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# Intra-molecular Reactions as a New Approach to Investigate Bio-Radical Reactivity: A Case Study of Cysteine Sulfinyl Radical

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**Herein, we demonstrated the use of gas-phase intra-molecular reactions facilitated by collisional activation to investigate bi-molecular reactions with inherent low reactivity. Reactions between sulfinyl radical (-SO•) toward free thiol (-SH) were employed as a model system. A new reaction channel, i.e. sulfinyl exchange with thiol was observed under beam-type collision-induced dissociation (CID), which was not detectable from traditional ion/molecule reactions.**

Cysteine, a sulfur containing amino acid, is one of the most reactive sites within a protein upon radical attack.[1] Cysteinyl peptides such as glutathione act as redox buffers[2] and antioxidants to prevent or repair damage to cellular components caused by reactive oxygen species.[3] Cysteine sulfinyl radical ($^{SO\bullet}$Cys) is a reactive intermediate involved in the inactivation of enzymes (i.e. pyruvate formate-lyase) utilizing the glycyl/thiyl radical in their catalytic functions upon exposure to air.[4] Partially owing to the difficulty of detecting and characterizing transient species such as bio-radicals at low concentrations under physiological conditions, detailed knowledge is limited with regard to the reactivity and structures of protein radicals. Studying the gas-phase chemistry of bio-radicals provides direct experimental evidences of their intrinsic chemical properties, which can be helpful in unveiling the fate of bio-radical species including intra- or inter-molecular radical transfer after the initial formation.

Gas-phase ion/molecule reactions have been well established as a useful means to study bio-organic radical reactivity, structure, and migration.[5-11] In our previous report, gas-phase ion/molecule reactions were utilized to investigate the reactivity of peptide sulfinyl radical ions with organic disulfides, thiols, and oxygen in a linear ion trap mass spectrometer.[12] Sulfinyl radical appeared to be the least reactive species relative to the thiyl and perthiyl radical. No detectable reaction products were observed from ion/molecule reactions of peptide sulfinyl ions with thiophenol.[12] A possible reason of not observing hydrogen abstraction by -SO• from –SH (Reaction 1) might stem from the relatively large endothermicity of the reaction. The bond dissociation energy (BDE) of an S-H bond (i.e., 69.8-81.4 kcal/mol in a variety of substituted thiophenols[13] and 81.4-88.5 kcal/mol in cysteine[14]) is typically much higher than that of the BDE of the SO-H bond (i.e., 68.6-73.1 kcal/mol for several sulfenic acids[15]). However, Reaction 2, which is the sulfinyl exchange with a thiol group, should be thermally neutral. If energetic collisions[16] can overcome the associated reaction energy barrier, this reaction could be potentially observed.

Peptide-SO• + RS-H → Peptide-SOH + RS• Reaction 1
Peptide-SO• + RS-H → Peptide-S-H + RSO• Reaction 2

In this study, we utilized ***intra-molecular reactions*** as an alternative way to study species of inherent low reactivity. The key concept is to place the two functional groups of interest within the same molecule scaffold. Such an ion is then subjected to additional activation (e.g. collisional activation) to enhance the interaction between the two functional groups (analogous to collisions) by overcoming the energy barrier necessary for conformational changes or reactions. Intra-molecular proton or hydrogen atom transfers in biomolecule ion systems have been investigated in a similar fashion.[17-19] The advantage of intra-molecular reactions as compared to bi-molecular reactions exists in that the chances of the two functional groups interacting are no longer limited by collision rates or number density of reagents.[20, 21] In order to test the feasibility of this intra-molecular reaction approach, we chose to investigate the reactions between cysteine sulfinyl radical and thiol (no reaction products were observed using traditional ion/molecule reactions).

Selectin binding peptide, a natural peptide with the first and last amino acid residue (cysteine) connected by an intrachain disulfide bond (***Pep 1***, sequence and structure shown in Fig. 1a), was used as a model system to form peptide ions consisting of both sulfinyl (-SO•) and thiol (-SH) functional groups. Radical reactions in a nanoelectrospray ionization (nanoESI) plume were utilized to form the peptide ion system containing both a sulfinyl radical and a thiol group.[22-24] In short, oxidative radicals (presumably OH radicals) produced by an atmospheric pressure low temperature helium plasma cleaved the intrachain disulfide bond of ***Pep 1*** via dissociative addition, leading to the formation of -SO• at one cysteine and -SH on the other one. Such a radical reaction product

