Radical Chemistry

Deutsche Ausgabe: DOI: 10.1002/ange.201506019
Internationale Ausgabe: DOI: 10.1002/anie.201506019

Radical Formation in the Gas-Phase Ozonolysis of Deprotonated Cysteine

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Abstract: *Although the deleterious effects of ozone on the human respiratory system are well-known, many of the precise chemical mechanisms that both cause damage and afford protection in the pulmonary epithelial lining fluid are poorly understood. As a key first step to elucidating the intrinsic reactivity of ozone with proteins, its reactions with deprotonated cysteine [Cys−H]⁻ are examined in the gas phase. Reaction proceeds at near the collision limit to give a rich set of products including 1) sequential oxygen atom abstraction reactions to yield cysteine sulfenate, sulfinate and sulfonate anions, and significantly 2) sulfenate radical anions formed by ejection of a hydroperoxy radical. The free-radical pathway occurs only when both thiol and carboxylate moieties are available, implicating electron-transfer as a key step in this reaction. This novel and facile reaction is also observed in small cys-containing peptides indicating a possible role for this chemistry in protein ozonolysis.*

Ozone (O_3), a triatomic allotrope of oxygen, plays two opposing roles for human life depending on its location in the Earth's atmosphere. In the upper atmosphere (stratosphere) O_3 is beneficial in preventing UV light from reaching the surface of the Earth, thereby minimizing human exposure to damaging wavelengths in the solar spectrum.[1] In contrast, in the lower atmosphere (troposphere) O_3 is a product of photochemical smog and its role changes to that of an air pollutant with deleterious effects to the human respiratory system.[1] Long-term exposure to elevated O_3 levels has been linked to increased risk of death from respiratory causes, which is more than three times greater in metropolitan areas with high tropospheric O_3 concentrations (62.5–104.0 ppb) compared to those with lower levels (33.3–53.1 ppb).[2] This association is concerning given predictions that background O_3 levels may exceed internationally accepted levels for human health this century.[3]

While the physiological and biochemical outcomes of O_3 inhalation are well studied, the underlying chemical mechanisms are less well characterized.[4] This is in large part because the interaction of O_3 with the lung surfactant layer is thought to give rise to a cascade of secondary reactive species, which may ultimately be responsible for the bulk of the damaging effects.[5] Significant effort has been expended to characterize the interaction of O_3 with unsaturated lipids present in pulmonary surfactant and indeed some of these processes have been shown to release detectable free radicals in vitro.[5] The nature of the interaction of O_3 with the peptides and proteins—also key components of lung surfactants—is less well understood. It has been hypothesized that thiols present in biomolecules such as pulmonary surfactant B and glutathione (GSH) could serve as sacrificial antioxidants giving rise to the largely benign sulfonates. Conversely, reaction of O_3 with glutathione has been shown to mediate the release of damaging hydroxy radicals. Although the precise mechanism of this process has not been fully elucidated, a key first step is believed to involve electron transfer (e.g., Eq. (1), where GS^- is deprotonated glutathione).[5]

$$GS^- + O_3 \rightarrow GS^{\bullet} + O_3^{\bullet -} \quad (1)$$

To better understand the effects of O_3 on the respiratory tract, recent studies have examined the ozonolysis of amino acids, peptides and proteins.[6–8] While most studies monitor the end products of solution-phase reactions, novel mass spectrometric approaches have probed the interaction of O_3 with biomolecules at an air–liquid interface.[8] These phase-boundary experiments confirm that cysteine residues are preferential sites of oxidation in peptides and proteins under conditions relevant to the interaction of ambient O_3 with lung surfactant. Thermospray ionization tandem mass spectrometry identified the following key intermediates produced in the interfacial ozonolysis of deprotonated cysteine: cysteine sulfenate (m/z 136, **1** of Scheme 1), cysteine sulfinate (m/z 152, **2**), and cysteine sulfonate (m/z 168, **3**). In addition, product ions at m/z 135 and m/z 271 were tentatively assigned

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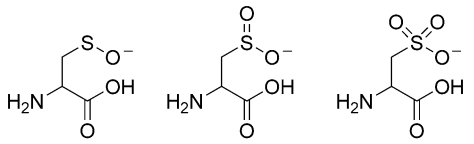
Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201506019.



