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On the Distinction between Nitroxyl and Nitric Oxide Using Nitronyl Nitroxides

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Abstract: A better understanding of the origins of NO and HNO and their activities and biological functions requires accurate methods for their detection and quantification. The unique reaction of NO with nitronyl nitroxides such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (C-PTIO), which yields the corresponding imino nitroxides, is widely used for NO detection (mainly by electron paramagnetic resonance spectroscopy) and for modulation of NO-induced physiological functions. The present study demonstrates that HNO readily reacts with nitronyl nitroxides, leading to the formation of the respective imino nitroxides and hydroxylamines via a complex mechanism. Through the use of the HNO donor Angeli's salt (AS) with metmyoglobin as a competing agent, the rate constant for C-PTIO reduction by HNO has been determined to be $(1.4 \pm 0.2) \times 10^5$ M^{-1} s^{-1} at pH 7.0. This reaction yields the corresponding nitronyl hydroxylamine C-PTIO-H and NO, which is trapped by C-PTIO to form $^{\bullet}NO_2$ and the corresponding imino nitroxide, C-PTI. $^{\bullet}NO_2$ oxidizes the nitronyl and imino nitroxides to their respective oxoammonium cations, which decay mainly via comproportionation with the nitronyl and imino hydroxylamines. When [AS] > [C-PTIO], the reduction of C-PTI by HNO proceeds, eventually converting C-PTIO to the corresponding imino hydroxylamine, C-PTI-H. Similar results were obtained for 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). It is concluded that nitronyl nitroxide is readily reduced by HNO to nitronyl hydroxylamine and is eventually converted into imino nitroxide and imino hydroxylamine. The yield of the imino hydroxylamine increases at the expense of the imino nitroxide as the ratio $[AS]_0/[\text{nitronyl nitroxide}]_0$ is increased. Since the reaction of NO with nitronyl nitroxide yields only the corresponding imino nitroxide, nitronyl nitroxide can discriminate NO from HNO only when present at a concentration much lower than the total production of HNO.

Introduction

Nitric oxide (NO) is an important mediator of both physiological and pathophysiological processes.[1] HNO, the 1-electron-reduced and protonated product of NO, has been suggested to be formed in cellular milieu via several routes.[2] A better understanding of the origins of NO and HNO and their activities and biological functions requires accurate methods for their detection and quantification. Electron paramagnetic resonance (EPR) methods have been developed that enable the stabilization of NO using endogenous and exogenous spin traps, such as iron *N*-methyl-D-glucamine dithiocarbamate[3−6] and nitronyl nitroxides.[7−10] It has been argued that iron *N*-methyl-D-glucamine dithiocarbamate can distinguish NO from HNO,[11] although the specificity of this method has been questioned.[12]

The most widely used nitronyl nitroxides are 2-phenyl-4,4,5,5-tetramethylimidazoline-l-oxyl 3-oxide (PTIO) and its water-soluble analogue 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (C-PTIO). The unique reaction 1 of nitronyl nitroxides with NO yields the corresponding imino nitroxides, which are detectable and distinguishable by EPR spectrometry.[7−10]

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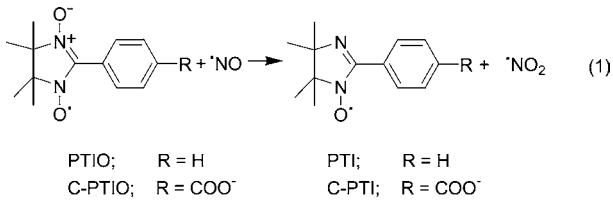