has a distinct mass (+ 17 Da) as compared to the intact peptide and therefore can be readily isolated in a mass spectrometer. It is convenient to generate an intra-molecular system containing both -SO• and -SH functional groups within a peptide using this method. However, ions formed in this manner may contain a mixture of peptide sulfinyl radical location isomers due to a possibility of forming SO•/SH at either disulfide sulfurs as indicated in Fig. 1a. The radical reaction product (*m/z* 532.3) of ***Pep 1*** was isolated and subjected to collisional activation. The on-resonance ion trap CID data of the radical reaction product (*m/z* 532.3) of ***Pep 1*** is shown in Fig. 1b. Peaks labelled as $^{H}b_n$ or $^{H}y_n$ indicate that fragment ions (*b* or *y* type) contain a thiol functional group at that cysteinyl residue, while the complimentary fragment ion, $^{O}b_n$ or $^{O}y_n$, indicates the presence of the sulfinyl radical at the other cysteinyl residue. The major fragment ions in Fig. 1b, such as $^{H}b_{5\text{-}8}$ and $^{O}y_{2\text{-}7}$, suggest that the sulfinyl radical is located at the C-terminal cysteine (having a structure of ***Pep 2-C***). The detection of $^{O}b_8^{2+}$ ions (45% relative intensity normalized to $^{H}b_8^{2+}$, the base peak) clearly suggests the presence of the sulfinyl radical location isomer (sulfinyl radical at the N-terminus, ***Pep 2-N***). This phenomenon is consistent with results obtained from a different linear ion trap mass spectrometer.[23] The population of the two isomers can be estimated based on the ratio of the total intensities of all fragment ions from each isomer. A ratio of 2.5 was calculated for ***Pep 2-C***: ***Pep 2-N***, corresponding to about 70% of ***Pep 2-C*** in the mixture of peptide sulfinyl radical isomers.

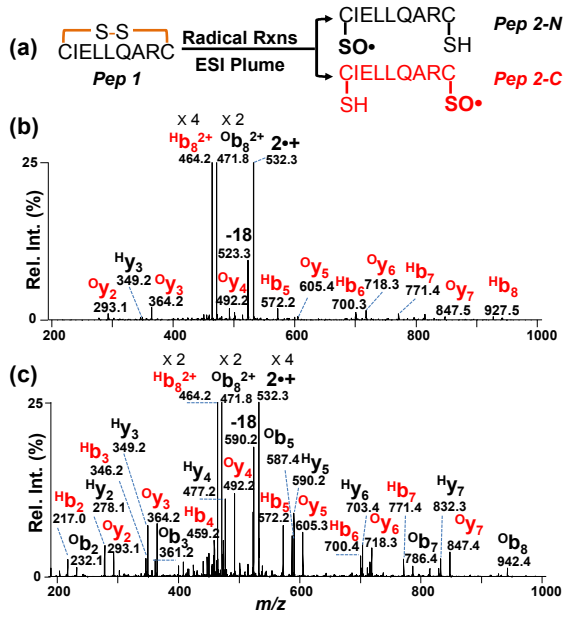


**Fig. 1.** **(a)** Method of forming peptide ions consisting of both sulfiny radical and thiol based on radical reactions of ***Pep 1***. $MS^2$ CID data of peptide sulfinyl radical ions (2+, *m/z* 532.3) via **(b)** ion trap CID, activation energy 60 mV and 200 ms activation time; and **(c)** beam-type CID, CE =17 V.

Beam-type CID data of the 2+ peptide sulfinly radical ions (*m/z* 532.3) is shown in Fig. 1c (Collision energy (CE), 17 V). Compared to on-resonance ion trap CID, beam-type CID offers higher collision energy (in tens of eV vs. meV, lab energy frame) and energy deposition happens in a shorter time scale (~ sub ms vs. hundreds of ms).[25] Sequence ions arising from sulfinyl radical located at the C-terminal cysteine (peaks labelled in red; $^{H}b_{2,\ 4\text{-}8}$ and $^{O}y_{2\text{-}7}$) and those arising from sulfinyl radical at the N-terminal cysteine (peaks labelled in black; $^{O}b_{2\text{-}8}$ and $^{H}y_{2\text{-}7}$) can both be detected in relatively high abundances. A ratio of 0.96 was obtained for ***Pep 2-C***: ***Pep 2-N***, indicating that there is almost an equal amount of each isomer. The ion trap and beam-type CID results were highly repeatable from experiments conducted on different days. Given the fact that the same population of parent peptide sulfinyl radical ions was sampled, isolated, and subjected to ion activation/dissociation on a same instrument, it is intriguing that different populations of peptide sulfinyl radical isomers are deduced from beam-type and ion trap CID. A possible cause leading to such a difference is that the sulfinyl radical reacts with a thiol within the peptide ion via a pathway proposed in Reaction 2 (exchange of sulfinyl radical) and therefore changes the population of isomers from their initial formation. However, without knowing the initial isomer population upon ion formation, it is difficult to characterize the factors that may affect Reaction 2 based on the data shown in Fig. 1.

