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Intra-molecular reactions between cysteine sulfinyl radical and a disulfide bond within peptide ions

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ABSTRACT

Cysteine sulfinyl radical ($^{SO\bullet}$Cys) is a reactive intermediate discovered in the inactivation of enzymes utilizing the glycyl/thiyl radical in their catalytic functions upon exposure to air. $^{SO\bullet}$Cys has been recently formed and investigated in the gas phase via mass spectrometry (MS), with the aim being to acquire direct experimental evidence of the radical's intrinsic chemical reactivity. Ion/molecule reaction studies showed that $^{SO\bullet}$Cys was relatively chemically inert toward thiol (S—H) and disulfide (S—S) functional groups under the explored experimental conditions. Herein, we utilized intra-molecular reactions aided by collision-induced dissociation (CID) to overcome the limitations associated with the traditional bimolecular reactions and explore the reactivity of $^{SO\bullet}$Cys. Our results revealed a new reaction pathway in which the sulfinyl radical exchanged with an intrachain or interchain disulfide bond within a peptide ion, leading to the formation of a new disulfide bond and a sulfinyl radical. As a consequence, CID of peptide disulfide regio-isomers consisting of $^{SO\bullet}$Cys led to enhanced sequence information, however the disulfide bond linkage patterns could not be accurately assigned. This reaction pathway also has implications on disulfide bond scrambling in proteins initiated by a radical intermediate.

1. Introduction

Radicals or reactive oxygen species (ROS) play crucial roles in biological systems [1]. They are constantly generated in cells and their concentrations (in nM range) are rigorously regulated via multiple mechanisms. It was also revealed that many of the radical species are involved in cell signaling [2] and can be used by the immune system to fight invading organisms [3]. However, due to their highly reactive nature, radicals can induce irreversible modification of biomolecules and lead to cell death when their local concentrations are too high [4]. Cysteine, a sulfur-containing amino acid, is known to be very susceptible to radical attack [5]. Cysteine-containing peptides such as glutathione (in disulfide reduced or oxidized forms) function as redox buffers and antioxidants to maintain cell redox homeostasis [6,7].

Cysteine sulfinyl radical ($^{SO\bullet}$Cys) has been detected as an intermediate during the inactivation of enzymes (i.e., pyruvate formate lyase) utilizing the glycyl/thiyl radical in their catalytic functions upon exposure to air [8]. Detailed knowledge of cysteine sulfinyl radical in protein systems such as their reactivity and structure is very limited from solution-phase studies partially owing to the transient nature and low concentration of bio-radicals under physiological conditions. Carefully examining the gas-phase chemistry of bio-radicals can provide experimental evidence of their intrinsic chemical properties; this information will also provide mechanistic insight of the fate of protein radical species including intra- or inter-molecular radical transfer after the initial formation. The formation of site-specific peptide sulfinyl radical ions in the gas phase has been reported by our group based on radical reactions in the nanoelectrospray ionization (nanoESI) plume and therefore allowed its fundamental chemical properties to be investigated in the gas phase in detail [9,10]. Gas-phase ion/molecule reactions have been used by many research groups as a means to study peptide radical reactivity, structure, and migration [11–17]. The reactivity of peptide cysteine sulfinyl radical ions with organic disulfides, thiols, and molecular oxygen in a linear ion trap mass spectrometer was investigated via ion/molecule reactions recently [18]. The sulfinyl radical appeared to be the least reactive relative to the thiyl and perthiyl radical, which agreed with studies in the condensed phase [19–21]. The low reactivity was in-part due to the delocalization of the spin between the sulfur and oxygen atom within $^{SO\bullet}$Cys as shown by theoretical calculations [22]. This

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chemical property of ^{SO}•Cys makes it difficult to further characterize its reaction products due to low reaction yields (typically <1%) and expand the scope of investigation to other reactions.

Note that the reaction yield of gas-phase ion/molecule reactions is affected by several factors including (1) collision rate constant ($k_{collision}$ ~10^{-9} cm^3/(molecule s)) [23–27], (2) the number density of neutral reagents, (3) inherent reaction efficiency (the fraction of collisions leading to reaction), and (4) reaction time (ms to s). For a reaction with inherent "low reactivity", increasing the partial pressure of the neutral reagent (to increase the number density) or reaction time will help, however, only to a limited degree. We recently utilized a new approach, "intra-molecular reactions", as an alternative means to study reactions having inherent low reactivity. The two functional groups intended for reaction are placed within the same peptide ion and collisional activation is used to overcome energy barriers associated with reactions and peptide conformation changes. In such a system, the chance of the two functional groups interacting is determined by the vibrational frequency of the peptide scaffold. This frequency is independent of the collisional rate constant in traditional ion/molecule reactions, and can be tuned to a much larger magnitude due to vibrational excitation of the ion from collisional activation. We have successfully applied this approach to probe the reaction between cysteine sulfinyl radical and free thiol. When sulfinyl radical was allowed to react with free thiol using the intra-molecular reaction approach, a new reaction channel, sulfinyl exchange with thiol was observed (Peptide-SO• + RS—H → Peptide-S—H + RSO•) [28]. This reaction channel required high activation energy (such as in beam-type CID) and was absent from ion/molecule reactions performed in a linear ion trap.

