The Kinetics of Thiol-mediated Decomposition of S-Nitrosothiols

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ABSTRACT

The reaction of sulfhydryl (SH)-containing molecules (thiols) with S-nitrosothiols (RSNO) has been shown to be of biological importance. Biologically or therapeutically relevant thiols generally have a pK_a value ranging from 8 to 10 for the SH group. In addition, some of these thiols contain a carboxyl group and are acidic, which should be considered in studying the reaction between RSNO and thiols. In the present study, the kinetics of thiol-mediated decomposition of RSNO was investigated in a commonly used phosphate buffer, phosphate buffered saline (PBS; containing 6.9 mM phosphates; buffer capacity = 3.8 mM/pH). The thiols studied can be divided into 2 groups, depending on their pH perturbation capacity. The kinetics was studied using a wide range of thiol concentrations (ie, from 0.1 to 10 mM). A high-performance liquid chromatography (HPLC) method was used to determine RSNO concentrations. The results showed that the acidic thiols, including glutathione, captopril, N-acetylcysteine, and tiopronin, stimulated RSNO decomposition at low millimolar concentrations up to 2 mM. The stimulatory effect, however, became attenuated at concentrations higher than 2 mM in PBS. Increasing the concentration of acidic thiols caused a decrease in solution pH, which was attributable to the inhibitory effect at high thiol concentrations. The effect of thiols on the pH of reaction solution, and the resulting bell-shaped rate profiles, can be predicted by a quantitative analysis, from which a comparison of the intrinsic reactivity toward RSNO, among 8 thiols, was possible. The intrinsic reactivity in general followed the Brønsted relation.

KEYWORDS: kinetics, nitric oxide, S-nitrosothiols, thiol, thiolate anion, S-transnitrosation

INTRODUCTION

S-nitrosothiols (RSNO), the S-nitros(yl)ated products of thiols, have been extensively investigated as the important intermediate for nitric oxide (NO)-mediated biological actions.[1-9] Numerous studies have suggested that RSNO is the carrier, or the storage form, of NO in vivo, thereby serving as a reservoir for NO bioactivity.[10-15] The study of chemical stability of RSNO therefore provides basic information about RSNO as potential NO carriers or donors.[16-18] Several factors have been attributed to the acceleration of RSNO decomposition, including heat, UV light, certain metal ions, superoxide, seleno compounds, ascorbate, and thiols.[19-21]

Two facets of thiols (RSH) as NO modulators have been extensively studied, given the abundance of thiols present in the biological system. First, the reaction between thiols and reactive nitrogen species, via S-nitrosation, forms RSNO.[22-25] Second, thiols may accelerate the decomposition of S-nitrosothiols, via S-transnitrosation (Equation 1).[16,26-29]

$$RSH + R'SNO \longleftrightarrow R'SH + RSNO. \quad (1)$$

Mechanistically, thiolate anions (RS^-) have been proposed as the key reactive forms involved in S-transnitrosation reaction, therefore the kinetics of thiol-RSNO reaction is dependent on the pK_a of thiols and the pH of solution.[29-32] Kowaluk and Fung[9] studied the effect of added thiol on the decomposition of RSNO and found that the rate of RSNO decomposition was increased in the presence of the added thiol. Singh et al[16] studied the mechanism of NO release from RSNO and showed that S-transnitrosation can stimulate RSNO decomposition in the presence of transition metal ions. The chemical study of S-nitrosoglutathione (GSNO)/glutathione (GSH) couple by Singh et al[33] showed that incubation of GSNO (1 mM) with GSH at various concentrations (1-10 mM) in phosphate buffer (pH 7.4) for 40 hours yielded oxidized glutathione, nitrite, nitrous oxide, and ammonia as end products. The study of Dicks et al[34] confirmed the finding by Singh et al[33] and demonstrated that ammonia but not NO is the main "nitrogen" product in RSNO decomposition at high concentrations of RSH.

To study the reactivity of various thiolate anions toward RSNO, the pH of the reaction solution may be raised to >12, because the pK_a of most biologically or therapeutically relevant thiols is approximately in the range of 8 to 10. The alkaline pH of the reaction solution may result in confounding side reactions, which might be significant when high concentrations of thiols (ie, several millimoles) were used. In addition, the reaction may be too fast to be determined, since the reaction rate is generally proportional to thiolate concentrations. The use of low thiol concentrations may solve this problem; however, it may require a more sensitive analytical method and may compromise the desire to study

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the kinetics at a concentration range that covers the physiological concentrations for thiols such as reduced GSH.

