Mechanism of the Nitrosation of Thiols and Amines by Oxygenated •NO Solutions: the Nature of the Nitrosating Intermediates

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Abstract: The nitrosation of various thiols and morpholine by oxygenated •NO solutions at physiological pH was investigated. The formation rates and the yields of the nitroso compounds were determined using the stopped-flow technique. The stoichiometry of this process has been determined, and is given by $4^{\bullet}NO + O_2 + 2RSH/2RR'NH \rightarrow 2RSNO/2RR'NNO + 2NO_2^- + 2H^+$. Kinetic studies show that the rate law is $-d[O_2]/dt = k_1[^{\bullet}NO]^2[O_2]$ with $k_1 = (2.54 \pm 0.26) \times 10^6\ M^{-2}\ s^{-1}$ and $-d[^{\bullet}NO]/dt = 4k_1[^{\bullet}NO]^2[O_2]$ with $4k_1 = (1.17 \pm 0.12) \times 10^7\ M^{-2}\ s^{-1}$, independent of the kind of substrate present. The kinetic results are identical to those obtained for the autoxidation of •NO, indicating that the rate of the autoxidation of •NO is unaffected by the presence of thiols and amines. The nitrosation by •NO takes place only in the presence of oxygen, and therefore the rate of the formation of *S*-nitrosothiols from thiols and oxygenated •NO solution is relatively slow in biological systems. Under physiological conditions where $[^{\bullet}NO] < 1\ \mu M$ and $[O_2] < 200\ \mu M$, the half-life of the nitrosation process exceeds 7 min. Therefore, this is an unlikely biosynthetic pathway for the formation of *S*-nitrosothiols. As such, *S*-nitrosothiols cannot serve as carrier molecules of •NO *in vivo*. The rate-determining step of the nitrosation of thiols and amines by oxygenated •NO solution is the formation of ONOONO (or $ONONO_2$ or O_2NNO_2), which is the precursor of $^{\bullet}NO_2$ and N_2O_3. The stoichiometry of the nitrosation process suggests that $^{\bullet}NO_2$ and/or N_2O_3 are the reactive species. We have demonstrated that $^{\bullet}NO_2$ initiates the nitrosation process unless it is scavenged faster by •NO to form N_2O_3. The latter entity is also capable of directly nitrosating thiols and amines with rate constants exceeding $6 \times 10^7\ M^{-1}\ s^{-1}$.

Introduction

Nitric oxide (•NO) is formed enzymatically from L-arginine by many types of cells and is an important mediator in several physiological processes.[1] In neuronal and endothelial cells, •NO is an intercellular messenger, effecting signal transduction by stimulation of heme-containing soluble guanylate cyclase.[1] •NO is unusual as a biochemical messenger and effector because of its small size, high diffusibility, and high chemical reactivity as a free radical. It reacts with superoxide,[2] oxygen,[3] and heme and non-heme iron,[4,5] which are all present in the medium. Therefore, it has been argued that •NO is stabilized by the reaction with a carrier molecule that prolongs its half-life and preserves its biological activity.[6−9] Important classes of potential carrier molecules are those containing sulfhydryl (RSH) and amine (RR′NH) functional groups. It has been demonstrated that oxygenated •NO solutions nitrosate thiols and amines at physiological pH to form *S*-nitrosothiols (RSNO) and *N*-nitrosoamines (RR′NNO), respectively.[10−13] These nitroso compounds are more stable than •NO,[6−8,10,11,14] and certain *S*-nitrosothiols are potent vasodilators and platelet inhibitors,[6−8,14] similar to endothelium-derived relaxing factor (EDRF),[15] which has been identified as •NO.[16,17] A recent comparison of the physiological properties of *S*-nitrosothiols to those of •NO and EDRF has shown that they differ in their stability and reactivity with oxyhemoglobin.[18] However, there is currently no convincing experimental evidence to indicate that EDRF is *S*-nitrosothiol rather than •NO.

Although •NO is a major participant in a large number of physiological processes,[1] excess production of •NO can be toxic.[10,11,18−23] The cytotoxic effects of •NO have been at-

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(1) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.
(2) Goldstein, S.; Czapski, G. *Free Radical Biol. Med.* **1995**, *19*, 505.
(3) Awad, H. H.; Stanbury, D. M. *Int. J. Chem. Kinet.* **1993**, *25*, 375.
(4) Reddy, D.; Lanaster, J. R.; Cornforth, D. P. *Science* **1983**, *221*, 769.
(5) Tsai, A.-L. *FEBS Lett.* **1994**, *341*, 141.
(6) Ignarro, J. L. *Annu. Rev. Pharmacol. Toxicol.* **1990**, *30*, 535.
(7) Stamler, J. S.; Simon, D. L.; Osborne, J. A.; Mullins, M. E.; Jarkai, O.; Michel, T.; Singel, D. J.; Loscalzo, J. *Proc. Natl. Acad. U.S.A.* **1992**, *89*, 444.
(8) Stamler, J. S.; Jaraki, O.; Osborne, J.; Simon, D. I.; Keaney, J.; Vita, J.; Singel, D.; Valeri, C. R.; Loscalzo, J. *Proc. Natl. Acad. U.S.A.* **1992**, *89*, 7674.
(9) Czapski, G.; Goldstein, S. *Free Radical Biol. Med.* **1995**, *6*, 785.
(10) Wink, D. A.; Kasprzak, K. S.; Maragos, C. M.; Elespuru, R. K.; Misra, M.; Dunams, T. M.; Cebula, T. A.; Koch, W. H.; Andrews, A. W.; Allen, J. S.; Keefer, L. K. *Science* **1991**, *254*, 1001.
(11) Nguyen, T.; Crespi, C. L.; Penman, B. W.; Wishnok, J. S.; Tannenbaum, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3030.
(12) Wink, D. A.; Nims, R. W.; Darbyshire, J. F.; Christodoulou, D.; Hanbauer, I.; Cox, G. W.; Laval, F.; Laval, J.; Cook, J. A.; Krishna, M. C.; DeGraff, W. G., Mitchell, J. B. *Chem. Res. Toxicol.* **1994**, *7*, 519.
(13) Lewis, R. S.; Tannenbaum, S. R.; Deen, W. M. *J. Am. Chem. Soc.* **1995**, *117*, 3933.
(14) Ignarro, L. J.; Lipton, H.; Edwards, J. C.; Baricos, W. H.; Hyman, A. L.; Kadowitz, P. J.; Gruetter, C. A. *J. Pharmacol. Exp. Ther.* **1981**, *218*, 739.
(15) Furchgott, R. F.; Zawadski, J. V. *Nature (London)* **1980**, *288*, 373
(16) Palmer, R. M. J.; Ferrige, A. J.; Moncada, S. *Nature* **1987**, *327*, 524.
(17) Ignarro, L. J.; Buga, J. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265.
(18) Feelisch, M.; te Poel, M.; Zamora, R.; Deussen, A.; Moncada, S. *Nature* **1994**, *368*, 62.
(19) Hibbs, J. B., Jr.; Taintor, R. R.; Vavrin, Z.; Rachlin, E. M. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 87.
(20) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1620.
(21) Nowicki, J. P.; Duval, D.; Poignet, H.; Scatton, B. *FEBS Lett.* **1991**, *204*, 339.