Nitronyl nitroxides have also been widely employed as specific NO scavengers in various experimental in vitro and in vivo models[13−21] to attenuate[17,22−31] or even, surprisingly, potentiate[19,32] NO-induced physiological effects. Conversely, the failure of C-PTIO to affect a system was taken as proof excluding the intermediacy of NO.[33] Several studies even proposed a therapeutic potential of nitronyl nitroxides against NO-induced neurotoxicity.[15,34,35] However, some of the observations could not be readily settled with the reported selectivity of nitronyl nitroxides toward NO.[25,32] In cases where NO or HNO donors induced similar biological effects, the use of C-PTIO could not clearly distinguish NO from HNO.[16,30] It was therefore suggested that HNO is oxidized to NO by C-PTIO, albeit slightly,[16] but later that reaction was examined and excluded.[36] However, the feasibility of HNO oxidation by nitronyl nitroxides is supported by the observation that HNO readily reduces 4-hydroxyl-2,2,6,6 tetramethylpiperidine-*N*-oxyl (Tempol),[37] which at pH 7.0 has a lower reduction potential than that of PTIO/C-PTIO (i.e., $E_{1/2} = 130$ compared with 270 mV vs NHE, respectively).[38,39]

We previously reported that the mechanism of the reaction of NO with C-PTIO and PTIO involves the oxidation of PTIO and C-PTIO by $^{\bullet}NO_2$ to their respective oxoammonium cations, which can be reduced back to the parent nitroxides by NO.[39] Here we demonstrate that C-PTIO and PTIO are readily reduced by HNO and eventually converted into their respective imino nitroxides or imino hydroxylamines.

Materials and Methods

All of the chemicals were of analytical grade and used as received. Water for preparation of the solutions was purified using a Milli-Q purification system. Angeli's salt ($Na_2N_2O_3$, AS) and C-PTIO were purchased from Cayman Chemical Co. and PTIO from ALEXIS Biochemicals. A stock solution of AS was prepared in 10 mM NaOH, and the concentration was determined by the absorbance at 248 nm ($\varepsilon = 8$ mM^{-1} cm^{-1}). Metmyoglobin ($MbFe^{III}OH_2$) was prepared by addition of excess ferricyanide to myoglobin in 10 mM phosphate buffer (PB) at pH 7.0 followed by chromatographic separation through a Sephadex G-25 column. The concentration of $MbFe^{III}OH_2$ was determined spectrophotometrically using $\varepsilon_{408} = 188$ mM^{-1} cm^{-1}.[40] NO was purchased from Matheson Gas Products and purified by passing it through a series of scrubbing bottles containing deaerated 50% NaOH and purified water in that order. NO solutions were prepared in gas-tight syringes containing 10 mM PB, and the concentration of NO (1.9 mM/atm at 22 °C) was accurately determined on the basis of its known temperature- and pressure-dependent solubility in aqueous systems.

C-PTI was prepared by mixing NO-saturated solution with a deaerated solution of C-PTIO in 10 mM PB at pH 7.0. Oxidized and reduced nitronyl and imino nitroxides were prepared electrochemically. Briefly, the electrochemical cell consisted of a working electrode of graphite grains packed inside a porous Vycor glass tube (5 mm i.d.) through which the solution of nitronyl or imino nitroxides in 10 mM PB (pH 7.0) was pumped (2−4 mL min^{-1}). An outer glass cylinder contained 10 mM PB in which a Pt auxiliary electrode and a Ag/AgCl (3.5 M) reference electrode were immersed. A BAS100B Electrochemical Analyzer was used to control the voltage.

Oxidation of imino hydroxylamines was achieved either electrochemically or by addition of 1 mM ferricyanide as described previously.[41] In systems containing residual AS, a mixture of 10 mM H_2O_2 and 5 μM $CuSO_4$ was added prior to the addition of ferricyanide.

Cyclic voltammetry (CV) was performed with a BAS100B electrochemical analyzer using a three-electrode system consisting of a glassy carbon working electrode, a Pt wire auxiliary electrode, and a Ag/AgCl (3.5 M) reference electrode.

Stopped-flow kinetic measurements were carried out using an Applied Photophysics Bio SX-17MV sequential stopped-flow apparatus having a 1 cm optical path.

Kinetic measurements were carried out at 25 °C using an HP 8452A diode-array spectrophotometer coupled with a thermostat (HP 89075C Programmable Multicell Transport).