GSH and some other therapeutic thiols are acidic in water owing to their carboxyl groups. The solution pH, therefore, may not be maintained when these thiols are dissolved in a weak buffer. For the use of phosphate buffers, high concentrations of phosphates (eg, >50 mM[16,35]) may be needed to obtain adequate buffer capacity. Phosphates have been shown to inhibit N-nitrosation in the GSH/S-nitrosoglutathione system.[16] It is, however, not clear as to whether high phosphate concentrations would directly affect thiol-RSNO reaction kinetics. Since the cytoplasmic phosphate concentration is ~5 mM,[36] the use of low phosphate buffer may be more appropriate, but the problem of low buffer capacity should be addressed.

The present study investigated the kinetics of RSNO decomposition mediated by thiols in phosphate buffered saline (PBS). Owing to a high-performance liquid chromatography (HPLC) method used, the concentrations of RSNO were in the micromolar range and the major reaction involved was shown to be S-transnitrosation between RSNO and thiols. A total of 8 biologically and therapeutically relevant thiols were included, which were divided into 2 groups according to their pH perturbation capability and rate profile. Kinetic analysis provided a measure by which to normalize these 2 groups of thiols, so that the rate constants for various thiols reacting with RSNO could be compared.

Experimental Procedures

Reagents

S-nitrosocaptopril (CapNO) was purchased from Calbiochem (La Jolla, CA). Penicillamine and mesna (sodium 2-mercaptoethanesulfonate) were purchased from Fluka (Buchs, Switzerland). All other chemicals including S-nitroso-N-acetyl-penicillamine (SNAP), S-nitrosoglutathione (GSNO), GSH, N-acetyl-penicillamine (NAP), cysteine, N-acetyl-cysteine, captopril, tiopronin (N-(2-mercaptopropionyl)glycine), sodium thioglycolate, and DTPA (diethylenetriaminepentaacetate) were purchased from Sigma (St Louis, MO). All chemicals used were of the highest grade and purity available.

High-performance Liquid Chromatography Determination of S-nitrosothiols

HPLC methods were developed for the determination of RSNO concentrations. The instrumentation consisted of a solvent delivery system (BAS-PM 80, Bioanalytical Systems Inc, West Lafayette, IN), a loop injector (Rheodyne 7125, Rohnert Park, CA), a reverse-phase C18 column (Merck Lichrospher RP-select B [5 μm], Merck Inc, Whitehouse Station, NJ), a UV detector (Soma S-3702, Tokyo, Japan), and an integrator (SIC Chromatocorder 12, SIC, Tokyo, Japan). For studying the decomposition kinetics of SNAP and CapNO, the same chromatographic conditions were used, which included a mobile phase (0.1% phosphoric acid:methanol:acetonitrile = 65.5:27:7.5) at a flow rate of 1.0 mL/min and UV detection at either 220 nm or 210 nm (for the experiment using 5 μM SNAP). The condition was slightly modified for studying the decomposition of GSNO (mobile phase: 0.1% phosphoric acid:methanol = 80:20; flow rate = 0.4 mL/min). For all RSNOs, an aliquot of a 20-μL sample was injected into the HPLC system in each chromatographic analysis. The retention times of RSNOs and thiols were verified using authentic standards.

RSNO Decomposition Kinetics

In a typical kinetic study, the reaction was run in a reaction buffer made from PBS (pH 7.4, [phosphate] = 6.9 mM), and DTPA (0.1 mM), at 25°C. Initially, an aliquot of 100-μL stock solution of thiol (1-100 mM in deionized water) was added to the reaction buffer to obtain a target thiol concentration (0.1-10 mM). To start the reaction, 990 μL of the thiol-containing reaction buffer was added to a 1.5-mL tube preloaded with 10 μL of RSNO (10-mM stock solution in water, freshly prepared and stored at −80°C). The initial concentration of RSNO in the reaction buffer was 100 μM. The reaction was kept in the dark and was typically followed for a 30-minute period—during which 4 samples each of 50 μL were taken from the reaction buffer at 0.5, 10, 20, and 30 minutes, and were injected (20 μL) directly and immediately into the HPLC system. A preliminary test was conducted to assure that the reaction was completely quenched in the acidic mobile phase during the HPLC analysis. For a fast reaction mediated by some thiols, reaction was followed for a shorter period of time.

