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# Equilibrium and Kinetics Studies of Transnitrosation Between *S*-Nitrosothiols and Thiols

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**Abstract**—Using UV–vis spectrometrical measurements, equilibrium constants for NO transfer between *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and different thiols as well as kinetic data for NO transfer from *S*-nitroso bovine serum albumin (BSANO) to thiols have been obtained. NO transfer from SNAP to other primary/secondary thiols are thermodynamically favorable, whereas other *S*-nitrosothiols exhibit similar NO transfer potential. The obtained Gibbs free energy, enthalpy and entropy data indicated that NO transfer reactions from SNAP to four thiols are exothermic with entropy loss. The kinetic behavior of BSANO/RSH transfer can be related to both the acidity of sulfhydryl group and the electronic structure in thiol. 

In recent years, tremendous attention has been focused on the chemistry and biology of nitric oxide (NO) due to the discovery that NO plays a key role in a wide variety of human physiological processes.[1] It has been suggested that *S*-nitrosothiols (RSNOs) may be directly involved in many of the biological functions of nitric oxide.[2–4] RSNOs have been detected in human airway lining fluid plasma, platelets and neutrophils,[5] therefore they can act as an NO carrier in the form of free thiol or cysteine containing protein in biological systems.[6] These endogenous *S*-nitrosothiols may play an important role in NO storage, transport, and delivery. Some RSNO compounds, such as *S*-nitroso-*N*-acetyl-penicillamine (SNAP)[7] and *S*-nitrosocaptopril,[8] can indeed be used as therapeutic drugs for treatment of angina and other circulation problems, their functions also rely on their ability to generate NO in vivo.[9]

Fully understanding the biological roles of NO requires fundamental knowledge of the kinetics and thermodynamics of the NO-related reactions. NO transfer from one NO donor, especially *S*-nitrosothiol, to another thiol has been a focus of interest due to their role in NO transfer in vivo. Mechanistic and kinetics studies on transnitrosation from RSNOs to thiols have been reported previously by Williams,[10] Meyer,[11] Rossi,[12] and Means,[13] using UV–vis spectroscopic methods. Recently using HPLC method, Hogg[14] reported equilibrium and kinetics study on transnitrosation. As an effort to offer a thermodynamic view of NO transfer both in vitro and in vivo, we reported herein our investigation on the equilibrium of the transnitrosation between SNAP and thiols as well as kinetic study on the transnitrosation between *S*-nitroso bovine serum albumin (BSANO) and thiols.

## Equilibrium Study of NO Exchange Between SNAP and Thiols

In the present study, SNAP was selected as the NO donor since it has a maximum absorption at 590 nm while $\lambda_{max}$ for primary *S*-nitrosothiols is around 540 nm, transnitrosation between SNAP and primary thiols can be readily analyzed using UV–vis spectrometric measurement (Fig. 1). UV–vis spectra of reaction mixtures were obtained by adding an equimolar amount of thiol to a cuvette containing RSNO. After the equilibrium was reached (Fig. 2), the concentrations of the reactants and products can be calculated from the change of the absorption of SNAP at 590 nm according to the Beer–Bouger–Lambert law. Equilibrium constant ($K_{eq}$) of NO transfer between SNAP and different thiols can be determined from the calculated concentrations as

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shown in eq (1). Equilibrium constants for the reverse reactions ($K_{eq}^{-1}$) reflect the NO donating potential of the corresponding RSNO. The results were summarized in Table 1.

$$\mathrm{SNAP} + \mathrm{RSH} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{NAP} + \mathrm{RSNO}$$

$$k_{eq} = k_2/k_{-2} = \frac{[\mathrm{NAP}][\mathrm{RSNO}]}{[\mathrm{SNAP}][\mathrm{RSH}]} \quad (1)$$

As can be seen from Table 1, overall, there exists a tendency of NO transfer from SNAP to all other thiols which is either primary or secondary ($K_{eq}>1$). This can largely be attributed to the steric repulsion between two methyl groups and −SNO moiety in tertiary SNAP, as suggested by our ab initio density functional theory calculation.[15]

Among the eight reactions, $K_{eq}^{-1}$ for the reaction between SNAP and *N*-acetyl cysteine is the largest, indicating that *S*-nitroso-*N*-acetyl cysteine has the highest NO donating ability. The reaction with 2-mercaptoethanol has the smallest $K_{eq}^{-1}$ of 0.12, suggesting that *S*-nitroso-2-mercaptoethanol is less capable of transferring NO to another thiol. All other *S*-nitrosothiol compounds have similar NO transferability with $K_{eq}^{-1}$s ranging from 0.2 to 0.3. It is worth noting that *N*-acetylation of cysteine resulted in an increase of NO donating ability ($K_{eq}^{-1}$ from 0.28 to 0.68).