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tributed partially to the formation of oxidizing and nitrosating agents that are formed via the reaction of •NO with $O_2^{\bullet-}$ and O_2. Thus, the formation of NO adducts may have several roles *in vivo*, either as carrier molecules of •NO or as effective scavengers that may further lead to toxicity, e.g., deamination of DNA[10,11] and *S*-nitrosation of enzymes.[22]

The autoxidation of •NO in aqueous solution is a very complex process.[3,9,24,25] It has been suggested by several groups that the nitrosating species in the $^{\bullet}NO/O_2$ system is N_2O_3,[10,11,13,24] whereas Wink et al.[12] have suggested that the nitrosating species is an unidentified NO_x species. The rates of the formation of the nitroso compounds by $^{\bullet}NO/O_2$ and the nature of the nitrosating intermediates are of great importance from the biological point of view. The identification of these species is crucial for understanding the various biological roles of •NO *in vivo*.

In this study we will demonstrate that the rate of the autoxidation of •NO is unaffected by the presence of various thiols and amines. We will show that the nitrosation process is initiated by $^{\bullet}NO_2$ unless $^{\bullet}NO_2$ is scavenged more rapidly by •NO to form N_2O_3, which is capable of directly nitrosating these compounds.

Experimental Section

Materials. All chemicals were of analytical grade and were used as received. Nitric oxide, C.P., was purchased from Matheson Gas Products. Solutions were prepared with deionized water that was distilled and purified using a Milli-Q water purification system. Glutathione (GSH), cysteine (CysSH), penicillamine (PenSH), *N*-acetylpenicillamine (NAPenSH), captopril (CapSH), dithiothreitol (DTT), morpholine (MorNH), *N*-nitrosomorpholine (MorNNO), *S*-nitrosoglutathione (GSNO), and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from Sigma.

Oxygen-saturated solutions (1.12 mM at 25 °C and 690 mmHg, which is the barometric pressure in Jerusalem)[26] were prepared by bubbling gas-tight syringes with oxygen for 30 min. The •NO gas was purified by passing it through a series of scrubbing bottles containing 50% NaOH and distilled water in this order. The solutions in the traps were first deaerated by purging them with helium for 1 h. Nitric oxide solutions were prepared in gas-tight syringes by purging first 1 mM phosphate buffer solutions (pH 7.4) with helium to remove O_2, followed by bubbling for 30 min with •NO. The •NO-saturated solutions (1.72 mM at 25 °C and 690 mmHg)[26] were stored in syringes and subsequently diluted with helium-saturated solutions to the desired concentrations by the syringe technique. Stock solutions of thiols were daily prepared and were used after neutralization. Acetate, phosphate, and borate buffers were used for pH 4−5, 6−8, and 9−10, respectively.

S-Nitrosothiols were prepared by acid-catalyzed *S*-nitrosation of the thiol with sodium nitrite at 2 °C as previously described.[27] Equimolar concentrations of $NaNO_2$ and thiol in 0.1 N sulfuric acid were mixed and allowed to react for a few minutes. Dilutions were made with 0.1 M phosphate buffer at pH 7.4. *S*-Nitrosothiols have two absorption maxima at 330−340 and 544 nm, except SNAP, which absorbs at 590 nm. The extinction coefficients are as follows: (for GSNO) $\epsilon_{336} = 770 \pm 30\ M^{-1}\ cm^{-1}$ and $\epsilon_{544} = 15.1 \pm 0.6\ M^{-1}\ cm^{-1}$; (for SNAP) $\epsilon_{340} = 815 \pm 50\ M^{-1}\ cm^{-1}$ and $\epsilon_{590} = 11.6 \pm 0.3\ M^{-1}\ cm^{-1}$; (for CapSNO) $\epsilon_{332} = 940 \pm 40\ M^{-1}\ cm^{-1}$ and $\epsilon_{544} = 16.7 \pm 0.5\ M^{-1}\ cm^{-1}$.[28] When $[NO_2^-]_o \geq 2[DTT]_o$, $\epsilon_{336} = 1685 \pm 60\ M^{-1}\ cm^{-1}$ and $\epsilon_{544} = 30 \pm 1.6\ M^{-1}\ cm^{-1}$, and when $[DTT]_o \geq [NO_2^-]_o$, $\epsilon_{332} = 825 \pm 25\ M^{-1}\ cm^{-1}$ and $\epsilon_{544} = 13.8 \pm 1.4\ M^{-1}\ cm^{-1}$, which indicates the formation of two nitroso groups in the presence of excess nitrite over DTT. The absorption maxima of GSNO, SNAP, and MorNNO were also determined by dissolving commercial materials at pH 7.4 (5 mM phosphate buffer) and are at 338 nm ($\epsilon = 760 \pm 20\ M^{-1}\ cm^{-1}$), 340 nm ($\epsilon = 930 \pm 30\ M^{-1}\ cm^{-1}$) and 340 nm ($\epsilon = 100 \pm 1\ M^{-1}\ cm^{-1}$), respectively.

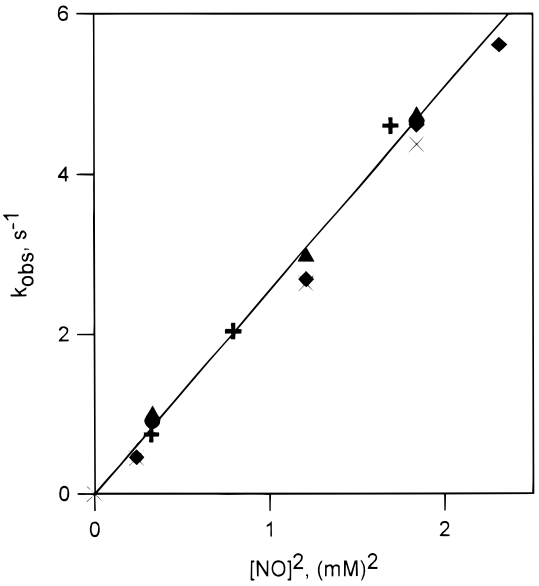

Figure 1. Observed first-order rate constant of the formation of RSNO and MorNNO as a function of $[^{\bullet}NO]^2$ in the presence of 20.4−102 μM O_2 at pH 7.4 ± 0.1 (1−2 mM phosphate buffer), and the following substrates: (◆) GSH; (×) CysSH; (▲) NAPenSH; (+) DTT; (●) MorNH. $[^{\bullet}NO]$ was taken as $[^{\bullet}NO]_o - 2[O_2]_o$.

Methods. Stopped-flow kinetic measurements were carried out using the Bio SX-17MV sequential stopped-flow apparatus from Applied Photophysics. A xenon lamp (Osram XBO 150W) produced the analyzing light, and a Hammamatsu R928 photomultiplier was used for measurements in the near UV and visible regions. The formation of the nitroso compounds was followed at 333−340 nm. The optical path length was 1 cm. All measurements were carried out at 22 °C. Each value given is an average of at least five measurements.

