-H atom transfer required to interconvert $[GCG]^+$ to $[G'CG]^+$. This radical migration is predicted to occur starting from **10d**, which is ca. 24.0 kcal.mol⁻¹ higher in enthalphy than **02a**, and then proceeding via **TS10d-02e** to **02e**. The forward and reverse barriers for this rearrangement relative to **10d** and **02c** are 54.6 and 56.3 kcal.mol⁻¹, respectively.

Scheme 4 combines the key energies of Figures 4 and 5 to provide an overview of the barriers associated with radical migration between the four possible radical location sites, and for dissociation from these sites to

Figure 5. (U)B3-LYP/6-31++G(d,p) calculated reaction pathways and associated structures for key structures involved in interconversions between: (a) $[G^+CG]^{+\bullet}$ and $[GC^+G]^{+\bullet}$; (b) $[GC^+G]^{+\bullet}$ and $[GCG]^{+\bullet}$; (c) $[GCG]^{+\bullet}$ and $[G^+CG]^{+\bullet}$. For structures and cartesian coordinates of each species, refer to Figure S5 of the Supplementary Material. All numbers (in kcal.mol^{-1}) are relative to **02a**.

yield b_2 -type ions. From an examination of this scheme, two conclusions can be made: (1) radicals which are initially located on the sulfur, amino or internal α -carbon will not migrate, as the barriers for radical migration are greater than those of the mobile proton pathways required to form $[b_2-H]^{+\bullet}$ ions; and (2) radicals generated on the C-terminal α -carbon will migrate via a 1,6 HAT reaction to yield a sulfur-centered radical, which subsequently fragments into a $[b_2-H]^{+\bullet}$ ion. This suggests that the two $[b_2-H]^{+\bullet}$ ions observed for $[GC(S)G]^{+\bullet}$ and $[GCG]^{+\bullet}$ (Figure 2b and e) originate from different precursor ions

and their formation occurs solely via mobile proton pathways. The latter point is consistent with observation of Siu and coworkers [65] whereby a two-step proton migration involving canonical $[GGG]^{+\bullet}$ was proposed to catalyze the interconversion from $[GGG]^{+\bullet}$ to $[G^+GG]^{+\bullet}$.

Blocking Competing Mobile Proton Pathways by Sequestering the Proton onto an Arginine Residue

Since the DFT studies indicated that interconversion between the tautomers of $[GCG]^{+\bullet}$ before fragmentation

Scheme 4. (U)B3-LYP/6-31++G(d,p) calculated enthalpies and local minima involved in the interconversions and dissociations of the various isomers of $[GCG]^+$. For structures and cartesian coordinates of each species, refer to Figure S5 of the Supplementary Material. All numbers (in kcal.mol^{-1}) are relative to **02a**.

is unlikely due to the energetically more favorable charge-directed fragmentation pathways, we were interested in examining a system in which competing mobile proton pathways were blocked. Through the introduction of an arginine residue, the proton is sequestered, potentially allowing for HAT processes and radical-driven cleavages. An examination of the resultant MS^3 spectra of the site-specific hydrogen-deficient GCR radical cation $[GC(S)R]^+$ (Figure 6a) shows that it fragments in an identical manner to the previously studied $[GCR]^+$ (Figure 6b), [60] with radical-directed losses of $\text{HS}^\cdot$ (m/z 301), and CH_2S (m/z 288). The loss of $\text{HS}^\cdot$ is most likely to occur via HAT from the α -carbon of the central residue, followed by an α -cleavage reaction, whilst the previously discussed CH_2S loss is initiated from the sulfur-centered sulfhydryl radical. The presence of both of these losses for the two systems indicates that radical migration occurs for both systems, with tautomerism of $[GCR]^+$ to $[GC(S)R]^+$ required for loss of $\text{HS}^\cdot$. Interestingly, a minor ion corresponding to protonated N-formyl-arginine is observed at m/z 203, confirmed via accurate mass measurements and comparison of the CID spectrum of this ion (Figure 6c) with

that of the $[M + H]^+$ of an independently synthesized sample of N-formyl-arginine (Figure 6d).