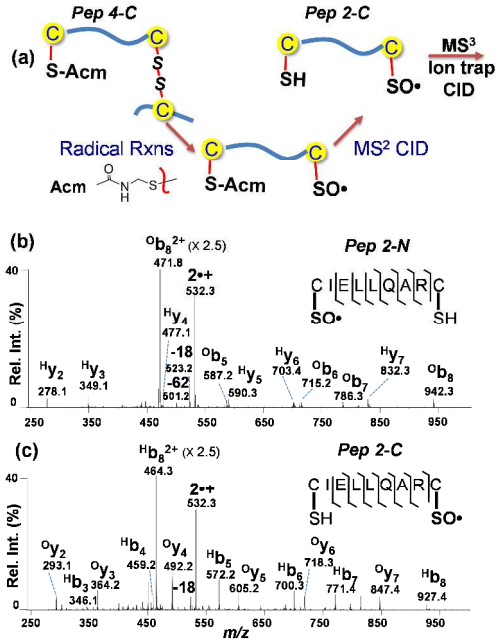


**Fig. 2.** **(a)** Method of forming peptide ions consisting of site-specific sulfinyl radical and thiol. $MS^2$ ion trap CID data of *m/z* 532.3 (2+) of **(b)** ***Pep 2-N***, activation energy 65 mV and 200 ms activation time and **(c)** ***Pep 2-C***, activation energy 70 mV and 200 ms activation time.

In order to form a peptide system containing a site-specific -SO• and -SH (i.e., ***Pep 2-C*** and ***Pep 2-N*** in Fig. 1a), multiple variants of ***Pep 1*** were employed. Fig. 2a shows one of the approaches via radical reactions of the interchain linked disulfide peptides in a nanoESI plume.[26, 27] Using ***Pep 4-C*** as an example, a ***Pep 1*** variant with an Acm (acetamidomethyl) protecting group at the N-terminal cysteine and an interchain disulfide linkage at the C-terminal cysteine, the sulfinyl radical was formed at the C-terminus due to the selective S-S cleavage from radical reactions. This peptide sulfinyl radical ion species (*m/z* 567.8, 2+) was mass-selected and further subjected to collisional activation. Gentle collisional activation allowed removal of the Acm protecting group and formation of a site-specific thiol group at the N-terminus (***Pep 2-C***, *m/z* 532.3, 2+). The sulfinyl radical location isomer, ***Pep 2-N*** (sulfinyl radical at the N-terminus and thiol at the C-terminus), was formed using the same approach shown in Fig. 2a, however, started

from ***Pep 4-N***. The data showing the formation of ***Pep 2-N*** and ***2-C*** via $MS^2$ CID are presented in Fig. S1, Supporting Information. $MS^3$ ion trap CID data of the peptide sulfinyl radical location isomers (***Pep 2-N*** and ***Pep 2-C***) are shown in Fig. 2b and 2c, respectively. No products arising from hydrogen abstraction by sulfinyl radical or sulfinyl radical exchange with a thiol (Reaction 1 or 2, respectively) were observed after careful examination of the spectra (details in Supporting Information). The above data suggest that intra-molecular reactions are not induced under ion trap CID conditions.