Scheme 1. Structures of oxidation products formed by ozonolysis of deprotonated cysteine as identified by Colussi and co-workers.[8d]

as being due to $[M-2H]^{2-}$ and $[M-H]^-$ of cystine oxide, respectively.[8d]

These observations raise intriguing questions as to the: mechanism of the reaction(s) involved; precise nature of the oxidant in each case (i.e., O_3 or secondary oxidants); identity of all the reaction intermediates and products. To address these questions, we have studied the gas-phase ion–molecule reaction of deprotonated cysteine (and its derivatives) with O_3 using a combination of multistage mass spectrometry experiments, deuterium and structural labeling, and high-resolution mass spectrometry (HRMS).[9]

Using linear ion-trap mass spectrometers recently modified to examine ion–molecule reactions,[9] deprotonated cysteine ($[Cys-H]^-$ at m/z 120) was transferred to the gas phase by electrospray ionization, mass-selected and allowed to react with O_3 within the ion trap, resulting in a product-rich spectrum (Figure 1 a). The product ions observed include the previously reported oxides $[Cys-H+O]^-$ (**1** m/z 136), $[Cys-H+2O]^-$ (**2** m/z 152), $[Cys-H+3O]^-$ (**3** m/z 168) together with a range of other species at m/z 135 (**4**), 88 (**5**), and 74 (**6**). Sequential isolation of the oxides and subsequent reactions with O_3 (Figure 1 b–d) reveal that:

1) the higher oxides can be formed by sequential oxygen atom transfer reactions [Eqs. (2)–(4)];

$$[Cys-H]^- + O_3 \rightarrow [Cys-H+O]^- + O_2 \quad (2)$$

$$[Cys-H+O]^- + O_3 \rightarrow [Cys-H+2O]^- + O_2 \quad (3)$$

$$[Cys-H+2O]^- + O_3 \rightarrow [Cys-H+3O]^- + O_2 \quad (4)$$

2) ozonolysis terminates with $[Cys-H+3O]^-$, which does not react further with O_3 [Figure 1 d and Eq. (5)];

$$[Cys-H+3O]^- + O_3 \rightarrow \text{no reaction} \quad (5)$$

3) the ion observed at m/z 88 in Figure 1 a arises at least in part by loss of SO_2 from nascent $[Cys-H+2O]^-$ [Eq. (6)];

$$[Cys-H+2O]^{-*} \rightarrow [Cys-H-S]^- + SO_2 \quad (6)$$

4) the remaining product ions at m/z 74 and 135 are either primary product ions in the ozonolysis of deprotonated cysteine, or fragments formed from activated $[Cys-H+O]^-$ [Eq. (7)].

$$[Cys-H]^- + O_3 \rightarrow [Cys-2H+O]^{\bullet -} + HO_2^{\bullet} \quad (7)$$