General Comparison of the Fragmentation Reactions of Protonated and Radical Cations of the Cysteine-Containing Peptides CG, GC, CGG, GCG, and GGC

To examine the effect of removing the hydrogen atom on the sulfhydryl moiety to yield a radical center, the fragmentation reactions of protonated and sulfur-centered radical cations were briefly compared. CID of $[CG + H]^+$ and $[GC + H]^+$ in the linear ion trap proceeds via losses of NH_3 and H_2O , respectively (Supplementary Material Figure S6A and B), and the spectra are very similar to those found in a three-dimensional ion trap [73, 74]. Both losses occur through intramolecular nucleophilic attack by the cysteinyl side-chain thiol onto the α -carbon and carbonyl-carbon, respectively. In contrast, the presence of a sulfur-centered radicals, $[C(S)G]^+$ and $[GC(S)]^+$, disfavors dissociation via these neighboring group pathways,

Figure 6. LTQ collision-induced dissociation of (a) the sulfur centered radical cation $[GC(S)R]^+$ generated via CID of its S-nitrosylated derivative; (b) the α -carbon-centered radical cation $[G^\bullet CR]^+$; formed via oxidative dissociation of the ternary metal complex $[Cu^{II}(2,2':6',2''\text{-terpyridine})GCR]^{2+}$; (c) protonated N-formyl-arginine synthesized in the gas-phase via MS⁴ of $[GC(S)R]^+$; (d) protonated N-formyl-arginine synthesized in the condensed phase. The double dagger symbol ($\ddagger$) indicates that the ion has been confirmed via HRMS.

yielding the immonium ions instead (Supplementary Material Figure S6C and D). The same trend is observed for N- or C-terminal cysteine-containing tripeptides: losses corresponding to b_2 and H_2O are observed for $[CGG + H]^+$ and $[GGC + H]^+$, respectively (Supplementary Material Figure S7A and C), whilst the radical cations $[C(S)GG]^+$ and $[GGC(S)]^+$ (Supplementary Material Figure S7D and F) dissociate to give sequence and non-sequence ions that occur primarily via radical-driven processes. Systems containing an internal cysteine residue, $[GCG + H]^+$ and $[GC(S)G]^+$, (Supplementary Material Figure S7B and E) dissociate in a similar manner to yield b_2 -type ions, although H_2O loss is not observed for the radical system. Thus, as has been previously noted for the library of tripeptides, GXR, radical cations can fragment quite differently from their protonated counterparts [60].

Conclusions

Despite decades of work, mass spectrometry-based studies on peptides continue to provide new and fascinating fragmentation chemistry. For example, here we

have discovered a novel fragmentation reaction that occurs at a cysteine residue to give a truncated N-formyl peptide, corresponding to an even-electron x_n ion. Apart from the inherent structural diversity of peptides, one of the reasons that new types of fragmentation reactions continue to appear is that the types of peptide ions that are examined can be manipulated. For instance, the nature of the charge (cation versus anion) can be varied; metal complexes of peptides can be examined; a range of charge states are available; numerous ways of forming radical ions have been developed.

The current work further highlights that the CID spectra of canonical/hydrogen-deficient radical cations are complementary to those of the even-electron protonated ions. The presence of both a charge and a radical makes unraveling the mechanisms of the fragmentation reaction of the former systems challenging. The simplest peptide radical cations appear to be those where the charge is sequestered (i.e., non-mobile) on a basic residue such as arginine, allowing charge-remote, HAT, and radical-driven fragmentation reactions to proceed. In cases where the peptide lacks a basic residue, mobile-proton- and charge-directed fragmentation reactions

can compete with HAT and radical-driven fragmentation reactions. The CID spectra of the radical cations of CG, GC, CGG, GCG, and GGC show that a combination of proton- and radical-driven processes can occur, and that these are dependent on both the position of the cysteine residue and the initial site of the radical center.

When does the mobile proton “win” over the mobile radical? Although this question cannot yet be fully answered, the following observations can be made: (1) both mobile proton and mobile radical processes can only occur if the barriers to proton or hydrogen atom transfer are below the barriers for fragmentation at the original charge or radical site; (2) the typical energetic requirement to cleave amide bonds or eliminate neutral molecules in protonated peptides with mobile protons lies in an energy range between 25–40 kcal mol⁻¹ [80]. If radical pathways such as HAT and radical directed cleavages are to compete, the barriers associated with the processes need to fall within the energy range; (3) the initial site of radical generation can play a role, as illustrated for [GGC]⁺. Whilst mobile-proton-type losses are only observed for [G[•]GC]⁺, [GGC(S)]⁺ dissociates via a combination of the mobile-proton-type losses observed for [G[•]GC]⁺, and via odd-electron processes to form unique fragments. This former requires tautomerization of the radical to occur, with the similar peak abundances and identities suggesting that radical interconversion from [GGC(S)]⁺ to [G[•]GC]⁺ occurs.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2010.01.027](https://doi.org/10.1016/j.jasms.2010.01.027).