Results

When aerated solutions of RSH (GSH, CysSH, PenSH, NAPenSH, CapSH, DTT) or MorNH at pH 7.4 ± 0.1 were mixed with •NO-saturated solution to yield final concentrations of 0.57−1.56 mM •NO, 20.4−102 μM O_2, 1−2 mM phosphate buffer, and 0.2−40 mM RSH or 0.5−150 mM MorNH, a rapid formation of an absorption with a maximum at 332−340 nm was observed, which was attributed to the formation of RSNO or MorNNO. Under these conditions the rate of the formation of RSNO and MorNNO was first-order. The observed first-order rate constant was linearly dependent on $[^{\bullet}NO]^2$, yielding a slope of $(2.54 \pm 0.26) \times 10^6\ M^{-2}\ s^{-1}$ for all compounds studied (Figure 1). This third-order rate constant is within experimental error identical to that determined for the autoxidation of •NO and for the oxidation of ferrocyanide and ABTS by the $^{\bullet}NO/O_2$ under limiting concentrations of O_2.[25] No formation of absorbance at 332−340 nm was observed when

(22) Molina y Vedia, L.; McDonald, B.; Reep, B.; Brune, B.; Di Silvio, M.; Billiar, T. R.; Lapetina, E. G. *J. Biol. Chem.* **1992**, *267*, 24929.
(23) Dawson, T. M.; Snyder, S. H. *J. Neurosci.* **1994**, *14*, 5147.
(24) Pires, M.; Rossi, M. J.; Ross, D. S. *Int. J. Chem. Kinet.* **1994**, *26*, 1207.
(25) Goldstein, S.; Czapski, G. *J. Am. Chem. Soc.* **1995**, *117*, 12078.
(26) *Lange's Handbook of Chemistry*, 13th ed.; Dean, J. A., Ed.; McGraw-Hill: New York, 1985; pp 10−15.
(27) Loscalzo, J.; Smick, D.; Andon, N.; Cooke, J. *J. Pharmacol. Exp. Ther.* **1989**, *249*, 726.

(28) It has been reported that the absorption spectrum of CapSNO consists of four absorption maxima: 333 nm ($\epsilon = 1890\ M^{-1}\ cm^{-1}$), 404 nm ($\epsilon = 339\ M^{-1}\ cm^{-1}$), 512 nm ($\epsilon = 7.9\ M^{-1}\ cm^{-1}$), and 545 nm ($\epsilon = 13.3\ M^{-1}\ cm^{-1}$).[27] However, we did not observe any absorption peak at 404 nm and determined $\epsilon_{333} = 940\ M^{-1}\ cm^{-1}$, which differs from than that determined in ref 27, but is similar to those of all *S*-nitrosothiols that have been measured so far as well as to those of many other thionitrites in nonaqueous solutions (Oae; et al. *J. Chem. Soc., Perkin Trans. 1* **1978**, 913).

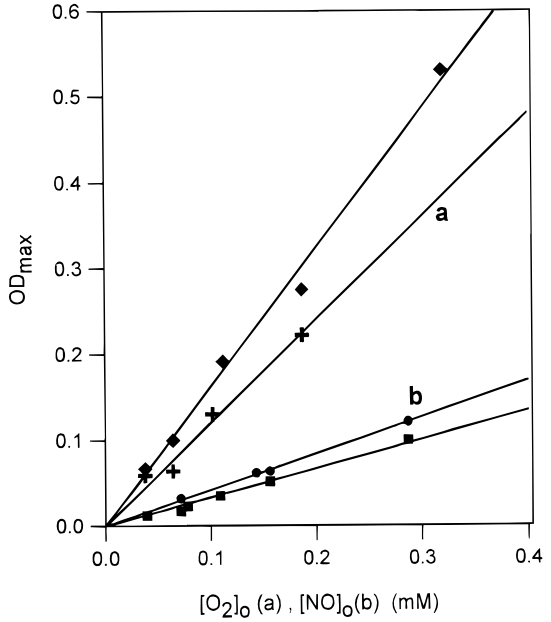

Figure 2. Measured OD at the end of the formation of SNAP and CysSO at 340 and 338 nm, respectively, in the presence of 1−4 mM thiol at pH 7.4 ± 0.1 (1.2−1.9 mM phosphate buffer) and (a) under limiting concentration of O_2, (◆) SNAP, (+) CysNO and (b) under limiting concentration of •NO, (●) SNAP, (■) CysSNO.

Table 1. Slopes of the Lines of the Maximun Absorbance of RSNO and MorNNO versus O_2 and •NO Concentrations at pH 7.4 ± 0.1 (1−2 mM Phosphate Buffer)[a]

substrate	slope a, M^{-1} cm^{-1} $[•NO]_o > [O_2]_o$	slope b, M^{-1} cm^{-1} $[O_2]_o > [•NO]_o$	slope a/slope b
GSH	1583	387	4.09
CysSH	1245	323	3.85
NAPenSH	1621	425	3.82
PenSH	1625	423	3.84
CapSH	1653	350	4.72
DTT	1530	391	3.91
MorNH	182	46	3.96

[a] Slope a = The slope of the line as in Figure 2a, obtained under limiting concentrations of O_2. [b] Slope b =The slope of the line as in Figure 2b, obtained under limiting concentrations of •NO.

•NO-saturated solutions were mixed with deaerated solutions containing the various thiols or morpholine at pH 7.4, indicating that O_2 is essential for the nitrosation process as previously reported.[12,29]

Maximum nitrosation yields were obtained when $[RSH]_o \geq 2[O_2]_o$ in the case of CysSH, GSH, PenSH, and DTT, but decreased when $[RSH]_o$ exceeded 4 mM. In the case of NAPenSH, CapSH, and MorNH, maximum yields were obtained for $[RSH]_o$ > 15, 1, and 2 mM, respectively. The yields of RSNO and MorNNO were linearly dependent on $[O_2]_o$ (Figure 2a). The slopes obtained for the various substrates from plots similar to those given in Figure 2a are summarized in Table 1.

When oxygen-saturated solutions containing RSH and MorNH were mixed with •NO solutions to yield final concentrations of 0.3−1.02 mM O_2, 39−287 μM •NO, 1−2 mM phosphate buffer (pH 7.4 ± 0.1), and 0.2−100 mM RSH or 0.5−480 mM MorNH, the rate of the formation of RSNO and MorNNO was second-order. The observed second-order rate constant was linearly dependent on $[O_2]_o$ (Figure 3). The third-order rate constants obtained for the formation of RSNO and MorNNO from the dependence of k_{obs} on $[O_2]_o$ and the known or measured ϵ_{max}, are summarized in Table 2. The rate of the formation of

(29) Pryor, W. A.; Curch, D. F.; Govindan, C. K.; Crank, J. *J. Org. Chem.* **1982**, *47*, 156.