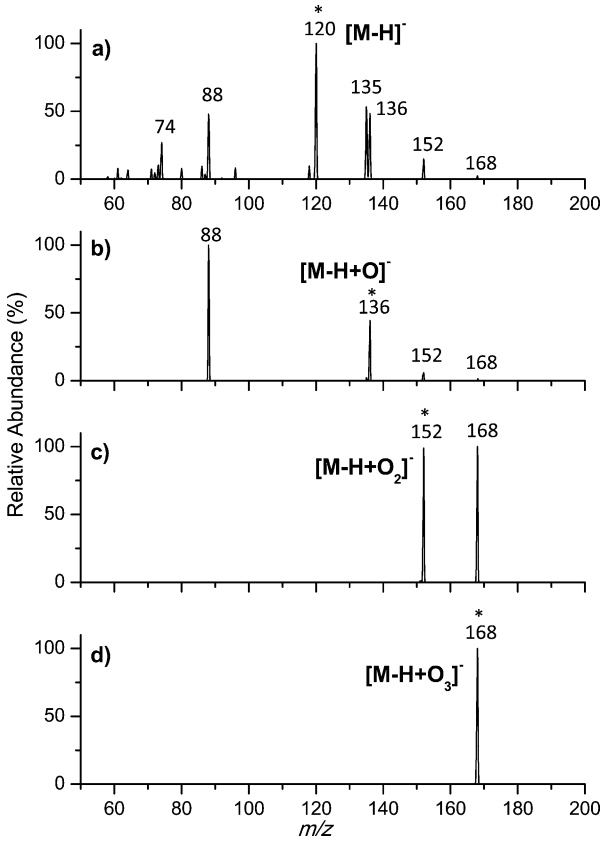


Figure 1. Mass spectra obtained following isolation of the following cysteine derived anions (marked with *) in the presence of ozone: a) $[Cys-H]^-$ at m/z 120; b) $[Cys-H+O]^-$ at m/z 136; c) $[Cys-H+2O]^-$ at m/z 152; and d) $[Cys-H+3O]^-$ at m/z 168.

HRMS allowed assignment of the empirical formulas of m/z 74 (**6**) and 88 (**5**) as being the even-electron species C_2H_4NS and $C_3H_6NO_2$, while m/z 135 (**4**) was confirmed to be a radical anion, $C_3H_5NO_3S$ (see Table S1 in the Supporting Information). A recent report on the gas-phase reaction of deprotonated cysteine and singlet molecular oxygen ($^1\Delta_g$) assigned a m/z 135 product ion to the sulfinyl radical anion $NH_2CH(CH_2SO^{\bullet})CO_2^-$ which is consistent with the high-resolution data here, but attributed a m/z 74 product ion to the glycyl anion $NH_2CH_2CO_2^-$ suggesting it has been miss-assigned or there is significant divergence in the chemical reactions of the two oxygen allotropes with the cysteine anion.[10] The possible origins of the products **4**–**6** arising from ozonolysis were investigated by isolating each of the oxides $[Cys-H+nO]^-$ ($n=1$–3) and subjecting them to activation by collision-induced dissociation (CID) (see Figure S1 in the Supporting Information). $[Cys-H+O]^-$ dissociates to give both m/z 74 and 88, whereas m/z 88 is the sole fragment of $[M-H+2O]^-$. The CID spectrum of $[Cys-H+3O]^-$ is dominated by loss of ammonia, with no formation of products at m/z 74 and 88. These tandem mass spectra are consistent with those reported by Colussi and co-workers for cysteine oxides formed upon ozonolysis at the air–liquid interface[8d] and confirm the identity of these ions as cysteine sulfenate (**1**), sulfinate (**2**), and sulfonate (**3**) (Scheme 1). These experiments further suggest that the even-electron ions at m/z 74

and 88 observed in Figure 1a arise from spontaneous dissociation of vibrationally hot $[Cys-H+O]^-$ and $[Cys-H+2O]^-$. In contrast, the $[Cys-2H+O]^{\bullet-}$ radical anion at m/z 135 cannot be formed by dissociation of any of the cysteine oxides, $[Cys-H+nO]^-$ ($n=1$–3), suggesting that it is a primary product formed by addition of O_3 with ejection of the hydroperoxy radical [Eq. (7)].