References

- Burlet, O.; Orkiszewski, R. S.; Ballard, K. D.; Gaskell, S. J. Charge Promotion of Low-Energy Fragmentations of Peptide Ions. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 658–662.
- Jones, J. L.; Dongre, A. R.; Somogyi, A.; Wysocki, V. H. Sequence Dependence of Peptide Fragmentation Efficiency Curves Determined by Electrospray Ionization/Surface-Induced Dissociation Mass Spectrometry. *J. Am. Chem. Soc.* **1994**, *116*, 8368–8369.
- Dongre, A. R.; Jones, J. L.; Somogyi, A.; Wysocki, V. H. Influence of Peptide Composition, Gas-Phase Basicity, and Chemical Modification on Fragmentation Efficiency: Evidence for the Mobile Proton Model. *J. Am. Chem. Soc.* **1996**, *118*, 8365–8374.

- Tsapraillis, G.; Nair, H.; Somogyi, A.; Wysocki, V. H.; Zhong, W.; Futrell, J. H.; Summerfield, S. G.; Gaskell, S. J. Influence of Secondary Structure on the Fragmentation of Protonated Peptides. *J. Am. Chem. Soc.* **1999**, *121*, 5142–5154.
- Wysocki, V. H.; Tsapraillis, G.; Smith, L. L.; Breci, L. A. Mobile and Localized Protons: A Framework for Understanding Peptide Dissociation. *J. Mass Spectrom.* **2000**, *35*, 1399–1406.
- O'Hair, R. A. J. The Role of Nucleophile-Electrophile Interactions in the Unimolecular and Bimolecular Gas-Phase Ion Chemistry of Peptides and Related Systems. *J. Mass Spectrom.* **2000**, *35*, 1377–1381.
- Harrison, A. G. To b or Not to b: The Ongoing Saga of Peptide b Ions. *Mass Spectrom. Rev.* **2009**, *28*, 640–654.
- Schlosser, A.; Lehmann, W. D. Five-Membered Ring Formation in Unimolecular Reactions of Peptides: A Key Structural Element Controlling Low-Energy Collision-Induced Dissociation of Peptides. *J. Mass Spectrom.* **2000**, *35*, 1382–1390.
- Polce, M. J.; Ren, D.; Wesdemiotis, C. Dissociation of the Peptide Bond in Protonated Peptides. *J. Mass Spectrom.* **2000**, *35*, 1391–1398.
- Paizs, B.; Suhai, S. Fragmentation Pathways of Protonated Peptides. *Mass Spectrom. Rev.* **2005**, *24*, 508–548.
- Barlow, C. K.; O'Hair, R. A. J. Gas-Phase Peptide Fragmentation: How Understanding the Fundamentals Provides a Springboard to Developing New Chemistry and Novel Proteomic Tools. *J. Mass Spectrom.* **2008**, *43*, 1301–1319.
- Barton, S. J.; Whittaker, J. C. Review of Factors that Influence the Abundance of Ions Produced in a Tandem Mass Spectrometer and Statistical Methods for Discovering These Factors. *Mass Spectrom. Rev.* **2009**, *28*, 177–187.
- Huang, Y.; Wysocki, V. H.; Tabb, D. L.; Yates, J. R. The Influence of Histidine on Cleavage C-Terminal to Acidic Residues in Doubly Protonated Tryptic Peptides. *Int. J. Mass Spectrom.* **2002**, *219*, 233–244.
- Tabb, D. L.; Smith, L. L.; Breci, L. A.; Wysocki, V. H.; Lin, D.; Yates, J. R. III Statistical Characterization of Ion Trap Tandem Mass Spectra from Doubly Charged Tryptic Peptides. *Anal. Chem.* **2003**, *75*, 1155–1163.
- Breci, L. A.; Tabb, D. L.; Yates, J. R. III; Wysocki, V. H. Cleavage N-Terminal to Proline: Analysis of a Database of Peptide Tandem Mass Spectra. *Anal. Chem.* **2003**, *75*, 1963–1971.
- Kapp, E. A.; Schutz, F.; Reid, G. E.; Eddes, J. S.; Moritz, R. L.; O'Hair, R. A. J.; Speed, T. P.; Simpson, R. J. Mining a Tandem Mass Spectrometry Database To Determine the Trends and Global Factors Influencing Peptide Fragmentation. *Anal. Chem.* **2003**, *75*, 6251–6264.
