The objective of this study was to investigate the mechanism of S-nitrosothiol formation under physiological conditions. A mechanism is proposed by which nitric oxide (NO) reacts directly with reduced thiol to produce a radical intermediate, R-S-N-O-H. This intermediate reduces an electron acceptor to produce S-nitrosothiol. Under aerobic conditions O2 acts as the electron acceptor and is reduced to produce superoxide (O2−). The following experimental evidence is provided in support of this mechanism. Cysteine accelerates the consumption of NO by 2.5-fold under physiological conditions. The consumption of O2 in the presence of NO and cysteine is increased by 2.4-fold. The reaction orders of NO and cysteine are second and first order, respectively. The second order of reaction for NO may result from interaction between NO and O2 to form peroxynitrite. In the presence of Cu,Zn-superoxide dismutase, the reaction of NO with cysteine generates hydrogen peroxide, indicating that the reaction generates O2−. Finally, the formation of S-nitrosothiol is demonstrated in an anaerobic environment and, as predicted by the mechanism, is dependent on the presence of an electron acceptor. These results demonstrate that under physiological conditions NO reacts directly with thiols to form S-nitrosothiol in the presence of an electron acceptor.

S-Nitrosothiols are important physiological regulators capable of producing vasodilation and inhibition of platelet aggregation (1–4). An increasing number of proteins such as albumin, glutathione, and p21ras have been found to be S-nitrosylated in vivo (5–9). With the discovery of new S-nitrosylated proteins, it becomes evident that the formation of S-nitrosothiols may be important in such diverse processes as signal transduction, DNA repair, and blood-pressure regulation. However, at present the mechanism of the biosynthetic pathway for the formation of S-nitrosothiols is unclear (10).

It has been shown previously that the reaction of NO1 with sulfhydryl groups under anaerobic conditions at neutral pH does not produce S-nitrosothiol (11–13). This has led to the conclusion that S-nitrosothiols are formed by the autoxidation of NO, a second order reaction with respect to NO, to higher oxides of nitrogen (NOx), by metal catalysis (12, 14, 15), or by the action of dinitrosyl-iron complexes (16). However, the reaction of NO and oxygen is slow, approximately 3 to 300 pmol/s, at physiological concentrations of NO (0.1–1.0 μM), and the availability of redox metal is unclear (17, 18). Although dinitrosyl-iron complexes represent one possible mechanism for the formation of S-nitrosothiols, the reaction mechanism under physiological conditions also remains unclear (10).

Here we propose a novel mechanism for S-nitrosothiol formation that would operate at physiological concentrations of NO. In this mechanism NO reacts directly with a reduced thiol to produce a radical intermediate, R–S–N–O–H (Equation 1). In the presence of an electron acceptor, such as oxygen, this intermediate can be converted to S-nitrosothiol by the reduction of the acceptor.

The reaction of NO and cysteine in buffer under aerobic conditions will form superoxide via the reduction of O2− by R–S–N–O–H (Equation 2). Superoxide reacts at a nearly diffusion-limited rate with NO to form peroxynitrite (19) (Equation 3). The overall reaction mechanism is shown below (Equation 4).

\[
R - SH + NO \rightleftharpoons R-S-N-O-H \quad (Eq. 1) \\
R-S-N-O-H + O_2 \rightarrow R-S-N + O + O_2 \quad (Eq. 2) \\
O_2 + NO \rightarrow ONOO^- \quad (Eq. 3) \\
R-SH + 2'NO + O_2 \rightarrow R-S-N + O + ONOO^- \quad (Eq. 4)
\]

From this reaction mechanism the following testable predictions can be made: first, that free thiol will accelerate the decomposition of NO and will result in the formation of a NO donor; second, that thiol will accelerate the consumption of O2 by NO and generate H2O2 in the presence of Cu,Zn-superoxide dismutase; and third, that the reaction will proceed under anaerobic conditions in the presence of an electron acceptor. Here experimental evidence is provided for each of the above predictions in support of the proposed mechanism.