Kinetic Analyses

Under the study conditions (pseudo-first-order, pre-equilibrium), thiol-mediated S-nitrosothiol decomposition may be assumed to follow a 2-step reaction in which the backward reaction of S-transnitrosation is negligible:

$$RSH \overset{K_a}{\longleftrightarrow} RS^- + H^+ \tag{2}$$

$$RS^- + R'SNO \xrightarrow{k_2} RSNO + R'S^-, \tag{3}$$

where K_a is the acidic constant of thiol and k_2 the second-order rate constant for the reaction between the thiolate anion and S-nitrosothiol. Accordingly, the rate law for S-nitrosothiol decomposition can be expressed as

$$\begin{aligned} -\frac{d[R'SNO]}{dt} &= k_2 \cdot [RS^-][R'SNO] \\ &= k_2 \cdot f_{thiolate} \cdot [RSH]_T[R'SNO], \end{aligned} \tag{4}$$

where $[RSH]_T$ is the total thiol concentration at any given time, and $f_{thiolate}$, the fraction of the thiolate anion:

$$f_{thiolate} \equiv \frac{[RS^-]}{[RSH]_T} = \frac{K_a}{K_a + [H^+]} = \frac{1}{1 + 10^{(pK_a - pH)}}. \quad (5)$$

Since pseudo-first-order kinetics was observed, Equation 4 can be simplified as

$$-\frac{d[R'SNO]}{dt} = k_{obs}[R'SNO], \quad (6)$$

where k_{obs} is the observed pseudo-first-order rate constant—which, according to Equation 4 and assuming under the study condition that $[RSH]_T \cong [RSH]_0$ (ie, the initially added thiol concentrations)—can be expressed as

$$k_{obs} = k_2 \cdot [RS^-] \quad (7)$$

or

$$k_{obs} = k_2 \cdot f_{thiolate} \cdot [RSH]_0 = k_2' \cdot [RSH]_0, \quad (8)$$

where

$$k_2' = k_2 f_{thiolate} = \frac{k_2}{1 + 10^{(pK_a - pH)}}. \quad (9)$$

To predict the effect of an acidic thiol on the pH of the reaction buffer, we considered a solution system, in which the acidic thiol (HS-R-COOH)—acting as a weak acid—is added to a buffer made from the conjugate acid-base pair, $H_2PO_4^-/HPO_4^{2-}$. In such a system, the effect of SH group on the pH can be ignored (since in this study, pK_a (SH) >8 versus pK_a (COOH) ~3.5). Therefore, an equation for pH calculation, based on the principle of ionic equilibria, can be derived[37]:

$$\begin{aligned} x^4 + (K_1 + K_2 + C_{b1})x^3 + [K_1(K_2 - C_{a1}) \\ + K_2(C_{b1} - C_{a2}) - K_W]x^2 - [K_1K_2(C_{a1} + C_{a2}) \\ + K_W(K_1 + K_2)]x - K_1K_2K_W = 0, \end{aligned} \quad (10)$$

where x represents $[H^+]$; C_{a1} and C_{b1} are the concentrations for the conjugate acid-base pair and C_{a2}, the concentration of an added thiol; K_1 (= $10^{-7.2}$) and K_2 (= $10^{-3.5}$) are the acidity constants for $H_2PO_4^-$ and the acidic thiol (HS-R-COOH), respectively; and K_w is the dissociation constant of water. The pH value upon the addition of acidic thiols can be estimated by solving Equation 10 for x (Mathematica 4.2, Wolfram Research, Champagne, IL).

Data Analyses

Least-squared linear regressions were used throughout the study to obtain the rate constants. At least 3 kinetic data points for each thiol concentration were used for the initial estimation of k_{obs}.

Results and Discussion

The chemical structures of various thiols studied are shown in Figure 1. The thiols of interest can be divided into 2 groups,