Given the available MS instrument setting, beam-type CID can only be performed at $MS^2$ stage and therefore it is impossible to directly compare ion trap and beam-type CID on the same population of sulfinyl radical ions using the method described in Fig. 2. To overcome this limitation, ***Pep 5-C*** (A Chain: CIELLQARC/B Chain: Cys, A9-B1 disulfide) and ***5-N (***A Chain: CIELLQARC/B Chain: Cys, A1-B1 disulfide) variants of ***Pep 1*** consisting of a free thiol and an interchain disulfide linkage with an N-acetylated -L-cysteine-methyl ester, were utilized to form ***Pep 2-C*** and ***Pep 2-N*** correspondingly in $MS^1$ step . Fig. 3a depicts such a process using ***Pep 5-C*** as an example to form ***Pep 2-C*** sulfinyl radical ions on-line, which are further subjected to either beam-type or ion trap CID (mass spectra shown in Fig. S2, Supporting Information). The data were re-organized in Fig. 3b to reflect the population of ***Pep 2-C*** at individual cleavage sites under ion trap and beam-type CID conditions. ***Pep 2-C*** % is calculated as the total intensity of complementary $^{H}$b and $^{O}$y ions from ***Pep 2-C*** at a given cleavage site over the sum of backbone fragments at that site from both ***Pep 2-C*** and ***Pep 2-N*** if there is sulfinyl transfer. The list of ions and their intensities are provided in Table S3 and S4 (Supporting Information).

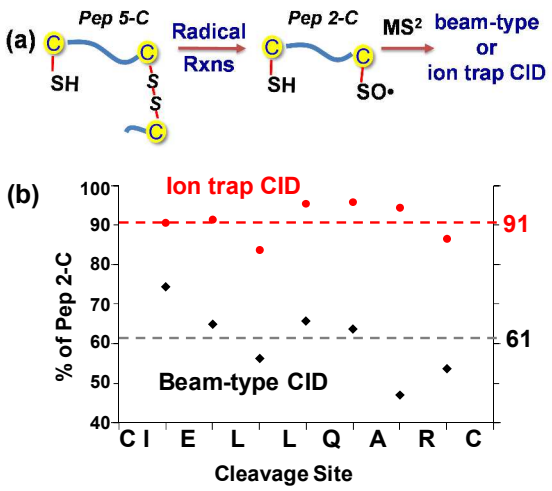


**Fig. 3.** (**a**) Method of forming **Pep 2-C** via **Pep 5-C**. (**b**) Graph showing the Pep 2-C% at each fragment site from $MS^2$ beam-type CID (diamond) and ion trap CID (circle).

As shown in Fig 3b, the detected fragments reflect that ***Pep 2-C*** is the major ion form, accounting for 91% of the total ion population under ion trap CID condition. Contrary to the ion trap CID data, when the same population of ions were subjected to beam-type CID, ***Pep 2-C*** % decreases to 61% in the total ion population. This change of ***Pep 2-C*** % provides unambiguous evidence that sulfinyl radical has transferred from C-terminus, the initial site, to N-terminus under higher energy activation conditions. Activation energies as well as degrees of parent ion consumption were not found to significantly affect the reproducibility of CID spectra of Pep 2-C and they provided consistent isomer ratios (Table S5, Supporting Information). Since the data in Fig. 2 suggest that there should be no sulfinyl transfer under ion trap CID conditions, the 91% purity of ***Pep 2-C*** in Fig. 3b is likely due to a small degree of isomerization of ***Pep 5-C*** to ***Pep 5-N*** before reactions during sample preparation (vacuum drying or storage). It has been shown that the free thiol group is very sensitive to air oxidation and pH changes and disulfide scrambling may occur.[28] This is probably also the reason that a less pure ***Pep 2-N*** is obtained from ***Pep 5-N*** (data not shown).

In summary, we report a new approach utilizing "intra-molecular reactions" to facilitate reactions between cysteine sulfinyl radical ($^{SO\bullet}$Cys) and thiol (-SH). Energetic collisional activation allows the two functional groups to come close enough for reactions and also supplies energies to overcome associated reaction activation barriers. Many of the limitations (reaction rates, low product yields, etc…) of bimolecular ion/molecule reactions can be circumvented using intra-molecular reactions. A new reaction channel involving the exchange of sulfinyl radical and thiol was observed and characterized for the first time utilizing this approach. Note that this reaction channel was not observed using traditional ion/molecule reactions even though the reaction should be thermally neutral. This reaction phenomenon is of special interest as it may have implications on how a radical (sulfinyl) intermediate migrates in a protein system and attacks other functional groups, resulting in conformational or functional changes. In addition, it also suggests that this oxidative damage can be potentially repaired by reacting with a nearby thiol group. Although in this study we chose the reaction between sulfinyl radical and thiol for proof-of-principle demonstrations, this approach can be easily applied for a variety of reactions as long as the two reaction functional groups can be tethered within a same molecule.