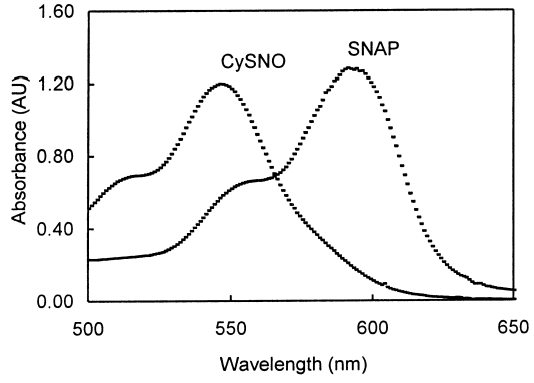


**Figure 1.** UV absorption of SNAP and CysNO.

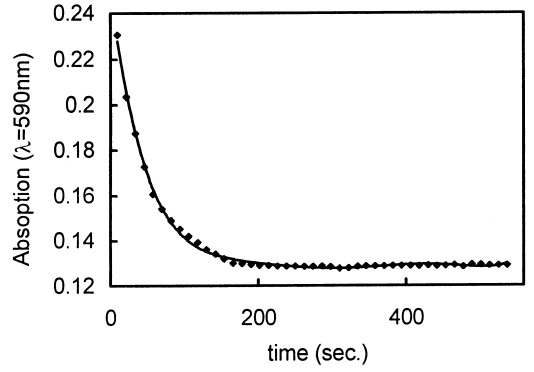


**Figure 2.** Kinetics of NO transfer between SNAP and L-cysteine.

Transnitrosation between SNAP and four thiols was also carried out at different temperatures. Gibb free energy ($\Delta G^\circ$), enthalpy ($\Delta H^\circ$), and entropy ($\Delta S^\circ$) were thus calculated. The results are summarized in Table 2.

Table 2 showed that $\Delta H^\circ$s for these reactions are negative, again indicating that the NO transfer from SNAP to these thiols is thermodynamically favorable. While $\Delta H$s of reactions **1**, **2** and **3** are similar, ranging from −14.6 to −21.6 kJ/mol, the $\Delta H^\circ$ for reaction **4** is fairly high, accompanied by significant entropy loss (−208 J $mol^{-1}$ $K^{-1}$) for NO transfer from SNAP to cysteineamine. This may be related to the difference between *S*-nitrosocysteineamine and other RSNO compounds in terms of structure and thermal stability. The *S*-nitrosocysteineamine exists largely as the protonated form ($H_3N^+CH_2CH_2SNO$) at pH 7.4, and there exists significant intramolecular electrostatic interaction between the ammonium group and nitroso oxygen atom, which leads to an energetically preferred cyclic form and significant entropy loss.[15] On the other hand, intramolecular H-bonding between sulfhydryl and $-COO^-$ in other thiols may lower both enthalpy and entropy, and this may eventually lead to smaller $\Delta H^\circ$ and $\Delta S^\circ$ after *S*-nitrosation.

**Table 1.** Equilibrium constants of SNAP–thiol exchange reactions at pH 7.4

| Thiol | $K_{eq}$ | $K_{eq}^{-1}$ |
|---|---|---|
| 2-Mercaptoethanol | 8.44 | 0.12 |
| 3-Mercaptoproprionic acid | 5.04 | 0.20 |
| 2-Propethiol | 5.04 | 0.20 |
| L-Cysteine ethyl ester | 4.99 | 0.20 |
| Cysteineamine | 3.89 | 0.26 |
| L-Cysteine | 3.63 | 0.28 |
| Glutathione | 3.34[a] | 0.30 |
| *N*-Acetyl-cysteine | 1.47 | 0.68 |

