Reactions of PTIO and Carboxy-PTIO with 'NO, 'NO₂, and O₂*

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Nitronyl nitroxides, such as derivatives of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIOs), react with 'NO to form the corresponding imino nitroxides (PTIs) and 'NO₂. PTIOs are considered as monitors of 'NO, stoichiometric sources of 'NO₂, biochemical and physiological effectors, specific tools for the elimination of 'NO, and potential therapeutic agents. However, a better understanding of the chemical properties of PTIOs, especially following their reaction with 'NO, is necessary to resolve many of the reported discrepancies surrounding the effects of PTIOs and to better characterize their potential therapeutic activity. We have generated electrochemically the oxidized and reduced forms of PTIO and carboxy-PTIO (C-PTIO), characterized their absorption spectra, and determined the reduction potentials for the o xoammonium/nitroxide and nitroxide/hydroxylamine couples. The rate constants for the reaction of 'NO₂ with PTIO and C-PTIO to form the corresponding o xoammonium cations (PTIO’s) and nitrite (Scheme 1). We have also shown that the reactions of PTIO’s with 'NO form PTIOs and 'NO₂. The rate constants for these reactions are approximately 30-fold higher than those for the reactions of PTIOs with 'NO or O₂. The present results show that (i) the reaction of PTIOs with 'NO forms solely PTIs and 'NO₂ where [NO₂] /[PTI] varies between 1 and 2 depending on the steady-state concentrations of 'NO. Consequently, quantitation of 'NO is valid only at sufficiently low fluxes of 'NO (ii) the reaction of PTIOs with 'NO can be used as a valid source of 'NO₂ only when the latter is effectively scavenged by an appropriate reductant; and (iii) the formation of peroxynitrite cannot be efficiently inhibited by PTIOs even under relatively low fluxes of 'NO and O₂ and millimolar levels of PTIOs.

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§ The abbreviations used are: 'NO, nitric oxide; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; DAF, 4,5-di-1-oxyl-2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonate).

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\[
\begin{align*}
\text{PTIO;} & \quad R = H \\
\text{C-PTIO;} & \quad R = \text{COO}^+ \\
\text{PTI;} & \quad R = H \\
\text{C-PTI;} & \quad R = \text{COO}^-
\end{align*}
\]

\text{Reaction 1}

Nitric oxide ('NO)1 is synthesized by 'NO synthase from the substrate l-arginine in a wide variety of cell types and is a major participant in numerous beneficial physiological functions such as blood pressure regulation, inhibition of platelet aggregation, and neurotransmission (1). However, high steady-state levels of 'NO function critically in modulating inflammatory, infectious, and degenerative diseases (2–6). The reaction of 'NO with metalloproteins can abrogate the function of proteins (7, 8). Moreover, the reactions of 'NO with oxygen and superoxide convert 'NO into other reactive nitrogen species such as ONOO⁻, 'NO₂, or N₂O₅, which can react with virtually all of the classes of biomolecules including amino acids, lipids, DNA, thios, and metals (5, 6, 9–11). One therapeutic approach to ameliorate 'NO-induced biological damage is to use 'NO scavengers, preferably selective scavengers that distinguish between free 'NO and species exhibiting 'NO-like biological activity.

