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Post-translational modification in the gas phase: mechanism of cysteine S-nitrosylation via ion-molecule reactions

Sandra Osburn¹, Richard A.J. O'Hair², Stephen M. Black³, and Victor Ryzhov^{1,*}

¹Department of Chemistry and Biochemistry, and Center for Biochemical and Biophysical Studies, Northern Illinois University, DeKalb, IL 60115, USA

²School of Chemistry, University of Melbourne; Bio21 Institute of Molecular Science and Biotechnology, University of Melbourne; and ARC Centre of Excellence for Free Radical Chemistry and Biotechnology, Melbourne, VIC 3010, Australia

³Pulmonary Vascular Disease Program, Vascular Biology Center, Georgia Health Sciences University, Augusta, GA 30912, USA

Abstract

The gas-phase mechanism of S-nitrosylation of thiols was studied in a quadrupole ion trap mass spectrometer. This was done via ion-molecule reactions of protonated cysteine and many of its derivatives and other thiol ions with neutral *tert*-butyl nitrite or nitrous acid. Our results showed that the presence of the carboxylic acid functional group, –COOH, in the vicinity of the thiol group is essential for the gas-phase nitrosylation of thiols. When the carboxyl proton is replaced by a methyl group (cysteine methyl ester) no nitrosylation was observed. Other thiols lacking a carboxylic acid functional group displayed no S-nitrosylation, strongly suggesting that the carboxyl hydrogen plays a key role in the nitrosylation process. These results are in excellent agreement with a solution-phase mechanism proposed by Stamler *et al.* (J. S. Stamler, E. J. Toone, S. A. Lipton, N. J. Sucher. *Neuron* 1997, 18, 691–696) who suggested a catalytic role for the carboxylic acid group adjacent to cysteine residues and with later additions by Ascenzi *et al.* (P. Ascenzi, M. Colasanti, T. Persichini, M. Muolo, F. Polticelli, G. Venturini, D. Bordo, M. Bolognesi. *Biol. Chem.* 2000, 381, 623–627) who postulated that the presence of the carboxyl in the cysteine microenvironment in proteins is crucial for S-nitrosylation. A concerted mechanism for the gas-phase S-nitrosylation was proposed based on our results and was further studied using theoretical calculations. Our calculations showed that this proposed pathway is exothermic by 44.0 kJ mol^{–1}. This is one of the few recent examples when a gas-phase mechanism matches one in solution.

S-Nitrosylation of cysteine residues (substitution of a thiol hydrogen by NO), first brought to light by Murad in 1986,^[1] is an important post-translational protein modification that can be carried out by a wide range of NO donors.^[2,3] It not only plays a crucial role in many signaling pathways, but has also been implicated in neurodegenerative disorders,^[4–6] antiviral mechanisms,^[7–11] intermediate filament (IF) collapse,^[12] as well as other signaling pathways.^[13–18] Due to the wide range of functions of S-nitrosylated proteins, a substantial effort has been made to elucidate which cysteines in a protein are susceptible to nitrosylation. A number of studies have noted that while a given protein may possess several cysteine residues, not all of them are susceptible to nitrosylation – indeed in some cases only a single cysteine is nitrosylated.^[19–24] An acid/base primary sequence motif was first

*Correspondence to: V. Ryzhov, Department of Chemistry and Biochemistry, Northern Illinois University, DeKalb, IL 60115, USA. ryzhov@niu.edu.

proposed by Stamler *et al.*, who suggested that the cysteines which are targets for S-nitrosylation are often flanked by acidic or basic groups [(K,R,H,D,E)C(D,E)].^[25] They proposed that the basic residue facilitates the deprotonation of cysteine and the acidic residue facilitates the protonation of the nitrosylating agent enhancing its ability to donate NO (Scheme 1). Furthermore, they found that the acidic group on the C-terminal side of the cysteine was the most important component suggesting it may play a critical role in the nitrosylation mechanism.

