Two-step reaction mechanism reveals new antioxidant capability of cysteine disulfides against hydroxyl radical attack

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Cysteine disulfides, which constitute an important component in biological redox buffer systems, are highly reactive toward the hydroxyl radical (·OH). The mechanistic details of this reaction, however, remain unclear, largely due to the difficulty in characterizing unstable reaction products. Herein, we have developed a combined approach involving mass spectrometry (MS) and theoretical calculations to investigate reactions of ·OH with cysteine disulfides (Cys–S–S–R) in the gas phase. Four types of first-generation products were identified: protonated ions of the cysteine thyl radical (+Cys–SOH), cysteine (Cys–SH), cysteine sulfynil radical (Cys–SO), and cysteine sulfenic acid (+Cys–SOH). The relative reaction rates and product branching ratios responded sensitively to the electronic property of the R group, providing key evidence to deriving a two-step reaction mechanism. The first step involved ·OH conducting a back-side attack on one of the sulfur atoms, forming sulfenic acid (–SOH) and thyl radical (–S•) product pairs. A subsequent H transfer step within the product complex was favored for protonated systems, generating sulfynil radical (–SO) and thiol (–SH) products. Because sulfenic acid is a potent scavenger of peroxyl radicals, our results implied that cysteine disulfide can form two lines of defense against reactive oxygen species, one using the cysteine disulfide itself and the other using the sulfenic acid product of the conversion of cysteine disulfide. This aspect suggested that, in a nonpolar environment, cysteine disulfides might play a more active role in the antioxidant network than previously appreciated.


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The authors declare no competing interest.

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Significance

In this work, we harnessed mass spectrometry for online reaction monitoring of hydroxyl radical (·OH) attack to cysteine disulfide and acquired mechanistic details that had not been achieved previously. Our findings suggest that ·OH substitution at the disulfide bond is a fast and predominant reaction channel in the gas phase, while subsequent hydrogen transfer within the product pairs can be favorable in protonated systems. Notably, by reacting with ·OH cysteine disulfide converts itself to a more potent antioxidant, sulfenic acid (–SOH), thus forming two lines of defense against reactive oxygen species (ROS). These results provide insight into studying the antioxidant roles of cysteine disulfide in nonpolar biological environments, such as at the interface of lipid membrane and plasma.
described a relatively simple picture, involving addition of \( ^{1} \text{OH} \) onto the disulfide bond in R–S–S–R followed by rapid scission of the disulfide bond into R–S and RSOH (18). Butkovskaya and Setzer (19) later detected two product pairs: CH3SH/CH3SO\(^{\bullet} \) and CH3SCH3SOH, with the former pair being found more favorable from reactions of dimethyl disulfide with \(^{1} \text{OH} \). Bil et al. suggested H atom transfer in the product complex [CH3SOH–*SCH\(_{3} \)] to be a key step to account for the detection of CH3SH/HSCCH\(_{3} \) (20). However, the product pair CH\(_{3} \)SCH3SOH was not detected and thus ruled out in their mechanism. Obviously, the difficulties in identifying and quantifying the reactive intermediates have constituted a major hurdle for mechanistic studies of radical reactions.

While electron paramagnetic resonance and other spectroscopic methods are useful tools for detecting radical species (21), mass spectrometry (MS), which offers a distinct advantage in providing detailed molecular information, is increasingly being used to investigate the structure and reactivity of bio-radical ions in the gas phase (22–25). The recently developed electrospray ionization (ESI)–MS and direct MS for online reaction monitoring have provided additional powerful tools for elucidating transient radical species in solution reactions (26–29). Given the importance of –SS/–SH/–S cycle in biology, we previously developed an MS method to synthesize and characterize cysteinyl (Cys–S\(^{\bullet} \)), perthyl (Cys–S\(^{\bullet} \)), and sulfanyl (Cys–SO\(^{\bullet} \)) radicals in the gas phase (30–32). The method is based on performing radical reactions in the plume region of a nanoelectrospray ionization (nano-ESI) source right before the sampling interface of a mass spectrometer. Such a setup provides a short sampling time (submicrosecond to microsecond) so that bio-radicals can be detected and subsequently characterized using MS. For instance, two pairs of reaction products, pep–S\(^{\bullet} \)pep–SOH and pep–SH/pep–SO\(^{\bullet} \) resulting from disulfide bond cleavage in peptides (pep–S–S–pep), have been identified from reactions with \(^{1} \text{OH} \) (33). These results were consistent with the formation of two pairs of products upon disulfide bond cleavage from gas-phase studies and also suggested the feasibility of using MS to monitor the presence of sulfur radicals during reactions.