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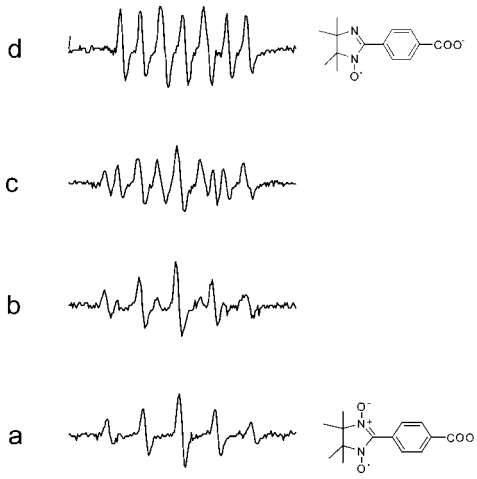

Figure 1. Conversion of C-PTIO to C-PTI upon exposure of 170 μM C-PTIO to 350 μM AS in an aerated solution containing 40 mM PB at pH 7.0: (a) $t = 0$, gain = 7.9; (b) $t = 1$ min, gain = 7.9; (c) $t = 8$ min, gain = 16; (d) $t = 38$ min, gain = 20. A spectrum identical to (d) was observed at $t = 57$ min with and without the addition of 1 mM ferricyanide.

EPR spectra were recorded using a Varian E-9 spectrometer and repeated using JEOL X-band JES-RE3X and Bruker X-band spectrometers operating at 9.25 GHz with the following settings: center field, 3290 G; modulation frequency, 100 kHz; modulation amplitude, 1 G; time constant, 0.128 s; field sweep, 25 G/min; incident microwave power, 4−16 mW. The reaction mixture was transferred to a gas-permeable Teflon capillary (Zeus Industries, Orangeburg, SC) having an i.d. of 0.81 mm, a wall thickness of 0.38 mm, and a length of 15 cm. Each capillary was folded twice, inserted into a narrow quartz tube that was open at both ends (2.5 mm inner diameter), and placed within the EPR cavity. All of the experiments were carried out at room temperature.

Results and Discussion

The reaction of HNO with PTIO and C-PTIO was studied using the HNO donor AS[42,43] in aerated solutions at pH 7.0. Upon exposure of 170 μM C-PTIO to 350 μM AS, the seven-line EPR spectrum of C-PTI appeared at the expense of the five-line EPR spectrum of C-PTIO (Figure 1). Figure 1b,c shows the buildup of the nine-line EPR spectrum indicative of a mixture of C-PTIO and C-PTI. Upon exposure of 180 μM C-PTIO to 1 mM AS, the EPR spectrum of C-PTI appeared and was progressively lost during the decay of AS (Figure 2a−d). However, C-PTI was fully restored upon oxidation (Figure 2e), indicating that the EPR-silent final product was C-PTI-H. The same results were observed in the case of PTIO. Huang et al.[36] reported that AS at 10 μM had no effect on the EPR spectrum of C-PTIO at 4 μM. However, a closer examination of their findings clearly reveals the emergence of the C-PTI signal.

Knowledge of the spectral characteristics of C-PTIO, C-PTI, and their reduced and oxidized forms (Figure 3) also enables the study of the reaction of HNO with C-PTIO through monitoring of the UV−vis absorption of C-PTIO upon exposure to AS. Figure 4 shows the decay of C-PTIO (190 μM) to C-PTI upon exposure to 350 μM AS in an aerated solution at pH 7.0. The addition of 1 mM ferricyanide did not restore the absorption

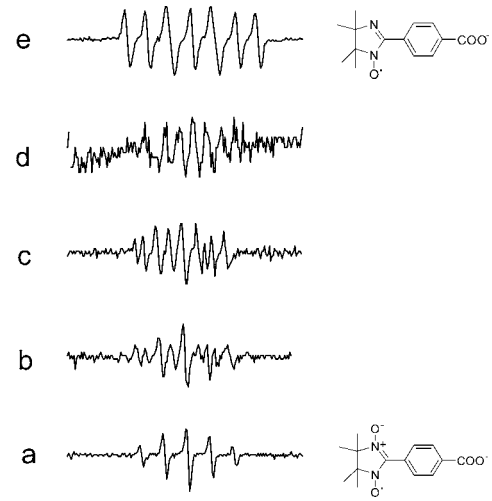

Figure 2. Conversion of C-PTIO to C-PTI-H upon exposure of 180 μM C-PTIO to 1 mM AS in an aerated solution containing 100 mM PB at pH 7.0: (a) $t = 0$, gain = 12.5; (b) $t = 4$ min, gain = 12.5; (c) $t = 8$ min, gain = 40; (d) $t = 28$ min, gain = 400; (e) $t = 62$ min, gain = 12.5, obtained after the addition of $CuSO_4/H_2O_2$/ferricyanide.

