Supporting Information

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

Kirt L. Durand, Xiaoxiao Ma, and Yu Xia*

Department of Chemistry, Purdue University, West Lafayette, IN, 47907-2084

Address reprint requests to:
Dr. Yu Xia
Department of Chemistry
Purdue University
West Lafayette, IN, USA 47907-2084
Phone: (765) 494-1142
Fax: (765) 494-9421

E-mail: yxia@purdue.edu

Table of Contents

Experimental

Materials

Selectin binding peptide (**Pep 1**, Table S1) was purchased from AnaSpec (Freemont. CA). The acetamidomethyl protected thiols (**Pep 3-N** and **3-C**, Table S1) were synthesized by LifeTein, (South Plainfield, NJ). Reduced glutathione, N-acetyl-l-cysteine methyl ester (Cys) and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO).

Table S1. Peptide systems used to study intra-molecular reactions between sulfinyl and thiol. Cys refers to N-acetylated-L-cysteine-methyl ester, while Acm refers to acetamidomethyl, the thiol protecting group.

Pep	Structure	Pep	Structure
1	C I E L L Q A R C (cyclic)	1-R	C I E L L Q A R C (SH, SH)
2-N	C I E L L Q A R C (SO•, SH)	2-C	C I E L L Q A R C (SH, SO•)
3-N	C I E L L Q A R C (SH, Acm)	3-C	C I E L L Q A R C (Acm, SH)
4-N	C I E L L Q A R C (ECG, Acm)	4-C	C I E L L Q A R C (Acm, ECG)
5-N	C I E L L Q A R C (Cys, SH)	5-C	C I E L L Q A R C (SH, Cys)

*Synthesis of **Pep 4-N/4-C** and **5-N/5-C***

Pep 4-N and **4-C** (Table S1) were synthesized from **Pep 3-N** and **3-C** via hydrogen peroxide (H_2O_2) oxidation in the presence of reduced glutathione (single letter sequence: γECG). Briefly, aqueous H_2O_2 solution (1% v/v) was added to the dissolved peptide (0.5 mg/mL) in a molar ratio of 2:1 ~ 5:1 (H_2O_2: peptide). The reaction was allowed to proceed at room temperature for 1-4

hours and monitored via MS. Acetic acid was used to terminate the reaction by adjusting the pH to 4. The resulting isomers were separated via Reverse Phase–High Performance Liquid Chromatography (RP-HPLC) and vacuum dried overnight. **Pep 5-N** and **5-C** were synthesized by reacting reduced selectin binding peptide (**Pep 1-R**) (0.5 mg/mL) with N-acetyl-L-cysteine methyl ester (0.5 mg/mL) in a 1: 2 molar ratio. The reaction was subjected air oxidation overnight and products were separated via RP-HPLC.

RP-HPLC

All peptides were separated using an Agilent 1200 series RP-HPLC system (Agilent Technologies, Santa Clara, CA). Separation was carried out on a Zobrax C18 column at a flow rate of 0.5 mL/min with a stepwise gradient of 25-27% solvent B over 45 min. Briefly, An isocratic gradient (25-25 % B) was used from 0-10 minutes followed by 25-27 % B from 11-45 minutes. Solvent A was a mixture of 0.085 % TFA in water and solvent B contained 0.085 % TFA in 100% CH_3CN (acetonitrile). The eluent was detected at a wavelength of 206 nm. The collected eluent was vacuum dried overnight using a centrivap concentrator (Labconco, Kansas City, MO).

Mass Spectrometry

Peptide solutions for nanoelectrospray ionization (nanoESI)[1] were prepared at a concentration of 10 μM in 50/49/1.0 of water/methanol/ acetic acid (v/v/v). All experiments were carried out on a 4000QTRAP tandem mass spectrometer (Applied Biosystems/SCIEX, Toronto, Canada). The hybrid triple quadrupole/linear ion trap configuration allows for two types of collisional activation methods, i.e., beam-type CID and ion trap CID. For beam-type CID, parent ions are isolated by Q1 quadrupole and accelerated to Q2 quadrupole for collisional activation with a neutral gas (nitrogen in this case). The collision energy (CE), defined by the DC potential difference between

Q0 and Q2, was optimized and typically within the range of 15 - 20 V. Ion trap CID was carried out in Q3 linear ion trap, where a dipolar excitation was used for on-resonance collisional activation. The activation amplitudes were within the range of 40 – 70 mV and activation time of 200 ms was used. The characteristic parameters of the mass spectrometer during this study were set as follows: spray voltage, 1200 - 1800 V; curtain gas, 10 psi, and declustering potential, 20 V. Mass analysis was achieved by using Q3 as a linear ion trap at a scan rate of 1000 Th/s. Data acquisition, processing, and instrument control were performed using Analyst 1.5 software.