Leveraging the detailed molecular information that MS can provide for radical reactions, in the current work we aimed to delineate the mechanism of the reaction of \(^{1} \text{OH} \) with the cysteine disulfide bond in the gas phase. Gaining this type of knowledge is important for understanding the intrinsic reactivity of the disulfide bond, and it is relevant to reactions occurring in nonpolar microenvironments of biological systems. A series of cysteine disulfide derivatives was investigated (Cys–S–S–R, structure shown in Scheme 1) using a combined experimental and theoretical approach. The electronic character of the disulfide bond was modulated by changing the inductive effect of the connecting R group. For MS experiments, we focused on optimizing the formation of the first-generation products and performing a cross-group comparison of the reaction reactivities resulting from the R substituents. We were interested in describing the effect of the electronic character of the disulfide bond on its reactivity and the product branching ratio. High-level theoretical calculations were carried out to provide detailed descriptions of the thermodynamics and kinetics of the reactions. A two-step reaction mechanism was derived, with this mechanism involving rapid \(^{1} \text{OH} \) substitution cysteine on either sulfur atom, leading to cleavage of the disulfide bond and formation of Cys–SOH/R–S\(^{-} \) and Cys–S\(^{-} \)R–SOH. For protonated reaction systems, subsequent transfer of H within the product complex was indicated to be a significant process, and to be assisted by a stabilizing effect of hydrogen bonding in the transition states.

**Results and Discussion**

**Reaction Phenomena of \(^{1} \text{OH} \) and Cys–S–S–R.** Experimental details for conducting the reactions between \(^{1} \text{OH} \) and Cys–S–S–R in the plume region of a nano-ESI source have been reported previously and are supplied in SI Appendix. In brief, solutions containing equimolar amounts of the model compound and the internal standard (O-ethylated cystine, I–S–I–, 10 \( \mu \)M each in water) were ionized using nano-ESI in positive ion mode and subjected to online reactions with \(^{1} \text{OH} \) radicals produced by performing dielectric barrier discharge of helium in ambient air (SI Appendix, Fig. S1) (31). In this study, we chose to perform reactions in positive ion mode because higher ionization efficiency for Cys–S–S–R was obtained than that from negative ion mode and the internal standard could not be ionized in negative ion mode. It should be noted that similar reaction phenomena of \(^{1} \text{OH} \) attack to cysteinyl disulfide bond were observed regardless of the ion polarity (33). Although minor amounts of reactive species such as H atoms, O atoms, singlet oxygen, and ozone were also expected to have formed under the discharge conditions (34), their contribution to the observed reaction phenomena were expected to be insignificant. This expectation was due to a couple of factors: H and O atoms having short (nanosecond-scale) lifetimes and being quickly converted to \(^{1} \text{OH} \) upon collisions with water molecules (35), and ozone (36) or singlet oxygen (37) reacting very sluggishly with cystine disulfide. According to the ion evaporation model, developed to explain the ionization of small molecules by ESI (38), cysteine disulfides should most likely be in the form of protonated ions when encountering \(^{1} \text{OH} \). Note that because of the lack of basic functional groups for protonation in the tested R groups (except for R = cysteine), the disulfide cleavage products involving the R side chain, viz. R–S\(^{-} \), R–SH, R–SO\(^{-} \), and R–SOH, could not be detected using MS. For this reason, the same types of ionic products were expected to be observed from the disulfide cleavages of all of the tested Cys–S–S–R compounds, albeit with perhaps different relative abundances of these ionic products due to the impact of the R substituent. Fig. 1A summarizes the expected main reaction products from \(^{1} \text{OH} \) attack on different sulfur atoms in the disulfide bond. The attack of \(^{1} \text{OH} \) on the
sulfur atom connected to R should produce protonated cysteine thyl radical ("Cys-S\(^{+}\), m/z 121) and cysteine ("Cys-SH, m/z 122), while protonated cysteine sulfanyl radical ("Cys-SO\(^{+}\), m/z 137) and cysteine sulfenic acid ("Cys-SOH, m/z 138) should result from an attack on the Cys side of sulfur atom. Possible reaction products involving C–S bond cleavage or H abstraction were not detected above noise level; thus, these reaction channels were not investigated in this study.