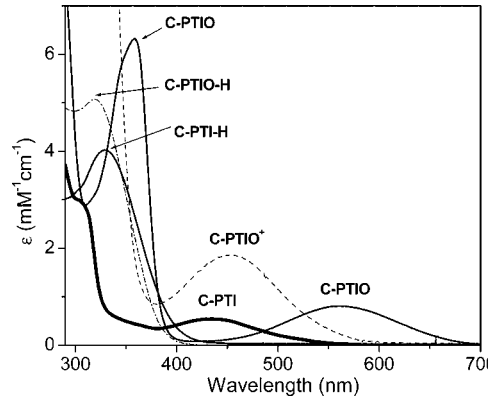

Figure 3. UV−vis spectra of C-PTI, C-PTI-H, C-PTIO, C-PTIO-H, and C-PTIO$^+$. C-PTI, C-PTI-H, C-PTIO-H, and C-PTIO$^+$ were prepared as described in Materials and Methods.

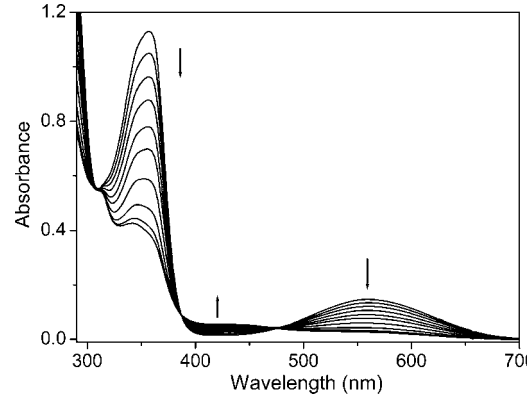

Figure 4. UV−vis spectra obtained upon exposure of 190 μM C-PTIO to 350 μM AS in an aerated solution containing 40 mM PB (pH 7.0, 25 °C) at $t = 30$, 60, 90, 130, 190, 250, 350, 450, 550, and 1100 s.

at 560 nm, indicating that C-PTIO-H did not accumulate during this process. The AS-induced decay of C-PTIO followed at 560 nm and 25 °C obeyed first-order kinetics with a rate constant of $k = (2.6 \pm 0.2) \times 10^{-3}$ s^{-1}, whereas the decay of AS alone followed at 240 nm demonstrated $k = (1.25 \pm 0.05) \times 10^{-3}$

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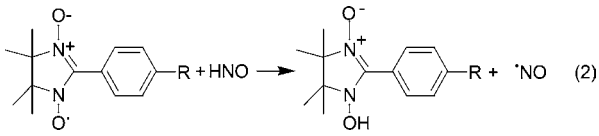
s^{-1}. These rate constants were unaffected by the presence of 100 μM diethylenetriaminepentaacetic acid (DTPA).

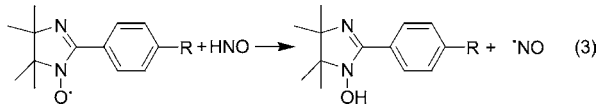
The results described above demonstrate that C-PTIO and PTIO are fully converted to the respective imino nitroxides and imino hydroxylamines upon exposure to AS. We suggest that the initiating reaction of nitronyl nitroxide with HNO yields the corresponding nitronyl hydroxylamine and NO (reaction 2):



(2)

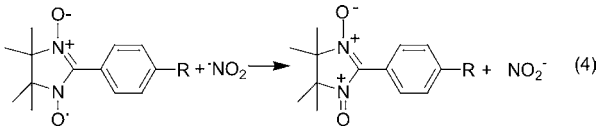
The NO is then trapped by nitronyl nitroxide to form the corresponding imino nitroxide and $^{\cdot}NO_2$ (reaction 1).

