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KINETIC STUDIES OF THE REACTION OF S-NITROSO-L-CYSTEINE WITH L-CYSTEINE

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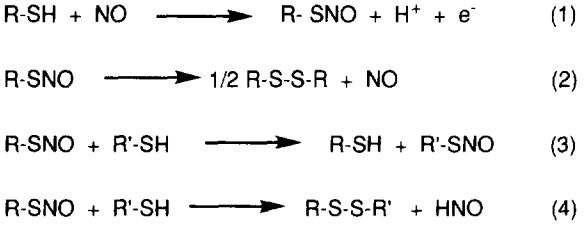
Abstract: Rates for the reaction between L-S-nitrosocysteine and L-cysteine were found to be first order with respect to concentrations of the two reactants in the pH range of 4.85 - 9.41. The second order rate constant was determined to be 3.10×10^{-2} $M^{-1}s^{-1}$ at pH = 7.4 at 37 °C. Spontaneous decomposition of L-S-nitrosocysteine proceeded with the first order rate constant, 1.46×10^{-5} s^{-1} under the same conditions.

A S-nitrosothiol has been considered to be one of most plausible candidates for the endothelium-derived relaxing factor (EDRF).[1] Finally, however, EDRF was identified to be NO producing via NO-synthase instead of a S-nitrosothiol.[1-3] Meanwhile, the following observations have been accumulated which suggest that NO is reserved and transported as nitrosothiols, the carriers, which then give their NO to metal prosthetic groups or cysteine residues of the target proteins to transduce signaling: (a) NO reacts readily with thiols to give the corresponding S-nitrosothiols (reaction 1).[4-8] (b) S-nitrosothiols are much more stable than NO, which is a labile free radical in the physiological milieu. Cys-34 of serum albumin is nitrosylated to form long lived S-nitrosoalbmin which may act as a reserver and carrier of NO in plasma.[9,10] (c) Thiols potentiate the action of NO. For example, relaxant action of NO on the rat anococcygeus muscle is much greater and more prolonged in the presence of cysteine (Cys-SH) than in its absence.[8] S-nitrosocysteine (Cys-SNO) is a more potent relaxant of endothelium-denuded stripes of rabbit aorta than NO. S-nitrosothiols add NO to soluble guanylate cyclase.[3] The NO binding to the heme moiety of the guanylate cyclase triggers the generation of cyclic GMP, the next messenger in the NO-mediated signaling pathway. (d) Nitoso group transfer, transnitrosation (reaction 3) between S-nitrosothiols and thiols takes place readily.[10-12] (e) S-nitrosation of cysteine residues of the particular proteins modulates their functions.[13-17]



Since all physiological events are rate processes, understanding the biological roles of S-nitrosothiols requires rates and mechanisms of the key reactions 1-4 of S-nitrosothiols. Recently kinetic data and mechanisms for the Cys-SNO-forming reaction of NO with cysteine (reaction 1) have been reported by two groups.[18,19] When the reaction 1 is carried out under aerobic conditions, O_2 acts as the electron acceptor.[18,19] Kinetic studies on the transition metal ion-catalyzed NO-regenerating process from S-nitrosothiols (reaction 2) have been investigated by Williams et al.[20] Arnelle and Stamler showed in their *in vitro* semi-quantitative studies of the reaction 2 that the apparent life times of S-nitrosothiols vary depending on the analytical methods.[11] The kinetics and mechanisms of the transnitrosation from S-nitrosothiols to thiols (reaction 3) have been extensively studied by Williams and coworkers.[12]

In this way, thiols produce S-nitrosothiols in biological systems, however, they also destroy S-nitrosothiols to give disulfides (reaction 5) as reported by Oae et al.[21] On the contrary, recently Feelisch et al. reported that the life time of Cys-NO is prolonged by the presence of cysteine in a concentration-dependent manner.[3] Since the thiol concentration level in normal living cells is pretty high (ca. 5 mM)[22] the decomposition reaction of S-nitrosothiol with thiols (reaction 4) is of importance but its kinetics study has not as yet been reported. Thus, we initiated the kinetic studies of the degradation reaction of Cys-SNO in the presence and absence of Cys-SH to solve these problems.