- Tabb, D. L.; Huang, Y.; Wysocki, V. H.; Yates, J. R. III Influence of Basic Residue Content on Fragment Ion Peak Intensities in Low-Energy Collision-Induced Dissociation Spectra of Peptides. *Anal. Chem.* **2004**, *76*, 1243–1248.
- Elias, J. E.; Gibbons, F. D.; King, O. D.; Roth, F. P.; Gygi, S. P. Intensity-Based Protein Identification by Machine Learning from a Library of Tandem Mass Spectra. *Nat. Biotechnol.* **2004**, *22*, 214–219.
- Huang, Y.; Triscari, J. M.; Tseng, G. C.; Pasa-Tolic, L.; Lipton, M. S.; Smith, R. D.; Wysocki, V. H. Statistical Characterization of the Charge State and Residue Dependence of Low-Energy CID Peptide Dissociation Patterns. *Anal. Chem.* **2005**, *77*, 5800–5813.
- Barton, S. J.; Richardson, S.; Perkins, D. N.; Bellahn, I.; Bryant, T. N.; Whittaker, J. C. Using Statistical Models to Identify Factors That Have a Role in Defining the Abundance of Ions Produced by Tandem MS. *Anal. Chem.* (Washington, DC) **2007**, *79*, 5601–5607.
- Huang, Y.; Tseng, G. C.; Yuan, S.; Pasa-Tolic, L.; Lipton, M. S.; Smith, R. D.; Wysocki, V. H. A Data-Mining Scheme for Identifying Peptide Structural Motifs Responsible for Different MS/MS Fragmentation Intensity Patterns. *J. Proteome Res.* **2008**, *7*, 70–79.
- Savitski, M. M.; Kjeldsen, F.; Nielsen, M. L.; Zubarev, R. A. Relative Specificities of Water and Ammonia Losses from Backbone Fragments in Collision-Activated Dissociation. *J. Proteome Res.* **2007**, *6*, 2669–2673.
- Andersson, C. O. Mass Spectrometric Studies on Amino Acids and Dipeptide Derivatives. *Acta Chem. Scand.* **1958**, *12*, 1353.
- Stubbe, J. Radicals with a Controlled Lifestyle. *Chem. Commun.* **2003**, 2511–2513.
- Davies, M. J. The Oxidative Environment and Protein Damage. *Biochim. Biophys. Acta Prot. Proteom.* **2005**, *1703*, 93–109.
- Ly, T.; Julian, R. R. Ultraviolet Photodissociation: Developments Towards Applications for Mass-Spectrometry-Based Proteomics. *Angew. Chem. Int. Ed.* **2009**, *48*, 7130–7137.
- Zubarev, R. A.; Kelleher, N. L.; McLafferty, F. W. Electron Capture Dissociation of Multiply Charged Protein Cations. A Nonergodic Process. *J. Am. Chem. Soc.* **1998**, *120*, 3265–3266.
- Syka, J. E. P.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. Peptide and Protein Sequence Analysis by Electron Transfer Dissociation Mass Spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9528–9533.
- Zubarev, R. A. Reactions of Polypeptide Ions with Electrons in the Gas Phase. *Mass Spectrom. Rev.* **2003**, *22*, 57–77.
- Cooper, H. J.; Håkansson, K.; Marshall, A. G. The Role of Electron Capture Dissociation in Biomolecular Analysis. *Mass Spectrom. Rev.* **2005**, *24*, 201–222.
- Syrstad, E. A.; Tureček, F. Toward a General Mechanism of Electron Capture Dissociation. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 208–224.
- Gunawardena, H. P.; He, M.; Chrisman, P. A.; Pitteri, S. J.; Hogan, J. M.; Hodges, B. D. M.; McLuckey, S. A. Electron Transfer Versus Proton Transfer in Gas-Phase Ion/Ion Reactions of Polyprotonated Peptides. *J. Am. Chem. Soc.* **2005**, *127*, 12627–12639.