The use of nitronyl nitroxides, such as 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), as selective traps for 'NO is rapidly increasing as reflected by the numerous publications in the recent biomedical literature. PTIO and its derivatives were shown to react with 'NO to form the corresponding imino nitroxides and 'NO₂ (12–16), where \( k_1 = (0.5 - 16) \times 10^7 \text{ M}^{-1} \text{s}^{-1} \) (Reaction 1) (12, 14, 16). Both the nitronyl nitroxide and the imino nitroxide are detectable and distinguishable by EPR spectroscopy, and various PTIOs are used as spin traps for 'NO (13, 14, 16–18). In addition, Akaike et al. (12) demonstrated that excess of 'NO₂ had no effect on the EPR spectrum of PTIOs and, subsequently, Reaction 1 has been adopted to generate 'NO₂ (19, 20). Likewise, direct scavenging of 'NO by PTIOs has been shown to inhibit endothelium-derived relaxing factor (EDRF) in bioassay in vitro (12), to cause cell cycle alteration, to result in oxidative stress and apoptosis in pulmonary cells (21), and to exhibit a potent therapeutic effect in cases of endotoxin shock (22). Therefore, PTIOs are not only useful as spin traps of 'NO and generators of 'NO₂ but also provide insight into the physiology of 'NO and potential use as a target specific therapeutic to intervene in various diseases caused by excess production of 'NO. However, as is the case with other cyclic nitroxides, nitronyl nitroxide may undergo one-electron redox reactions to yield the respective hydroxylamine and oxoammonium cation (Scheme 1).

We recently demonstrated that cyclic nitroxides are readily oxidized by 'NO₂ to form the respective oxoammonium cations (23), which are reduced by 'NO back to the parent nitroxides (24). If this is also the case with PTIOs, their use as selective scavengers for 'NO and generators of 'NO₂ seems to meet a serious limitation. Peffleier et al. (25) have already indicated
that the effects of C-PTIO are diverse and questioned its claimed specificity as 'NO scavenger. Espey et al. (19) used Reaction 1 to produce NO\(_2\) and measured the nitrosation yield of 4,5-diaminofluorescein (DAF) in the presence of excess PTO. They found that the nitrosation yield formed via Reaction 1 is considerably higher than that formed during autoxidation of 'NO in aqueous solutions and concluded that NO\(_2\) is not formed as an intermediate during autoxidation of 'NO. This conclusion contradicts the results obtained by many other studies (26–31). Also, there is a discrepancy concerning the reactivity of PTIOs with superoxide radicals.Haseloff et al. (32) reported that the rate constant for the reduction of nitronyl nitroxides by O\(_2^-\) is more than two orders of magnitude higher than that for Reaction 1 and suggested that PTIOs can protect against 'NO-induced damage by preventing the formation of peroxynitrite. Conversely, Rosen et al. (16) found that the half-life of 5 \(\mu\)M C-PTIO exposed to a flux of 10 \(\mu\)M/min of O\(_2^-\) at pH 7.4 is \(-10\) min, which indicates that the rate of the reduction of C-PTIO is considerably lower than that reported by Haseloff et al. (32). This demonstrates the need for a detailed investigation of the reactivity of PTIOs with 'NO, NO\(_2\), and superoxide radicals. In this study, the reactions of these radicals with PTIO, C-PTIO, and their corresponding oxoammonium cations were investigated using pulse radiolysis and rapid-mixing stopped-flow techniques.

**Experimental Procedures**

**Materials**—All of the chemicals were of analytical grade and were used as received. Water for preparation of the solutions was distilled and purified using a Milli-Q purification system. PTO and C-PTIO were purchased from Sigma and Cayman Chemical Company, respectively. Xanthine oxidase, catalase, hypoxanthine, ferricytochrome c, and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS\(^{3-}\)) were purchased from Sigma. Nitric oxide was purchased from Matheson Gas Products and was purified by passing it through a series of scrubbing bottles containing deaerated 50% NaOH and purified water in this order. Nitric oxide solutions were prepared in gas-tight syringes, and the solubility of NO in water at 22 °C was taken to be 1.9 ms/atm. The oxoammonium cations and the respective hydroxylamines were prepared using an electrochemical reactor. The cell consisted of a working electrode of graphite grains packed inside a porous Vycor glass tube (5-mm inner diameter) through which 0.1 mM PTO in 4 mM phosphate buffer (PB) at pH 6.8 was pumped (4 ml min\(^{-1}\)). An outer glass cylinder contained 0.1 M NaCl in which a Pt auxiliary electrode and Ag/AgCl wire as a reference electrode were immersed. A BAS100B electrochemical analyzer controlled the voltage. Immediately following electro-oxidation or reduction, the solution was pumped through a 1-cm optical flow cell positioned within a HP 8453 diode array spectrophotometer.