Experimental

Preparation of Cys-SNO: Cys-SH was allowed to react with an equimolar amount of nitrous acid in a aqueous solution of pH 1-2 at 25 °C for 3 min at room temperature. The pH of the solution was raised to 7 by adding a NaOH solution. The solution of Cys-SNO was treated with Vio-rad Chelex 100 ion exchange resin in an ice bath to remove any trace heavy metal ions which might be contained in the solution. Buffer solutions and Cys-SH solution were also treated with Chelex 100, since heavy metal ions, especially Cu^+, catalyzes the decomposition of nitrosothiols as noted by Williams et al.[20] All the solutions contained 0.1 mM ethylendiaminetetraacetate (EDTA) to protect Cys-SNO from the heavy metal catalyzed decomposition. The solution of Cys-SNO thus prepared is stable for a few days in an ice bath.

$$\text{Cys-SH} + \text{HONO} \longrightarrow \text{Cys-SNO} + H_2O \qquad (5)$$

Molar extinction coefficient of Cys-SNO (e): Equimolar amounts of known concentration of Cys-SH in 0.1 M HCl solution and aqueous $NaNO_2$ solution were mixed in a quartz UV cuvette and the increasing absorbance at 543 nm was recorded. After 3 min the absorbance reached to plateau. The value of e was calculated from the maximum absorbance of 852 $M^{-1}cm^{-1}$ (λ_{max} 335 nm) and 16.8 $M^{-1}cm^{-1}$ (λ_{max} 543 nm) by assuming that Cys-SH was converted to Cys-SNO quantitatively. The experiment was repeated 3 times and was reproducible. The same value was obtained under argon.

UV-visible spectra of Cys-SNO were recorded on a JASCO Ubest-50 spectrophotometer equipped with a quartz UV cuvette with a water jacket to which water of 37± 0.1 °C was circulated from a NESLAB RTE-210 thermostatted bath. When microsyringes with stainless steel needle and piston to transfer Cys-SNO into the UV cuvette was used, metal ion catalyzed decomposition of Cys-SNO took place rapidly at a nonreproducible rate. Therefore, pipetting of the solutions was done by an eppendorf-pipette.

All the pH values were measured at 37 °C by Horiba M 13 pH meter. The following buffer solutions were used : pH 4.5-5.5, sodium acetate; pH 6-7.8, sodium phosphates; pH 8-9, sodium borate.

Results

Since most of thiols contained in peptides and proteins come from cysteine residues, kinetics of the reaction of Cys-SNO with Cys-SH (reaction 6) have been studied. Both the nucleophilic substitutions on the nitroso-nitrogen (reaction 3) and on the sulfur (reaction 4) of a S-nitrosothiol with a thiol are orbital controlled reactions[23,24] and therefore these reactions may occur smoothly. The reversible transnitrosation reaction 3 and the irreversible reaction 4 take place competitively.[11] However, since R and R' are the same in this work, the reaction 3 does not interfere in the kinetic investigation of the reaction 6. Three fates await the HNO formed by the reaction 6, i.e. the condensation reaction 7 to give dinitrogen oxide, single electron oxidation affording NO (reaction 8),[25] and the reduction by thiol affording hydroxylamine (reaction 9).[11] In accordance with the observation by Arnelle and Stamler,[11] no hydroxylamine was detected in the reaction mixture of Cys-SNO with Cys-SH, suggesting that HNO disappears via the reactions 7 and 8 .

$$\text{Cys-SNO} + \text{Cys-SH} \longrightarrow \text{Cys-SS-Cys} + H^+ + NO^- \quad (6)$$

$$2\,NO^- + 2H^+ \longrightarrow N_2O + H_2O \ (\text{pKa of HNO} = 4.7)^{26} \quad (7)$$

$$NO^- + \text{ox} \longrightarrow NO + \text{ox}^- \quad (8)$$

$$NO^- + 2\,\text{Cys-SH} + H^+ \longrightarrow H_2NOH + \text{Cys-SS-Cys} \quad (9)$$

