Kinetics of Nitrosation of Thiols by Nitric Oxide in the Presence of Oxygen*

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Nitrosothiols are powerful vasodilators. They act by releasing nitric oxide, which activates the heme protein guanylate cyclase. We have studied the kinetics of nitrosation of glutathione, cysteine, N-acetylcysteine, human serum albumin, and bovine serum albumin upon reaction with nitric oxide (NO) in the presence of oxygen. These studies have been made at low pH as well as at physiological pH. At pH 7.0, contrary to published reports, nitric oxide by itself does not react with thiols to yield nitrosothiol. However, formation of nitrosothiols is observed in the presence of oxygen. For all thiols studied, the rates of nitrosothiol formation were first order in O2 concentration and second order in NO concentration and at lower concentrations (<5 mM thiol) also depended on thiol concentrations. Analysis of the kinetic data indicated that the rate-limiting step was the reaction of NO with oxygen. Analysis of the reaction products suggests that the main nitrosating species is N2O3:RSH + N2O3 → RSNO + NO2 + H+. Rate constants for this reaction for glutathione and several other low molecular weight thiols are in the range of 3-1.5 × 109 M-1 s-1, and for human and bovine serum albumins 0.3 × 109 M-1 s-1 and 0.06 × 109 M-1 s-1, respectively. The data further indicate that the reaction rate of the nitrosating species N2O3 with thiols is competitive with its rate of hydrolysis. At physiological concentrations nitrosoglutathione formation represents a significant metabolic fate of N2O3, and at glutathione concentrations of 5 mM or higher almost all of N2O3 formed is consumed in nitrosation of glutathione. Implications of these results for in vivo nitrosation of thiols are discussed.

The abbreviations used are: HSA, human serum albumin; BSA, bovine serum albumin; RSH, reduced thiol in general; NEM, N-ethylmaleimide; HPLC, high performance liquid chromatography; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)-propane-1,3-diol.

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NO + GC(heme) ⇌ GC(heme-NO)

REACTION 2

GC(heme-NO) ⇌ GC(heme-NO)*

REACTION 3

Combination reactions of NO with five coordinate ferro heme proteins are fast, and the NO dissociation reaction is slow (Moore and Gibson, 1976). GC(heme-NO)* represents the NO-activated form of the enzyme.

Stamler et al. (1992) have reported that naturally produced nitric oxide circulates in plasma primarily complexed as S-nitrosothiol species, principal among which is S-nitroso-serum albumin. Although nitrosothiols (both low M, and protein) have endothelium-derived relaxation factor or NO-like properties, the mechanism by which circulating or cellular RSNO is synthesized has not been described. At pH 7.0, contrary to the published reports, nitric oxide, by itself, does not react with thiols to yield nitrosothiol. However, formation of nitrosothiol observed in the presence of oxygen suggested that nitrosation proceeds via the formation of oxides of nitrogen (N2O4, N2O3, etc.).

In view of the fact that in aqueous solutions the rates of hydrolysis of these oxides are high, it is not known how much nitrosation of low M, thiols such as glutathione and protein thiols such as HSA1 can be expected to proceed via this mechanism. The potential reactivity of NO with the sulfhydryl group is also of interest when one considers that during its diffusion from endothelial cells to the target molecule guanylate cyclase in neighboring muscle cells, NO will be exposed in the cytoplasm to mM concentrations of the tripeptide glutathione (Meister and Anderson, 1983). One wonders how NO is able to avoid reaction with such a large excess of an intracellular thiol. To answer some of these questions would require knowing the mechanism of nitrosation of thiols in aqueous solutions in the presence of oxygen and the relevant rate constants. In this article we present the results of our kinetic studies on the reaction of nitric oxide with several thiols in the presence of oxygen. In agreement with earlier studies it was observed that NO reacts with thiols via the rate-limiting step of nitric oxide's reaction with oxygen and formation of the nitrosating species N2O3 (Wink et al., 1994). The reaction of N2O3 with thiols is competitive with its reaction with water. The novel finding of the present study is that physiologically attainable concentrations (5 mM or higher) of glutathione compete successfully with water for reaction with N2O3. Nitrosation of HSA, on the other hand, is less significant by this reaction path.
obtained with eluent A (10 mM sodium phosphate, pH 6.0) and eluent B (0.1 M sodium phosphate) over the pH range 5–8 and with NO from fresh KOH pellets or by bubbling through aqueous NaOH. Incubations were conducted in a deaerated spectrophotometer with the Kontron spectrophotometer using 1-cm-pathlength cuvettes. Spectral analysis and kinetic experiments were made in solutions both with and without 1 mM EDTA. Analytical grade sodium phosphate from Fisher was used in the present study. Like most other brands of sodium phosphate, it contained iron as a contaminant. In 0.1 M phosphate buffers used in the present study, iron contamination ranged between 0.6 and 1 μM. Therefore, all buffers were made in 1 mM in EDTA. In the time domain of the kinetic experiments made in this study, only in the case of cysteine inclusion of EDTA affected the kinetics of the reaction.