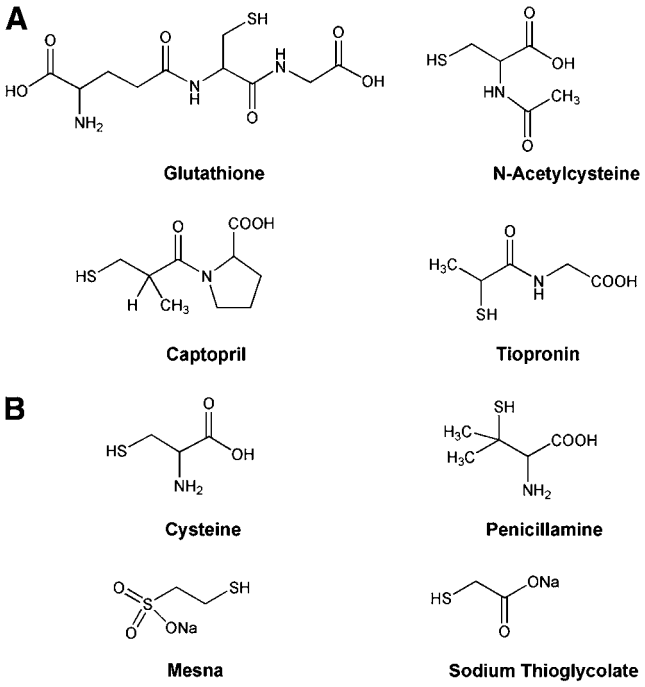

Figure 1. The chemical structures of thiols: (A) acidic thiols; (B) "neutral" thiols.

based on their pH perturbation capacity in aqueous solution. The first group (group A) includes GSH, N-acetylcysteine, captopril, and tiopronin, which, at high concentrations, significantly reduced the pH of the PBS solution. The second group (group B), however, includes thiols that are either "neutral" amino acids (cysteine and penicillamine) or salts of weak acids (mesna and sodium thioglycolate) that comparably have less effect on the pH. For group B thiols, pH perturbation at 10 mM was less than 0.35 pH unit.

The HPLC method described offered an accurate and rapid determination of RSNO decomposition in different reaction couples of RSNO/R′SH. The typical HPLC chromatograms for the kinetic study of RSNO decomposition are shown in Figure 2; in this particular example of SNAP/GSH couple, only SNAP and its denitrosylated thiol product, N-acetylpenicillamine (NAP), can be measured kinetically. Since GSNO and GSH in the case just described cannot be simultaneously determined under the same HPLC condition, it is important to demonstrate, using another reaction couple (SNAP/captopril), that S-transnitrosation was indeed observed (Figure 3). In the SNAP/captopril couple, all reactants (SNAP, captopril) and products (NAP, S-nitrosocaptopril) were measured during a kinetic study that lasted for 4 hours (Figure 3).

The kinetic profiles of RSNO decomposition are illustrated in Figure 4, using the SNAP/GSH couple as an example. The log-linear kinetic data indicate that, under the conditions tested, first-order kinetics was apparent for RSNO decomposition. The slopes of the log-linear profiles were used for estimating the observed pseudo-first-order rate

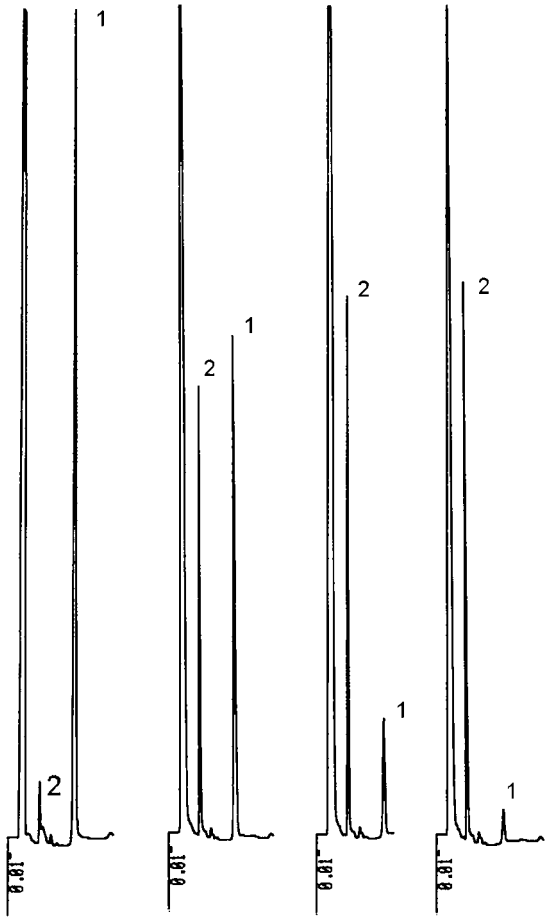

Figure 2. Typical HPLC chromatograms for the kinetic study. (A) SNAP (peak 1, retention time = 5.6 minutes) and its decomposition product, N-acetylpenicillamine (peak 2, retention time = 2.6 minutes) in the presence of GSH (1 mM). Each trace represents the HPLC recording at different reaction time (from left to right: 0.5, 10, 20, and 30 minutes).

constants (k_{obs}) for RSNO decomposition, according to Equation 6. Figure 5 shows that the concentration profiles of k_{obs} versus thiol were bell shaped for all RSNO/R′SH couples, with the effect of thiols on RSNO breakdown being almost completely blocked ($k_{obs} \rightarrow 0$) at a thiol concentration of 10 mM.