33. Tureček, F. N–Ca Bond Dissociation Energies and Kinetics in Amide and Peptide Radicals. Is the Dissociation a Non-ergodic Process? *J. Am. Chem. Soc.* **2003**, *125*, 5954–5963.
34. Becker, C. H.; Wu, K. J. On the Photoionization of Large Molecules. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 883–888.
35. Weinkauff, R.; Schanen, P.; Yang, D.; Soukara, S.; Schlag, E. W. Elementary Processes in Peptides: Electron Mobility and Dissociation in Peptide Cations in the Gas Phase. *J. Phys. Chem.* **1995**, *99*, 11255–11265.
36. Grottemeyer, J.; Schlag, E. W. Peptides Investigated by Laser Desorption-Multiphoton Ionization Mass Spectrometry. Part VII. *Org. Mass Spectrom.* **1988**, *23*, 388–396.
37. Cui, W.; Hu, Y.; Lifshitz, C. Time Resolved Photo Dissociation of Small Peptide Ions—Combining Laser Desorption with Ion Trap/Reflectron TOF Mass Spectrometry. *Eur. Phys. J. D* **2002**, *20*, 565–571.
38. Chu, I. K.; Rodriguez, C. F.; Lau, T.-C.; Hopkinson, A. C.; Siu, K. W. M. Molecular Radical Cations of Oligopeptides. *J. Phys. Chem. B* **2000**, *104*, 3393–3397.
39. Barlow, C. K.; Wee, S.; McFadyen, W. D.; O'Hair, R. A. J. Designing copper(II) Ternary Complexes to Generate Radical Cations of Peptides in the Gas Phase: Role of the Auxiliary Ligand. *Dalton Trans.* **2004**, 3199–3204.
40. Bagheri-Majidi, E.; Ke, Y.; Orlova, G.; Chu, I. K.; Hopkinson, A. C.; Siu, K. W. M. Copper-Mediated Peptide Radical Ions in the Gas Phase. *J. Phys. Chem. B* **2004**, *108*, 11170–11181.
41. Barlow, C. K.; McFadyen, W. D.; O'Hair, R. A. J. Formation of Cationic Peptide Radicals by Gas-Phase Redox Reactions with Trivalent Chromium, Manganese, Iron, and Cobalt Complexes. *J. Am. Chem. Soc.* **2005**, *127*, 6109–6115.
42. Hopkinson, A. C.; Siu, K. W. M. Peptide Radical Cations. In *Principles of Mass Spectrometry Applied to Biomolecules*; Laskin, J.; Lifshitz, C., Eds.; John Wiley and Sons, Inc.: Hoboken, NJ, 2006; p. 301–335.
43. Hopkinson, A. C. Radical Cations of Amino Acids and Peptides: Structures and Stabilities. *Mass Spectrom. Rev.* **2009**, *28*, 655–671.
44. Tureček, F. Copper-Biomolecule Complexes in the Gas Phase. The Ternary Way. *Mass Spectrom. Rev.* **2007**, *26*, 563–582.
45. Joly, L.; Antoine, R.; Broyer, M.; Dugourd, P.; Lemoine, J. Specific UV Photodissociation of Tyrosyl-Containing Peptides in Multistage Mass Spectrometry. *J. Mass Spectrom.* **2007**, *42*, 818–824.
46. Fung, Y. M. E.; Kjeldsen, F.; Silivra, O. A.; Chan, T. W. D.; Zubarev, R. A. Facile Disulfide Bond Cleavage in Gaseous Peptide and Protein Cations by Ultraviolet Photodissociation at 157 nm. *Angew. Chem. Int. Ed.* **2005**, *44*, 6399–6403.
47. Ly, T.; Julian, R. R. Residue-Specific Radical-Directed Dissociation of Whole Proteins in the Gas Phase. *J. Am. Chem. Soc.* **2008**, *130*, 351–358.
48. Liu, Z.; Julian, R. R. Deciphering the Peptide Iodination Code: Influence on Subsequent Gas-Phase Radical Generation with Photodissociation ESI-MS. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 965–971.
49. Sun, Q.; Nelson, H.; Ly, T.; Stoltz, B. M.; Julian, R. R. Side Chain Chemistry Mediates Backbone Fragmentation in Hydrogen Deficient Peptide Radicals. *J. Proteome Res.* **2009**, *8*, 958–966.
50. Lioe, H.; O'Hair, R. A. J. Comparison of Collision-Induced Dissociation and Electron-Induced Dissociation of Singly Protonated Aromatic Amino Acids, Cysteine, and Related Simple Peptides Using a Hybrid Linear Ion Trap-FT-ICR Mass Spectrometer. *Anal. Bioanal. Chem.* **2007**, *389*, 1429–1437.