[a] $K = 3.75$–$5.32$ reported by Hogg.[14]

**Table 2.** Thermodynamic parameters for reaction between SNAP and thiols

| Thiol | Temp (C) | $K_{eq}$ | $\Delta G^{\circ a}$ (kJ $mol^{-1}$) | $\Delta H^{\circ b}$ (kJ $mol^{-1}$) | $\Delta S^\circ$ (J $mol^{-1}K^{-1}$) |
|---|---|---|---|---|---|
| Glutathione | 25 | 3.69 | −3.23 | | |
| | 30 | 3.27 | −2.99 | −21.6 | −61 |
| | 33 | 3.00 | −2.80 | | |
| | 37 | 2.63 | −2.49 | | |
| L-Cysteine | 20 | 4.18 | −3.38 | | |
| | 25 | 3.66 | −3.21 | −20.9 | −59 |
| | 30 | 3.35 | −3.05 | | |
| | 33 | 2.58 | −2.44 | | |
| L-Cysteine ethyl ester | 25 | 4.56 | −3.76 | | |
| | 30 | 4.15 | −3.59 | −14.6 | −36 |
| | 33 | 3.92 | −3.48 | | |
| | 37 | 3.60 | −3.32 | | |
| Cysteineamine | 25 | 3.89 | −3.37 | | |
| | 30 | 3.22 | −2.94 | −66.4 | −208 |
| | 33 | 2.56 | −2.39 | | |
| | 37 | 1.33 | −0.73 | | |

[a] Obtained from $\Delta G^\circ = -RT\ln K_{eq}$ (3).
[b] $\ln K_{eq} = -\Delta H^\circ/(RT) + \Delta S^\circ/R$ (4). The $\Delta H^\circ$ and $\Delta S^\circ$ values can be obtained from a plot of $\ln K_{eq}$ versus $1/T$.

## Kinetics Study of NO Transfer Between BSANO and Thiols

It should be noted that other reactions, mainly *S*-thiolation of RSNO compounds in the presence of thiols which gives disulfides, would compete with the investigated transnitrosation process.[16] Nevertheless it has been reported that *S*-thiolation rates are generally several orders of magnitude smaller than *S*-nitrosation and therefore the equilibrium and kinetics study reported here would not be interfered.[17] To probe the kinetics of NO transfer between *S*-nitrosylated proteins and thiols, we also examined transnitrosation between BSANO and several thiol compounds (Table 3).

It has been established that the transnitrosation occurs as nitroso-nitrogen atom is attacked by the thiolate nucleophile.[10] Therefore, the reaction rate is dependent on the reactivity of the thiol, which is directly correlated with p$K_a$ of the sulfhydryl group and stereoelectronic factors in thiol. From Table 3 one can find that transnitrosation rate constant increases with increased acidity of -SH, and a linear relationship exists between p$K_a$ and lg$k_2$ as shown in Figure 3. The slope of the correlation is less than unity, indicating that other factors may also affect the reactivity of thiolate. Noted is that the thiols exist as the ionized form at pH 7.4: cysteine ethyl ester (protonated); cysteine and penicillamine (zwitterionic); entries 4–6 (deprotonated). This exhibits roughly an increase in negative charge from entry 1 through 6. Thus it appears that negative charge neighboring thiolate favors nucleophilic attack, and therefore this partly compensates for the loss in acidity of sulfhydryl. Since the p$K_a$'s of protein thiols are greatly affected by their environment, it is conceivable that NO transfer in vivo may be particularly efficient from *S*-nitrosothiols such as GSNO or BSANO to certain proteins.

**Table 3.** Kinetic data for reaction between BSANO and thiols

| Entry | RSH | p$K_a$[a] | $k_2$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| 1 | L-Cysteine ethyl ester | 6.50 | $6.45\times10^{-1}$ |
| 2 | L-Cysteine | 8.30 | $6.12\times10^{-2}$ |
| 3 | D-Penicillamine | 8.53 | $3.01\times10^{-2}$ |
| 4 | Glutathione | 8.75 | $1.68\times10^{-2}$ |
| 5 | *N*-Acetyl cysteine | 9.52 | $8.41\times10^{-3}$ |
| 6 | *N*-Acetyl-D-penicillamine | 9.90 | $9.90\times10^{-3}$ |

[a]Ref 18.