Superoxide radicals were generated by the addition of xanthine oxidase (0.002 units/ml) to aerated solutions containing 2.5 mM hypoxanthine, 130 units/ml catalase, 50 \(\mu\)M DTPA, and 160 mM phosphate buffer at pH 5.5–8.5. The flux of O\(_2^-\) under these experimental conditions was 2 \(\mu\)M/min as determined by monitoring the reduction of ferricytochrome c at 550 nm as described previously (33, 34).

**Methods**—Cyclic voltammetry was performed with a BAS100B electrochemical analyzer. We used a three-electrode system consisting of a graphite (2-mm inner diameter) working electrode, a Pt wire auxiliary electrode, and an Ag/AgCl wire as a reference electrode. In a typical experiment, the electrodes were immersed into solutions containing 0.1 M NaCl, 10 mM PB, and 1 mM PTO or C-PTIO. The solutions were purged with purified argon, and an argon atmosphere was maintained over the solution throughout the measurements. The potentials quoted versus normal hydrogen electrode were corrected for Ag/AgCl in 0.1 M NaCl against normal hydrogen electrode (i.e. +288 mV).

Stopped-flow kinetic measurements were carried out using the Bio SX-17MV sequential stopped-flow from Applied Photophysics with a 1-cm optical path. Anaerobic experiments were carried out while flushing the syringe cups with inert gas. The final pH in each experiment was measured at the outlet of the stopped-flow. All of the experiments were carried out at 25 °C.

Pulse radiolysis experiments were carried out using a 5-MeV Varian 7715 linear accelerator (0.05–0.5-\(\mu\) electron pulses, 200-mA current). The dose per pulse was determined with N\(_2\)O-saturated solutions containing 5 mM KSCN. A 200-watt Xe lamp produced the analyzing light. Appropriate cut-off filters were used to minimize photochemistry. All of the measurements were made at room temperature using a 1-cm spectroscopy cell and applying three light passes. EPR spectra were recorded using a JEOL X band JES-REX3X spectrometer operating at 9.5 GHz with the center field set at 3362 G (100 kHz modulation frequency; 1-G modulation amplitude; 4-milliwatt incident microwave power). The nitroxide concentration was calculated from the EPR signal intensity using standard solutions of the nitroxide.

**Results**

**Cyclic Voltammetry**—The redox properties of PTO and C-PTIO were investigated by cyclic voltammetry at varying pH values. Cyclic voltammograms for PTIO at pH 3.5 are shown in Fig. 1 and demonstrate the difference between the first and the second cycle upon increasing the voltage from 250 mV into the positive direction. An anodic peak at 658 mV and a cathodic one at 588 mV reflect a midpoint potential of \(E_{1/2}^{\text{NO}} = 623\) mV, which is attributed to the reversible oxidation of the nitroxide to the oxoammonium cation.

The scan of the negative potential region shows an anodic peak at 230 mV only in the second cycle (scan rate 100 mV s\(^{-1}\)).
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where $E^{red}_{1/2} = 190 \text{ mV}$ at pH 3.5. The voltammetric behavior of C-PTIO was similar to that of PTIO. Reversible oxidation of both PTIO and C-PTIO was found at pH 1.5–8.8, resulting in $E^{red}_{1/2} = 627 \pm 7$ and $648 \pm 15 \text{ mV}$, respectively (i.e., $915 \pm 10$ and $936 \pm 21 \text{ mV}$ versus normal hydrogen electrode). Both the reduction and reoxidation peaks of PTIO and C-PTIO varied with the pH value. Fig. 2 illustrates the cyclic voltammograms of C-PTIO at various pH values.

Reversible one-electron redox reaction was obtained only below pH 5 where the peak separation is less than 100 mV. The calculated half-wave potentials, $E^{red}_{1/2}$, as a function of pH are given in Fig. 3, resulting in two straight lines that intersect each other around pH 3.5.