51. Ly, T.; Yin, S.; Loo, J. A.; Julian, R. R. Electron-Induced Dissociation of Protonated Peptides Yields Backbone Fragmentation Consistent with a Hydrogen-Deficient Radical. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 2099–2101.
52. Hodyss, R.; Cox, H. A.; Beauchamp, J. L. Bioconjugates for Tunable Peptide Fragmentation: Free Radical Initiated Peptide Sequencing (FRIPS). *J. Am. Chem. Soc.* **2005**, *127*, 12436–12437.
53. Masterson, D. S.; Yin, H.; Chacon, A.; Hachey, D. L.; Norris, J. L.; Porter, N. A. Lysine Peroxycarbamates: Free Radical-Promoted Peptide Cleavage. *J. Am. Chem. Soc.* **2004**, *126*, 720–721.
54. Chacon, A.; Masterson, D. S.; Yin, H.; Liebler, D. C.; Porter, N. A. N-Terminal Amino Acid Side-Chain Cleavage of Chemically Modified Peptides in the Gas Phase: A Mass Spectrometry Technique for N-Terminus Identification. *Bioorg. Med. Chem.* **2006**, *14*, 6213–6222.
55. Wee, S.; Mortimer, A.; Moran, D.; Wright, A.; Barlow, C. K.; O'Hair, R. A. J.; Radom, L.; Easton, C. J. Gas-Phase Regiocontrolled Generation of Charged Amino Acid and Peptide Radicals. *Chem. Commun. (Cambridge, UK)* **2006**, 4233–4235.
56. Hao, G.; Gross, S. S. Electrospray Tandem Mass Spectrometry Analysis of S- and N-Nitrosopeptides: Facile Loss of NO and Radical-Induced Fragmentation. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1725–1730.
57. Seymour, J. L.; Tureček, F. Structure, Energetics, and Reactivity of Ternary Complexes of Amino Acids with Cu(II) and 2,2'-bipyridine by density functional theory. A Combination of Radical-Induced and Spin-Remote Fragmentations. *J. Mass Spectrom.* **2002**, *37*, 533–540, 1 plate.
58. Laskin, J.; Yang, Z.; Lam, C.; Chu, I. K. Charge-Remote Fragmentation of Odd-Electron Peptide Ions. *Anal. Chem.* **2007**, *79*, 6607–6614.
59. Wee, S.; O'Hair, R. A. J.; McFadyen, W. D. Side-Chain Radical Losses from Radical Cations Allows Distinction of Leucine and Isoleucine Residues in the Isomeric Peptides Gly-XXX-Arg. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 884–890.
60. Wee, S.; O'Hair, R. A. J.; McFadyen, W. D. Comparing the Gas-Phase Fragmentation Reactions of Protonated and Radical Cations of the Tripeptides GXR*. *Int. J. Mass Spectrom.* **2004**, *234*, 101–122.
61. Rauk, A.; Yu, D.; Taylor, J.; Shustov, G. V.; Block, D. A.; Armstrong, D. A. Effects of Structure on α C–H Bond Enthalpies of Amino Acid Residues: Relevance to H Transfers in Enzyme Mechanisms and in Protein Oxidation. *Biochemistry* **1999**, *38*, 9089–9096.
62. Viehe, H. G.; Janousek, Z.; Merenyi, R.; Stella, L. The Captodative Effect. *Acc. Chem. Res.* **1985**, *18*, 148–154.
63. Pingitore, F.; Bleiholder, C.; Paizs, B.; Wesdemiotis, C. Unimolecular Chemistry of Metal Ion-Coordinated α -Dipeptide Radicals. *Int. J. Mass Spectrom.* **2007**, *265*, 251–260.
64. Chu, I. K.; Zhao, J.; Xu, M.; Siu, S. O.; Hopkinson, A. C.; Siu, K. W. M. Are the Radical Centers in Peptide Radical Cations Mobile? The Generation, Tautomerism, and Dissociation of Isomeric α -Carbon-Centered Triglycine Radical Cations in the Gas Phase. *J. Am. Chem. Soc.* **2008**, *130*, 7862–7872.
65. Siu, C.-K.; Zhao, J.; Laskin, J.; Chu, I. K.; Hopkinson, A. C.; Siu, K. W. M. Kinetics for Tautomerizations and Dissociations of Triglycine Radical Cations. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 996–1005.
