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Decomposition of S-nitrosocysteine via S- to N-transnitrosation

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Abstract

S-Nitrosothiols are thought to be important intermediates in nitric oxide signaling pathways. These compounds are unstable, in part, through their ability to donate NO. One model S-nitrosothiol, S-nitrosocysteine is particularly unstable. Recently, it was proposed that this compound decomposed via intra- and intermolecular transfer of the NO group from the sulfur to the nitrogen to form N-nitrosocysteine. This primary nitrosamine is expected to rapidly rearrange to ultimately form a reactive diazonium ion intermediate. To test this hypothesis, we demonstrated that thiirane-2-carboxylic acid is formed during the decomposition of S-nitrosocysteine at neutral pH. Acrylic acid was another product of this reaction. These results indicate that a small but significant amount of S-nitrosocysteine decomposes via S- to N-transnitrosation. The formation of a reactive intermediate in this process indicates the potential for this reaction to contribute to the toxicological properties of nitric oxide.

Introduction

Nitric oxide (NO¹) plays a critical role in both normal and pathological cellular processes. The biological half-life of NO is very short. Therefore, the biological effects of this important signalling molecule are thought to be mediated, in part, through the formation of longer lived S-nitrosothiols (1). S-Nitrosothiols not only serve as an endogenous NO pool but also play a role in NO signalling and nitrosative stress (1).

Because S-nitrosothiols are unstable, elucidation of decomposition pathways and the corresponding organic and inorganic intermediates and products will provide insight into their biological functions. One major in vitro decomposition pathway is the reaction of S-nitrosothiols with other thiols to form disulfides and HNO (2). In the presence of metals or UV light, they undergo decomposition to NO and oxidized thiol (3,4). S-Nitrosothiols will also donate NO to another thiol resulting in S- to S-transnitrosation (5), which is thought to be important for NO signaling (1). S- to N-transnitrosation is possible (6,7) but the biological relevance of this reaction is not known.

S-Nitrosocysteine (**1**) is one of the more unstable biologically relevant S-nitrosothiols (8) but the organic chemistry of its decomposition pathways has not been well explored. The instability of **1** has been attributed to the presence of the free amino group two carbon atoms away from the S-NO group (6,9). Primarily on the basis of extensive kinetic studies, Adam et al have

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¹Abbreviations: NO, nitric oxide

recently proposed that *S*-nitrosocysteine decomposes via intra- and intermolecular transfer of the NO group from the sulfur to the nitrogen to form *N*-nitrosocysteine (**2**, Scheme 1) (10). On the basis of well-known instability of primary *N*-nitrosoamines, **2** is expected to rapidly rearrange to a diazohydroxide (**3**) which, in turn, would generate a diazonium intermediate **4**. This intermediate will then lead to the formation of thiirane-2-carboxylic acid (**5**), 2-hydroxy-3-mercapto-propionic acid (**6**) and 3-mercapto-acrylic acid (**7**, Scheme 1). While compound **5** has been reported as a product of *N*-nitrosation of cysteine under acidic conditions (11), it is not known if this or the other proposed products of *N*-nitrosation are formed when *S*-nitrosocysteine decomposes under physiological conditions. In this report, provide direct evidence for involvement of reactive intermediates in the decomposition of *S*-nitrosocysteine at neutral pH. These reactive intermediates may contribute nitrosative stress.

Experimental Procedures

S-Nitrosocysteine and thiirane-2-carboxylic acid were prepared as previously described.(8, 11) All solutions were stored in brown glass vials at 23 °C.

Decomposition of *S*-nitrosocysteine

Solutions of 20 mM *S*-nitrosocysteine were freshly prepared by reacting cysteine with equal molar concentrations of sodium nitrite in 0.1 N HCl. After 5 min, more than 90% of the cysteine had been converted to *S*-nitrosocysteine. At this point, the solution was neutralized with 1/10 volume 1 M potassium phosphate, pH 7.2, containing 10 mM EDTA. The solution was then diluted to 1.9 mM *S*-nitrosocysteine. Due to the known instability of *S*-nitrosocysteine (8), it was not feasible to purify it prior to monitoring its degradation. The subsequent decomposition of *S*-nitrosocysteine and the formation of *N*-nitrosocysteine reaction products were determined by HPLC analysis, monitoring at 330 nm and 210 nm, respectively. The Phenomenex Synergi column (4.6 × 250 mm, 4 micron, Torrance, CA) was isocratically eluted with 10 mM ammonium phosphate, pH 2.7 (retention times: **1**, 3.5 min; **8**, 6.8 min and **5**, 8.9 min) or 10 mM ammonium formate, pH 2.8 (retention times: **1**, 3.5 min; **8**, 7.2 min and **5**, 9.8 min) at a flow of 1 mL/min. Concentrations of **1** and **8** were determined by comparison of the peak areas present in the reaction mixtures to standard curves generated with solutions of **1** and **8** of known concentration. The levels of **5** were estimated by generating a standard curve for this compound using a solution containing both **5** and **8** in which the relative concentrations of these two compounds were determined via ¹H NMR analysis.