EXPERIMENTAL PROCEDURES

Materials—Cu,Zn-superoxide dismutase was obtained from Fluka (Switzerland), and DEANO was obtained from Cayman Chemical (Ann Arbor, MI). All other chemicals were obtained from Sigma. Spectrophotometric measurements were made using a UV diode array spectrophotometer (Hewlett-Packard), and NO in solution was measured using a

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’NO-specific electrode (World Precision Instruments, Sarasota, FL).

Synthesis and Measurement of NO—Nitric oxide was synthesized by bubbling nitrogen through KNO₂ that was acidified with HCl. Nitric oxide and other nitrogen oxides formed in the reaction flask were forced through a gas-washing column containing 1 M NaOH. Nitric oxide was then collected in a nitrogen-purged, sealed vessel containing double distilled H₂O that was passed through a Chelex-100 column to remove metal contaminants.

Nitric oxide measurements were continuously monitored by means of a specific electrode in a 1-ml solution of PBS, pH 7.4, 100 μM DTPA at a constant stirring velocity and at constant temperature (22 °C) and in the presence or absence of cysteine. All measurements were made in an open system at a constant stirring speed and temperature (22 °C). Oxygen was continuously monitored by electrochemical reduction using a recessed micro-electrode with a gold cathode polarized at –0.65 V relative to an Ag/AgCl reference electrode (20). The current sensitivity of the micro-electrode was 1.2 pA/μM. Hydrogen peroxide was measured by electrochemical oxidation using a 25-μm diameter platinum wire sealed in glass that was polarized at 0.65 V relative to an Ag/AgCl reference electrode (21). The electrode was covered with a thin membrane by dip coating in a solution of 5% Nafion dissolved in aliphatic alcohols (Aldrich). The current sensitivity of the electrode was 0.32 nA/μM. Hydrogen peroxide was also measured using the horseradish peroxidase-mediated oxidation of o-phenylenediamine; 25 μg of horseradish peroxidase and 1 mM o-phenylenediamine were added to a 2-ml reaction mixture, and the mixture was vortexed and incubated at room temperature for 30 min. The mixture was acidified by adding 60 μl of 5 M HCl, and the absorbance at 420 nm was measured (22).

Reaction of NO and Cysteine in an Anaerobic Environment—2 ml of PBS, 100 μM DTPA was placed in a 4-ml sealed vial. The solution was degassed by bubbling through N₂ for approximately 30 min. 750 μM cysteine and/or 10 mM Na⁺ were added to the solution prior to degassing. After degassing, 2 μl of 37 mM DEANO, stored under N₂, was injected into the vial via a gas-tight syringe with a Teflon seal. The solution was incubated at room temperature for 30 min. Under these conditions DEANO decomposes to release NO at a rate of approximately 6 μM/min (23). After incubation the vial was unstoppered and 1 ml of the solution was monitored for the release of NO by electrode as described above. Another 1 ml was placed immediately in a quartz cuvette, and absorbance at 336 nm was measured.

RESULTS AND DISCUSSION

Cysteine Accelerates the Decomposition of NO in Solution—In order to test the prediction that thiol will accelerate the decomposition of NO, we examined the loss of NO from an open aerobic system in the presence and absence of cysteine. Fig. 1 shows that cysteine accelerates the decomposition of NO from buffer under aerobic conditions. In the absence of cysteine the initial rate of NO loss was approximately 1.6 nM/s, whereas in the presence of cysteine the rate of NO loss was approximately 3.8 nM/s. These experiments were performed at a constant stirring speed and temperature (22 °C) and in the presence of a metal chelator. We have previously found that these factors are critical in determining the rate of loss of NO in an open system.⁵

The acceleration of NO decomposition is dependent on the relative concentrations of NO and cysteine. It was necessary for the thiol concentration to exceed the NO concentration by approximately 100-fold. In addition, the effect of thiol on NO decay was only relevant at NO concentrations of less than 50 μM. At higher concentrations of NO the addition of thiol did not alter the decomposition of NO, as has been reported previously (12). The reaction of NO with O₂, which is second order with respect to NO, becomes increasingly relevant at higher concentrations of NO (17). Therefore, at higher concentrations of NO a NO₂⁻-based mechanism for S-nitrosothiol formation and other nitrosation reactions becomes increasingly relevant (12, 14, 24).