Nitric oxide (99.0% pure) was purchased from Matheson. Two methods were used to purify NO: passing it through a column filled with fresh KOH pellets or by bubbling through a solution of 0.5 M NaOH. Such procedures were used to purify NO: passing it through a column filled with fresh KOH pellets or by bubbling through a solution of 0.5 M NaOH. However, nitrosothiol formation was not observed when a solution of unpurified NO was bubbled through GSH solution for 6 min. A small absorbance change (Δ absorbance) around 350 nm, which is different and is discussed separately. Nitrosating species was seen when solutions of unpurified NO were prepared in deaerated phosphate solutions prior to reaction with thiols. Nitrosating species was seen when solutions of unpurified NO were prepared in deaerated phosphate solutions prior to reaction with thiols. These results indicate that an oxidation product of NO present as a contaminant in unpurified NO is the nitrosating species and that it decomposes when aqueous solutions of NO are prepared prior to reaction with thiols.

Buffer Effect—With some buffers, longer living, buffer-derived nitrosating species may form. Bis-Tris (0.1 M, pH 7.0) buffered solutions of unpurified NO yielded measurable GSNO when freshly prepared and added to GSH. The nitrosating activity of this solution decreased to background level over a period of 6 h. This reactivity correlated with the appearance of multiple absorbance bands at 330–370 nm, upon saturating deaerated bis-Tris with unpurified NO, and then with a time-dependent decrease in intensity of these bands. When purified NO was used, neither the nitrosating activity nor the bands at 330–370 nm were observed. Apparently, in the reaction of
unpurified NO with bis-Tris a nitrosated product forms, which is capable of nitrosating thiols and which is relatively stable. Due to these complications, bis-Tris buffer was not used further. All subsequent experiments were conducted in phosphate buffer.

**Kinetic Studies**—The reaction order with respect to each of the starting reactants, NO, O₂, and GSH, was determined in single mixing stopped-flow experiments by varying the initial concentration of a single reactant while keeping the concentration of the others constant. In these experiments a solution containing GSH and O₂ was mixed with a solution of NO. The slope of ln(ΔGSNO)/dt_init vs ln[reactant] plots yielded the reaction order with respect to the reactant being varied.

Three sets of experiments were performed to determine the order of the reaction with respect to each of the reactants. 1) A solution of constant GSH (10.8 mM) and O₂ concentrations (0.72 mM) was mixed with a solution of NO. The slope of ln(ΔGSNO)/dt_init versus ln(NO) (Fig. 2) plot was 2, which demonstrates a second order concentration dependence of reaction rates on NO concentration. 2) Solutions with constant GSH concentrations (10.4 mM) and varying concentrations of O₂ (0.24–1.2 mM) were mixed with a solution of constant nitric oxide concentration (0.2 mM). These data yielded first order dependence of reaction rates on O₂ concentration (Fig. 3). 3) Solutions of varying GSH concentrations (0.6–60 mM) and constant O₂ concentration (0.72 mM) were mixed with a solution of constant NO concentration (0.2 mM). In Fig. 4, ln R_init is plotted as a function of ln[GSH]. It is clear that at lower GSH concentrations initial rates depend on GSH concentration. At higher GSH concentrations (>5 mM) initial rates become independent of GSH concentration.