Chemical characterization of the decomposition products

A solution of 20 mM *S*-nitrosocysteine in 100 mM potassium phosphate, pH 7.2, containing 1 mM EDTA which had been incubated at 23 °C for two weeks was acidified and immediately extracted with *t*-butyl methyl ether. Following concentration, the extract was dissolved in D₂O. The resulting residue was dissolved in either DMSO-*d*₆ or D₂O for NMR analysis. Preparative purification of the extract components was achieved on a Phenomenex Synergi column (4.6 × 250 mm, 4 micron, Torrance, CA). The column was isocratically eluted with 50 mM ammonium formate, pH 2.8 (retention times: **8**, 7.2 min and **5**, 9.8 min) at a flow of 1 mL/min. The collected fractions were extracted with methyl-*t*-butyl ether, dried over magnesium sulphate, filtered and concentrated. The resulting residue was dissolved in DMSO-*d*₆ for NMR analysis.

Thiirane-2-carboxylic acid (5**)**—¹H NMR DMSO-*d*₆ δ: 3.38 (dd, 1H, C2-H), 2.67 (dd, 2H, C3-H₂); ¹³C NMR DMSO-*d*₆ δ: C1, 171; C2, 29; C3, 24.

Acrylic acid (8**)**—¹H NMR DMSO-*d*₆ δ: 6.2 (dd, 1H, C3-H), 6.1 (dd, 2H, C2-H), 5.9 (dd, 1H, C3-H); ¹³C NMR DMSO-*d*₆ δ: C1, 171; C3, 131; C2, 129.

Results and Discussion

If *S*- to *N*-transnitrosation is occurring during the decomposition of *S*-nitrosocysteine, then several carbon based products may be formed, among them, thiirane-2-carboxylic acid (**5**), 2-hydroxy-3-mercapto-propionic acid (**6**) and 3-mercapto-acrylic acid (**7**, Scheme 1) (11). To test this hypothesis, we determined if these products were formed during the decomposition of **1** under neutral conditions. Their presence will serve as markers for the formation of **2** during the decomposition of **1**.

To determine if the products of *N*-nitrosation of cysteine (**5**, **6** and **7**) were formed when *S*-nitrosocysteine decomposed under neutral conditions, solutions of *S*-nitrosocysteine were monitored by HPLC. The degradation of *S*-nitrosocysteine was first order with respect to *S*-nitrosocysteine concentration; the half-life of 1.9 mM *S*-nitrosocysteine was approx. 3.75 days. This half-life was unexpectedly long (6,9). When the reaction was performed in clear glass or in the absence of 0.1 mM EDTA, the half-life of *S*-nitrosocysteine was less than 30 min confirming that light and/or metals destabilize it (3,4). The decomposition of *S*-nitrosocysteine was accompanied by the formation of at least two new peaks in the HPLC trace which eluted at 7.2 and 9.8 min (Figure 1A). The formation of these two peaks was time dependent and correlated to the decomposition of *S*-nitrosocysteine in the solution (Figure 1B).

To characterize the *N*-nitrosocysteine reaction products, a solution of 20 mM *S*-nitrosocysteine in 100 mM potassium phosphate, pH 7.2, containing 1 mM EDTA which had been incubated at 23 °C for two weeks was acidified and immediately extracted with *t*-butyl methyl ether. Following concentration, the extract was dissolved in D₂O. HPLC analysis of this extract indicated that it contained primarily the two compounds that were formed upon the decomposition of *S*-nitrosocysteine. ¹H NMR analysis confirmed that the compound eluting at 9.8 min was thiirane-2-carboxylic acid (**5**) (11). While this compound has been observed when cysteine is reacted with an excess of sodium nitrite in acidic conditions (11), ours is the first report that this compound is formed during the decomposition of *S*-nitrosocysteine at neutral pH.