The conversion of the imino nitroxide to imino hydroxylamine occurred when the nitronyl nitroxide was exposed to 1 mM AS but not to 0.35 mM AS, suggesting that the imino nitroxides are reduced by the excess HNO to their corresponding imino hydroxylamines (reaction 3):



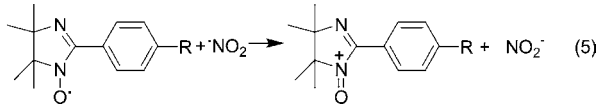
(3)

C-PTIO was fully converted to C-PTI and C-PTI-H, indicating that C-PTIO-H, which is formed via reaction 2, does not accumulate. The possibility that $^{\cdot}NO_2$, which is formed via reaction 1, oxidizes C-PTIO-H to C-PTIO has been proposed previously.[41,44] However, this reaction proceeds slowly ($k \leq 1 \times 10^5$ M^{-1} s^{-1})[41,45] and cannot efficiently compete with the oxidation of PTIO and C-PTIO by $^{\cdot}NO_2$ to their respective oxoammonium cations $PTIO^+$ and C-$PTIO^+$ [reaction 4; $k_4 = (1.5 - 2.0) \times 10^7$ M^{-1} s^{-1}].[39]



(4)

The CV of an aerated solution containing 0.25 mM C-PTI, 0.1 M KNO_3, and 10 mM PB (pH 7.0) exhibited a reversible oxidation of C-PTI to C-PTI^+ having $E_{1/2} = 550 \pm 7$ mV vs NHE. This value is significantly lower than that for C-PTIO (936 $\pm$ 21 mV vs NHE),[39] suggesting that k_5, the rate constant for reaction 5, might be even larger than k_4.



(5)

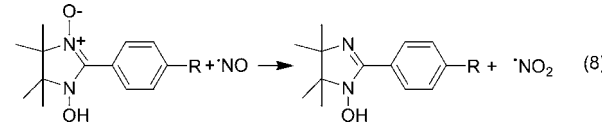
The failure of C-PTIO-H to accumulate could be due to its reaction with C-$PTIO^+$ (reaction 6) and/or C-PTI^+ (reaction 7):

$$\text{PTIO-H} + \text{PTIO}^+ \rightarrow 2\text{PTIO} + \text{H}^+ \qquad (6)$$

$$\text{PTIO-H} + \text{PTI}^+ \rightarrow \text{PTIO} + \text{PTI} + \text{H}^+ \qquad (7)$$

We have previously shown that the comproportionation in the case of cyclic nitroxides (e.g., Tempol) proceeds mainly through the deprotonated form of the corresponding hydroxylamines.[45] Since C-PTIO-H ($pK_a = 3.5$)[39] is a stronger acid than Tempol-H ($pK_a = 6.9$),[45] the apparent rate constants of reactions 6 and 7 are expected to be significantly larger than that of Tempol-H comproportionation with its corresponding oxoammonium cation at pH 7.0 (i.e., 24 M^{-1} s^{-1} [45]).

In addition to reactions 6 and 7, C-PTIO-H having a nitronyl group can react with NO similarly to C-PTIO, forming C-PTI-H and $^{\cdot}NO_2$ (reaction 8):



(8)

To support or exclude the proposed mechanism, the kinetics of reactions 2, 6, 7, and 8 were studied. Reaction 2 was studied using competition kinetics against metmyoglobin (reaction 9):

$$\text{MbFe}^{\text{III}}\text{OH}_2 + \text{HNO} \rightarrow \text{MbFe}^{\text{II}}\text{NO} + \text{H}^+ + \text{H}_2\text{O} \qquad (9)$$