The slopes of the linear plots in the pH above and below the intersection are $-58$ and $-121 \text{ mV/pH}$, respectively, and reflect the participation of one and two hydrogen ions, respectively, in the electrode reaction (Scheme 2).

Following Reactions 2–4, the potential of the electrode, $E^{red}_{1/2}$, depends on $K_2$, $K_3$, and the pH value, and is expressed by Equation 1,

$$\text{PTIO(C-PTIO)} + e^{-} \rightleftharpoons \text{PTIO}^{-} \left(\text{C-PTIO}^{-}\right) \quad E_0$$

$$\frac{1}{K_2}$$

$$\text{PTIO}^{-} \left(\text{C-PTIO}^{-}\right) + \text{H}^{+} \rightleftharpoons \text{PTIO-H} \left(\text{C-PTIO-H}\right)$$

$$\frac{1}{K_3}$$

$$\text{PTIO-H} \left(\text{C-PTIO-H}\right) + \text{H}^{+} \rightleftharpoons \text{PTIO-H}_2 \left(\text{C-PTIO-H}_2\right)$$

Reactions 2–4

$$E^{red} = E_0 + \frac{(RT/F)\ln([\text{PTIO}]/[\text{PTIO}^{-}])}{f} = E_0 - \frac{(RT/F)\ln(K_2K_3)}{f}$$

$$+ \frac{(RT/F)\ln(K_3[H^+] + [H^2]^+)}{f}$$

$$+ \frac{(RT/F)\ln([\text{PTIO}]/[\text{PTIO}^{-}])}{f} \quad \text{(Eq. 1)}$$

where $[\text{PTIO}^{-}] = [\text{PTIO}^{-}] + [\text{PTIO-H}] + [\text{PTIO-H}_2]$. Equation 2 is obtained by assuming that the diffusion coefficients of all of the species involved are equal.

$$E^{red}_{1/2} = E_0 - \frac{(RT/F)\ln(K_3K_4)}{f} + \frac{(RT/F)\ln(K_3[H^+] + [H^2]^+)}{f} \quad \text{(Eq. 2)}$$

Thus, the plot of $E^{red}_{1/2}$ versus pH should have a slope of $-2RT/F = -118 \text{ mV at } [H^+] \gg K_4$, a slope of $-RT/F = -59 \text{ mV at } K_3 \gg [H^+] \gg K_4$, and $E^{red}_{1/2}$ should remain constant at $[H^+] \ll K_3$. Under our experimental conditions, the folding of the plots at $K_3$ was not observed. The intersection of the two lines yields $pK_4 = 3.5$ for PTIO-H and C-PTIO-H, indicating that these hydroxylamines are stronger acids than (CH$_3$)$_2$NOH ($pK_4 = 5.2$) (35).

Bulk Electrolysis—PTIO and C-PTIO were electro-oxidized to the corresponding oxoammonium cations in aerated solutions at pH 7.0 (10 mM PB) by applying a voltage of 0.8 V (versus Ag/AgCl) (0.1 m). The oxidation yield was determined using ferrocyanide, which is rapidly oxidized by oxoammonium cations ($\epsilon_{420}(\text{Fe(CN)}_6^{3-}) = 1,000 \text{ m} \cdot \text{cm}^{-1}$) (23). Similarly, the nitroxides were reduced in anoxia by reversing the voltage and the reduction yield was determined using ferricyanide, which can efficiently oxidize hydroxylamines to their corresponding nitroxides. Both the oxidation and reduction yields approached 100%. The UV-visible spectra of the nitroxides and their oxidized and reduced forms are given in Figs. 4 and 5. The respective differential spectra are presented in the inset of these figures and demonstrate that the formation and decay of PTIO$^+$ and C-PTIO$^+$ can be readily followed spectrophotometrically around 300 and 450 nm.