Formation of peptide sulfinyl radical ions

Details of the experimental setup for radical reactions in ESI plume has been shown previously.[2] The nanoESI tips were pulled from borosilicate glass capillaries (1.5 mm o.d. and 0.86 mm i.d.) using a Model P-1000 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA). The nanoESI emitter was inserted into the main arm of a T-shaped tube and was kept in line with the inlet of the mass spectrometer at a distance of 8 –10 mm. A low temperature helium plasma was initiated in the side arm of the T-shape tube to form hydroxyl radicals.[3] The nanoESI plume of peptides was allowed to interact with hydroxyl radicals and the products formed *in situ* were analyzed on-line by mass spectrometry.

Results

*Ion trap CID of **Pep 4-N** and **Pep 4-C***

Fig. S1 shows the ion trap CID of **Pep 4-N/4-C** after radical reactions allowed for the formation of a site specific sulfinyl radical at the disulfide cleavage site. CID was used to gently remove Acm protecting group (labeled in red) leading to the formation radical ions **Pep 2-N** (*m/z* 532.3, Fig. S1a.) and **Pep 2-C** (*m/z* 532.3, Fig. S1b), respectively. All the sequence ions observed in Fig. S1a/b agreed well with the structures shown.

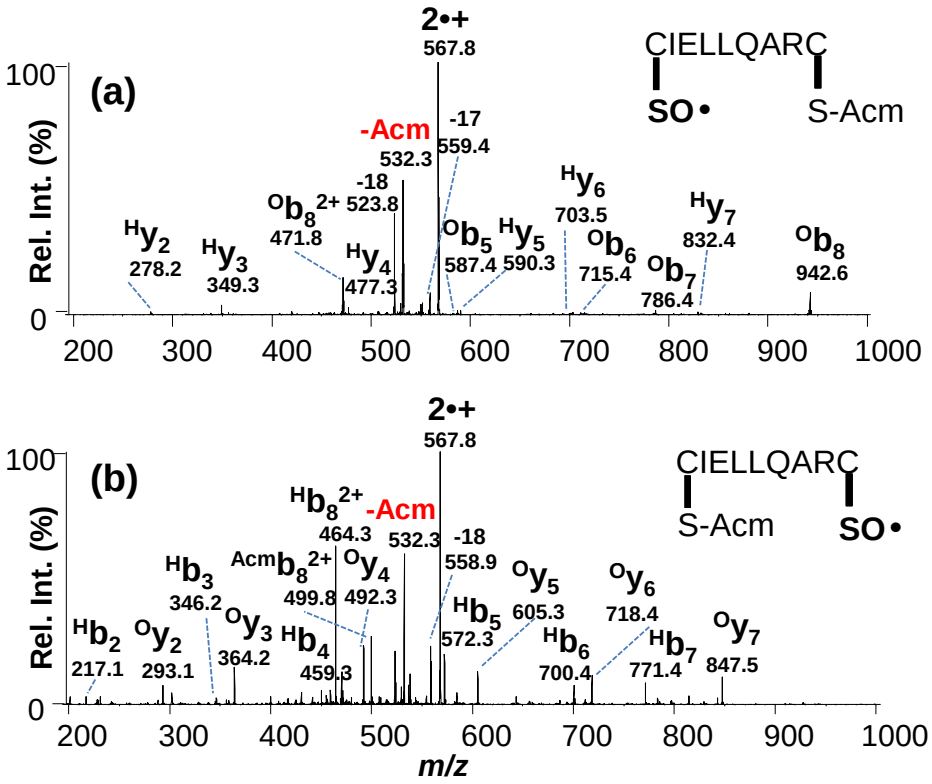

Fig. S1. Formation of **Pep 2-N** and **2-C** (*m/z* 532.3, 2+) via MS^2 ion trap CID of site specific sulfinyl radical ions derived from **(a) Pep 4-N**: activation energy 45 mV and 200 ms activation time and **(b) Pep 4-C**: activation energy 40 mV and 200 ms activation time.