The addition of 44 μM AS to 10 or 30 μM $MbFe^{III}OH_2$ in aerated buffered solutions resulted in the same initial rate of $MbFe^{II}NO$ formation, which was followed at 425 nm. This observation implies that under such experimental conditions the self-decomposition of HNO ($k = 8 \times 10^6$ M^{-1} s^{-1})[46] and its reaction with O_2 ($k \sim 3 \times 10^3$ M^{-1} s^{-1})[37] are negligible. The initial rate of $MbFe^{II}NO$ formation was measured upon the addition of 44 μM AS to aerated buffered solutions containing 10 μM $MbFe^{III}OH_2$ in the absence (V_0) and presence (V) of various concentrations of C-PTIO or PTIO. The effect of added nitronyl nitroxide on the initial rate follows eq 10 if only reactions 2 and 9 take place:

$$\frac{1}{V} = \frac{1}{V_0} + \left(\frac{k_2}{k_9 V_0}\right)\frac{[\text{nitronyl nitroxide}]}{[\text{MbFe}^{\text{III}}\text{OH}_2]} \qquad (10)$$

Accordingly, a plot of $V_0/V - 1$ versus [nitronyl nitroxide] should be linear, as demonstrated in Figure 5. The slope of the line in Figure 5 yields $k_2/k_9 = 0.18 \pm 0.02$ for both PTIO and C-PTIO, and thus, $k_2 = (1.4 \pm 0.2) \times 10^5$ M^{-1} s^{-1} using $k_9 = 8 \times 10^5$ M^{-1} s^{-1}.[37]

The kinetics of reactions 6 and 7 were studied under limiting concentrations of C-$PTIO^+$ or C-PTI^+ using the stopped-flow technique. The decays of C-$PTIO^+$ and C-PTIO-H were followed at 450 and 320 nm, respectively. Both reactions obeyed pseudo-first-order kinetics, and k_{obs} was linearly dependent on [C-PTIO-H], yielding $k_6 = (1.2 \pm 0.1) \times 10^6$ M^{-1} s^{-1} and $k_7 = (2.1 \pm 0.3) \times 10^7$ M^{-1} s^{-1} (Figure 6).

The kinetics of the reaction of C-PTIO-H with NO was studied using the stopped-flow technique under limiting concentrations of C-PTIO-H by following its decay at 320 nm. The decay of the absorption obeyed pseudo-first-order kinetics, and k_{obs} was linearly dependent on [NO], yielding $k_8 = (1.7 \pm 0.2) \times 10^3$ M^{-1} s^{-1} (Figure 7). The product of this reaction was identified as C-PTI-H, as it was oxidized electrochemically to C-PTI, where $[\text{C-PTI}] = [\text{C-PTIO-H}]_0$. This result indicates that

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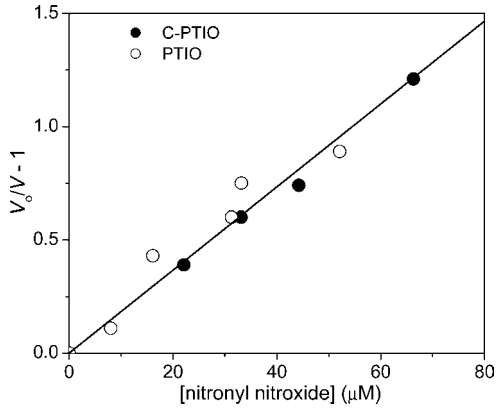


Figure 5. Effects of nitronyl nitroxide on the initial rate of $MbFe^{II}NO$ formation analyzed according to eq 10. The rate of $MbFe^{II}NO$ formation was measured at 425 nm upon exposure of 10 μM $MbFe^{III}OH_2$ to 44 μM AS in aerated solutions containing 10 mM PB at pH 7.0 and 22 °C.

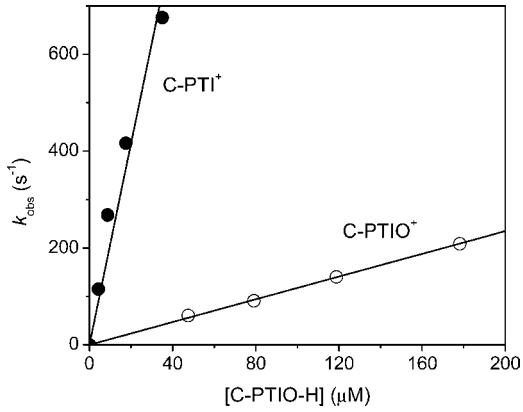


Figure 6. Dependence of the pseudo-first-order rate constants for the decays of $C\text{-}PTIO^+$ and $C\text{-}PTI^+$ on [C-PTIO-H] at pH 7 and 22 °C. Equal volumes of C-PTIO-H (9−356 μM) and $C\text{-}PTIO^+$ (20 μM) or $C\text{-}PTI^+$ (2−20 μM), all in 10 mM PB, were mixed using the stopped-flow apparatus, and the decay of the absorption was followed at 450 or 320 nm, respectively.