Reaction of $'NO_2$ with PTIO and C-PTIO—Nitrogen dioxide was generated by pulse-irradiation of N$_2$O-saturated (−25 mM) aqueous solutions containing 1–4 mM sodium nitrite and 8 mM PB (pH 6.9) through Reactions 5–7.

$$\text{H}_2\text{O} \rightleftharpoons e^{-}(2.6), \text{OH}(2.7), \text{H}(0.6), \text{H}_2\text{O}^{+}(2.6), \text{H}_2\text{O}_2(0.72)$$

Reaction 5

The numbers in parentheses are G-values, which represent the concentrations of the species (in $10^{-7} \text{ M} \cdot \text{Gy}^{-1}$), and are −7% higher in the presence of higher solute concentrations.
obtained by flushing the deaerated solution of C-PTIO with 

The radiolytically formed H (i.e., ~10% of the total radical yield at pH > 3) adds rapidly to NO₂ (k = 1.6 × 10⁹ M⁻¹ s⁻¹) to form 'NO in a process catalyzed by acids and bases (37).

The reaction of NO₂ with an excess of PTIO or C-PTIO was studied by following the absorption changes at 290–500. A rapid first-order formation of a transient species was observed, which was identified as PTIO⁺ or C-PTIO⁺, respectively. The formation of the oxoammonium cations was followed by a second-order decay. The observed rate constants were determined by following the absorption changes at 290, 360, and 445 nm (see the insets in Figs. 4 and 5). The observed first-order rate constant for the formation of the oxoammonium cations increased with increasing [nitroxide]₀ at a constant [NO₂]₀, and the second-order decay was linearly dependent on [nitroxide]₀² at a constant [NO₂]₀ (Fig. 6).

Reactions 6 and 7

\[
\begin{align*}
\text{e}_\text{aq} + \text{N}_2\text{O} &\rightarrow \text{N}_2 + \text{OH}^- + \text{OH}^- & k_0 = 9.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \quad (36) \\
\text{OH}^- + \text{NO}_2^- &\rightarrow \text{NO}_2 + \text{OH}^- & k_1 = 5.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \quad (36)
\end{align*}
\]

Assuming that Reaction 8 rapidly approaches equilibrium, the rate-limiting step for the decay of the oxoammonium cation is Reaction 9 and rate Equation 3 is obtained.

\[
-\frac{d[\text{PTIO}^+]}{dt} = \frac{2k_a[\text{NO}_2^-][\text{PTIO}^+]}{K_d[\text{PTIO}^+]^2} \quad (Eq. 3)
\]

Hence, plots of k_{obs} versus [NO₂]² at a constant [PTIO] or versus [PTIO]² at a constant [NO₂] should be linear (Fig. 6). The results demonstrate that K_d = 145 ± 15 for both PTIO and C-PTIO, independent of being determined from the slope of the lines in Fig. 6, A or B. Hence, the reduction potential of the PTIO/PTIO⁺ and C-PTIO/C-PTIO⁺ couples is the same and was calculated using K_d and E°(NO₂/NO₂⁻) = 1.04 V to be 912 mV. The latter value is in agreement with the mid-potentials, which were determined by cyclic voltammetry to be 915 ± 10 and 936 ± 21 mV, respectively.
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To determine accurately the rate constant of Reaction 8, we used ABTS²⁻ as an efficient scavenger of oxoammonium cations (23). The 'NO₂-induced formation of ABTS⁻ in the presence of 250 μM ABTS²⁻ was followed at 416 nm (ε₄₁₆ = 38,000 M⁻¹ cm⁻¹) where [NO₂]₀ = -1 μM. The yield of ABTS⁻ was unaffected upon the addition of PTIO or C-PTIO, whereas k_{obs} = (4.0 ± 0.1) × 10⁷ M⁻¹ s⁻¹ in the absence of the nitroxides increased upon their addition (Fig. 7). From the slope of the lines in Fig. 7, we determined k₂ = (2.0 ± 0.1) × 10⁷ M⁻¹ s⁻¹ for PTIO and (1.5 ± 0.1) × 10⁷ M⁻¹ s⁻¹ for C-PTIO.