To further explore the mechanism associated with the formation of the radical anion **4**, deuterium and structural labelling was carried out. Condensed phase hydrogen-deuterium exchange of all the four labile, heteroatom protons of cysteine (i.e., at -NH_2, -CO_2H and -SH) followed by electrospray ionization produced the labeled $[[D_4]\text{-}Cys-D]^-$ anion at m/z 123. This isotopologue was found to react with O_3 to yield, amongst other products, $[[D_4]\text{-}Cys-2D+O]^{\bullet-}$ at m/z 137 and $[[D_4]\text{-}Cys-D+O]^-$ at m/z 139 (see Table S2 in the Supporting Information) thus confirming the exclusive loss of one of the four exchangeable protons in the formation of **4**. Since previous gas-phase studies have suggested that the two most acidic sites in cysteine are the -CO_2H and -SH moieties,[11] derivatives in which each of these sites are blocked by methylation were investigated. Ozonolysis of deprotonated *S*-methyl cysteine (m/z 134) produces $[Cys(SCH_3)-H+O]^-$ (m/z 150) as the major product and no odd-electron product ions are observed (see Figure S2a in the Supporting Information). The reaction is poorly efficient ($\Phi=3\%$, Table 1) compared to the cysteine archetype ($\Phi=89\%$). The deprotonated cysteine *O*-methyl ester shows an initial rapid reaction with O_3 that leads to a 40% loss of ion signal. Following this initial phase a persistent, non-reactive m/z 134 ion population is observed (see Figure S3 in the Supporting Information). HRMS measurements (data not shown) exclude the presence of isobars, suggesting that two isomers are present within the ion population or that O_3 catalyzes the isomerization of the nascent $[Cys(OCH_3)-H]^-$ anions. Significantly, the absence of abundant odd-electron product ions in the reactions of both $[Cys(SCH_3)-]^-$ and $[Cys(OCH_3)-H]^-$ implicates both the carboxylic acid and thiol functional groups in the free-radical pathways observed for $[Cys-H]^-$. In contrast, blocking of the amine moiety in *N*-acetyl cysteine, was found to yield odd and even-electron products analogous to those observed for cysteine (see Figure S2b). The effect of structural variation on reaction efficiency was also measured. In general, anions with a free thiol were found to react efficiently with O_3 (e.g., $[Cys-H]^-$, and $[Cys(OCH_3)-H]^-$ see Table 1) with the efficiency dropping markedly for sulfides (e.g., $[Cys(SCH_3)-H]^-$ and $[Met-H]^-$) and slowly reducing with increasing oxidation of the sulfur (e.g., $[Cys-H+O]^-$, $[Cys-H+2O]^-$).

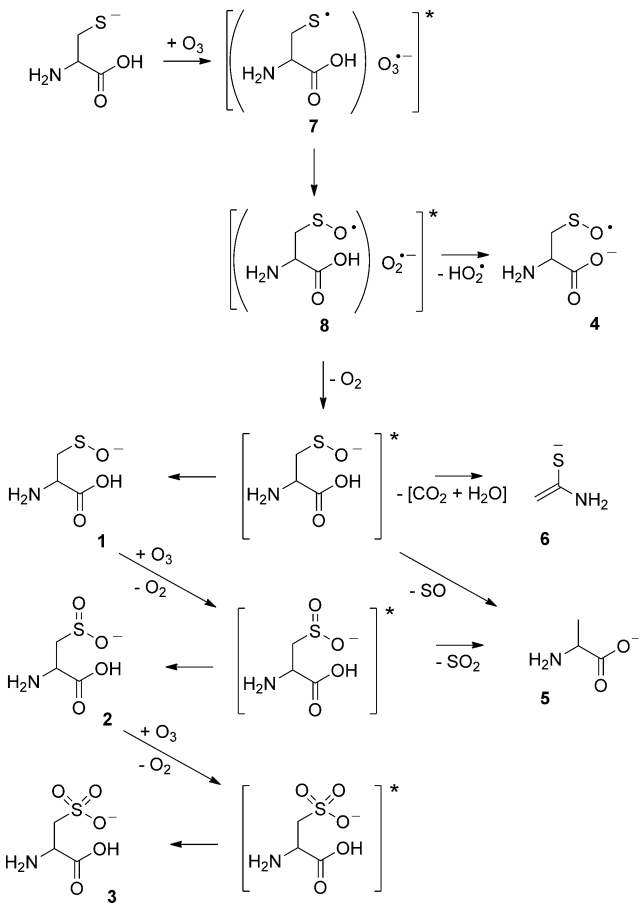
The presence of cysteine in a deprotonated di- or tripeptide facilitates efficient reaction with O_3; even as the carboxylate and thiol moieties are separated along the peptide chain (e.g., CysGly and CysGlyGly in Table 1). Ozonolysis of cysteine-containing peptides also yields abundant odd-electron $[M-2H+O]^{\bullet-}$ ions (Table S3). Similar sulfinyl radicals have been reported from radical-driven cleavage of disulfide-linked peptides in the gas phase[12] and have also been detected as reactive intermediates in some biochemical transformations.[13]