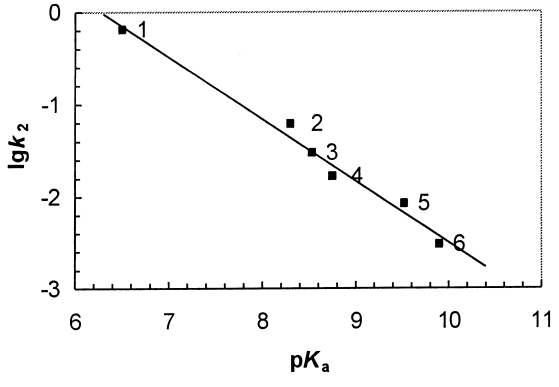


**Figure 3.** Correlation of lg$k_2$ and p$K_a$(RSH).

In summary, an equilibrium study of NO transfer between SNAP and different thiols shows that this transfer is thermodynamically favorable, and the difference between thiols was generally insignificant. This is further supported by the observed enthalpy change for NO transfer reaction. Entropy loss was found for NO transfer from SNAP to four biologically relevant thiols. These results can be rationalized in terms of stereoelectronic factors. Kinetics of the transnitrosation between BSANO and low-molecular-weight thiols can be directly related to the p$K_a$ of the parent thiols. It is suggested that electrostatic interaction may also contribute to the observed relative NO transfer kinetics behavior.

## Experimental

### General

All the reagents were purchased from commercial suppliers and were used as received. Thin-layer chromatography was conducted on Baker Si250f silica gel TLC plates with a fluorescent indicator. Bovine serum albumin was purchased from Sigma Co. Dialysis was performed against deionized water using dialysis tubing (8000 MW cutoff). $^1$H and $^{13}$C NMR spectra were recorded on a Varian Unity 500 MHz NMR instrument. UV–visible measurements were carried out on a HP 8453 UV–visible spectrometer (Hewlett Packard Co.).

***S*-Nitrosopenicillamine (SNAP) preparation.**[19] To a solution of *N*-acetyl-DL-penicillamine (10 mmol) in MeOH (20 mL), 1 N HCl (20 mL), and concentrated $H_2SO_4$ (2 mL) was added $NaNO_2$ (20 mmol) in water (20 mL) in a period of 20 min with vigorous stirring at 0 °C. Stirring was continued for another 15 min, the liquid was separated and the solid was washed with cold water and dried in vacuum. The product SNAP was obtained as deep green crystals.

**BSANO Preparation.**[14] BSA was dissolved in PBS buffer to 150 mg/mL followed by addition of 10 mM dithiothreithiol and 10 mM EDTA. After 1 h at 20 °C, dithiothreitol and EDTA were removed by dialysis against PBS buffer. The sulfhydryl-to-protein ratio of BSA is 0.6. *S*-Nitrosylation of the BSA was then achieved by incubation with a 5-fold molar excess of isobutyl nitrite at 37 °C until no further increase in $A_{335}$ was observed. BSANO concentration was determined by the UV spectrometer ($\varepsilon_{335} = 3869$ $M^{-1}\cdot cm^{-1}$).[20]

**Equilibrium study of transnitrosation between SNAP and thiols.** The equilibrium constants ($K_{eq}$) for transnitrosation were determined by reacting equimolar amounts of thiols and SNAP. Briefly, *S*-nitrosothiol (29 mM) in phosphate buffer (pH 7.4, 1 mM EDTA) was placed in a quartz cell at different temperatures. $A_{330-370}$ or $A_{500-600}$ nm was monitored for 5 min to obtain the slow decomposition rate. An equimolar amount of thiol was added and the exchange reaction was monitored until equilibrium was reached. $K_{eq}$ was calculated based on

the change of the UV absorption. Experiments were repeated at least once.

**Kinetics study of transnitrosation between BSANO and thiols.** The NO transfer reactions were carried out at 25 °C. BSANO (0.20 mM) in PBS buffer (pH 7.4, 1 mM EDTA) was placed in a quartz cell. Thiol (3.0 mM) was added and the NO exchange reaction was monitored at $A_{335}$ for 10 min. The second order rates $k_2$ can be derived from the pseudo-first order rates $k_{off}$ as $k_{off}/[RSH]_0$.

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