In order to perform relative reaction rate comparisons, we first investigated the reaction of \(^{16}\)OH with the internal standard (I–S–S–I, O-ethylated cystine). Fig. 1B shows the reaction spectrum; as predicted, four types of disulfide cleavage product ions were detected: \(^{16}\)I–S–I (m/z 149), \(^{16}\)I–SH (m/z 150), \(^{16}\)I–SO\(^{+}\) (m/z 165), and \(^{16}\)I–SOH (m/z 166). Due to an overlap of the signal of the doubly protonated form of I–S–S–I (m/z 149.1) with the signal of singly protonated \(^{16}\)I–S form (also m/z 149.1), the actual amount of \(^{16}\)I–S produced was likely lower than that indicated by the spectrum. By changing the rate of the helium gas flow in the discharge area and changing the position of the nano-ESI tip relative to the location of the discharge, different degrees of reaction of I–S–S–I with \(^{16}\)OH were tested (SI Appendix, Fig. S2).

We found that by keeping the amount of \(^{16}\)SO\(^{+}\) formed at \(\sim 10\%\) of that of the remaining I–S–S–I ions (m/z 297, Fig. 1B), the first-generation reaction products predominated while the subsequent reaction products, i.e., I–SO\(^{+}\)H (m/z 182.1, Fig. 1B), contributed relatively little to the spectrum. Therefore, we kept the relative ion abundance of \(^{16}\)I–SO\(^{+}\) at a constant 10\% for all reactions to ensure that the same reaction conditions were reproduced for each individual Cys–S–S–R compound even though the actual number density of \(^{16}\)OH and the reaction time could not be precisely controlled using our current experimental methods. It is worth noting that doubly protonated ions were only observed from I–S–S–I at relatively low ion abundances but not for the rest of Cys–S–S–R compounds. Their presence should not cause interferences to the measurements of reactions involving Cys–S–S–R because the degree of reaction was monitored using %\(^{16}\)I–SO\(^{+}\)/I–S–S–I and their m/z value (m/z 149) was different from reaction products involving Cys–S–S–R.

Reactions of Cys–S–S–Cys, Cys–S–S–CH\(_3\), and Cys–S–S–C(O)–OCH\(_3\) were used as examples to demonstrate the characteristics of reactions of a symmetric disulfide bond vs. asymmetric disulfide bond connected to an electron-donating group (EDG) and electron-withdrawing group (EWG), respectively. For Cys–S–S–Cys, the four expected reaction products were clearly detected (Fig. 1C), showing patterns similar to that of the internal standard; while for Cys–S–S–CH\(_3\) (Fig. 1D), the \(^{16}\)Cys–S– and Cys–SH signals were stronger than the \(^{16}\)Cys–SO\(^{+}\) and \(^{16}\)Cys–SOH signals. The spectrum resulting from the reaction of Cys–S–S–C(O)–OCH\(_3\) (Fig. 1D), however, presented a drastically different product partitioning: \(^{16}\)Cys–SO\(^{+}\) was detected as the predominant reaction product, while the signals corresponding to the other three ionic products were much weaker. Overall, our results were consistent with the product branching ratios being sensitive to the electronic properties of the R group.