Reaction of 'NO with PTIO and C-PTIO and Their Corresponding Nitroxides—The reaction of excess of 'NO with PTIO⁺ or C-PTIO⁺ was studied in deaerated solutions at pH 6.8 ± 0.1 (7–10 mM PB). The first-order decay of the oxoammonium cations monitored at 310 nm was followed by a slower first-order build-up. The observed rate constants of both processes were linearly dependent on [NO⁻] (Fig. 8).

Therefore, we attribute the first process to the reduction of the oxoammonium cations back to the nitroxides by 'NO (Reaction 10), which is followed by the slower reaction of the nitroxides with 'NO (Reaction 1).

NO + PTIO⁺ (C-PTIO⁺) + H₂O → PTIO (C-PTIO) + NO₂⁻ + 2H⁺

Reaction 10

Fig. 8. Reaction of 'NO with PTIO⁺ or C-PTIO⁺. The decay of PTIO⁺ was followed at 290 nm upon mixing equal volumes of deaerated solution containing 0.4–1.8 mM NO in 10 mM PB with deaerated solution containing 50–100 μM PTIO⁺ in 4 mM PB. The decay of C-PTIO⁺ was followed at 310 nm upon mixing 0.21–1.8 mM NO in 10 mM PB with 90 μM C-PTIO⁺ in 10 mM PB at a 5:1 volume ratio. The final pH was 6.8 ± 0.1.

The rate constants for Reaction 1 and Reaction 10 were obtained from the slopes of the lines in Fig. 8 and are summarized in Table I. These values agree with those determined previously by stopped-flow EPR and spectrophotometric methods (12, 14) and are somewhat lower than that determined using hemoglobin as a competing agent (16). The literature data also are summarized in Table I.

The reaction of NO with PTIO or C-PTIO was also studied in the absence and presence of ABTS²⁻. The formation of PTIO or C-PTIO was followed at 300 nm in the absence of ABTS²⁻ and was found to obey first-order kinetics where k_{obs} increased linearly with [NO⁻], resulting in k₁ = (4.1 ± 0.1) × 10⁸ and (6.0 ± 0.2) × 10⁸ M⁻¹ s⁻¹, respectively (Fig. 9).

In the presence of an excess of ABTS²⁻ over the PTIOs, the build-up of the absorption at 416 nm was observed and the measured yield of ABTS⁻ was identical to that of [PTIOs]₀. In addition, the rate of the formation of ABTS⁻ was first order with the same dependence on [NO⁻] as in the absence of ABTS²⁻ (Fig. 9 and Table I). Finally, the latter experiment demonstrates the formation of NO₂ via Reaction 1.

Reaction of Superoxide Radicals with PTIO and C-PTIO—The reaction of ~1 μM O2 with 50 μM PTIO or C-PTIO was studied upon pulse-irradiation of oxygenated solutions containing 0.1 mM formate at pH 3.6 in the absence and presence of 100 μM ABTS²⁻. Under such conditions where all of the primary radicals formed by the radiation (Reaction 5) are converted into HO₂ (pKₐ(HO₂) = 4.8), the corresponding oxoammonium cation or ABTS⁻ was not observed. The apparent rate constant for the dismutation of HO₂ is ~1 × 10⁶ M⁻¹ s⁻¹ at pH 3.6, and therefore, the rate constant for the reaction of HO₂ with PTIO or C-PTIO cannot exceed 2 × 10⁴ M⁻¹ s⁻¹. The reaction with O₂ was studied upon pulse-irradiation of oxygenated solutions containing 20 mM formate and 8 mM PB at pH 8.5. In the case of PTIO, the bleaching of the absorption was observed both at 355 and 560 nm where ΔA₁₅₀/ΔA₅₆₀ = ~6. The absorption of C-PTIO at 360 nm is relatively higher than that of PTIO (Figs. 4 and 5), and therefore, the bleaching of the absorption was followed at 370–375 and 560 nm where ΔA₁₃₇/ΔA₅₆₀ = ~2. These observations indicate that O₂ reduced PTIO and C-PTIO to their respective hydroxylamines (see Figs. 4 and 5) via Reaction 11.