Orders of Reaction for Cysteine and NO—The apparent orders of reaction for the loss of NO were calculated from the initial rate of decay measured at varying concentrations of NO and cysteine. Fig. 2 shows the plots of log[(d[NO]/dt)] versus log[substrate], where (d[NO]/dt) is expressed as the initial rate of decay in the presence of cysteine minus the initial rate in the absence of cysteine. As stirring velocity, ionic strength, and temperature are kept constant the difference between the initial rates in the presence and absence of cysteine represents the rate of reaction of NO with cysteine. The slopes of the linear regression lines shown in Fig. 2 represent the apparent orders of reaction for NO (Fig. 2A) and cysteine (Fig. 2B). The reaction orders for NO and cysteine approximate to second and first order, respectively. The apparent second order for NO can be explained by the production of O₂⁻ upon decomposition of the radical intermediate. Superoxide will react with a second molecule of NO in an almost instantaneous fashion.

The Product of the Reaction Is S-Nitrosocysteine—Spectrophotometric examination of the reaction mixture containing cysteine and NO reveals an absorbance peak at 336 nm, which is typical of S-nitrosothiol (5). The absorbance at 336 nm is dependent on the quantity of NO added (Fig. 3A). However, the product yield expected from these experiments is very low, on the order of 1 μM or less, because of the low concentrations of NO in the reaction mixture. In addition, the molar absorptivity of S-nitrosothiol is low, 3869 M⁻¹ cm⁻¹. As a result the absorbances measured are at the limit of spectrophotometric detection and are hence quite variable. In order to confirm that the product of the reaction was S-nitrosothiol, we assayed its ability to act as a NO donor, a recognized property of S-nitrosothiol.

Cysteine-based nitrosothiols release NO in the presence of reduced thiol (25, 26). In our experiments, cysteine was in approximately 100-fold excess and, therefore, any S-nitrosothiol formed will readily decompose to release free NO. Fig. 3B shows that the reaction of NO and cysteine results in the

⁵ Gow, A. J., Thom, S. R., Brass, C., and Ischiropoulos, H. (1997) Microchem. J., in press.
production of a rapidly decaying NO donor. In this reaction NO is added to PBS either in the presence or absence of cysteine. After 1.5 min the reaction mixture is vortexed to remove any residual NO and then monitored for release of NO by electrode. In the absence of cysteine no release is measured, whereas in the presence of cysteine a peak of NO is observed. The presence of the characteristic S-nitrosothiol absorbance and the ability of the product to release NO indicate that the reaction of physiological concentrations of NO and cysteine forms S-nitrosocysteine.

Oxygen Consumption and Superoxide Production—In order to test the second prediction of the model, the consumption of O2 by the decomposition of NO as measured by the electrode at the time of cysteine addition (t = 45 s). The rate of NO decomposition is the initial rate of decline in NO concentration upon addition of cysteine and is expressed as moles per second. A, varying concentrations of NO were treated with 750 µM cysteine in 1 ml of PBS, 100 µM DTPA. B, varying concentrations of cysteine were added to 1 ml of PBS, 100 µM DTPA containing 4 µl of 1.5 mM NO.

To further confirm that superoxide was generated by the reaction of thiol and NO, the production of H2O2 in the presence of Cu,Zn superoxide dismutase was monitored by means of a specific electrode (21). The addition of approximately 6 µM NO had no effect on the current within the electrode in the absence of cysteine. However, in the presence of 750 µM cysteine the mixture was added to 1 ml of PBS, 100 µM DTPA, and 6 µM NO. The mixture was incubated for 1.5 min at 22 °C and vortexed vigorously. The mixture was then monitored for NO by electrode. Solid line represents no addition of cysteine, and dashed line represents addition of cysteine.

Formation of S-Nitrosocysteine under Anaerobic Conditions—In order to confirm the final prediction of the model, NO was reacted with cysteine in an anaerobic environment in the presence of an electron acceptor, NAD+.

FIG. 2. Reaction orders of NO and cysteine. Substrate concentrations are given as log moles per liter and represent concentration of NO as measured by the electrode at the time of cysteine addition (t = 45 s). The slope of the regression line was 0.59 ± 0.018 (n = 4). In the absence of either cysteine (A420 = 0.018) or superoxide dismutase (A420 = 0.020), oxidation of o-phenylenediamine was not observed. These results indicate that the reaction of NO and cysteine in the presence of O2 produces superoxide.