66. Rauk, A.; Yu, D.; Armstrong, D. A. Toward Site Specificity of Oxidative Damage in Proteins: C–H and C–C Bond Dissociation Energies and Reduction Potentials of the Radicals of Alanine, Serine, and Threonine Residues—An Ab Initio Study. *J. Am. Chem. Soc.* **1997**, *119*, 208–217.
67. Schoeneich, C. Mechanisms of Protein Damage Induced by Cysteine Thiol Radical Formation. *Chem. Res. Toxicol.* **2008**, *21*, 1175–1179.
68. Nausser, T.; Schoeneich, C. Thiol Radicals Abstract Hydrogen Atoms from the (α)C–H Bonds in Model Peptides: Absolute Rate Constants and Effect of Amino Acid Structure. *J. Am. Chem. Soc.* **2003**, *125*, 2042–2043.
69. Nausser, T.; Pelling, J.; Schoeneich, C. Thiol Radical Reaction with Amino Acid Side Chains: Rate Constants for Hydrogen Transfer and Relevance for Post-Translational Protein Modification. *Chem. Res. Toxicol.* **2004**, *17*, 1323–1328.
70. Mozziconacci, O.; Sharov, V.; Williams, T. D.; Kerwin, B. A.; Schoeneich, C. Peptide Cysteine Thiol Radicals Abstract Hydrogen Atoms from Surrounding Amino Acids: The Photolysis of a Cysteine Containing Model Peptide. *J. Phys. Chem. B* **2008**, *112*, 9250–9257.
71. Mozziconacci, O.; Williams, T. D.; Kerwin, B. A.; Schoeneich, C. Reversible Intramolecular Hydrogen Transfer Between Protein Cysteine Thiol Radicals and α C–H Bonds in Insulin: Control of Selectivity by Secondary Structure. *J. Phys. Chem. B* **2008**, *112*, 15921–15932.
72. Nausser, T.; Cusi, G.; Koppenol, W. H.; Schoeneich, C. Reversible Intramolecular Hydrogen Transfer Between Cysteine Thiol Radicals and Glycine and Alanine in Model Peptides: Absolute Rate Constants Derived from Pulse Radiolysis and Laser Flash Photolysis. *J. Phys. Chem. B* **2008**, *112*, 15034–15044.
73. Reid, G. E.; Simpson, R. J.; O'Hair, R. A. J. A Mass Spectrometric and Ab Initio Study of the Pathways for Dehydration of Simple Glycine and Cysteine-Containing Peptide [M + H]⁺ Ions. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 945–956.
74. O'Hair, R. A. J.; Styles, M. L.; Reid, G. E. Role of the Sulfhydryl Group on the Gas Phase Fragmentation Reactions of Protonated Cysteine and Cysteine Containing Peptides. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1275–1284.
75. Castro, L.; Faus, J.; Julve, M.; Gleizes, A. Complex Formation Between Oxalate and (2,2':6',2''-Terpyridyl)Copper(II) in Dimethyl Sulfoxide Solution. Synthesis and Crystal Structures of Mono- and Dinuclear Complexes. *Dalton Trans.* **1991**, 1937–1944.
76. Sheehan, J. C.; Yang, D.-D. H. The Use of N-Formylamino Acids in Peptide Synthesis. *J. Am. Chem. Soc.* **1958**, *80*, 1154–1158.
77. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R. Jr.; Montgomery, J. A.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03*, Revision E 01; Gaussian, Inc.: Wallingford CT, 2004.
78. Becke, A. D. Density-Functional Thermochemistry. III. The Role of Exact Exchange. *J. Chem. Phys.* **1993**, *98*, 5648–5652.
79. Lee, C.; Yang, W.; Parr, R. G. Development of the Colle-Salvetti Correlation-Energy Formula into a Functional of the Electron Density. *Phys. Rev. B* **1988**, *37*, 785–789.
80. Ryzhov, V.; Lam, A. K. Y.; O'Hair, R. A. J. Gas-Phase Fragmentation of Long-Lived Cysteine Radical Cations Formed Via NO Loss from Protonated S-Nitrosocysteine. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 985–995.
81. Yu, D.; Rauk, A.; Armstrong, D. A. Radicals and Ions of Glycine: An Ab Initio Study of the Structures and Gas-Phase Thermochemistry. *J. Am. Chem. Soc.* **1995**, *117*, 1789–1796.