Although the actual number density of \(^{16}\)OH and the reaction time could not be precisely measured, the use of the internal standard allowed us to compare the reactivities of all seven tested Cys–S–S–R model compounds. The %\(\text{rel. }\)reaction of each Cys–S–S–R was defined as the sum of the peak areas of all first-generation reaction products divided by the peak area of the remaining Cys–S–S–R ions. This value was further normalized to the %\(\text{rel. }\)reaction of I–S–S–I to provide %\(\text{rel. }\)reactivity of each Cys–S–S–R compound. All experiments were repeated three times; the average %\(\text{rel. }\)reactivity values and associated SDs were reported. As shown in Fig. 2A, Cys–S–S–R showed higher reactivity toward \(^{16}\)OH with R, being an EDG (C\(_3\)H\(_7\) (100 ± 2%), C\(_2\)H\(_4\) (110 ± 17%), Ph (120 ± 6%), than with it being an EWG, i.e., CH\(_2\)CF\(_3\) (23 ± 10%), C(O)CH\(_3\) (64 ± 40%), C(O)OCH\(_3\) (39 ± 7%). Interestingly, Cys–S–S–Cys showed a lower reactivity (66 ± 16%) than the internal standard (100%), despite the two compounds only differing in the modification of the ethyl ester at the carboxylic group. These results indicated that the \(^{16}\)OH group in the carboxylic acid might play a role in affecting the energetics of disulfide bond cleavage.

The preference of \(^{16}\)OH to attack the cysteiny sulfurl (Cys–S) (%selectivity) in Cys–S–S–R was evaluated by calculating the summed ion abundances of Cys–S–SO\(^{+}\) and Cys–SOH from all first-generation products. A value of 50\% selectivity would suggest that the \(^{16}\)OH radical has an equal probability of attacking either sulfur atom of the disulfide bond. Approximately such a value (specifically 53 ± 12\%) was in fact found for Cys–S–S–Cys, while 45 ± 4\% was found for that of I–S–S–I (SI Appendix, Fig. S2B). A selectivity value greater than 50\% would indicate that the \(^{16}\)OH preferentially reacts with the cysteiny sulfur (Cys–S), while a value less than 50\% would indicate a preference for the S–R moiety. As shown in Fig. 2B, the %selectivity values were clearly clustered into two groups based on whether an EWG or EDG R group was used. Specifically, \(^{16}\)OH showed a high preference to attack the cysteiny sulfurl when R was an EWG, with the %selectivity values here at 70 ± 9\% (CH\(_2\)CF\(_3\)), 68 ± 13\% [C(O)CH\(_3\)], and 91 ± 1\% [C(O)OCH\(_3\)]. For all of the cases in which R was an EDG, the %selectivity values were all less than
reaction mechanism to be the predominant pathway for both neutral and protonated Cys–S–S–R compounds (SI Appendix, Figs. S3–S7). The distance between the approaching oxygen atom and the attacked sulfur atom was indicated from our calculations to, in general, range between 1.82 and 2.17 Å, and the OSS angle generally ranged between 132° and 155°, similar to the reaction of *OH with hydperoxides (41). In addition, intramolecular hydrogen bonds were indicated to play a role in stabilizing the transition states. Shorter hydrogen bonds, indicative of stronger bonds, were calculated for the protonated species than for the corresponding neutral species. For example, the N–H–O bond in the low-lying transition state of reaction 2b of *Cys–S–S–CH3 showed a length of 1.794 Å (Fig. 3a), while O–H–O showed a length of 1.867 Å in that of 2a of Cys–S–S–CH3 (Fig. 3B).

A new reaction channel, involving proton-coupled electron transfer (PCET), was found to be competitive for the protonated species, and to lead to the formation of [Cys–S–S–R]**:

\[
\text{Cys}^*\text{–S–S–R} + \text{OH} \rightarrow [\text{Cys–S–S–R}]^{**} + \text{H}_2\text{O} \quad \text{(PCET)}.
\]

The calculations indicated a PCET branching ratio between 22% and 32%, but also indicated further dissociation at the S–S bond or S–R bond to not be energetically favorable. In addition, ions corresponding to [Cys–S–S–R]** were not detected experientially. Therefore, this process was concluded to not contribute significantly to disulfide bond cleavage.

Table 1 summarizes kinetics data together with branching ratios for reactions 1 and 2 from the calculations. When the neutral cysteine disulfides were considered, the summed rate constants for disulfide cleavage (k_{total}) ranged between 5.9 \times 10^{10} and 4.2 \times 10^{12} M^{-1} s^{-1}, with R groups of –C(O)OCH3 and –CH3 yielding the slowest and fastest reactions, respectively. For reactions involving

50%, viz. 26 ± 5% for –CH3, 38 ± 5% for –C3H7, and 40 ± 3% for –Ph.