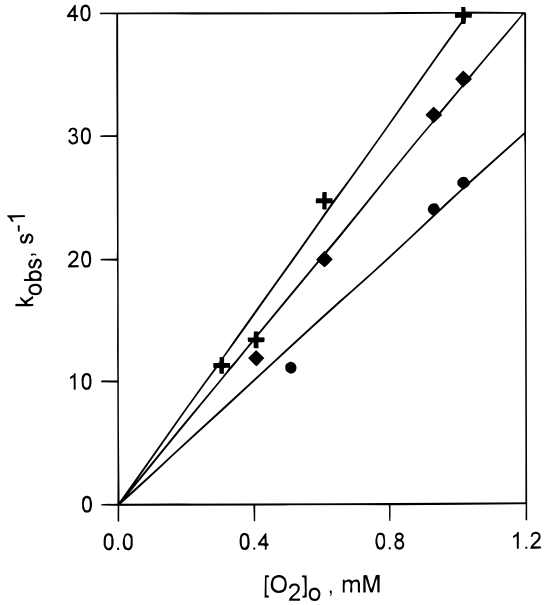

Figure 3. Observed second-order rate constant of the formation of RSNO as a function of $[O_2]_o$ at pH 7.4 ± 0.1: (+) [CysSH] = 2 mM; (◆) [GSH] = 4 mM; (●) [NAPenSH] = 15 mM.

Table 2. Observed Second-Order and the Third-Order Rate Constants of the Formation of RSNO and MorNNO under Limiting Concentrations of •NO

substrate	$k_{obs}/[O_2] = k/\epsilon l$, M^{-1} s^{-1}	k, M^{-2} s^{-1} (ϵ from Table 1)	k, M^{-2} s^{-1} (ϵ determined directly)
GSH	3.34×10^4	2.64×10^7	2.58×10^7
CysSH	3.86×10^4	2.40×10^7	nd
NAPenSH	2.52×10^4	2.04×10^7	2.34×10^7
PenSH	2.62×10^4	2.14×10^7	nd
CapSH	2.62×10^4	2.16×10^7	2.46×10^7
DTT	2.80×10^4	2.18×10^7	2.32×10^7
MorNH	2.13×10^5	1.96×10^7	2.14×10^7

all the nitroso products under limiting concentrations of •NO is $(2.34 \pm 0.24) \times 10^7$ M^{-2} s^{-1}, which is about 8 times the value determined under limiting concentrations of O_2.

Maximum nitrosation yields were obtained when $[RSH]_o \geq [•NO]_o/2$ in the case of CysSH, GSH, PenSH, and DTT, which decreased when $[RSH]_o$ exceeded 4 mM. In the case of NAPenSH, CapSH, and MorNH, maximum product yields were obtained for $[RSH]_o$ > 15, 1, and 2 mM, respectively. The maximum yields of RSNO and MorNNO were linearly dependent on $[•NO]_o$ (Figure 2b). The slopes obtained for the various substrates from plots similar to those given in Figure 2b are summarized in Table 1. The ratio of the slopes obtained under limiting concentrations of O_2 to those obtained under limiting concentrations of •NO is 4.0 ± 0.2 (Table 1).

The nitrosation yields decreased with the increase in phosphate buffer concentrations and with the decrease in pH (Table 3). The effect of phosphate on the product yields in the •NO/O_2 system has already been observed in the presence of MorNH,[13] ferrocyanide,[25] and ABTS.[25]

Discussion

The rate of the nitrosation of thiols and amines by oxygenated •NO solution is second-order in [•NO] and first-order in $[O_2]$. The third-order rate constant of the nitrosation is identical to that of the autoxidation of •NO, indicating that the rate-determining step of the nitrosation by oxygenated •NO solution is the same as that of the autoxidation of •NO. The ratio of the nitrosation yields under limiting concentrations of O_2 to those under limiting concentrations of •NO is 4. Therefore, the

Table 3. Effect of Phosphate Buffer and pH on the Nitrosation Yields

substrate	$[RSH]_o$, mM	$[NO]_o$, mM	$[O_2]_o$, mM	[Pi], mM	pH	[RSNO], μM	$[RSNO]/([RSNO]_{max} - [RSNO])$
PenSH	0.56	1.37	0.0363	1.6	7.4	71	
PenSH	0.56	1.37	0.0363	40	7.4	48	2.09
PenSH	0.56	1.37	0.0363	80	7.4	40	1.29
PenSH	0.56	1.37	0.0363	160	7.4	34	0.92
PenSH	0.56	1.37	0.0363	1.6	6.5	60	
PenSH	0.56	1.37	0.0363	−	4.1	15	
PenSH	0.56	1.37	0.0363	−	10	82	
GSH	2	0.156	1	1.6	7.4	74	
GSH	2	0.156	1	16	7.4	61	4.7
GSH	2	0.156	1	160	7.4	43	1.38
GSH	2	0.156	1	480	7.4	29	0.64
GSH	2	0.156	1	10	6.2	49	
GSH	2	0.156	1	−	10	72	

stoichiometry of the whole nitrosation process is given by eq 1, whereas that of the autoxidation of •NO is given by eq 2.[3,24,25]

$$4^{\bullet}NO + O_2 + 2RSH/2RR'NH \rightarrow 2RSNO/2RR'NNO + 2NO_2^- + 2H^+ \quad (1)$$

$$4^{\bullet}NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (2)$$

We have recently shown that the rate-determining step of the autoxidation of •NO is the formation of ONOONO, and the whole process can be described by reactions 3−7.[25] According

$$^{\bullet}NO + O_2 \rightleftharpoons ONOO^{\bullet} \text{ (or } NO\cdots O_2) \quad (3)$$

$$O_2NO^{\bullet} \text{ (or } NO\cdots O_2) + {}^{\bullet}NO \rightarrow ONOONO \text{ (or } ONONO_2 \text{ or } O_2NNO_2) \quad (4)$$

$$ONOONO \rightarrow 2^{\bullet}NO_2 \quad (5)$$

$$^{\bullet}NO_2 + {}^{\bullet}NO \rightleftharpoons N_2O_3 \quad k_6 = 1.1 \times 10^9\ M^{-1}\ s^{-1};\ k_{-6} = 8.1 \times 10^4\ s^{-1}\ ^{33} \quad (6)$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad k_7 = 530\ s^{-1}\ ^{33} \quad (7)$$

to this mechanism, rate equation 8 is obtained, where $k_{-3} > k_4[^{\bullet}NO]$ and $k_1 = k_3k_4/k_{-3} = (2-2.9) \times 10^6\ M^{-2}\ s^{-1}$.[3,24,25,32]

$$-\frac{1}{4}\frac{d[^{\bullet}NO]}{dt} = -\frac{d[O_2]}{dt} = \frac{k_3k_4[^{\bullet}NO]^2[O_2]}{k_{-3} + k_4[^{\bullet}NO]} = k_1[^{\bullet}NO]^2[O_2] \quad (8)$$