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**Notes and references**

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1. K. Takamoto and M. R. Chance, *Annu. Rev. Biophys. Biomol. Struct.*, 2006, **35**, 251-276.
2. J. R. Winther and U. Jakob, *Nat. Chem. Biol.*, 2013, **9**, 69-70.
3. I. Ceballos-Picot, V. Witko-Sarsat, M. Merad-Boudia, A. T. Nguyen, M. Thévenin, M. C. Jaudon, J. Zingraff, C. Verger, P. Jingers and B. Descamps-Latscha, *Free Rad. Bio. Med.*, 1996, **21**, 845-853.
4. A. Becker and W. Kabsch, *J. Bio. Chem.*, 2002, **277**, 40036-40042.
5. K. M. Stirk, L. K. M. Kiminkinen and H. I. Kenttamaa, *Chem. Rev.*, 1992, **92**, 1649-1665.
6. G. Gryn'ova, D. L. Marshall, S. J. Blanksby and M. L. Coote, *Nat. Chem.*, 2013, **5**, 474-481.
7. S. Osburn and V. Ryzhov, *Anal. Chem.*, 2012, **85**, 769-778.
8. B. N. Moore, S. J. Blanksby and R. R. Julian, *Chem. Commun.*, 2009, 5015-5017.
9. C. K. Barlow, A. Wright, C. J. Easton and R. A. J. O'Hair, *Org. Biomol. Chem.*, 2011, **9**, 3733-3745.
10. S. Li, M. K. Fu, S. C. Habicht, G. O. Pates, J. J. Nash and H. I. Kenttamaa, *J. Org. Chem.*, 2009, **74**, 7724-7732.

11. S. Osburn, G. Berden, J. Oomens, R. A. J. O'Hair and V. Ryzhov, *J. Am. Soc. Mass. Spectrom.*, 2012, **23**, 1019-1023.
12. L. Tan and Y. Xia, *J. Am. Soc. Mass. Spectrom.*, 2013, **24**, 534-542.
13. Y. Fu, B.-L. Lin, K.-S. Song, L. Liu and Q.-X. Guo, *J. Chem. Soc., Perkin Trans. 2*, 2002, **2**, 1223-1230.
14. F. Himo and P. E. Siegbahn, *Chem. Rev.*, 2003, **103**, 2421-2456.
15. V. Vaidya, K. U. Ingold and D. A. Pratt, *Angew. Chem. Int. Ed. Engl.*, 2009, **48**, 157-160.
16. X. H. Cheng and C. Fenselau, *J. Am. Chem. Soc.*, 1993, **115**, 10327-10333.
17. I. K. Chu, J. Zhao, M. Xu, S. O. Siu, A. C. Hopkinson and K. W. M. Siu, *J. Am. Chem. Soc.*, 2008, **130**, 7862-7872.
18. A. K. Y. Lam, V. Ryzhov and R. A. J. O'Hair, *J. Am. Soc. Mass.*, 2010, **21**, 1296-1312.
19. J. F. Zhao, T. Song, M. J. Xu, Q. Quan, K. W. M. Siu, A. C. Hopkinson and I. K. Chu, *Phys. Chem. Chem. Phys.*, 2012, **14**, 8723-8731.
20. T. Su and M. T. Bowers, *J. Am. Chem. Soc.*, 1973, **95**, 7611-7613.
21. T. Su, E. C. F. Su and M. T. Bowers, *Int. J. Mass Spectrom. Ion Processes* 1978, **28**, 285-288.
22. X. Ma, C. B. Love, X. Zhang and Y. Xia, *J. Am. Soc. Mass. Spectrom.*, 2011, **22**, 922-930.
23. Y. Xia and R. G. Cooks, *Anal. Chem.*, 2010, **82**, 2856-2864.
24. C. B. Love, L. Tan, J. S. Francisco and Y. Xia, *J. Am. Chem. Soc.*, 2013.
25. S. A. McLuckey and D. E. Goeringer, *J. Mass Spectrom.*, 1997, **32**, 461-474.
26. L. Tan and Y. Xia, *J. Am. Soc. Mass. Spectrom.*, 2012, **23**, 2011-2019.
27. C. A. Stinson and Y. Xia, *Analyst*, 2013, **138**, 2840-2846.
28. R. C. Fahey, *Annu. Rev. Microbiol.*, 2001, **55**, 333-356.



 

**Utilizing gas-phase intra-molecular reactions facilitated by energetic collisions, a new reaction channel, sulfinyl radical exchange with thiol within a polypeptide, was observed for the first time.**