These labeling experiments together with known thermochemistry can be used to infer a mechanism for free-radical formation in the ozonolysis of $[Cys-H]^-$ (Scheme 2). While there has been much discussion related to the gas-phase structure of $[Cys-H]^-$, stationary points for deprotonation at both -CO_2H and -SH have been identified and lie within a few kcal mol^{-1} of each other.[10,15] The flat potential-energy surface allows wide sampling of anion structures within the ion-dipole complex. Accessible conformers with a formal charge on sulfur will have lower electron binding energies than isomeric carboxylate anions (i.e., 2.4 eV for $[Cys(OCH_3)-H]^-$ versus 3.4 eV for $[Cys(SCH_3)-H]^-$).[11a] The comparative stability of sulfinyl radicals means that the intracomplex electron transfer (ET) from cysteine to ozone ($EA[O_3]=2.1$ eV)[16] is only slightly endothermic and can readily be fueled by the initial complexation energy. The participation of the S-centered anion is confirmed by structural labeling showing that the free-radical products are not observed for deprotonated *S*-methyl cysteine (see Figure S2a). Following ET, oxygen atom abstraction yields the modified ion–molecule complex **8**, which can dissociate by either 1) electron transfer to give molecular oxygen and $[Cys-H+O]^-$ or 2) proton transfer to give the hydroperoxy radical and **4**. In the absence of an acidic hydrogen atom in $[Cys(OCH_3)-H]^-$, the latter pathway is shut down and only even-electron products are observed. Scheme 2 also outlines the subsequent oxidation of the cysteine sulfenate anion (**1**) to yield the higher sulfinate (**2**) and sulfonate (**3**) homologues but the absence of the free sulfur deactivates the radical-forming pathway. The importance of initial ET in this mechanism is supported by the absence of reactivity of the protonated form of cysteine, $[Cys+H]^+$ under the same reaction conditions (Figure S4). The slow oxygen atom

Table 1: Second-order rate constants and efficiencies measured for the reaction of deprotonated cysteine analogs with ozone in the gas phase. Rate constants were determined by measuring the decay of the precursor ion in the presence of ozone using increasing reaction times in a modified linear ion-trap mass spectrometer. The concentration of ozone inside the ion trap was determined using reference reactions (see the Supporting Information).

Reactant anion[a]	m/z	Rate constant, k^{II} [a,d]	Reaction efficiency, Φ [%][b]
$[Cys-H]^-$	120	77 ($\pm$19)	89
$[Cys-H+O]^-$	136	50 ($\pm$12)	59
$[Cys-H+2O]^-$	152	18 ($\pm$4)	22
$[Cys(SCH_3)-H]^-$	134	3 ($\pm$1)	3
$[Cys(OMe)-H]^-$	134	$>$77 ($\pm$19)[c]	$>$89
$[Cys(NAc)-H]^-$	162	17 ($\pm$4)	21
$[Met-H]^-$	148	3 ($\pm$1)	3
$[CysGly-H]^-$	177	35 ($\pm$9)	43
$[CysGlyGly-H]^-$	234	30 ($\pm$7)	38

[a] Uncertainties in the second-order rate constants represent a least squares analysis of the standard error in the fit of the pseudo-first-order decay and the quoted uncertainty of $\pm$25% in the reference reaction.[14] [b] Reaction efficiencies based on theoretical collision rates (Supporting Information). [c] See discussion in the text and Figure S3. [d] Given in 10^{-11} cm^3 molecules^{-1} s^{-1}.