Similar concentration dependence with respect to NO, O₂, and thiol was observed for N-acetylcysteine.

On the basis of these kinetic observations and earlier studies by Wink et al. (1993, 1994), we can tentatively write the following reaction mechanism:

\[
\begin{align*}
2\text{NO} + \text{O}_2 & \rightarrow 2\text{NO}_2 \\
\text{REACTION 4} \\
\text{NO}_2 + \text{NO} & \rightarrow \text{N}_2\text{O}_3 \\
\text{REACTION 5} \\
\text{N}_2\text{O}_3 + \text{H}_2\text{O} & \rightarrow 2\text{H}^+ + 2\text{NO}_2 \\
\text{REACTION 6} \\
\text{N}_2\text{O}_3 + \text{RS} & \rightarrow \text{H}^+ + \text{NO}_2 + \text{RSNO} \\
\text{REACTION 7}
\end{align*}
\]

Justification for the individual steps is provided under "Discussion."

**Kinetics of Nitrosothiol Formation by Cysteine**

The reaction time course for the reaction of NO with cysteine in the presence of O₂ was complex and very different than for GSH described above. Initial rates, d(Cys-NO)/dt, were dependent only on NO concentration in a first order manner, although the presence of O₂ was essential for the reaction to take place. Later in the reaction, reaction rates depended both on NO and O₂ concentrations, approaching second and first order dependence on [NO] and [O₂], respectively. This behavior was observed in 0.1 M phosphate buffer of pH 7.0 and not in Tris or dilute phosphate buffers. Atomic absorption spectra analysis of the buffer solution indicated micromolar amounts of iron impurity; a solution of cysteine in water did not show this impurity. These observations suggest the involvement of an iron-mediated redox system responsible for the initial first order (in [NO]) reaction:

\[
\text{NO} \rightarrow \text{NO}^+ \text{ (slow)}
\]

\[
\text{REACTION 8}
\]
NO$^+$ + RS$^-$ → RSNO (fast)

**Reaction 9**

The approximate value of $k_9$ was 1.5 s$^{-1}$. However, it has not been possible to obtain quantitatively consistent results over a large enough concentration range of the reactants to give some confidence to the rate law and a reaction mechanism that may be postulated. Observations such as the effect of buffers on the reaction rates are difficult to explain even qualitatively. McAninley et al. (1993) made similar observations in their study of nitrosothiol decomposition and attributed these effects to the presence of metal ion contaminants. The effect disappeared on inclusion of EDTA in the reaction mixture. The reaction of cysteine with NO in the presence of O$_2$ therefore was studied in detail only in the presence of 50 mM EDTA in 0.1M phosphate buffer. Under these conditions the reaction follows the same kinetics as those observed for the formation of GSNO and N-acetylcyesteine-NO.

**Nitrosothiol Formation by HSA and BSA**

For both of these proteins nitrosothiol formation could be observed only in the presence of oxygen, indicating that the kinetics of nitrosothiol formation is qualitatively similar to that observed for the low M$_1$ thiols. In the"Discussion" we point out that nitrosothiol formation is competitive with the hydrolysis of the nitrosating reagent N$_2$O$_3$ (see Reactions 6 and 7). Therefore, high concentrations of HSA and BSA (>8 mM) were needed to observe measurable absorbance changes due to nitrosothiol formation. Such solutions are very viscous and pose mixing difficulties in stopped-flow experiments. For these reasons kinetic studies with HSA and BSA were not made. However, estimates of rate constants for the reaction of these proteins with N$_2$O$_3$ could be made from static spectrophotometric measurements. The data (plots of [NO] added versus [RSNO] formed) for BSA and HSA along with those for other thiols are shown in Fig. 5 and are described in the next section.