Theoretical Calculations. In order to rationalize these MS results, we carried out a series of theoretical calculations. These computational methods consisted of a combination of the molecular mechanics (MM) methods, ab initio density functional theory with the BH&HLYP functional, and domain-based local pair-natural orbital coupled cluster including perturbative triplet excitation methods [DLPNO–CCSD(T)]. The kinetics calculations were carried out in the framework of multiconformer transition state theory (MC-TST). The following elemental reactions were envisaged as the first steps of the disulfide bond cleavage:

\[
\begin{align*}
\text{Cys–S–S–R} + \text{OH} & \rightarrow \text{Cys–SOH} + S–R, \\
\text{Cys–S–S–R} + \text{OH} & \rightarrow \text{Cys–S}^* + \text{HOS–R}.
\end{align*}
\]

We also considered protonated species (*Cys–S–S–R) in the reactions, with the proton bound to the primary amine of the cysteine moiety:

\[
\begin{align*}
*\text{Cys–S–S–R} + \text{OH} & \rightarrow *\text{Cys–SOH} + S–R, \\
*\text{Cys–S–S–R} + \text{OH} & \rightarrow *\text{Cys–S}^* + \text{HOS–R}.
\end{align*}
\]

Regarding R substituents, –CH3 and –phenyl were chosen to represent EDG groups, while –CH2CF3 and –C(O)OCH3 were used as examples of weak and strong EWG groups, respectively. Previous reports showed that a carbon-centered radical or H atom can attack either of the sulfur atoms in a disulfide bond, following either a back-side mechanism (with an attacking angle between 140° and 180°) or a front-side mechanism (~90° attacking angle), but with the back-side attack being more favorable (39–42). The results of our calculations indicated the back-side
protonated Cys–S–R species, the charged species was calculated to react about two times slower than the neutral species containing the –CH3 group, whereas for the –CH2CF3, –phenyl, and –C(O)OCH3 groups, the reaction of the charged species was predicted to be, respectively, about three times, four times, and two orders of magnitude faster than the corresponding neutral species. These differences can be attributed to the presence of stabilizing hydrogen bond interactions, which are in general stronger in reactions involving protonated species. Experimentally, *Cys–S–Cys was found slightly less reactive than +Cys–S–S–R with R being an EDG (R = –CH3, C6H5–Ph, Fig. 24). This difference might be due to both the electron-donating character and small steric hindrance of these R groups as compared to Cys. It is worth pointing out that because reactions happened in the plume region of nanoeSSI, a fraction of ions of Cys–S–S–R might be microsolvated when reacting with +OH. They could adopt different ionic structures such as zwitterionic form, and thus contribute to differences in reactivities observed from experiments to that of calculations.

Analysis of the branching ratios for reactions 1 and 2 (Table 1) suggested a general preference of +OH to attack the more electron-rich S atom for both neutral and protonated systems. For instance, for *Cys–S–S–Ph, the branching ratio for the attack of +OH on the sulfur atom bonded to R (S2, ΓS2) was calculated to be 66%. The calculation using –C(O)OCH3 as the R group, in contrast, indicated a preference for attack on the S atom bonded to the Cys+ moiety (S1), with an ΓS1 of 85%; and that using –CH3 as the R indicated a high preference for S2, which may have been due to both the electron-donating character and small steric hindrance of this R group considering that +OH prefers back-side attack to the disulfide bond. These data showed a good linear correlation with experimental results, with coefficients of correlation (R2) of 0.923 and 0.969 for neutral and protonated systems, respectively (plots shown in SI Appendix, Figs. S9 and S10:)

\[
\text{Cys–SOH + +S–R} \rightarrow \text{Cys–SO}^+ + \text{HS–R}, \quad [3a]
\]

\[
\text{+Cys–SOH + +S–R} \rightarrow +\text{Cys–SO}^+ + \text{HS–R}, \quad [3b]
\]

\[
\text{Cys–S}^+ + \text{HOS–R} \rightarrow \text{Cys–SH} + \text{+OS–R}, \quad [4a]
\]

\[
\text{+Cys–S}^+ + \text{HOS–R} \rightarrow \text{+Cys–SH} + \text{+OS–R}. \quad [4b]
\]