The stoichiometry of the nitrosation process is given by eq 1. Thus, rate equation 9 is obtained irrespective of the detailed mechanism. The rate of the nitrosation process was determined

$$-\frac{1}{4}\frac{d[^{\bullet}NO]}{dt} = -\frac{d[O_2]}{dt} = \frac{1}{2}\frac{d[RSNO]}{dt} = k_1[^{\bullet}NO]^2[O_2] \quad (9)$$

by following the formation of RSNO/RR′NNO. Under limiting concentrations of O_2, each O_2 yields 2RSNO, and therefore rate equation 9a is obtained for the formation of RSNO. Under limiting concentrations of •NO, each •NO yields 0.5RSNO, and therefore rate equation 9b is obtained for the formation of RSNO. Thus, irrespective of the detailed mechanism of the nitrosation process, the rate constant determined by following the formation of RSNO under limiting concentrations of O_2 is k_1 (Figure 1), and under limiting concentrations of •NO $8k_1$ (Figure 3, Table 2), and hence $k_1 = (2.73 \pm 0.19) \times 10^6\ M^{-2}\ s^{-1}$. This value is in excellent agreement with the value of $k_1 = (2.9 \pm 0.1) \times 10^6\ M^{-2}\ s^{-1}$, which has been determined for the autoxidation of •NO.[25]

$$d[RSNO]/dt = 2k_1[^{\bullet}NO]^2[O_2] = 2k_1[0.5RSNO] = k_1[^{\bullet}NO]^2[RSNO] \quad (9a)$$

$$d[RSNO]/dt = 2k_1[^{\bullet}NO]^2[O_2] = 2k_1[O_2][2RSNO]^2 = 8k_1[O_2][RSNO]^2 \quad (9b)$$

Since the rate of the autoxidation of •NO is identical to that of the nitrosation of thiols and amines by $^{\bullet}NO/O_2$, the nitrosating species must be ONOONO (or $ONONO_2$ or O_2NNO_2), $^{\bullet}NO_2$, or N_2O_3. If ONOONO is the nitrosating agent, one expects that reactions 10−13 will take place. The direct oxidation of

$$ONOONO + RS^- \rightarrow RSNO + ONOO^- \quad (10)$$

$$ONOO^- + RSH \rightarrow RS^{\bullet} + {}^{\bullet}NO_2 + OH^- \quad (11)$$

$$^{\bullet}NO_2 + RS^- \rightarrow RS^{\bullet} + NO_2^- \quad (12)$$

$$RS^{\bullet} + {}^{\bullet}NO \rightarrow RSNO \quad (13)$$

cysteine to cystine by peroxynitrite in aerated solution has been demonstrated by Radi et al.,[33] the stoichiometry being 2.2 mol of cysteine/mol of peroxynitrite. This process is mediated by one-electron transfer with the formation of thiyl radical and most probably $^{\bullet}NO_2$.[34] In this case reactions 11 and 12 are followed by reactions 14−16, where $O_2^{\bullet-}$ dismutates faster than it reacts

$$RS^{\bullet} + RS^- \rightleftharpoons RSSR^{\bullet-} \quad (14)$$

$$RS^{\bullet} + RS^{\bullet} \rightarrow RSSR \quad (15)$$

$$RSSR^{\bullet-} + O_2 \rightarrow RSSR + O_2^{\bullet-} \quad (16)$$

with the thiol.[35] The addition of molecular oxygen to $RS^{\cdot}$ does not compete efficiently with reactions 14 and 16,[36] and can be ignored. We assume that, in the $^{\bullet}NO/O_2$ system, reaction 13

(30) McKee, M. L. *J. Am. Chem. Soc.* **1995**, *117*, 1629.
(31) Gratzel, Von, M.; Taniguchi, S.; Henglein, A. *Ber. Bunsen-Ges. Phys. Chem.* **1970**, *74*, 488.
(32) Ford, A. C.; Wink, D. A.; Stanbury, D. M. *FEBS Lett.* **1993**, *326*, 1.
(33) Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. *J. Biol. Chem.* **1991**, *266*, 4244.
(34) Gatti, R. M.; Radi, R.; Augusto, O. *FEBS Lett.* **1994**, *348*, 287.
(35) Bielski, B. H. J.; Shiue, G. G. *Oxygen Free Radicals and Tissue Damage*; Ciba Foundation Symposium 65 (new series); Excerpta Medica: New York, 1979, p. 43−56.
(36) Monig, J.; Asmus, K.-D.; Forni, L. G.; Willson, R. L. *Int. J. Radiat. Biol.* **1987**, *52*, 589.

competes efficiently with reactions 14−16,[37,38] and therefore, the stoichiometry of the nitrosation process will be given by eq 17, which differs from the stoichiometry of eq 1. If, however,

$$4^{\bullet}NO + O_2 + 3RSH/3RR'NH \rightarrow 3RSNO/3RR'NNO + NO_2^- + H^+ + H_2O \quad (17)$$

peroxynitrite does not oxidize the substrate via reaction 11, but decomposes into nitrate, or reaction 18 takes place, the net

$$RS^- + ONONO_2 \text{ (or } O_2NNO_2) \rightarrow RSNO + NO_3^- \quad (18)$$

nitrosation process will be given by eq 19, which also differs from the stoichiometry of eq 1. If ONOONO (or $ONONO_2$ or

$$2^{\bullet}NO + O_2 + RSH/RR'NH \rightarrow RSNO/RR'NNO + NO_3^- + H^+ \quad (19)$$

O_2NNO_2) is not the nitrosating species, one should consider both $^{\bullet}NO_2$ and N_2O_3 as the active intermediates. $^{\bullet}NO_2$ may nitrosate thiols and amines via reactions 12 and 13, which are both feasible.[37,39−42] The nitrosation of thiols and amines by $^{\bullet}NO_2$ has been demonstrated previously,[29,42,43] even at physiological pH.[43] However, if reaction 12 does not compete efficiently with reaction 6, N_2O_3 will be formed. This latter species is a strong nitrosating agent that can directly nitrosate thiols and amines via reaction 20. The stoichiometry of the

$$N_2O_3 + RS^- \rightarrow RSNO + NO_2^- \quad (20)$$

nitrosation process via $^{\bullet}NO_2$ or N_2O_3 as the active intermediate is in agreement with our experimental results (eq 1).

The reduction potentials of $RS^{\bullet}/RS^-$ and $RS^{\bullet},H^+/RSH$ are about 0.73 and 1.35 V, respectively, whereas that of $^{\bullet}NO_2/NO_2^-$ is 1.04 V.[44] The only exception is DTT, which has an unusually low reduction potential of −0.33 V at pH 7.[45] Therefore, from the thermodynamic point of view, $^{\bullet}NO_2$ and most probably N_2O_3 ($E°(NO^+/^{\bullet}NO) = 1.21$ V)[44] are capable of oxidizing/nitrosating the unprotonated forms of thiols. In the case of DTT the protonated form is also available for oxidation/nitrosation by $^{\bullet}NO_2$ and N_2O_3. Thus, of great importance is the concentration of the unprotonated form, which at a given pH is $[RS^-] = [RSH]_o/(1 + 10^{pK-pH})$.[13,42,43] The alternative nitrosation pathways that yield eq 1 are as follows:

Pathway I. The nitrosation will take place exclusively by $^{\bullet}NO_2$. This will be the case when reaction 12 competes efficiently with reactions 6 and 7 ($k_{12}[RS^-] \gg k_6k_7[^{\bullet}NO]/(k_{-6} + k_7)$). If this condition is not fulfilled, the nitrosation yield will be given by eq 21, provided that $k_7 > k_{20}[RS^-]$. A plot of

$$\frac{[RSNO]}{[RSNO]_{max}} = \frac{k_{12}[RS^-]}{k_{12}[RS^-] + k_6k_7[^{\bullet}NO]/(k_{-6} + k_7)} \quad (21)$$

1/[RSNO] as a function of $1/[RS^-]$ should yield a straight line with slope/intercept $= S/I = k_6k_7[^{\bullet}NO]/(k_{-6} + k_7)k_{12}$.