$$\text{Cys-SNO} \longrightarrow 1/2\,\text{Cys-S-S-Cys} + NO \quad (10)$$

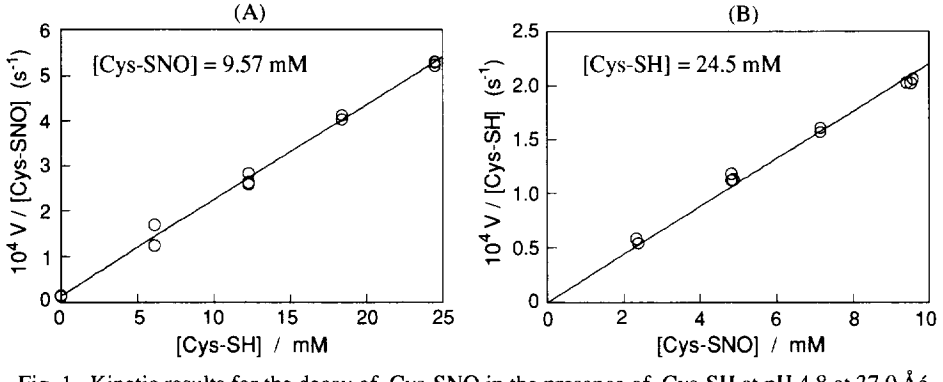


Fig. 1. Kinetic results for the decay of Cys-SNO in the presence of Cys-SH at pH 4.8 at 37.0 Åé.

The kinetic measurements of the reaction between Cys-SNO and Cys-SH have been carried out by adding the Cys-SH solution to the Cys-SNO solution in the UV cuvette at 37.0 °C and recording the absorbance of Cys-SNO at 335 nm or 543 nm. The rates were calculated from the slope of the time courses of the absorbance at the initial stage. When [Cys-SNO] was fixed to a value of 9.5 mM, the initial decay rates of

Cys-SNO increased linearly with [Cys-SH] as shown in Fig. 1A. In the absence of Cys-SH the very slow unimolecular spontaneous decomposition of Cys-SNO proceeded with the first order rate constant (1.46 ± 0.08) x 10^{-5} s^{-1}. With a fixed [Cys-SH] of 24.5 mM, the initial rates of Cys-SNO decay correlated linearly with [Cys-SNO] as shown in Fig. 1B. From these observations one obtains the rate equation 11.

$$V = k_1[\text{Cys-SNO}] + k_2[\text{Cys-SNO}][\text{CysSH}] \qquad (11)$$

The second order rate constant (k_2) was calculated from the slope of the line in Fig. 1A to obtain (2.11 ± 0.07) x 10^{-2} $M^{-1}s^{-1}$. From eqn. 11, the slope of the line in Fig. 1B, (2.20 ± 0.03) x 10^{-2} $M^{-1}s^{-1}$, is equal to {k_2 + k_1/[Cys-SH]}. Substituting k_1 and [Cys-SH] with 1.46 x 10^{-5} s^{-1} and 24.5 mM in this equation, one gets k_2 = 2.14 x 10^{-5} s^{-1}. The good agreement of the two k_2 values calculated based on the different kinetic data sources, i.e. Figures 1A and 1B, substantiates that Cys-SNO disappears through the Cys-SH-induced bimolecular decomposition pathway (reaction 3) accompanied with minor Cys-SH-independent unimolecular decomposition process (reaction 4). The k_1 value was found to be independent of the pH of the solution.

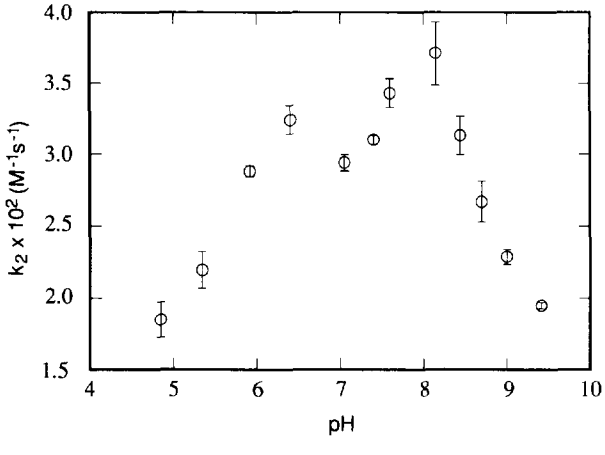


Fig. 2. The pH-rate profile for the reaction 3 at 37 °C.

Table 1. Second order rate constants for the reaction 6 at 37 °C.

pH	$k_2 \times 10^2$ ($M^{-1}s^{-1}$)
4.85	1.85 ± 0.21
5.34	2.20 ± 0.13
5.92	2.88 ± 0.04
6.40	3.24 ± 0.10
7.05	2.94 ± 0.06
7.40	3.10 ± 0.03
7.60	3.43 ± 0.10
8.15	3.71 ± 0.22
8.44	3.13 ± 0.14
8.70	2.67 ± 0.14
9.00	2.29 ± 0.05
9.41	1.95 ± 0.02

The values of k_2 were determined in the pH range of 4.85 - 9.41 with the initial concentrations of the reactants to be [Cys-SNO] = 9 mM, [Cys-SH] = 24 mM and [buffer] = 0.1 M. The kinetic measurement was repeated 3 - 5 times for each condition. The k_2 values determined in the solutions of different pH values at physiological temperature are listed in Table 1 and the pH-rate profile is illustrated in Fig. 2. It is difficult to explain such a small pH-effect, namely, that maximum value of k_2 at pH 8.15 is merely 2 folds greater than the minimum one at pH 4.85, although [H^+] between the two differs by 2 x 10^3 folds.