In general, for the various R groups, the reactions involving transfer of H from HO–S–R to +Cys–S–S–R to form +Cys–SH (reaction 4b) were computed to be the fastest reactions, with calculated rate constants between 8.0 × 1011 and 2.3 × 1012 M\(^{-1}\)s\(^{-1}\) except for R = –CH2CF3 where the computed rate constant was 2.4 × 109 M\(^{-1}\)s\(^{-1}\). Notably, these values are between 4 and 10 orders of magnitude faster than those of the corresponding

<table>
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<th>R</th>
<th>Reaction 3a</th>
<th>Reaction 4a</th>
<th>Reaction 3b</th>
<th>Reaction 4b</th>
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<td>–CH3</td>
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<td>8.0 × 10¹¹</td>
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<td>1.9 × 10⁷</td>
<td>6.9 × 10⁸</td>
<td>2.4 × 10¹²</td>
</tr>
<tr>
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<td>6.5 × 10⁴</td>
<td>3.2 × 10⁶</td>
<td>1.2 × 10⁸</td>
<td>2.4 × 10¹⁰</td>
</tr>
<tr>
<td>–C(O)OCH3</td>
<td>3.9 × 10⁴</td>
<td>9.0 × 10³</td>
<td>3.6 × 10¹¹</td>
<td>2.3 × 10¹²</td>
</tr>
</tbody>
</table>