The pH modifying effect of acidic thiols is shown in Figure 6. All acidic thiols exhibited similar pH-titration effect. The pH of PBS solution was reduced to ~3.5 when the thiol concentration reached 10 mM. The observed pH changes can be predicted by Equation 10, based on the assumption that the thiol carboxylates (HS-R-COOH) acted as weak acids in a buffer solution that contained a single conjugate acid-base pair ($H_2PO_4^-/HPO_4^{2-}$). The prediction was in general in agreement with the experimental data, with slight systematic overestimations of the pH value at lower thiol concentrations region. To demonstrate that solution pH was the artifactual factor for rate attenuation with high thiol concentrations, kinetic studies were performed in PBS solution,

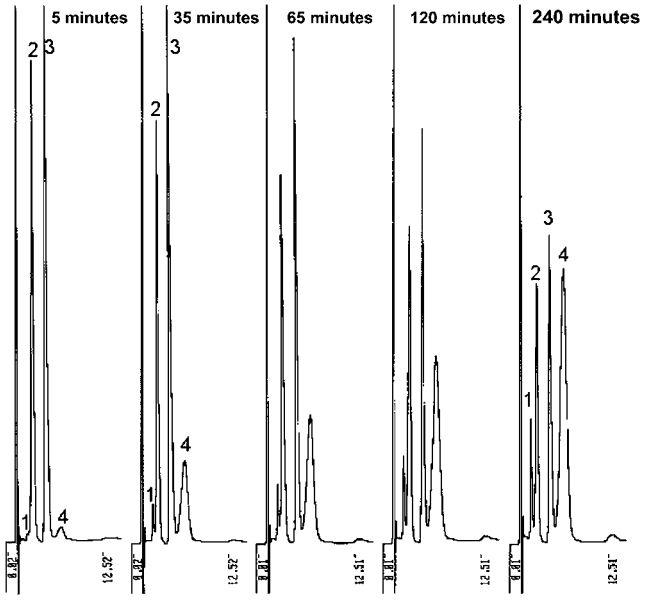

Figure 3. Kinetic HPLC recordings for the SNAP/captopril reactant couple and their products (N-acetylpenicillamine (NAP) and S-nitrosocaptopril (CapNO). The initial concentration for SNAP and captopril was 100 μM. HPLC chromatograms were recorded at 5, 35, 65, 120, and 240 minutes after starting the reaction. Peak 1: NAP; peak 2: captopril; peak 3: SNAP; peak 4: CapNO.

with pH adjusted back to 7.4 immediately after adding the thiols. For the purpose of comparison, kinetic studies were also performed for thiols that showed less effect on pH. The results in Figure 7 show that the rate profiles were linear for all acidic thiols with pH adjustment, and for all "neutral" thiols.

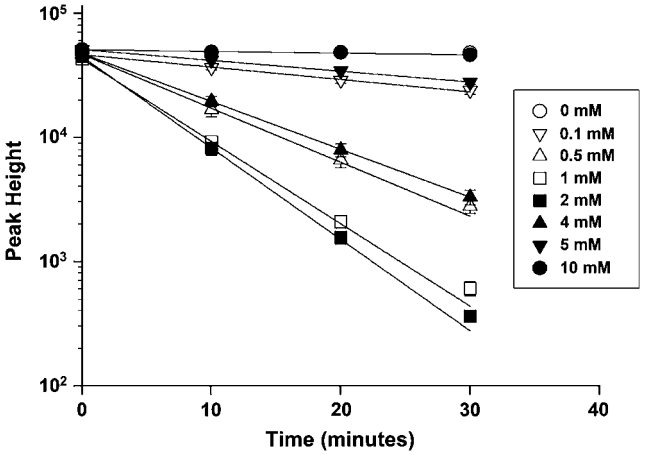

Figure 4. Decomposition kinetics of SNAP (initial [SNAP] = 100 μM) in the presence of GSH. The experiments were performed in a PBS-based reaction buffer (pH 7.4) containing DTPA (0.1 mM), at 25°C. The initial concentration of SNAP was 100 μM. The concentrations of GSH studied were 0, 0.1, 0.5, 1, 2, 4, 5, and 10 mM. Each data point represents mean ± SD of 3 independent experiments.