**Relationship between NO Added and RSNO Formed in the Reaction**

In Fig. 5 is shown a plot of [NO] added to an air-equilibrated solution of thiols versus [RSNO] formed when a solution of NO was rapidly mixed with the thiol solution on a stopped-flow spectrophotometer. In the case of protein thiols, the reaction was slower, and therefore similar experiments were made on a Kontron spectrophotometer. Concentration of thiols was much greater than NO concentration. Concentration of nitroso derivatives formed in the reaction was calculated from their published extinction coefficients (Park et al., 1993; Stamler et al., 1992; Byler et al., 1983). A linear relationship between [NO] added and [RSNO] formed was observed in mixing experiments. Wink et al. (1994) have already shown that in the presence of oxygen nitrosation of thiols proceeds more rapidly than of amines and tyrosine. We therefore can write the following:

\[
m[\text{NO}] \text{ added} = [\text{RSNO}] \text{ formed} \quad \text{(Eq. 1)}
\]

\[
\frac{[\text{RSNO}] \text{ formed}}{[\text{NO}] \text{ added}} = m \quad \text{(Eq. 2)}
\]

The coefficient $m$ represents the fraction of total NO used in nitrosation of thiols. The following can be shown:

\[
m = k_7 \times [\text{RS}][k_4[H_2O] + k_5[\text{RS}]] \quad \text{(Eq. 3)}
\]

**Product Identification Studies**

These studies were made for two reasons: 1) to ascertain the formation NO$_2^-$ in Reaction 7 and 2) to rule out any significant formation of GSSG in the reaction of NO with GSH in the presence of oxygen. We have already provided a kinetic argument against this possibility. Fig. 6 shows HPLC chromatograms of the following: A, a solution of GSNO (5 mM) + O$_2$ (0.4 mM) mixed with NO (0.5 mM); B, GSNO (5 mM) in air-equilibrated buffer; C, buffer blank + 1 mM NEM; and D, GSNO + GSSG + NaNO$_3$ + NaNO$_2$. Addition of the sulfhydryl-specific reagent NEM prior to HPLC analysis to yield the GS adduct of NEM had two effects: it blocked excess GSH and enabled measurement of GSSG, which otherwise elutes with GSH in this HPLC protocol (J. occl, 1972). The solutions were applied to the column within 5 min of mixing. In A, we observe the formation of GSSG (peak 3) and NO$_3^-$ (peak 5). The peak corresponding to GSSG has approximately the same area under it as in control GSNO solution (B, peak 5).

**Data Treatment**

Rate constants for Reactions 4–6 ($k_4 = 6.3 \times 10^6 \text{M}^{-2} \text{s}^{-1}$, Kharitonov et al. (1994); $k_5 = 1.1 \times 10^9 \text{M}^{-1} \text{s}^{-1}$, Grätzel et al. (1970); $k_6[H_2O] = 1.6 \times 10^9 \text{M}^{-1} \text{s}^{-1}$, Licht et al. (1988)) indicate that in aqueous buffers NO$_2^-$ and N$_2$O$_3$ should exist in very small amounts as reactive intermediates. Therefore, we can apply the steady-state approximation to their concentrations and obtain the following rate equation for the formation of GSNO:

\[
\frac{d[\text{GSNO}]}{dt} = k_4 \times \frac{k_5[\text{GSH}]}{2 \times [k_6[H_2O] + k_5[\text{GSH}]]} \times [\text{NO}]^2 \times [\text{O}_2], \quad \text{(Eq. 4)}
\]
At GSH concentrations 5 mM or higher, the rates of GSNO formation are independent of GSH concentration, indicating that \( k_7 \) (GSH) \( \geq k_6 \) (H\(_2\)O). Under these conditions the rate Equation 4 simplifies to the following:

\[
\frac{d[GSNO]}{dt} = k_4 \times [NO]^2 \times [O_2],
\]

(Eq. 5)

Using Equation 4 and the stoichiometric relationship \( \frac{3}{2} \Delta[NO] = \Delta[O_2] \), \( k_4 \) can be calculated from a reaction time course using the algorithm described earlier (Kharitonov et al., 1994). Over the entire range of concentrations of NO, O\(_2\), and thiols used in the present study, least squares analysis of reaction time courses (each consisting of more than 200 data points) yielded \( k_4 = 7.0 \pm 1 \times 10^6 \) (S.D.) M\(^{-2}\) s\(^{-1}\). This is a global average of \( k_4 \) for the three thiols used in this study. Fig. 7 shows typical observed and calculated reaction time courses for glutathione.