Computed using transition state theory with DLPNP–CCSD(T) energies and BH&HLYP free energy corrections. k3a stands for the attack of +OH on the S atom linked to Cys (reactions 1a and 1b); k3b stands for the attack of the +OH on the S atom linked to the R group (reactions 2a and 2b). A temperature of 298 K was used for all reactions.
neutral compounds (reaction 4a, Table 2). Analysis of these data suggested that protonated ions were likely responsible for the reaction products detected experimentally; moreover, the analyses indicated that formation of \( ^*\text{Cys-SH} \) should be detectable on the same timescale as disulfide bond cleavage (reactions 1 and 2, Table 1). In addition, these high values of the rate constants may be attributed to the combination of two factors, namely the strength of the hydrogen bond interaction, between the protonated amine and the oxygen atom of the carbonyl group when \( R = \text{OCH}_3 \) (1.745 Å, Fig. 3F), and the early transition state according the Hammond postulate, as in the case of reaction 4b for \( R = \text{CH}_3 \), where the \( S-H \) distance of the H atom being transferred is quite large (1.736 Å, Fig. 3E). The differences in the reactivity between compounds with different substituent in this reaction are mainly due to different contribution of these two effects. However, the reason for relatively low \( k_a \) associated with \( R = \text{OCH}_3 \) was not fully understood. The above-described predictions made were in good accordance with the substantial \%H transfer observed for \(^*\text{Cys-SH} \) formation according to the MS data (Fig. 2C). For reaction 3b, analysis of the results of calculations predicted quite scattered values of the rate constants, with a \( k \) value of \( 4.9 \times 10^7 \text{M}^{-1} \text{s}^{-1} \) for \( R = \text{CH}_3 \) and \( 3.6 \times 10^7 \text{M}^{-1} \text{s}^{-1} \) for \( R = \text{OCH}_3 \). These data were consistent with the experimental observations, in particular the highest \%H transfer observed for \( R = \text{OCH}_3 \) (88%, Fig. 2C). However, explaining such a large difference between the \%H transfer values of the different tested compounds by solely referring to the different inductive effects of their R substituents would be at odds with a previous study indicating a lack of any strong effect of the identity of R of alkyliols (RS-H) on the \( S-H \) bond dissociation energy (RS-H BDE: \~360 \text{kJ/mol}) (43). Analysis of the low-laying transition states of reaction 3b suggested an important role played by hydrogen bond interactions in stabilizing the transition state, thus favoring the formation of \(^*\text{Cys-SO}^*\). For instance, the hydrogen bond between the protonated amine and the carbonyl group was calculated to be 1.6 Å for the compound containing an R of \( \text{OCH}_3 \) (an early transition state, Fig. 3C), much shorter than the hydrogen bond in \( \text{H}_2\text{N-SH} \rightarrow \text{S-SH}_2 \) (2.2 Å), with its R being \( \text{CH}_3 \) (Fig. 3D). We noticed that for Cys-S-S-Cys and I-S-S-I, which contain a symmetric disulfide bond, the detected \%H transfer values were much smaller. In fact, H transfer leading to the formation of \(^*\text{Cys-SH} \) (70%) or \(^*\text{I-SH} \) (71%, \( \text{SI Appendix, Fig. S2B} \)) was higher than that of \(^*\text{Cys-SO}^* \) (43%) or \(^*\text{I-S}^* \) (34%, \( \text{SI Appendix, Fig. S2B} \)). This is because the disulfide bond is no longer symmetrical in these two compounds given that the proton can only reside on one side of the molecule. H transfer from \(^*\text{Cys-SH} \) to \(^*\text{Cys-SO}^* \) leads to the formation of \(^*\text{Cys-SO}^* \) (reaction 3b), while H transfer from Cys-SOH to \(^*\text{Cys-S}^* \) leads to the formation of \(^*\text{Cys-SH} \) (reaction 4b). Using the calculation results from \( R = \text{CH}_3 \) in Cys-S-S-R (Table 2) as a simplified estimation for Cys-S-S-Cys, it is clear that reaction 4b is significantly enhanced relative to reaction 3b when compared to their corresponding neutral reactions (reactions 4a and 3a). This difference might lead to the higher \%H transfer values for forming \(^*\text{Cys-SH} \) than \(^*\text{Cys-SO}^* \) or \(^*\text{I-S}^* \) (\( \text{SI Appendix, Scheme S2} \)). Hydrogen bond interactions discovered in the cysteinyl disulfide system also presented a distinct difference from the calculated results of \%OH reactions with small organic disulfides (20, 41). Combining the accumulated pieces of evidence from the experimental and computational studies, we derived a two-step reaction mechanism to account for \%OH attack on cysteine disulfides (Scheme 2). According to this proposed mechanism, in the first step, \(^*\text{OH} \) performs a back-side attack on one of the sulfur atoms, with a preference for the electron-richer one—with this attack leading to \(^*\text{OH} \) substitution and subsequent disulfide bond cleavage and hence forming the sulfenic acid (\(-\text{SOH}\)) and thyl radical (\(-\text{S}\)) pair of products. Note that hydroxyl radical substitution was indicated to be rapid for most neutral cysteine disulfides, with rate constants on the order of \(10^{12} \text{M}^{-1} \text{s}^{-1} \), but about two orders of magnitude slower for R being an EWG. For protonated compounds, however, the rate constant of \(^*\text{OH} \) substitution on a disulfide bond connected to an EWG was indicated to be increased to the same level as for R being an EDG, due to the stabilizing effect of hydrogen bonding for the low-lying transition states. Hydrogen bonding was also modeled to help stabilize the product complex and further facilitate rapid H transfer before product separation in protonated systems (reaction 4b), explaining the observation of \(^*\text{Cys-SO}^* \) and \(^*\text{Cys-SH} \) in the MS experiments. Analysis of our calculation results suggested that H transfer would not predominately for neutral species on the detection timescale used in this study.