FIG. 3. A, varying quantities of synthesized NO were added to buffer as above. After peak electrode output was reached, 7.5 µl of 10 mM cysteine was added. Two minutes after cysteine addition the solution was removed from the electrode and measured for absorbance at 336 nm in a UV-visible spectrophotometer. There is a significant correlation between NO added and absorbance at 336 nm (p < 0.05). B, 2.5 µl of 10 mM cysteine was added to 1 ml of PBS, 100 µM DTPA, and 6 µM NO. The mixture was incubated for 1.5 min at 22 °C and vortexed vigorously. The mixture was then monitored for NO by electrode. Solid line represents no addition of cysteine, and dashed line represents addition of cysteine.
the presence of 10 mM NAD+ the reaction of ‘NO and cysteine produced a compound that releases ‘NO at a steady rate. The decay of DEANO over the course of the anaerobic period will release approximately 18 µM ‘NO. However, the vial contains both a gaseous and an aqueous phase, so a considerable portion (approximately 80%) of the ‘NO released by DEANO may be in the gaseous phase. The ‘NO measured in solution after the anaerobic period is most likely the residual dissolved material left from the decomposition of DEANO. It is possible that some of the DEANO remains intact and is, therefore, releasing ‘NO after the anaerobic period. However, the half-life of DEANO under these conditions is 2 min and thus it is unlikely that a significant quantity of intact DEANO remains after 30 min (23).

In the absence of either cysteine or NAD+ the rate of loss of ‘NO from the buffer immediately after the anaerobic incubation period was 6.7 and 6.0 nM/s, respectively. This rate probably represents the loss of dissolved ‘NO from the solution. In contrast, the rate of loss of ‘NO was only 3.1 nM/s in the presence of cysteine and NAD+, indicating the presence of a ‘NO donor. The addition of excess reduced thiols confirmed the presence of a ‘NO donor, as it elicited an increase in the concentration of ‘NO within the reaction mixture only when both cysteine and NAD+ were present. Spectrophotometric analysis immediately after anaerobic incubation reveals that the product of the reaction possesses an absorbance peak of 0.05 unit at 336 nm, equivalent to 11 µM S-nitrosothiol. Previous work has shown that the anaerobic interaction of glutathione and ‘NO in the absence of an electron acceptor proceeds through the same radical intermediate but that the products are disulfide and N2O (13). These results indicate that S-nitrosothiol can be formed from the anaerobic reaction of cysteine and ‘NO only in the presence of an electron acceptor.

Previous work has shown that it is possible to form S-nitrosothiol via mechanisms involving either ‘NO reaction with molecular O2 or metal catalysis (12, 14, 15). However, at the concentrations of ‘NO used in this study, there is little or no production of higher oxides of nitrogen, and interaction with metals was avoided by using a metal chelator. Therefore, the formation of S-nitrosothiols under the conditions used in this study shows that S-nitrosothiols can be formed by a novel mechanism. An alternative to the mechanism proposed within this paper is that ‘NO reacts with oxygen to form a nitrosyl-dioxyl radical (Equation 5). The nitrosyl-dioxyl radical then reacts with a reduced thiol to produce S-nitrosothiol and superoxide (Equation 6).

\[
{\text{NO} + {\text{O}}_2 \rightarrow {\text{ONOO}}} \tag{5}
\]

\[
{\text{ONOO}}^- + {\text{R-SH}} \rightarrow \text{R-S-N} = {\text{O}} + {\text{O}}_2 \tag{6}
\]

This superoxide could then react with another molecule of ‘NO to produce peroxynitrite (Equation 3). This reaction mechanism would result in the same overall reaction equation described above (Equation 4). Therefore, this mechanism could explain all the data shown here except for the formation of S-nitrosothiol under anaerobic conditions, as oxygen is a requirement.

The reactions described here are significant because they operate at low concentrations of ‘NO and thus allow for the formation of S-nitrosothiols under physiological conditions. Our results and the already proven mechanisms of S-nitrosothiol formation indicate that S-nitrosothiols can be formed in vivo under a wide variety of pathophysiological conditions.

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