Pathway II. N_2O_3 is the only nitrosating species. This will be the case if $k_{12}[RS^-] \ll k_6k_7[NO]/(k_{-6} + k_7)$. With this mechanism, the nitrosation yields will be independent of [•NO], and will be given by eq 22. A plot of 1/[RSNO] as a function of $1/[RS^-]$ should yield a straight line with $S/I = k_7/k_{20}$.

$$\frac{[RSNO]}{[RSNO]_{max}} = \frac{k_{20}[RS^-]}{k_{20}[RS^-] + k_7} \quad (22)$$

Pathway III. $^{\bullet}NO_2$ and N_2O_3 are both responsible for the nitrosation process. In this case $k_{12}[RS^-]$ does not exceed $k_6k_7[^{\bullet}NO]/(k_{-6} + k_7)$, and the ratio $k_{12}[RS^-]/k_6k_7[^{\bullet}NO]/(k_{-6} + k_7)$ determines the amount of the nitrosation by $^{\bullet}NO_2$. The nitrosation yield will be given by eq 23. When $k_{12}[RS^-] > k_6k_7[NO]/(k_{-6} + k_7)$, eq 23 reduces to eq 21, and when $k_{12}[RS^-] < k_6k_7[NO]/(k_{-6} + k_7)$, eq 23 reduces to eq 22.

$$\frac{[RSNO]}{[RSNO]_{max}} = \frac{[RS^-]}{k_{12}[RS^-] + k_6k_7[^{\bullet}NO]/(k_{-6} + k_7)}\left\{k_{12} + \frac{k_{20}k_6k_7[NO]/(k_{-6} + k_7)}{k_{20}[RS^-] + k_7}\right\} \quad (23)$$

The kinetics and the stoichiometry of the nitrosation by $^{\bullet}NO/O_2$ are the same whether $^{\bullet}NO_2$ (pathway I) or N_2O_3 (pathway II) is the active intermediates. However, according to pathway I, the nitrosation yield decreases with the increase in [•NO] (eq 21), whereas according to pathway II, it is independent of [•NO] (eq 22).

The only known values of k_{12} are for $CysS^-$ ($k_{12} = 2.4 \times 10^8$ M^{-1} s^{-1} at pH 9.2,[39] $k_{12} > 5 \times 10^8$ M^{-1} s^{-1} at pH 7.9−9.5[40]), DTT ($k_{12} = 4.6 \times 10^8$ M^{-1} s^{-1} at pH 9),[41] and $PenS^-$ ($k_{12} = 2.8 \times 10^8$ M^{-1} s^{-1} at pH 10).[46] Though there is some discrepancy between the values of k_{12} for $CysS^-$, it is clear that reaction 12 is very fast at pH 7.4 as the p*K* values of CysSH and PenSH are 8.18 and 7.93, respectively,[47] and $^{\bullet}NO_2$ is capable of oxidizing both the protonated and unprotonated forms of DTT.

Under limiting concentrations of •NO or O_2 at pH 7.4, and in the presence of CysSH, GSH, PenSH, and DTT, full nitrosation yields were obtained for $[RSH]_o \geq [^{\bullet}NO]_o/2$ or $[RSH]_o \geq 2[O_2]_o$, respectively. Under these conditions $k_{12}[RS^-] > k_6k_7[^{\bullet}NO]/(k_{-6} + k_7)$, and therefore $^{\bullet}NO_2$ is the only species responsible for the nitrosation process in the presence of these thiols (pathway I). In these cases, the maximum nitrosation yields decrease at relatively high concentrations of RSH, most probably due to the competition of reactions 14−16 with reaction 13.

The nitrosation yields decreased with the increase in phosphate buffer concentrations (Table 3). This effect has also been reported in the presence of MorNH,[13] ABTS,[25] and ferrocyanide.[25] It has been suggested that phosphate reacts directly with N_2O_3,[13] or that HPO_4^- catalyzes the hydrolysis of N_2O_3.[25] According to both suggestions, $k_7 = (530 + k_p[Pi])$, and

(37) Williams, D. H. L. *Nitrosation*; Cambridge University Press: Cambridge, 1988.
(38) Eiserich, J. P.; Butler, J.; vander Vliet, A.; Cross, C. E.; Halliwell, B. *Biochem. J.* **1995**, *310*, 745.
(39) Prutz, W. A.; Monig, H.; Butler, J.; Land, E. J. *Arch. Biochem. Biophys.* **1985**, *243*, 125.
(40) Forni, L. G.; Mora-Arellano, V. O.; Packer, J. E.; Willson, R. L. *J. Chem. Soc., Perin Trans. 2* **1986**, 1.
(41) Elliot, A. J.; Sopchyshyn, F. C. *Radiat. Phys. Res.* **1982**, *19*, 417.
(42) Challis, B. C.; Kyrtopoulos, S. A. *Br. J. Cancer* **1977**, *35*, 693.
(43) Cooney, R. V.; Ross, P. D.; Bartonili, G. L. Ramseyer, J. *Environ. Sci. Technol.* **1987**, *21*, 77.
(44) Stanbury, D. M. *Adv. Inorg. Chem.* **1989**, *33*, 69.
(45) Cleland, W. W. *Biochemistry* **1964**, *3*, 480.
(46) N_2O-saturated solutions containing nitrite and penicillamine were pulse-irradiated at pH 7.6 (2 mM phosphate buffer) and 10. The formation of the absorbance at 330 nm was followed, and $k_{12} = (2.8 \pm 0.3) \times 10^8$ M^{-1} s^{-1} was obtained from a fit to a complex mechanism which includes reactions 12, 14, and 15 for which $k_{14} = 2.8 \times 10^9$ M^{-1} s^{-1}, $k_{-14} = 2.9 \times 10^6$ s^{-1}, and $k_{15} = 2.3 \times 10^9$ M^{-1} s^{-1} (Hoffman, H. Z.; Hayon, E. *J. Phys. Chem.* **1973**, *77*, 990). No reaction was observed at pH 6.
(47) *Stability Constants of Metal Ion Complexes: Part B, Organic Ligands*; Perrin, D. D., Ed.; IUPAC Chemical Data Series No. 22; Pergamon Press: Oxford−New York−Toronto−Sydney−Paris−Frankfurt, 1979.

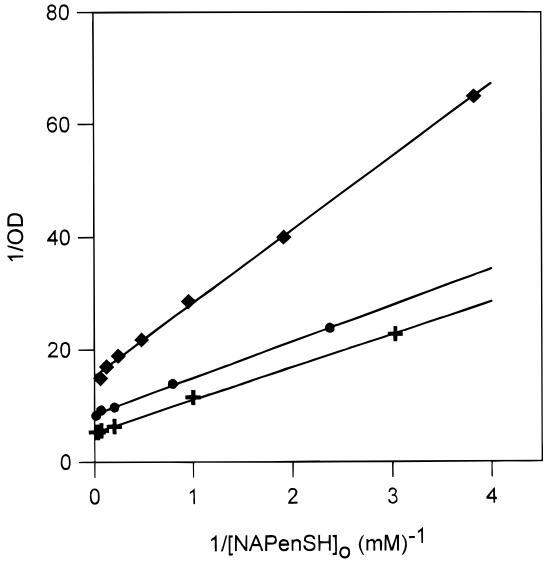

Figure 4. Double reciprocal plot of the absorbance of SNAP with varying concentrations of NAPenSH in the presence of (+) 0.86 mM •NO and 0.112 mM O_2 and (◆) 1.43 mM •NO and 37.3 μM O_2. All solutions contained 1.9 mM phosphate buffer at pH 7.4 ± 0.1.