The protons for the second compound resonate at 6.2, 6.1, and 5.9 ppm; each signal integrates for one proton. The protons at 6.2 and 5.9 ppm were located on the same carbon atom and were coupled to the proton at 6.1 ppm. The ¹H and ¹³C NMR spectra of this compound were identical to those obtained with commercial acrylic acid (**8**).

The extracts did not contain the expected 2-hydroxy-3-mercaptopropionic acid (**6**) or 3-mercapto-acrylic acid (**7**). While there was evidence that compounds were lost when the extracts were concentrated, another possible explanation for the absence of these two compounds is that they have likely undergone further reactions. Both compounds contain a free thiol group and, under our reaction conditions, they can be expected to be *S*-nitrosated as a result of *S*- to *S*-transnitrosation reactions or oxidized to disulfides. Preliminary capillary LC-MS/MS analysis of solutions of 10 mM *S*-nitrosocysteine in 100 mM potassium phosphate, pH 7.2, containing 1 mM EDTA indicates the presence of a compound with a molecular ion at *m/z* 150 which is consistent with the formation of *S*-nitroso-2-hydroxy-3-mercaptopropionic acid (MS² of *m/z* 150: 148, 120 and 102). At higher concentrations, a compound with a molecular ion at *m/z* 241 is also detected (MS² of *m/z* 241: 153, 121 and 119). This latter data is consistent with the formation of 2-hydroxy-3-mercaptopropionic acid disulfide.

On basis of these studies, we concluded that *S*- to *N*-transnitrosation occurs during decomposition of **1** and that acrylic acid (**8**) and thiirane-2-carboxylate (**5**) are products of this decomposition pathway under our reaction conditions. The source of acrylic acid is not clear. It may be formed from the reactive intermediate **4**, thiirane-2-carboxylic acid (**5**) or another product of this reaction in the presence of reducing agents. If **5** rearranged to form 2-mercapto-

acrylic acid, this product may form acrylic acid in the presence of a reducing agent (12). Cysteine and chloride ion are potential reducing agents present in the *S*-nitrosocysteine solutions in our studies.

These data demonstrate that *S*- to *N*-transnitrosation occurs to a small but significant extent in solutions of **1** at neutral pH. Since the other expected products of *N*-nitrosocysteine decomposition were subject to further reactions, the extent of *S*- to *N*-transnitrosation is likely greater than that indicated by the levels of **5** and **8**. Together, these two compounds represent 1–2% of the decomposed **1**. The major pathway of decomposition of this *S*-nitrosothiol under our experimental conditions appears to be denitrosation since cystine is a major product in our reactions; it precipitates from the reaction mixture as a result of its poor water solubility as previously reported (5,13,14).

The transfer of the NO group to the amino group occurs either inter- or intramolecularly. Based on the small yield of the reaction products, it is likely that transfer of the NO group from the thiol to the amino group of cysteine is slow relative to other mechanisms of decomposition (denitrosation) at pH 7.2. However, the *S*- to *N*-transnitrosation reaction may dominate in the presence of excess nucleophiles as was observed by Adam et al (10). In those studies, cysteine was present in large concentrations.

The intramolecular trapping of the reactive intermediate to form **5** supports a mechanism of decomposition of *N*-nitrosocysteine which involves a diazonium ion. If these reactions occur in vivo, this reactive intermediate may contribute to nitrosative stress (15). The reactive intermediate generated from *N*-nitrosation could react with DNA and protein to form covalent adducts. In addition, this mechanism of decomposition of **1** may provide an alternative mechanism for the modulation of protein activity by endogenous *S*-nitrosothiols. *S*-Nitrosation of cysteine sulfhydryls in proteins which also have a nearby amine, such as a lysine residue, could result in the transfer of the NO group from sulfur to the amino group generating a reactive molecule in the vicinity of a nucleophilic thiol. Such a situation could lead to irreversible inactivation of the protein via the formation of thiol-amine crosslinks. Therefore, this reaction has potential importance in the biochemical and toxicological properties of NO.

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Figure 1.

(a) Representative chromatogram of 18 mM *S*-nitrosocysteine in 100 mM potassium phosphate, pH 7.2, containing 1 mM EDTA incubated for 1 day at 23 °C. (b) Formation of *N*-nitrosation products **5** and **8** during the decomposition of 1.9 mM *S*-nitrosocysteine in 100 mM potassium phosphate, pH 7.2, containing 1 mM EDTA.

Scheme 1. Formation of cysteine *N*-nitrosation products from *S*-nitrosocysteine.