**Summary**

We have developed a combined experimental and theoretical approach to study reactions of the \(^*\text{OH} \) radical with a series of cysteine disulfides (Cys-S-S-R) in the gas phase. The ability to use MS to perform online identification and relative quantitation of first-generation reaction products provided mechanistic details that had not been achieved for this reaction previously. A two-step reaction mechanism has been proposed. In contrast to solution reactions where single-electron transfer is prevalent, \(^*\text{OH} \) substitution at the disulfide bond is a fast and predominant reaction channel in nonpolar environment, forming product pairs containing sulfenic acid (\(-\text{SOH}\)) and thyl radical (\(-\text{S}\)) at the cleavage site (Scheme 2). For protonated cysteine disulfides, subsequent H transfer within each of the product pairs could be competitive due to the stabilizing effect of hydrogen bonding in the transition states. Both experimental and theoretical results supported the idea that reactions of \(^*\text{OH} \) and cysteine disulfide compounds produce four types of sulfur species, namely \(-\text{SOH}, \ -\text{SO}^*, \ -\text{SH}, \) and \(-\text{S}^* \), which are important to the overall sulfur redox cycle. Of these species, \(-\text{SOH} \) is a potent scavenger for peroxyl radicals via H atom transfer (\( k = 3 \times 10^7 \text{M}^{-1} \text{s}^{-1} \)) (44, 45), presenting a contrast to its disulfide bond precursor and the reduced thiol both of which show limited reactivity toward peroxyl radicals (46). This implies that after the cysteine disulfide reacts directly with \(^*\text{OH} \), the in situ formed reaction product, \(-\text{SOH} \), can join the antioxidant network of other peroxyl scavengers (such as vitamin E), thus forming two lines of defense. Also of importance is the formation of a sulfinyl radical from H transfer within \(-\text{SOH}...\text{S} \) product complex. Because the sulfinyl radical is much less reactive than the thyl radical (32), this process basically detoxifies of thyl radical. However, in a nonpolar biological system, such as at the interface of lipid membrane or in the hydrophobic regions of protein tertiary structures, the local environment will largely modulate the reactivity of the cysteine disulfide toward \(^*\text{OH} \) as well as the rate of subsequent H transfer. In summary, while cysteine disulfide has been considered to be as an inefficient antioxidant, our study...
suggested that by reacting it with OH to form a more potent antioxidant in nonpolar environment, disulfide may be more actively involved in the antioxidant network to combat elevated levels of ROS than previously appreciated.

**Methods**

**Materials.** All reagents and solvents were purchased from commercial sources and were used without further purification. S-methyl methanethiosulfonate (MMTS), L-cysteine, α-cysteine, thioacetic acid, methoxy carbonyl chloride, benzyl mercaptan, 1-propanethiol, 2,2,2-trifluoroethanethiol, acetyl chloride, ethanol, anhydrous methanol, and trimethylamine were purchased from Sigma-Aldrich. Syntheses of cysteine cysteiny1 disulfides (Cys–S–S–R, Scheme 1) were achieved according to procedures described in the literature (details in Appendix) (47–49). The ethyl ester of cystine was synthesized and used as an internal standard to compare the reaction rates using different cysteine disulfide derivatives. Working solutions for positive mode nano-ESI were prepared in deionized H2O (ultrapure purification).

All MS data were collected on a 4000 QTRAP triple quadrupole/linear ion trap (LIT) mass spectrometer equipped with a nano-ESI source made in the laboratory. Analyst software 1.6.2 was used for data acquisition, processing, and instrument control. Typical MS parameters used during the study were a spray voltage of ±1,500 to 1,800 V, curtain gas pressure of 10 psi, declustering potential of ±20 V, and scan rate of 1,000 Da/s. MS1 mass analysis was performed in LIT mode in Q3. Beam-type collision-induced dissociation (CID) was performed by performing precursor ion selection in Q1 and ion acceleration into a Q2 collision cell followed by product analysis in a Q3 linear ion trap. Ion-trap CID consisted of precursor ion selection in Q1 and ion transfer through Q2 to Q3 with minimum activation energy followed by reisolation, accumulation, and application of di-polar interaction to effect CID in Q3. To induce radical reactions, the nano-ESI plume of disulfide was allowed to interact with OH in the afterglow region of an atmospheric-pressure helium low-temperature plasma enabled in a T-shaped glass tube placed in front of the entrance of the mass spectrometer (SI Appendix, Fig. S1) (16).

**Computational Details.** The computational methods employed in this work includes a combination of MM methods (50), ab initio density functional theory with the B3LYP functional (51), domain-based local pair natural orbital coupled cluster including perturbative triple excitations [DLPNO-CCSD(T)] methods (52), and kinetics calculations in the framework of MC-TST (53). A full detail of theoretical approaches used is discussed in SI Appendix, which also includes a detailed description of the electronic features of the processes investigated and the most relevant transition states of the different reactions investigated.

**Data Availability.** All data are included in the manuscript and SI Appendix.

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