Ascenzi *et al.* re-evaluated data on the sequence-specific cysteine nitrosylation and showed that it was not necessary for the acidic and basic groups to flank the cysteine in the primary sequence. Rather these groups need to be in close proximity to the cysteine residue.^[26–29] An excellent example of how the microenvironment rather than the primary sequence of the cysteine residue influences nitrosylation is methionine adenosyltransferase. Thus Perez-Mato *et al.* found that Cys121 could be nitrosylated without an acid/base motif in its primary sequence, but rather utilizing an aspartic acid (355) and two arginines (357 and 363) that are located in the structural vicinity of Cys121.^[29] They proposed that the guanidine moieties of the two arginines facilitated the deprotonation of the sulfur group and the carboxylic acid group of the aspartic acid facilitated the protonation of S-nitrosoglutathione (GSNO) which would enhance its ability to donate its NO group. In earlier work, we were able to nitrosylate cysteines in several model peptides irrespectively of their primary sequence around the cysteine.^[30]

The majority of these solution-phase studies indicate that there is an acid/base motif that plays either a direct or indirect (as in the case of trans-nitrosylation between proteins)^[31] role in the nitrosylation of cysteine. Ascenzi *et al.* suggested that acid/base catalysis may even occur through water molecules.^[26] Gas-phase studies of small model systems offer a unique approach to studying the mechanisms of a wide range of reactions.^[32–37] The absence of water (solvent) eliminates this potential source of acid/base catalysis. Looking at the amino acid or a small peptide level also eliminates the extensive three-dimensional (3D) structure of proteins, allowing the intrinsic chemical properties of certain cysteine microenvironments to be evaluated. As part of a series of studies on the gas-phase chemistry of protonated S-nitrosocysteine and its derivatives,^[34,38,39] in a recent study^[40] we have shown that protonated cysteine reacts with *n*-butyl nitrite in the gas phase via a nitrosylation reaction to give protonated S-nitrosocysteine. Here we report on the combined use of gas-phase ion-molecule reactions and theoretical calculations to gain further insight into the mechanism of S-nitrosylation of cysteine.

EXPERIMENTAL

Materials

All reagents were used as received. All thiols, methanol (HPLC grade) and *tert*-butyl nitrite (90%) were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used without further purification. Helium containing 1% NO was purchased from Air Liquide (LaPorte, TX, USA).

Ion-molecule reactions

Ion-molecule reactions were carried out on a modified commercial LCQ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) as described previously.^[41] Thiols were dissolved in methanol (about 1 mM) and diluted 10- to 100-fold using 50:50 methanol/water with 1% acetic acid and introduced into the electrospray ionization (ESI) source of the mass spectrometer at a flow rate of 5 $\mu\text{L min}^{-1}$. The sheath gas, capillary voltage and temperature were adjusted to ca. 10 arb. units, 3.0 kV and 250°C, respectively. Protonated

thiol ions were mass-selected using standard procedures, and then allowed to react with the *tert*-butyl nitrite delivered to the trap via the instrument helium line. The pressure of the *tert*-butyl nitrite was approximately 1×10^{-7} Torr in the ion-trap region. The scan delay was varied allowing the acquisition of mass spectra at different reaction time points.

Experiments involving nitrous acid as the neutral reagent were performed on a LTQ FT hybrid mass spectrometer (Thermo Scientific, Bremen, Germany) utilizing the linear ion trap (LTQ) of the mass spectrometer. Samples were introduced into the ESI source of the mass spectrometer at a flow rate of 5 mL min^{-1} . Sheath gas, capillary voltage, and temperature were adjusted to ca. 10 arbitrary units, 3.0 kV and 250°C , respectively. Protonated thiol ions were isolated and allowed to react with the nitrous acid in the linear trap for variable periods of time. Nitrous acid was formed via reactions of NO gas which was introduced into the trap as part (1%) of the He bath gas with ambient oxygen and water.

The presence of nitrous acid as one of the main neutral reagents in the LTQ was established by rapid, reversible addition of 47 Da (matching the molecular mass of HNO_2) to the deprotonated arginine anion among other reagents in the negative ion mode.

Computational methods

All geometry optimizations and harmonic vibrational frequencies were calculated using the Gaussian '03 suite of programs^[42] and the hybrid B3LYP functional. Initial geometry optimizations were performed using the relatively small 3-21G* basis set. All minima were re-optimized using the same functional and the 6-311++G(d,p) basis sets, since related basis sets have been shown to give reliable geometries.^[43] All transition state calculations were performed using the QST2 functions within Gaussian. In most cases intrinsic reaction coordinate calculations were used to confirm the transition states linked the correct minima that represent pre- and post-transformation structures. Vibrational frequency analysis were performed on the structures optimized at the B3LYP/6-311++G(d,p) level of theory and used to determine whether optimized structures were true minima (no imaginary frequencies) or transition states (one imaginary frequency); and for zero-point energy corrections to electronic energies (used unscaled).