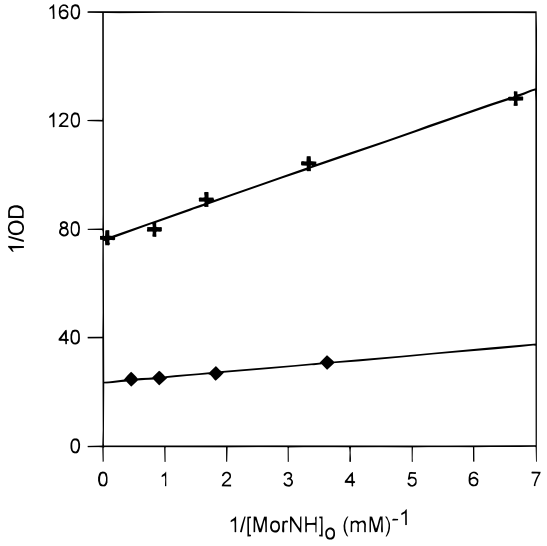

Figure 5. Double reciprocal plot of the absorbance of MorNNO with varying concentrations of MorNH in the presence of (+) 0.286 mM •NO and 0.933 mM O_2 (◆) 1.43 mM •NO and 0.187 mM O_2. All solutions contained 2 mM phosphate buffer at pH 7.4 ± 0.1.

phosphate shifts the whole nitrosation/oxidation process toward nitrite formation. When k_p[Pi] > 530 s^{-1}, eq 24 is obtained

$$\frac{[\mathrm{RSNO}]}{[\mathrm{RSNO}]_{max} - [\mathrm{RSNO}]} = \frac{k_{12}[\mathrm{RS}^-]}{k_6[\mathrm{NO}]}\left\{1 + \frac{k_{-6}}{k_p[\mathrm{Pi}]}\right\} \quad (24)$$

from eq 21. From the data given in Table 3, we determined k_p = 6.7 × 10^5 and 9.4 × 10^5 M^{-1} s^{-1} in the presence of PenSH and GSH, respectively. These values are in good agreement with the value of 6.4 × 10^5 M^{-1} s^{-1}, which has been determined by Lewis et al.[13] We have demonstrated[25] that HPO_4^{2-} catalyzes the hydrolysis of N_2O_3, and hence $k(N_2O_3 + HPO_4^{2-}) \sim 1 \times 10^6$ M^{-1} s^{-1} at pH 7.4.

In the presence of NAPenSH, CapSH, and MorNH, maximum nitrosation yields were obtained at substrate concentrations higher than 15, 1, and 2 mM, respectively. Plots of $1/OD_{340}$ as a function of $1/[\mathrm{NAPenSH}]_o$ (Figure 4), $1/[\mathrm{MorNH}]_o$ (Figure 5), and $1/[\mathrm{CapSH}]_o$ (results not shown) yield straight lines with S/I = 0.94 ± 0.16, 0.096 ± 0.009, and 0.30 ± 0.06 mM, respectively, independent of •NO concentrations. These results indicate that N_2O_3 is the main nitrosating species at pH 7.4 in the presence of NAPenSH, MorNH, and CapSH (pathway II). The nitrosation yields decreased substantially with the decrease in pH, indicating that the unprotonated forms of these compounds are the main species which are available for nitrosation. From the values of S/I, one calculates that $k_{20}/(1 + 10^{pK-pH})$ = (5.63 ± 0.95) × 10^5, (1.78 ± 0.36) × 10^6, and (5.52 ± 0.52) × 10^6 M^{-1} s^{-1} for NAPenSH, CapSH, and MorNH, respectively. The pK values of NAPenSH, CapSH, and MorNH are 10,[47] 9.7,[48] and 8.5,[49] and therefore k_{20} = 1.8 × 10^8, 3.5 × 10^8, and 7.5 × 10^7 M^{-1} s^{-1}, respectively. The latter value is in good agreement with the value of 6.4 × 10^7 M^{-1} s^{-1}, which has been determined by Lewis et al.[13]

Our results demonstrate that the rates of the oxidation of NAPenSH, CapSH, and MorNH by $•NO_2$ at pH 7.4 are too slow to compete effectively with reaction 6. The pK values of NAPenSH, and CapSH are 10 and 9.7, respectively, whereas those of CysSH, PenSH, and GSH are 8.18, 7.93, and 8.75, respectively. Therefore, even if k_{12} for all thiols is within the same order of magnitude, the concentrations of $NAPenS^-$ and $CapS^-$ are considerably lower than those of the other thiols at pH 7.4. Thus, the rate of reaction 12 in the case of NAPenSH and CapSH is considerably slower as compared to that of the other thiols, and reaction 6 competes efficiently with reaction 12. The pK of MorNH is 8.5, and therefore we have to assume that k_{12} for MorNH is more than an order of magnitude lower than k_{12} for thiols.

Wink et al.[12] have also studied the nitrosation of GSH and CysSH by $•NO/O_2$ at pH 7.4 (10 mM phosphate buffer), and concluded that the nitrosating species is an unidentified NO_x species. However, this conclusion was based on several kinetic errors and miscalculation of the extinction coefficients of RSNO (see the Appendix).

Piers et al.[24] measured the formation of 4-nitrophenol from phenol by $•NO/O_2$ at pH 12. They found that the net nitrosation process is described by eq 1, and concluded that the nitrosating species is N_2O_3. However, $•NO_2$ reacts with $C_6H_5O^-$ to form $C_6H_5O•$ with k_{12} = 8.6 × 10^6 M^{-1} s^{-1}.[50] The latter may react with •NO to yield nitrophenol, and therefore $•NO_2$ may initiate the nitrosation of phenol. The rate of the hydrolysis of N_2O_3 is pH dependent (k_7 = 2000 + 10^8[OH^-]),[51] and therefore, according to eq 21, the maximum nitrosation yield at pH 12 is expected if k_{12}[phenol] > k_6[•NO], regardless of whether k_7 = 530 or 2000 s^{-1} at neutral pH. Piers et al.[24] found that maximum nitrosation yields were obtained at [phenol] > 0.2 M in the presence of $[•NO]_o$ = 0.6 mM and $[O_2]_o$ = 0.4 mM at pH 12. This result is in accord with $•NO_2$ being the main species responsible for the nitrosation of phenol since 8.6 × 10^6 × 0.2 > 1.1 × 10^9[•NO] ([•NO] < 0.6 mM).