This value of \( k_4 \) should be compared with the value of \( k_4 = 6.3 \times 10^6 \) M\(^{-2}\) s\(^{-1}\) reported by us earlier for the auto-oxidation of NO (Kharitonov et al., 1994).

Calculation of \( k_7 \)

From the slope (m) of [NO\(_\text{added}\)] versus [RSNO\(_\text{formed}\)] plots and the literature value of \( k_6 \), \( k_7 \) can be calculated using the following equation:

\[
m = k_7 \times [RSH]/2 \times (k_2 \times [RSH] + k_6 \times [H_2O])
\]

(Eq. 6)

Nitrosothiol Formation at Low pH

These studies were undertaken in order to determine rate constants for nitrosation of protein thiols (BSA and HSA) via their reaction with nitrosative acid. Knowledge of these rate constants will allow us to estimate the extent to which this reaction pathway may lead to nitrosation of BSA and HSA under physiological conditions. Nitrosothiol formation by glutathione, N-acetylcysteine, and cysteine at low pH has been studied earlier (Williams, 1988). It was shown that the overall reaction is third order: d[RSNO]/dt = \( k_7 \) [RSH][HNO\(_2\)][H\(^+\)], where \( k_7 \) is the overall third order rate constant. However, there are no data for BSA and HSA. Kinetic studies of low pH nitrosothiol formation by HSA and BSA were made in phosphate buffer of pH 2.0, using sodium nitrite. At a constant pH, the reaction rates were first order with respect to both the thiol (or the thiol protein) and sodium nitrite. In the presence of excess nitrite, reactions become pseudo first order in thiol concentration. The reaction time courses under these conditions were used to calculate the pseudo first order constant \( k_{7\alpha} \) defined by the equation: d[RSNO]/dt = \( k_{7\alpha} \) [RSH]. This was achieved by making a least square fit of the data to a single exponential equation. The constant thus obtained, if divided by nitrite and proton concentrations, yields \( k_{7\alpha} \), the overall third order rate constant listed in Table I. In the same table, the data for glutathione obtained in this study is compared with the literature values.

DISCUSSION

Reactions 4–7 explain all qualitative and quantitative observations described under “Results.” First and second order dependence of reaction rates with respect to NO and O\(_2\) is explained by Reaction 4 and substantiated by data in Figs. 2 and 3. Dependence of reaction rates \( (d[GSNO]/dt) \) on thiol concentration arises from competition between Reactions 6 and 7. This should be obvious from the rate Equation 4 and is made further clear by Fig. 8, in which initial reaction rates corrected for hydrolysis of N\(_2\)O\(_3\) by Reaction 6 are plotted against thiol concentration (i.e. initial reaction rates are divided by the factor \( m \)). Rate Equation 4 predicts that 1) at high thiol concentrations the reaction rates should be independent of thiol concentration and 2) at low thiol concentrations the reaction rates should approach first order dependence on thiol concentration. Both of these predictions are consistent with the data in Fig. 4. These observations indicate that the rate-limiting step in Reactions 4–7 is Reaction 4.

In Reactions 4–7 there are two potential nitrosating agents: N\(_2\)O\(_4\), a dimer of NO\(_2\), and N\(_2\)O\(_3\). For electrophilic nitrosation, these two reagents should act as sources of NO\(^+\). In the case of N\(_2\)O\(_4\), this would lead to the formation of NO\(_3\). Nitrosation via N\(_2\)O\(_3\) would form NO\(_2\). The HPLC data support the latter possibility.