MS^3 Ion Trap CID of ***Pep 2-C/2-N*** *via* ***Pep 4-C/4-N***

MS^3 ion trap CID data of the peptide sulfinyl radical location isomers (**Pep 2-N** and **Pep 2-C**, *m/z 532.3*, 2+) are shown in Fig. 2b and 2c, respectively. All the sequence ions ($^{H}y_{2\text{-}7}$ *and* $^{O}b_{5\text{-}8}$) observed in Fig. 2b are consistent with the structure of Pep 2-N, where a sulfinyl radical is on the N-terminal cysteine. There is also a small neural loss of 62 Da (CH_2SO), which has been established as a signature loss for peptide sulfinyl radical ions.[2, 4, 5] Similar to **Pep 2-N**, all the sequence ions ($^{H}b_{3\text{-}8}$ and $^{O}y_{2\text{-}7}$) observed in **Pep 2-C** correspond to fragments with the sulfinyl radical at the C-terminal cysteine. The predicted fragment ions from Pep 2-C and Pep 2-N are listed in Table S2. 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. Indicative products from Reaction 1 would lead to fragments with a 1 Da increase of mass at the site of sulfinyl radical (to form sulfenic acid) and a 1 Da decrease at the site of thiol (to form thiyl radical). If there were products produced from sulfinyl radical exchange with a thiol (Reaction 2), there would be a distinctive mass difference of 15 Da between the corresponding *b* or *y* ions due to the exchange of H and O atoms. For instance, if **Pep 2-N** were to undergo Reaction 2, the $^{O}b_n$ ions would become $^{H}b_n$ ions (a 15 Da decrease) and the $^{H}y_n$ ions would become $^{O}y_n$ ions (a 15 Da increase).

Table S2. Predicted fragment ions for **Pep 2-C** and **Pep 2-N.**

Pep 2-C					**Pep 2-N**				
C(SH)	Frag	*m/z*	Frag	m/z	C(SO•)	Frag	*m/z*	Frag	m/z
I	$^{H}b_{1}$	104.0	$^{O}y_{8}$	960.5	I	$^{O}b_{1}$	119.0	$^{H}y_{8}$	945.5
E	$^{H}b_{2}$	217.1	$^{O}y_{7}$	847.4	E	$^{O}b_{2}$	232.1	$^{H}y_{7}$	832.4
L	$^{H}b_{3}$	346.1	$^{O}y_{6}$	718.4	L	$^{O}b_{3}$	361.1	$^{H}y_{6}$	703.4
L	$^{H}b_{4}$	459.2	$^{O}y_{5}$	605.3	L	$^{O}b_{4}$	474.2	$^{H}y_{5}$	590.3
Q	$^{H}b_{5}$	572.3	$^{O}y_{4}$	492.2	Q	$^{O}b_{5}$	587.3	$^{H}y_{4}$	477.2
A	$^{H}b_{6}$	700.4	$^{O}y_{3}$	364.2	A	$^{O}b_{6}$	715.4	$^{H}y_{3}$	349.2
R	$^{H}b_{7}$	771.4	$^{O}y_{2}$	293.1	R	$^{O}b_{7}$	786.4	$^{H}y_{2}$	278.1
C(SO•)	$^{H}b_{8}$	927.5	$^{O}y_{1}$	137.0	C(SH)	$^{O}b_{8}$	942.5	$^{H}y_{1}$	122.0

*Formation and fragmentation of **Pep 2-C** via **Pep 5-C***

Pep 5-N and **5-C** were subjected to radical reactions in the nanoESI spray plume and the sulfinyl radical location isomers, **Pep 2-N** and **2-C**, were formed, respectively. These ions were mass-isolated and further subjected to beam-type or ion trap CID to investigate possible intra-molecular reactions. In the ion trap CID data of **Pep 2-C** (Fig. S2a, activation energy 65 mV, activation time 200 ms), the most abundant fragments ($^{O}y_{2\text{-}7}$ and $^{H}b_{3\text{-}8}$) belonged to the original structure with sulfinyl radical at the C-terminal cysteine. The only peak that indicated sulfinyl radical exchange with the thiol group (Reaction 2) was $^{O}b_{8}^{2+}$, the intensity of which was seven times less than that of $^{H}b_{8}^{2+}$ (Fig. S2a inset). The appearance of the $^{O}b_{8}^{2+}$ ion could be due to a small degree of isomerization of **Pep 5-C** to **Pep 5-N** during sample processing. An average percentage 91% of was obtained for **Pep 2-C** in the total ion population.