RESULTS AND DISCUSSION

For the gas-phase nitrosylation of thiols, ions of protonated cysteine and many of its derivatives were trapped in the ion trap of the mass spectrometer and allowed to react with a volatile nitrosylating agent (*tert*-butyl nitrite or nitrous acid) present in the ion trap together with the helium buffer gas. The general reaction is shown in Eqn. (1):

where R is a *tert*-butyl group or a hydrogen. We reported this reaction of protonated cysteine earlier.^[40] Figure 1 shows the mass spectrum of protonated cysteine (m/z 122) reacting with *t*-BuONO to form the cation of protonated, nitrosylated cysteine (m/z 151).

This type of reaction was also performed on several of cysteine derivatives and other thiols. The summary of the reactivity data for these reactions is given in Table 1 and the corresponding mass spectra for those species that reacted (all similar to Fig. 1(a) in the addition of 29 Da to the thiol ions when the nitrosylation occurred) can be seen in Figs. 2(a)–2(e).

Some of the systems where reaction (1) did not proceed are discussed below. First, when the S-methylcysteine cation (CysSMeH^+) was mixed with the nitrosylating agent no

nitrosylation occurred. This is expected as in S-methylcysteine as compared to cysteine the thiol hydrogen is replaced with a $-\text{CH}_3$ group, thus rendering the nitrosylation of that sulfur atom impossible. A less obvious but important observation from looking at Table 1 is that the carboxylic acid group of the cysteine is essential for the nitrosylation of cysteine. For instance, the immonium ion of cysteine, formed via the combined loss of $\text{CO-H}_2\text{O}$ from protonated cysteine, as well as protonated 2-mercaptopyridine (which does not have a carboxylic acid group at all) did not undergo nitrosylation.

The role of the carboxylic acid group in the nitrosylation process was further tested in the cases of cysteine methyl ester and the cysteine anion. Figure 1 shows the mass spectrum of protonated cysteine methyl ester being exposed to the nitrosylating agent (same pressure as in Fig. 1) for 3 s. The spectrum shows that almost no nitrosylation occurs. A similar result was also found for the cysteine anion (Table 1). This suggests that it is the hydrogen on the carboxylic acid that plays an active role in the S-nitrosylation process.

All of these observations are in good agreement with the mechanism proposed for nitrosylation by Stamler *et al.*^[25] (Scheme 1). First, the hydrogen on the sulfur atom is essential, because deprotonation of the intermediate sulfonium ion is needed to complete nitrosylation (Scheme 2). Second, the hydrogen on the carboxyl group of the cysteine is essential because it protonates the nitrosylating agent and facilitates the transfer of its $-\text{NO}$ group to the sulfur (intramolecular acid catalysis). If this proton is not available, nitrosylation does not occur (Scheme 3).

In order to understand if the amine group may play a role in the nitrosylation process several derivatives were studied that had the amine group modified. *N*-Acetylcysteine and *N*-(2-mercaptopropionyl) glycine both reacted with the nitrosylating agent at or near the collision rate (Table 1). The ions formed by the loss of ammonia via collision-induced dissociation from protonated cysteine and cysteine methyl ester, $\text{CysH}^+-\text{NH}_3$ and $\text{CysOMe}^+-\text{NH}_3$, respectively, also both reacted with the nitrosylating agent at the collision rate (Table 1). These experimental results suggest that the amine group does not appear to play an active role in the nitrosylation process. This is also in agreement with the proposed mechanism (Scheme 2).

Further experiments showed that sodiated cysteine is also unreactive towards nitrosylation (see Table 1). This is in agreement with our proposed mechanism. While the carboxyl hydrogen is still present in CysNa^+ ions, the structure of this ion is such that both sulfur and carbonyl oxygen lone pairs participate in the sodium cation coordination.^[5,44,45] This does not allow for conformational flexibility of the thiol and carboxyl moieties needed for nitrosylation to proceed according to Scheme 2.

Protonated cysteine was also exposed to neutral $\bullet\text{NO}$ to see if it would result in direct nitrosylation in the gas phase. The lack of reactivity showed that no nitrosylation occurred (Eqn. (2)):

This indicates that radical mechanism of S-nitrosylation is unlikely, and $\bullet\text{NO}$ by itself cannot function as a nitrosylating agent.