$^{\bullet}NO_2$ formed in reaction 8 reacts with the excess NO to yield N_2O_3, which hydrolyzes to nitrite.

We have shown that C-PTIO is converted into C-PTI and C-PTI-H by HNO. The yield of C-PTI-H increases at the expense of C-PTI as the ratio $[AS]_0/[C\text{-}PTIO]_0$ increases. The mechanism of the reaction of HNO with PTIO and C-PTIO involves the participation of several reactive species, including NO, $^{\bullet}NO_2$, and oxoammonium cations, as demonstrated in Scheme 1 for PTIO. Reaction 8 has not been included in Scheme 1, as it does not compete efficiently with the comproportionation of PTIO-H with $PTIO^+$ and PTI^+. The mechanism is more complex than what is shown in Scheme 1, and quantitative analysis should also include the oxidation of the nitronyl and imino nitroxides by the strongly oxidizing hyponitrite ion radical $N_2O_2^{\bullet-}$, which is formed via the reaction of HNO with NO.[47,48]

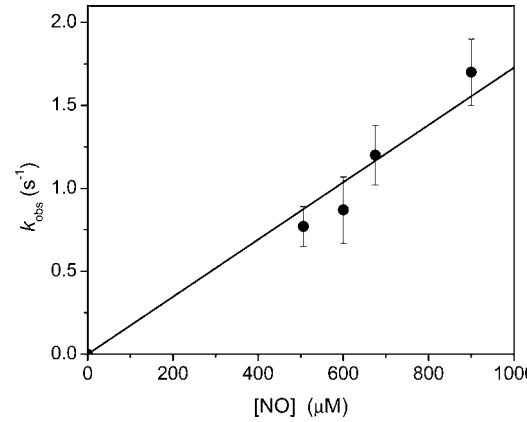
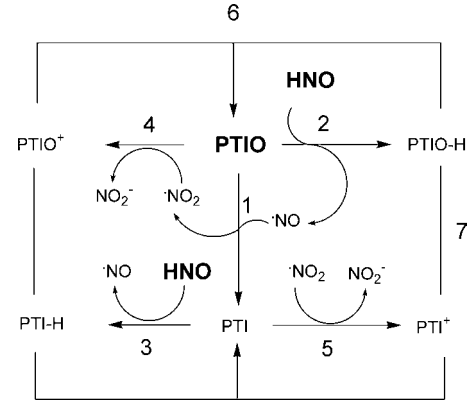


Figure 7. Dependence of the pseudo-first-order rate constant of the decay of C-PTIO-H on [NO] at pH 7.0 and 22 °C. Equal volumes of C-PTIO-H (120 μM, 10 mM PB, Ar-saturated) and NO (0.9−1.8 mM, 10 mM PB) were mixed using the stopped-flow apparatus, and the decay of C-PTIO-H was followed at 320 nm.

Scheme 1. Mechanism Underlying the Conversion of PTIO to PTI and PTI-H by HNO at pH 7.0[a]



[a] Reaction numbers are the same as listed in the text; reactants are written in enlarged bold text.

In conclusion, the reaction of HNO with nitronyl nitroxide forms the corresponding imino nitroxide and hydroxylamine. The yield of the imino hydroxylamine increases at the expense of the imino nitroxide with an increase in the ratio $[AS]_0/[\text{nitronyl nitroxide}]_0$, where $[AS]_0$ represents the total production of HNO. Since the reaction of NO with nitronyl nitroxide yields only the corresponding imino nitroxide, nitronyl nitroxide can discriminate between NO and HNO only when used at a concentration much lower than the total production of HNO.

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