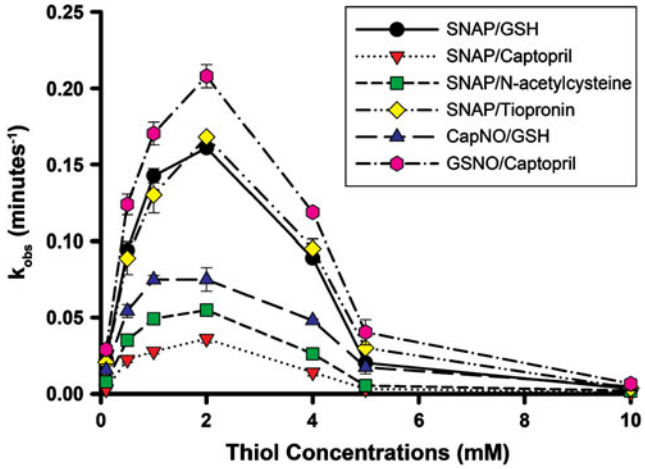

Figure 5. Bell-shaped plots of k_{obs} versus thiol concentrations for RSNO decomposition. The initial RSNO concentration was 100 μM.

According to the reaction mechanisms and equations described here, the second-order rate constant (k_2) for S-transnitrosation reaction between thiolate anions (RS^-) and RSNO can be estimated. Table 1 lists the estimated k_2 values for the 8 thiols that reacted with SNAP. Figure 8A shows that the magnitude of k_2 tended to increase with increasing basicity of the thiol, which (excluding penicillamine) can be described by the Brønsted relation:

$$\log k_2 = \beta_{nuc} \cdot pK_a + C, \qquad (11)$$

where β_{nuc} (~0.3) is the Brønsted coefficient and C is a constant. Structurally the SH group in penicillamine is attached to a tertiary carbon atom, which might account for the significantly lower intrinsic activity observed. Similar results have been reported for other nucleophilic reactions that involved thiolate anions.[38] The overall reactivity of thiols (RSH) at pH 7.4 was of interest, which can be estimated as

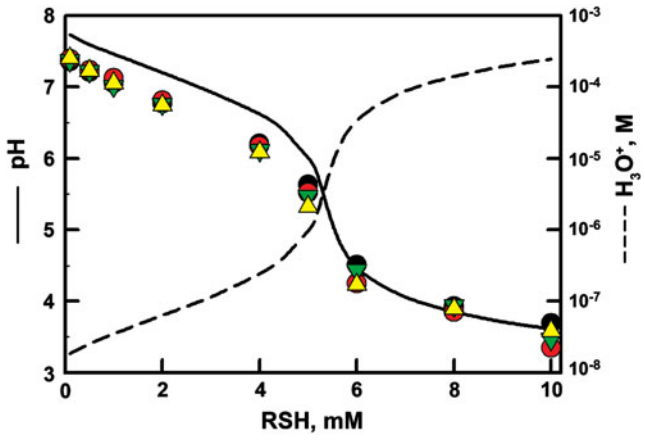

Figure 6. The pH-modifying effect of acidic thiols (GSH, captopril, N-acetylcysteine, and tiopronin). The symbols represent the experimental data and the lines are the theoretical predictions.

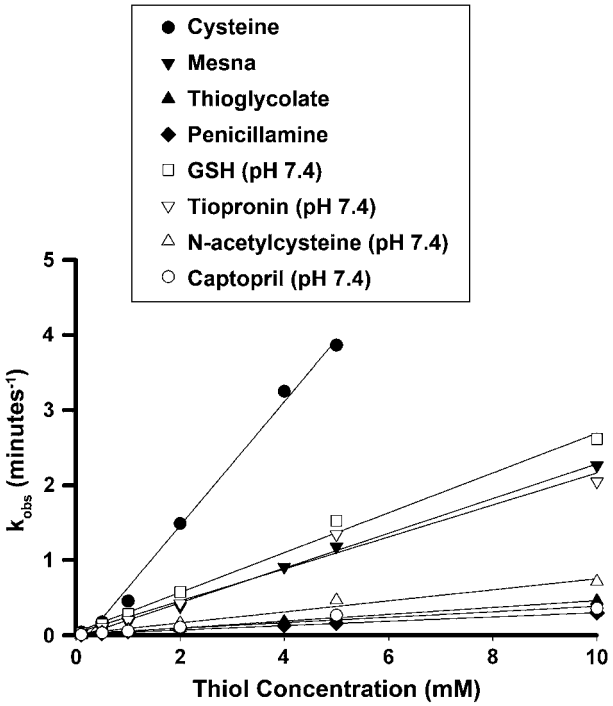

Figure 7. The plots of k_{obs} versus thiol concentrations for neutral thiols and for acidic thiols with pH adjustment to pH 7.4 before the kinetic measurements.