Conclusions

The rate of the autoxidation of •NO is unaffected by the presence of various thiols and morpholine. The stoichiometry of this process is identical for all thiols and morpholine and is given by eq 1. The kinetic studies show that the rate laws $-d[O_2]/dt = k_1[•NO]^2[O_2]$ with k_1 = (2.54 ± 0.26) × 10^6 M^{-2} s^{-1} and $-d[•NO]/dt = 4k_1[•NO]^2[O_2]$ with $4k_1$ = (1.17 ± 0.12) × 10^7 M^{-2} s^{-1} are independent of the kind of substrate present.

(48) Hughes, H. A.; Smith, G. L., Williams, D. R. *Inorg. Chim Acta* **1985**, *107*, 247.
(49) Hetzer, H. B.; Bates, R. G.; Robinson, R. A. *J. Phys. Chem.* **1966**, *70*, 2869.
(50) Huie, R. E.; Neta, P. *J. Phys. Chem.* **1986**, *90*, 1193.
(51) Treinin, A.; Hayon, E. *J. Am. Chem. Soc.* **1970**, *92*, 5821.

The rate-determining step of the nitrosation by $^{\bullet}NO/O_2$ is the formation of ONOONO (or $ONONO_2$ or O_2NNO_2), which is the precursor of $^{\bullet}NO_2$ and N_2O_3. The stoichiometry of this process suggests that $^{\bullet}NO_2$ and/or N_2O_3 are the only reactive species. We have demonstrated that $^{\bullet}NO_2$ initiates the nitrosation process unless it is scavenged by $^{\bullet}NO$ to form N_2O_3. The latter species is also capable of directly nitrosating thiols and amines with rate constants that exceed 6×10^7 M^{-1} s^{-1}. Full nitrosation yields are obtained either when $^{\bullet}NO_2$ rapidly oxidizes the substrate or when the nitrosation by N_2O_3 competes efficiently with the hydrolysis of N_2O_3 to nitrite.

It has been suggested that *S*-nitrosylation of proteins is a favorable reaction under physiologic conditions that prolongs the half-life of $^{\bullet}NO$ and preserves its biological activity. However, $^{\bullet}NO$ is incapable of nitrosating thiols and amines in the absence of oxygen at physiological pH, and the rate of the formation of *S*-nitrosothiols from thiols and oxygenated $^{\bullet}NO$ solution is relatively slow in biological systems. Under physiological conditions where $[^{\bullet}NO] < 1$ μM and $[O_2] < 200$ μM, the half-life of the nitrosation process exceeds 7 min ($t_{1/2} = 1/(1.17 \times 10^7[O_2][^{\bullet}NO]) > 7$ min). Thus, the formation of *S*-nitrosothiols from thiols and oxygenated $^{\bullet}NO$ solution is an unlikely biosynthetic pathway. We conclude that *S*-nitrosothiols cannot serve as carrier molecules of $^{\bullet}NO$ as $^{\bullet}NO$ will react with the various substrates present in the medium ($O_2^{\bullet-}$, hemoglobin, etc.) before it will nitrosate thiols.

Acknowledgment. This research was supported by The Council for Tobacco Research and by The Israel Science Foundation.

Appendix

Wink at al.[12] found that, under limiting concentrations of O_2, the observed first-order rate constant of the formation of RSNO was linearly dependent on $[^{\bullet}NO]^2$, and determined $k_1 = 7.3 \times 10^6$ and 8.1×10^6 M^{-2} s^{-1} for CysSH and GSH, respectively. However, under limiting concentrations of O_2, the third-order rate constant cannot exceed k_1, and should be $(2-2.9) \times 10^6$ M^{-2} s^{-1},[3,9,13,25,32] independent of the kind of substrate present. Wink et al.[12] did not calculate the concentration of $^{\bullet}NO$ in their stock solutions from the known solubility of $^{\bullet}NO$ in aqueous solution, but determined it with the use of ABTS, assuming that this reaction gives a 1:1 ratio between $ABTS^+$ and $^{\bullet}NO$. However, we have recently shown that this ratio is only 0.6:1,[25] and hence the concentration of $^{\bullet}NO$ in their stock solutions is 1.67 times higher ($[^{\bullet}NO]_{real} = 1.67[^{\bullet}NO]_{wink}$). As a result, their values for k_1 should be divided by $(1.67)^2 = 2.79$, and the corrected values are 2.6×10^6 and 2.9×10^6 M^{-2} s^{-1} for CysSH and GSH, respectively, in agreement with our results. Furthermore, they stated that, under limiting concentrations of O_2, the formation of RSNO was first-order, but the traces in their paper under these conditions follow second-order kinetics.

Wink et al.[12] measured the absorbance of RSNO as a function of $[^{\bullet}NO]$ in aerated solutions, and determined $\epsilon_{338}(max) = 1400 \pm 200$ and 1300 ± 200 M^{-1} cm^{-1} (per molar $^{\bullet}NO$) for GSNO and CysSNO, respectively. However, from the values given in their figures, one calculates that $\epsilon_{338} = 960$ and 970 M^{-1} cm^{-1} (per molar $^{\bullet}NO$) for GSNO and CysSNO, respectively. Thus, using $[^{\bullet}NO]_{real}$, the corrected values of ϵ_{338} are 575 and 581 M^{-1} cm^{-1}, respectively. We have demonstrated that 1 mol of $^{\bullet}NO$ forms 0.5 mol of RSNO, and therefore according to Wink's results, correcting the stoichiometry and the concentration, the real values of ϵ_{338} are 1150 and 1162 M^{-1} cm^{-1} for GSNO and CysSNO, respectively. These values are higher than those determined directly. We suspect that their method of mixing the solutions is less accurate than the stopped-flow technique as they added $^{\bullet}NO$ solutions via a gas-tight syringe to aerated stirred solutions containing the thiol at pH 7.4.

Their conclusion that NO_x is the nitrosating species was based on competitive kinetic studies. They measured the nitrosation yield as a function of [RSH] at constant $[^{\bullet}NO]$ and $[O_2]$ and as a function of [azide] at constant [RSH], $[^{\bullet}NO]$, and $[O_2]$. However, as noted above, their yields differ from those measured using the stopped-flow technique, and do not agree with the known extinction coefficients of RSNO and with the stoichiometry of the whole nitrosation process. Therefore, their proposal of an unknown NO_x has no justified basis.

Wink et al.[12] noted that, in the presence of 1 mM O_2, 0.09 mM $^{\bullet}NO$, and 1 mM GSH or CysSH, $k = 5 \times 10^3$ M^{-1} s^{-1} assuming $\epsilon_{338} = 1400$ M^{-1} cm^{-1}, and hence the third-order rate constant is 5×10^6 M^{-2} s^{-1}. If we use the correct value of $\epsilon_{338} = 760$ M^{-1} cm^{-1}, the third-order rate constant will be 2.7×10^6 M^{-2} s^{-1}, which is about an order of magnitude lower than our values (Table 2), and an order of magnitude lower than the rate of the autoxidation of $^{\bullet}NO$. In the same study, Wink et al.[12] reported that the rate of the decay of $^{\bullet}NO$ was not affected by the addition of 1 mM thiol, which supports our results and mechanism, and contradicts their experimental value.

Note Added in Proof. After submission of this paper, a related paper appeared: Kharitonov, V. G.; Sundquist, A. R.; Sharma, V. S. *J. Biol. Chem.* **1995**, *270* (47), 28158−28164.

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