Previous ion/molecule reaction studies showed that peptide sulfinyl radical (Peptide-SO•) ions did react with a disulfide bond (RS—SR) although with a very low yield (<0.2%, reaction time: 3–10 s, trap pressure: 6 mTorr) [18]. The detected reaction proceeded via alkyl sulfide (RS—) abstraction by sulfinyl radical leading to the formation of thiyl radical and alkylthio-sulfinyl (S—O—SR) (Reaction (1)). An alternative reaction pathway, Reaction (2), was not observed which would involve peptide sulfinyl radical exchange with a disulfide bond, causing the formation of a new disulfide bond and alkyl sulfinyl radical. This reaction should be thermally neutral; however, it might require overcoming a significant energy barrier and was therefore not observed under conventional ion/molecule reaction conditions. If energetic collisions are applied to overcome the associated reaction energy barrier [29], this reaction could be observed.

$$\text{Peptide-SO•} + \text{RS—SR} \rightarrow \text{Peptide-SO—SR} + \text{RS•} \quad (1)$$

$$\text{Peptide-SO•} + \text{RS—SR} \rightarrow \text{Peptide-S—SR} + \text{RSO•} \quad (2)$$

In this study, intra-molecular reactions aided by collisional activation were further utilized to study the reactions between cysteine sulfinyl radical and a disulfide bond (both intra and interchain). Experimental results obtained from model peptide systems which contained one disulfide bond and one cysteine sulfinyl radical all showed that both Reactions (1) and (2) happened upon collisional activation of the peptide ions. These two types of intramolecular reactions of cysteine sulfinyl radicals induced disulfide bond opening or scrambling within a peptide ion system. As a consequence, collisional activation of peptide disulfide regio-isomers consisting of ^{SO}•Cys led to enhanced sequence information from backbone regions covered by a disulfide bond; however the disulfide bond linkage patterns could not be accurately assigned.

2. Experimental

2.1. Materials

The peptides studied herein are listed in Table 1 with their single letter sequences and corresponding disulfide bond connecting patterns also indicated. The precursor for the **P1** peptide (^{1}CAEK^{5}CIEK^{9}CLVR^{13}C, C1-C13 and C5–C9 disulfide bonds) was synthesized by SynBioSci (San Francisco, CA). Selectin binding peptide (^{1}CIELLQAR^{9}C, C1–C9 disulfide bond) was purchased from AnaSpec (Freemont, CA) and was used as the precursor for **P3** peptide. Fully reduced **P4** peptide (single letter sequence: CARICAKLCLEVCK) was purchased from CPC Scientific (Sunnyvale, CA). Thermolysin, dichlorobis(ethylenediamine)platinum ($[Pt(en)_2Cl_2]$) and *N*-acetyl-L-cysteine methyl ester (Cys**) were purchased from Sigma–Aldrich (St. Louis, MO). **P4-II** was used as the precursor for the **P2** peptide.

2.2. Synthesis of disulfide bonds within peptides

The oxidizing agent $[Pt(en)_2(OH)_2Cl_2]$ was synthesized from $[Pt(en)_2Cl_2]$ in-house following the procedure described by Heneghan et al. [30]. $[Pt(en)_2(OH)_2Cl_2]$ was added to the dissolved peptide (1.0 mg/mL) in a molar ratio of 2:1–5:1 Pt(IV) to the peptide. The reaction was allowed to proceed at room temperature for 1–3 h and the reaction progress was monitored via MS. Following complete oxidation, the peptide disulfide bond isomers were separated via reverse phase-high performance liquid

Table 1
List of cysteinyl peptides studied herein.[a]

Peptide	Structure	Peptide	Structure
P1	Chain IEKC A CAEKC B LVRC C	P1N-SO•	Chain IEKC A (SO•C)AEKC B
		P1C-SO•	Chain CAEK(SO•C) B LVRC C
P2	Chain CAR A ICAKLCLEVCK B	P2C-SO•	Chain ICAKLCLEV(SO•C)K B
P3	Chain CIELLQARC A Cys** B	P3N-SO•	Chain (SO•C)IELLQARC A Cys** B
P4	CARICAKLCLEVCK	P4-I	CARICAKLCLEVCK
		P4-II	CARICAKLCLEVCK
		P4-III	CARICAKLCLEVCK

[a] The peptides are indicated with single letter sequences. The connection line between two "C"s within a peptide represents a disulfide linkage. The symbol, (^{SO}•C), stands for cysteine sulfinyl radical. *N*-acetyl-L-cysteine methyl ester is abbreviated as Cys**.