k_2' from Equation 9 (Table 1). The result generally indicates that k_2' is negatively correlated with the pK_a of thiols (except penicillamine, Figure 8B). Therefore, although thiols with a higher pK_a value were intrinsically better nucleophiles (ie, with higher k_2), their overall reactivity was offset by their comparably lower tendency to generate thiolate anions (RS^-). In previous studies, the second-order rate constant for the forward reaction between SNAP and GSH has been determined to be 5.39 $M^{-1}second^{-1}$ and 9.09 $M^{-1}second^{-1}$ at 37°C, using HPLC[27] and UV spectrophotometry,[16] respectively; for comparison, here k_2', equivalent to the forward rate constant mentioned, has a value estimated in our study of 5.46 $M^{-1}second^{-1}$ at 25°C (Table 1) and 10.4 $M^{-1}second^{-1}$ at 37°C, respectively.

At least 3 mechanisms are likely responsible for RSNO decomposition under the conditions tested. First, light and transition metal ions may mediate RSNO decomposition, resulting in the formation of disulfide and NO.[16] Second, nucleophilic attack of a thiol on the RSNO nitrogen results in the formation of an N-hydroxysulfenamide intermediate, consequently, via a free radical mechanism, giving rise to disulfide.[33] Third, the same N-hydroxysulfenamide intermediate is formed as a result of RSNO-thiol reaction, but the end products are another RSNO and thiol (the S-transnitrosation mechanism). Our data favor the third mechanism, given the following lines of reasoning: (1) The metal ion chelator, DTPA, was added in the reaction buffer and high purity reagents were used to avoid metal ion contamination;

Table 1. The Second-Order Rate Constants for the Reaction of S-nitroso-N-acetyl-penicillamine (SNAP) With Thiols at 25°C in Phosphate Buffered Saline

Thiol	pK_a*	k_2† ($M^{-1}s^{-1}$)	k_2' (pH = 7.4)‡ ($M^{-1}s^{-1}$)
Penicillamine	7.9§	3.77	0.906
Cysteine	8.35‖	180	18.2
Tiopronin	8.81¶	135	5.06
Glutathione	8.83#	152	5.46
Mesna	9.10**	231	4.52
N-acetylcysteine	9.52§	222	1.67
Captopril	9.80††	319	1.26
Thioglycolate	10.2‡‡	474	0.750

*For aminothiols such as cysteine and glutathione, microscopic pK_a values were used.
†Second-order rate constants for RS^-, using Equation 7. For each second-order rate constant, a regression analysis was performed to fit the rate data obtained for the 7 thiol concentrations studied. For each thiol concentration, the rate data were obtained from 3 kinetic measurements (n = 3). The coefficient of variation is generally within 10%.
‡Apparent second-order rate constants for RSH at pH 7.4, using Equation 9.
§Data from Friedman et al.[38]
‖Mean values of 8.15,[38] 8.21,[39] 8.50,[40] and 8.53.[41]
¶From http://medicine.cug.net/drug/07/07_02_01.htm.[42]
#Mean values of 8.72[39] and 8.93.[43]
**Data from Danehy and Noel.[44]
††From the Merck Index, monograph no 1780.[45]
‡‡Mean values of 9.82,[39] 10.01,[40] 10.22,[44] and 10.7.[46,47]