In order to gain further insight into the proposed mechanism we performed density functional calculations and modeled this reaction pathway. Figure 3 shows the potential energy surface (PES) reaction pathway and the structures associated with it. Structure S shows the lowest energy structures of the protonated cysteine and the nitrosylating agent

(nitrous acid) before they come into contact. The first step in this pathway involves a rotation so that the sulfur is facing the OH group of the carboxylic acid (I $\rightarrow$ II). This rotation requires 28.0 kJ mol⁻¹ of energy. The second step is a concerted reaction where the nitrosylation actually occurs (II $\rightarrow$ III). This step requires 10.8 kJ mol⁻¹ of energy and involves the transition state in which the S–N bond is formed, the HO–NO oxygen removes the hydrogen from the carboxyl group of the cysteine, water is eliminated, and the hydrogen from the sulfhydryl group is transferred to the carboxyl group, all in a concerted fashion as shown in Scheme 2. The third and final step in this pathway involves the rotation of the –S–NO moiety towards the carboxyl group and the rotation of the hydrogen atom on the OH group away from the sulfur (III $\rightarrow$ IV). These rotations require 23.9 kJ mol⁻¹ of energy compared to structure III. An alternative transition state for this step which included water separation before the rotation was calculated to be 42 kJ mol⁻¹ higher in energy (data not shown). The overall reaction is exothermic, with the protonated nitrosylated cysteine structure F being –44.0 kJ mol⁻¹ lower in energy than the protonated cysteine S, consistent with our previous report.^[40]

Stamler proposed that the presence of a base (side chain of histidine, arginine, or lysine) is needed for deprotonation of the thiol during S-nitrosylation (Scheme 1). In the reverse process of denitrosylation, the protonated base donates the proton back to the sulfur of cysteine. Since we are unable to examine endothermic ion-molecule reactions under our experimental conditions, we cannot comment on the denitrosylation process in the gas phase. However, the forward reaction (nitrosylation) seems to take advantage of the both the acidic and basic properties of the carboxyl moiety in the proposed concerted mechanism for the gas-phase S-nitrosylation.

CONCLUSIONS

Our results show that the carboxyl moiety of cysteine is essential for the gas-phase nitrosylation of cysteine. This is in agreement with the mechanisms proposed for protein S-nitrosylation^[25] in that an acidic residue needs to be in proximity of the cysteine in order to donate a proton to the nitrosylating agent. Our calculations for this reaction suggest that the concerted nitrosylation pathway has almost no energy barrier and that the overall S-nitrosylation process is slightly exothermic. Our findings provide a rare example of a biochemical reaction solution-phase mechanism matched in the gas phase. Hopefully, it will encourage more advances in using gas-phase experiments for solving condensed-phase problems.

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Figure 1.

Mass spectra showing the ion-molecule reactions of protonated precursor ions with *tert*-butyl nitrate at a pressure equivalent to 1×10^{-7} Torr: (a) formation of nitrosylated cysteine (m/z 151) from protonated cysteine (m/z 122). Reaction time is equal to 1 s (1000 ms); (b) protonated cysteine methyl ester (m/z 136) reacting to form trace amounts of nitrosylated cysteine methyl ester (m/z 175) after 3 s.

Figure 2.

Mass spectra of gas-phase reactions of several protonated thiols with the nitrosylating agent tBuONO ($P = 1 \times 10^{-7}$ Torr): (a) CysH⁺, reaction time 1 s; (b) N-AcCysH⁺, reaction time 40 ms; (c) CysH⁺-NH₃, reaction time 60 ms; (d) CysOMeH⁺-NH₃, reaction time 40 ms; and (e) [H₃C-CH(SH)-CO-NH-CH₂-COOH+H⁺]⁺, reaction time 40 ms.

Figure 3.

DFT calculations at the B3LYP/6-311++G(d,p) level of theory showing the Potential Energy Surface (PES) for the nitrosylation reaction of protonated cysteine with nitrous acid. Top panel shows the energy profile. Bottom panel shows key minima and transition state structures associated with the reaction.

Scheme 1.
Proposed mechanism for the S-nitrosylation of cysteine in a protein.

Scheme 2.

Mechanism for the S-nitrosylation of protonated cysteine in the gas phase.

Scheme 3.

Mechanism for the S-nitrosylation of protonated cysteine methyl ester in the gas phase.

Table 1

Table of reactivity data of cysteine and its derivatives

Reactivity Data

S-Nitrosylation
occurs

CysH⁻§
(slow)

N-AcCysH⁻
(fast)*

CysH⁻-NH₃
(fast)*

(fast)*

CysOMeH⁻-NH₃
(fast)*

S-Nitrosylation
Does NOT occur

CysOMeH⁻

CysSMeII

(Cys-H)⁻

CysNa⁻

(CysH⁻-CO-H₂O)

§ About 1% of the collision rate.

* At or near the collision rate.