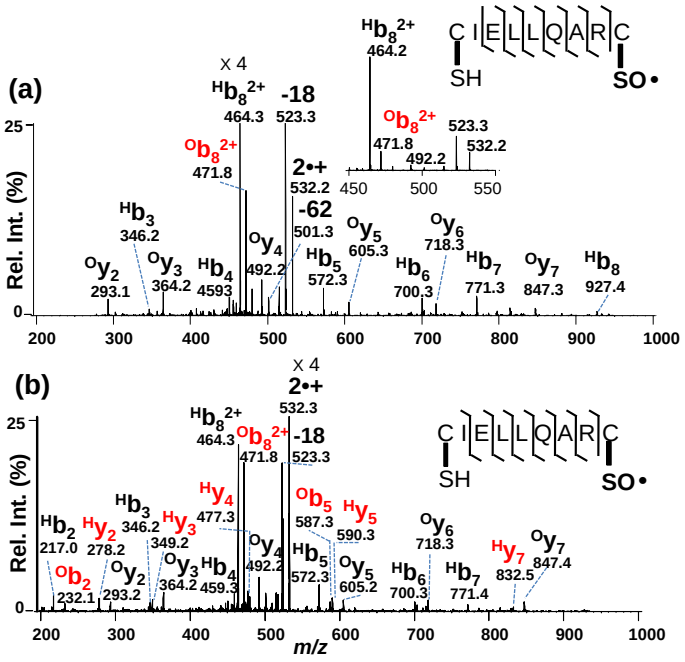

Fig. S2. MS^2 CID data of Pep 2-C (2+, *m/z* 532.3) derived from Pep 5-C **(a)** ion trap CID, activation energy 60 mV and 200 ms activation time. **(b)** Beam-type CID CE 15 V. inset in **(a)** shows the full scale spectra from *m/z 450-550*.

Beam-type CID of Pep 2-C (*m/z 532.3*, 2+, CE 15V) (Fig. S2b) produces a series of sequence ions ($^{H}b_{2\text{-}8}$ *and* $^{O}y_{2\text{-}7}$) consistent with the original structure in which the sulfinyl radical is formed at the C-terminus. However, there were also backbone fragments including $^{O}b_{2,5}$ and their complimentary fragment ions $^{H}y_{2\text{-}5,\,7}$, indicating the transfer of sulfinyl radical to the N-terminus (labeled in red in Fig. S2). Those fragments could only be the result of sulfinyl radical exchange with the thiol group (Reaction 2) and their intensities are almost at 1:1 ratio with fragments of the original structure (i.e. the ratio between $^{H}b_{8}$*:* $^{O}b_{8}$ is 1.1 while the ratio between $^{O}y_{5}$*:* $^{H}y_{5}$ is 0.9). Under beam type CID there was an average of 61% of ***Pep 2-C*** in the total ion population. Table S3 and S4 shows the list of fragment ions and their intensities used in the calculation of isomeric percentages.

Table S3. List of ions and intensities observed for ion trap CID of **Pep 2-C** derived from **Pep 5-C.** Intensity (counts per second, after background subtraction)