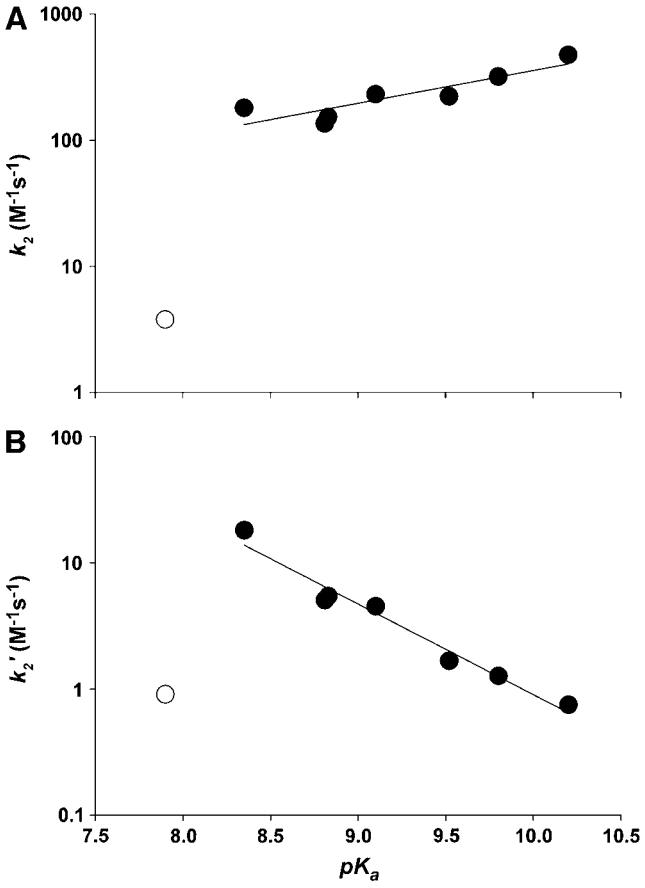

Figure 8. Dependence of second-order rate constants on thiol pK_a. (A) k_2. The line represents the least squares fit to the solid symbols ($\log k_2 = 0.26 \times pK_a - 0.0045$; $r = 0.881$, $P < 0.01$). (B) k_2'. (pH 7.4). The line represents the least-squares fit to the solid symbols ($\log k_2' = -0.72 \times pK_a + 7.15$; $r = 0.984$, $P < .001$).

(2) in the absence of thiols, RSNO was generally stable under the study conditions (data not shown); (3) formation of new RSNO and thiol was evident as exemplified by the SNAP/captopril couple (Figure 3); (4) significant disulfides formation is possibly ruled out, because we did not observe significant peak formation (usually with longer column retention) in the chromatographic analyses. The formation of disulfides, however, may be significant when thiol and RSNO, both at high concentrations, are reacting for a prolonged period of time (eg, >10 hour).[33,34] In our study, the reaction was monitored only for 30 minutes or less; and during this time period, >85% of the initially added RSNO was recovered as the parent RSNO and its S-denitrosylated product (data not shown), further suggesting that disulfides and other reaction products had minimal effect on our analysis.

What are the implications of the present study, which mainly deals with the physical chemical aspect of RSNO-thiol reaction? One immediately following question would be: can the observed phenomena of pH, and therefore rate reduction occur in biological systems or, specifically, in cells? Obviously, buffer capacity is the key factor that should be considered. According to the Koppel-Spiro-van Slyke equation,[37] the buffer capacity of the buffer used in our study is estimated to be 3.8 mM/pH. The estimation in literature put the buffer capacity for whole blood in the range of 25 to 39 mM/pH, and for plasma (mainly bicarbonate buffer), 3 mM/pH (reviewed in Martin's Physical Pharmacy[37]). In addition, the buffer capacities for the cellular subcompartments—endoreticulum (ER), Golgi complex, and endosome—were found to be between 6 and 50 mM/pH.[48-51] Since GSH concentrations determined in cellular organelles are in the range of 1 to 11 mM,[52-59] and the pH determined in different cellular compartments in the range of 5.5 to 7.5,[48,50,60-62] it is conceivable that S-transnitrosation profiles might be different in various organelles.

Conclusions

The present study investigated the kinetics of RSNO decomposition mediated by thiols of biological or therapeutic importance. While S-transnitrosation has often been treated as a reversible reaction,[20,27,29] the present study has focused on the forward reaction that contributes to RSNO decomposition in the presence of thiols. The reactant concentrations

and the reaction condition (pre-equilibrium; reaction time ≤30 minutes) have been carefully chosen so that pseudo-first-order decomposition kinetics of RSNO was analyzed. Acidic thiols such as GSH, captopril, N-acetylcysteine, and tiopronin can significantly modify the pH of a phosphate buffer that is so commonly used in the biological and pharmacological studies. The effect of thiols on solution pH results in artifactual bell-shaped rate profiles for RSNO-thiol reactions, which can be described by quantitative pH calculations. In conclusion, the present study demonstrates that RSNO-thiol reaction can be described by Brønsted relation, further supporting a nucleophilic mechanism for RSNO-thiol reaction.

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