PTIO (C-PTIO) + O₂⁻ + H⁺ → PTIO-H (C-PTIO-H) + O₂

Reaction 11

The bleaching of the absorption obeyed first-order kinetics, and k_{obs} increased upon increasing [PTIO] or [C-PTIO], thus resulting in k₁₁ = (2.0 ± 0.4) × 10⁶ or (9.3 ± 0.5) × 10⁵ M⁻¹ s⁻¹, respectively (Fig. 10). These rate constants are ~100-fold lower than that reported previously (32) (i.e. 8.8 × 10⁵ M⁻¹ s⁻¹ for PTIO at pH 7). Comments

| Reaction | PTIO | C-PTIO |
|----------|------|--------|
| k₁, M⁻¹ s⁻¹ | (4.1 ± 0.1) × 10⁷ | (6.0 ± 0.2) × 10⁸ |
| k₂, M⁻¹ s⁻¹ | (4.8 ± 0.1) × 10⁷ | (6.0 ± 0.1) × 10⁸ |
| k₃, M⁻¹ s⁻¹ | 9.15 ± 0.05 | 1.01 ± 0.05 |
| Kₛ | 2.5 ± 0.1 | 3.5 ± 0.1 |
| Eₐ₃, mV | 912 ± 2 | 912 ± 2 |
| Eₐ₂, mV | 915 ± 10 | 985 ± 21 |
| pH₄ | 3.5 | 3.5 |

Table I

Summary of all of the rate constants and reduction potentials (versus normal hydrogen electrode) determined in the present study

ND, not determined.
another derivative of PTIO where \( r = \text{N}(\text{CH}_3)_2 \) (32).

The reaction of superoxide radicals with PTIO and C-PTIO was also studied by exposure to a constant flux of \( \text{O}_2^\bullet^- \). The EPR signal of PTIO or C-PTIO was monitored as a function of time upon exposure of 8–10 \( \mu M \) nitronyl nitroxide to a flux of \( \text{O}_2^\bullet^- \) (2 \( \mu M/\text{min} \)). The initial rate of the decay of the EPR signal was considerably lower than the flux of \( \text{O}_2^\bullet^- \), indicating that the non-enzymatic dismutation of superoxide efficiently competes with its reaction with PTIOs. Complete reduction of PTIO or C-PTIO was not observed. Instead, a steady-state residual concentration of 1–1.4% was achieved after 30 min and persisted as long as the \( \text{O}_2^\bullet^- \) flux persisted. The failure of \( \text{O}_2^\bullet^- \) to reduce completely PTIO and C-PTIO suggests that under such conditions both \( \text{HO}_2 \) and \( \text{O}_2^- \) are capable of oxidizing the corresponding hydroxylamines. Our results agree with the observation reported for C-PTIO by Rosen et al. (16) A similar behavior has already been reported for other five-membered ring nitroxides (38, 39) and is currently under investigation.

**DISCUSSION**

The rate constants for the reaction of \( \text{NO}_2 \) with PTIO and C-PTIO to form the corresponding oxoammonium cations and nitrite were determined to be \((1.5 - 2) \times 10^7 \text{ M}^{-1} \text{s}^{-1} \). These rate constants are more than an order of magnitude lower than those determined previously for piperidine and pyrrolidine derivatives of nitroxides (i.e., \(-7 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \)) (23) and similar to those for thiols at physiological pH (11). We have further shown that PTIO’s oxidize \( \text{NO} \) forming PTIOs and \( \text{NO}_2 \) and that the rate constants for this reaction exceed by far those for the reaction of PTIOs with \( \text{NO} \) (i.e., \( k_{10} \sim 30 k_1 \) (Table 1)). Therefore, the mechanism of the reaction of PTIOs with \( \text{NO} \) is described by Reactions 1, 8, 10, 12, and 13.