In Table II are listed the calculated values of \( k_7 \). These depend on the value of \( k_6 \) used for calculating \( k_7 \) from Equation 6 and data in Fig. 5. Two values of \( k_6 \) differing by a factor of three have been reported \( (k_6[H_2O] = 5.3 \times 10^6 \) s\(^{-1}\), Grätz et al. (1970); and 1.6 \( \times \) \( 10^9 \) s\(^{-1}\), Licht et al. (1988)). We have used the more recent value reported by Licht et al. (1988). These calculations indicate that the second order rate constant \( k_7 \) is
lead to the conclusion that all of NO formed in vivo will be consumed to form RNO. Nitric oxide can react with various molecules found in biological systems. Those most often considered in this regard are oxygen, thiols, amines, tyrosine, free radicals, and ferric and ferro heme proteins. Kinetic studies of Wink et al. (1993, 1994) indicate that the third order reaction of nitric oxide with oxygen is slow and acts as the rate-limiting step in nitrosation reactions via the formation of N₂O₃. Reactions of NO with O₂ and ferro and ferric heme proteins, on the other hand, are very fast (Padmaja and Huie, 1993; Beckman and Crow, 1993; Cassoly and Gibson, 1975; Sharma et al., 1983, 1987). Therefore, in vivo only a small fraction of naturally produced NO will be able to react with oxygen to produce N₂O₃.

The novel finding of the present study is that almost all of N₂O₃ thus formed is consumed in GSNO formation in cells in which GSH concentrations are 5 mM or higher. At lower concentration of GSH, nitrosation of GSH by N₂O₃ will become progressively less significant.

In the case of the two protein thiols HSA and BSA, the situation is quite different. The rate constant for nitrosation is about 1/10 of that for the low molecular weight thiols, and their plasma concentration rarely exceeds 1 mM. Therefore, it seems that nitrosation of these two proteins via reaction with N₂O₃ may not be as significant. For protein thiols, alternative reaction mechanisms of nitrosation may be of more physiological significance than the reaction with N₂O₃. Keaney et al. (1993) have suggested the possibility of HSA nitrosation by reaction with the nitrosium ion, NO⁺. Metal-catalyzed formation of NO⁺ observed by us is consistent with this proposition. We have also observed that the reaction of NO with ferric heme-proteins can also be a potential source of nitrosation formation as shown:

\[ \text{Hb} + \text{NO} \rightarrow \text{Hb} + \text{NO}^+ \]

REACTION 10

Brackman and Smit (1965) have reported nitrosation via the mediation of copper salts and NO⁺ formation. Nitrosation of thiols and other reactive groups by metal ion-mediated formation of NO⁺ is likely to be considerably faster than via the reactions of NO with oxygen.

To summarize, in aqueous solutions of thiols, NO and O₂, nitrosation of the thiol proceeds via the rate-limiting step of formation of oxides of nitrogen. The nitrosating intermediate seems to be N₂O₃. Its reaction with thiols is competitive with the rate of its hydrolysis. Considerations based on the rate constants and in vivo concentrations of glutathione and HSA suggest that in the case of glutathione, significant nitrosation can take place by its reaction with N₂O₃ particularly in cells with high GSH concentration (≥5 mM). This reaction path seems to be less significant for the nitrosation of HSA.

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**Table I**

| RSH              | kₐ  |
|------------------|-----|
| GSH              | 1200–1080² |
| N-acetylcyesteine | 1500²  |
| Cys              | 450²  |
| HSA              | 7    |
| BSA              | 7    |

² Williams (1988).

**Table II**

Estimates of the rate constants for the reaction of thiols with N₂O₃ (T = 20°C, pH = 7.0)

| RSH              | kₐ × 10⁻⁵  |
|------------------|------------|
| GSH              | 2.9        |
| N-acetylcyesteine| 1.5        |
| Cys              | 2.6        |
| HSA              | 0.3        |
| BSA              | 0.06       |
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