Discussion

Spontaneous Unimolecular Decomposition of Cys-SNO: Based on $k_1 = 1.46 \times 10^{-5}$ s^{-1} one can calculate the half life of Cys-SNO in the absence of cysteine to be ca. 9 hr. at 37 °C. This value is much greater than previous ones, i.e. the values of ca. 1.5 hr, as calculated from the Park's second order rate constant if $[Cys\text{-}SNO]_0 = 10$ mM[27] and the values in the range of 1 - 2 hr, at room temperature as reported by Arnelle and Stamler.[11] These discrepancy would be due to the effect of trace amounts of transition metal ions, which was very carefully eliminated in this work as described in Experimental Section. Thus, the k_1 value determined in this work is the most reliable value for the uncatalyzed spontaneous decomposition of Cys-SNO. Such a small k_1 value reveals that the spontaneous NO release from S-nitrosothiols cannot be responsible for the biological NO transfer from S-nitrosothiols to soluble guanylate cyclase. Instead, the transition metal ions-catalyzed NO-forming reaction of S-nitrosothiol and/or direct NO transfer from nitrosothiols to the guanylate cyclase via bimolecular reactions of the two species may operate in the biological NO transfer from the S-nitrosothiol to the guanylate cyclase. The uncatalyzed decomposition of Cys-SNO is too slow to explain the NO-transfer from a NO-carrying S-nitrosothiol to cysteine residues of the target proteins via reaction 2 followed by reaction 1. The transnitrosation (reaction 3) may be responsible for this S-nitrosothiol function.

Cys-SH-Induced bimolecular Decomposition of Cys-SNO: S-nitrosothiols are thought to play the same roles as NO to control vasodilation and antiplatelet, and modulation of the reactivities of hemoglobin,[13] prostaglandin-H syntase,[14] creatine kinase,[15] glyceraldehyde-3-phosphate dehydrogenase[16] *etc.* by the transnitrosation reaction 2 to the respective thiol residues of these proteins. If these S-nitrosothiol-dependent reactions contribute in biological regulations, the S-nitrosothiol concentrations have to be greater than the threshold in the compartment sending the signal. Usual mammalian cells contain about 5 mM of the reduced form of glutatione.[22] Assuming that reactivities of intraceller S-nitrosothiols and thiols are the same as those of Cys-SNO and Cys-SH, one obtains the pseudo-first order rate constant for the decay of a intracellar S-nitrosothiol to be 1.55×10^{-4} s^{-1} at pH 7.4, at 37 °C from the k_2 value determined in this work. Under this condition, the half-life of the S-nitrosothiol is calculated to be 75 min. If the rate of S-nitrosothiol formation is greater than 1.55×10^{-4} s^{-1}, the intracellar concentration of S-nitrosothiol will increase.

On the other hand, the apparent second order rate constants of transnitrosation reaction 3 are in the order of 10 $M^{-1}s^{-1}$ at pH 7.4.[12] Therefore, in order that the S-nitrosation of the target protein with intracellar S-nitrosothiol (reaction 3) proceeds faster than the intracellar thiol dependent decomposition of the S-nitrosothiol (reaction 4), the concentration of the target protein should be greater than 10^{-5} M.

NO^- undergoes single electron oxidation with biological oxidant (even molecular oxygen) yielding NO. Fukuto et al. believe that NO^- is oxidized to NO (reaction 8) before binding to the guanylate cyclase. The

guanylate cyclase requires ferrous nitrosylheme, [PFe^{II}(NO)],[28] as a cofactor but not the ferric state. Hemin, [PFe^{III}], may accept NO^- to generate [PFe^{II}(NO)]. Then the reactions 1, 2, 4 and 8 would contribute partly and explain why Cys-SH prolongs and potentiates the vasodilation effect of NO and Cys-SNO.

In our experiments the presence of Cys-SH did not prolong the lifetime of Cys-SNO but merely decreased it in a concentration-dependent manner in contrary to the report by Feelish et al.[3]

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