Pep 2-C						**Pep 2-N**						**Pep 2-C%**
C(SH)	Frag	Int.	Frag	Int.	sum	C(SO•)	Frag	Int.	Frag	Int.	Sum	
I	$^{H}b_1$	n/a	$^{O}y_8$	n/a	n/a	I	$^{O}b_1$	n/a	$^{H}y_8$	n/a	n/a	n/a
E	$^{H}b_2$	n/a	$^{O}y_7$	4827	4827	E	$^{O}b_2$	n/a	$^{H}y_7$	500	500	91
L	$^{H}b_3$	3300	$^{O}y_6$	8500	11800	L	$^{O}b_3$	100	$^{H}y_6$	1000	1100	92
L	$^{H}b_4$	8000	$^{O}y_5$	9580	17580	L	$^{O}b_4$	1700	$^{H}y_5$	1700	3400	84
Q	$^{H}b_5$	18500	$^{O}y_4$	24500	43000	Q	$^{O}b_5$	500	$^{H}y_4$	1600	2100	95
A	$^{H}b_6$	12100	$^{O}y_3$	16500	28600	A	$^{O}b_6$	494	$^{H}y_3$	778	1278	96
R	$^{H}b_7$	14500	$^{O}y_2$	11500	26000	R	$^{O}b_7$	1063	$^{H}y_2$	494	1557	94
C(SO•)	$^{H}b_8$	539500	$^{O}y_1$	n/a	539500	C(SH)	$^{O}b_8$	84500	$^{H}y_1$	n/a	84500	87
All Frag intensities of **Pep 2-C**					671307	All Frag intensities of **Pep 2-N**					94429	**91**

Table S4. List of ions and intensities observed for beam-type CID of **Pep 2-C** derived from **Pep 5-C.** Intensity (counts per second, after background subtraction)

Pep 2-C						**Pep 2-N**						**Pep 2-C%**
C(SH)	Frag	Int.	Frag	Int.	sum	C(SO•)	Frag	Int.	Frag	Int.	Sum	
I	$^{H}b_1$	n/a	$^{O}y_8$	n/a	n/a	I	$^{O}b_1$	n/a	$^{H}y_8$	n/a	n/a	n/a
E	$^{H}b_2$	4662	$^{O}y_7$	2500	7162	E	$^{O}b_2$	1466	$^{H}y_7$	1000	2466	74
L	$^{H}b_3$	1700	$^{O}y_6$	3300	5000	L	$^{O}b_3$	800	$^{H}y_6$	1900	2700	65
L	$^{H}b_4$	4500	$^{O}y_5$	3900	8400	L	$^{O}b_4$	2379	$^{H}y_5$	4150	6529	56
Q	$^{H}b_5$	7962	$^{O}y_4$	9580	17542	Q	$^{O}b_5$	2801	$^{H}y_4$	6312	9113	66
A	$^{H}b_6$	2700	$^{O}y_3$	5680	8380	A	$^{O}b_6$	1000	$^{H}y_3$	3750	4750	64
R	$^{H}b_7$	1800	$^{O}y_2$	2700	4500	R	$^{O}b_7$	799	$^{H}y_2$	4300	5099	47
C(SO•)	$^{H}b_8$	51500	$^{O}y_1$	n/a	51500	C(SH)	$^{O}b_8$	44500	$^{H}y_1$	n/a	44500	54
All Frag intensities of **Pep 2-C**					102484	All Frag intensities of **Pep 2-N**					75157	61

*Effect of energy on **Pep 2-C** ion population*

Activation energies in beam-type and ion trap CID were not found to significantly affect the reproducibility of CID spectra of ***Pep 2-C*** or ***Pep 2-N***. We tested several activation energies (40 - 70 mV) in ion trap CID and the change in isomer population was minimal. A relatively constant ratio of sulfinyl radical transfer products was observed under CEs from 10-20 V in beam-type CID. Table S5 summarized the observed percentage of Pep 2-C isomer with varying energies under ion trap and beam-type CID.

Table S5. Effect of energy on **Pep 2-C** ion population.

Ion Trap CID CE (mV)	***Pep 2-C %***	**% of parent ion dissociated**	**Beam-type CID CE (V)**	***Pep 2-C %***
40	85	12	10	62
50	88	82	15	61
60	91	87	20	65

References

1. M. Wilm and M. Mann, *Anal. Chem.*, 1996, **68**, 1-8.
2. X. Ma, C. B. Love, X. Zhang and Y. Xia, *J. Am. Soc. Mass. Spectrom.*, 2011, **22**, 922-930.
3. Y. Xia and R. G. Cooks, *Anal. Chem.*, 2010, **82**, 2856-2864.
4. L. Tan and Y. Xia, *J. Am. Soc. Mass. Spectrom.*, 2012, **23**, 2011-2019.
5. C. B. Love, L. Tan, J. S. Francisco and Y. Xia, *J. Am. Chem. Soc.*, 2013, **135**, 6226-6233.