\[
\text{NO} + \text{NO}_2 = \text{N}_2\text{O}_3 \quad k_{12} = 1.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \quad k_{12} = 8.1 \times 10^8 \text{ s}^{-1} \quad (40)
\]

\[
\text{N}_2\text{O}_3 + \text{H}_2\text{O} = 2\text{NO}_2 + 2\text{H}^+ \quad k_{12} = 2 \times 10^3 \text{ s}^{-1} \quad (41)
\]

**CONCLUSIONS**

The reaction of PTIOs with \( \text{NO} \) forms solely PTIs and \( \text{NO}_2 \) as previously observed (25). However, the stoichiometric ratio of \([\text{NO}_2]/[\text{PTI}]\), which equals the ratio \([\text{NO}]/[\text{PTI}]\), varies between 1 and 2 depending on the steady-state concentrations of \( \text{NO} \). Upon bolus addition of \( \text{NO} \) or high fluxes of \( \text{NO} \), Reactions 12 and 13 can be ignored and the ratio approaches 2, whereas at relatively low fluxes Reaction 9 can be ignored and the ratio approaches 1. The variation of the stoichiometric ratio \([\text{NO}_2]/[\text{PTIs}]\) between 1 and 2 with the experimental conditions may explain the differences among the results reported in the literature (12, 13, 15). Consequently, quantitation of \( \text{NO} \) that is based on the yield of PTI is valid only at very low fluxes of \( \text{NO} \).

The present results explain why a large excess of \( \text{NO}_2 \) had no effect on the EPR spectra and signal intensities of PTIOs (12). These nitronyl nitroxides react rapidly with \( \text{NO}_2 \) to form PTIO’s, which are reduced back to PTIOs by nitrite (Reaction 8) where the latter is being formed mainly via the self-decomposition of the large excess of \( \text{NO}_2 \), in aqueous solutions (Reaction 9). Espey et al. (19) added 0.5 \( \mu M \) \( \text{NO} \) to aerated solutions containing 1 \( \mu M \) DAF in the absence and presence of 5 \( \mu M \) PTIO. They measured the nitrosation yield of DAF and found that the nitrosation yield in the presence of PTIO is considerably higher than that in its absence. This led them to exclude the intermediacy of \( \text{NO}_2 \) during autoxidation of \( \text{NO} \) in aqueous solution, which has been proposed by other studies (26–31). However, under their experimental conditions, PTIO competes efficiently with DAF for \( \text{NO}_2 \) and the resulting PTIO+ most probably rapidly oxidizes DAF to DAF+, which subsequently reacts readily with \( \text{NO} \) to form the nitrosation product (42). This process is more efficient than that occurring during autoxidation of \( \text{NO} \) and, therefore, the nitrosation yield in the presence of PTIO is considerably higher than that in its absence. Hence, Reaction 1 can be used as a stoichiometric source of \( \text{NO}_2 \) only when the latter is not scavenged by the nitronyl nitroxide itself.

Peroxynitrite ion is formed via the diffusion-limited reaction between \( \text{NO} \) and \( \text{O}_2^\bullet^- \) (43, 44), which is generally accepted as the main biological source of peroxynitrite. Because the rate constants for the reactions of \( \text{NO} \) and \( \text{O}_2^\bullet^- \) with PTIOs are \(-10^3\)-fold lower, even at millimolar levels, they cannot efficiently inhibit the formation of peroxynitrite under low fluxes of \( \text{NO} \) and \( \text{O}_2^\bullet^- \).
be used as a valid tool to form 'NO2 only when the latter is not scavenged by PTIOs themselves. The formation of peroxynitrite cannot be efficiently inhibited by PTIOs even under relatively low fluxes of 'NO and O2 and millimolar levels of PTIOs.

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