Scheme 2. Proposed mechanism for the formation of both $[Cys-H+O]^-$ and $[Cys-2H+O]^{\bullet-}$ from the reaction of ozone with $[Cys-H]^-$. Detailed mechanisms for formation of **6** and **5** are provided in Scheme S1. The asterisk (*) indicates energetic or activated intermediates.

abstraction reactions of $[Cys(SCH_3)-H]^-$ and $[Met-H]^-$ likely arise from other mechanistic pathways.

Modeling of the reaction kinetics provides a branching ratio (BR) of 4% for the free radical pathway resulting in **4** (see the Supporting Information, Figure S5, and Table S4). This compares to BRs of 10% and 36% for even-electron pathways resulting in product ions **1** and **6**, respectively. Thus almost half of all reactive encounters between $[Cys-H]^-$ and O_3 result in products undetectable by ion-trap MS, which may arise from efficient reaction pathways resulting in electron loss with concomitant formation of neutral free radical(s).

The observations of the gas-phase reactions between $[Cys-H]^-$ and O_3 demonstrates that 1) even-electron oxidation proceeds by facile, sequential oxygen atom abstraction reactions and 2) free-radical products are also formed by the addition of oxygen and the elimination of $HO_2^{\bullet}$. The products arising from the intrinsic gas-phase reactivity of cysteine toward O_3 show excellent agreement with prior observations of the interfacial ozonolysis suggesting the identity of the *m*/*z* 135 reaction product is the radical ion **4** rather than an even-electron dianion of cystine as previously proposed.[8d] Given the gas-phase ozonolysis of cysteine-containing peptides also yields free radicals, such pathways may also operate for the interfacial reactions of cysteine-containing proteins. The efficiency (i.e., 89%, Table 1) of the gas-phase reaction between $[Cys-H]^-$ and O_3 is approximately two orders of magnitude greater than for the ozonolysis of ionized unsaturated lipids under the same conditions.[17] This is consistent with observations of facile and selective oxidation of pulmonary surfactant protein B at the air–liquid interface even in the presence of an excess of unsaturated phospholipids,[8a] although further work is required to confirm free radical formation at the air–liquid interface of the lung.

The unequivocal identification of free-radical products in the intrinsic reaction of deprotonated cysteine (and its derivatives) with O_3 is consistent with the proposal of secondary reactive species being responsible for promulgating cellular damage in the lung upon O_3 exposure: the so-called "cascade mechanism" for O_3 toxicity.[18] Analogous chemistry occurring within the epithelial lining would release hydroperoxy radicals initiating lipid peroxidation[19] and cysteine sulfinyl radicals that have been implicated in enzyme inactivation pathways.[20]

Experimental Section

Experiments were carried out using a modified Thermo Fisher Scientific LTQ single stage linear ion-trap mass spectrometer (San Jose, CA, USA)[9a] and a modified Thermo LTQ-FT (Bremen, Germany).[9b] Full details of the experiments are described in the Supporting Information.

Acknowledgements

The authors are grateful to the Australian Research Council for funding through the Discovery Project (grant number DP140101237 S.J.B.) and the Centre of Excellence in Free Radical Chemistry and Biotechnology (grant number CE0561607). G.N.K. is an ARC Australian Research Fellow (grant number DP1096134) and G.d.S. is an ARC Future Fellow (grant number FT130101304).

Keywords: cysteine · gas-phase reactions · mass spectrometry · ozone · radicals

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 12947–12951
Angew. Chem. **2015**, *127*, 13139–13143

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Received: July 1, 2015
Published online: September 3, 2015

