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The use of cyclic nitroxide radicals as HNO scavengers

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ABSTRACT

Reduction of cyclic stable nitroxides (RNO) by HNO to the respective hydroxylamines (RNO-H) has been demonstrated using EPR spectrometry. HNO shows low reactivity toward piperidine, pyrrolidine and nitronyl nitroxides with rate constants below 1.4×10^5 M^{-1} s^{-1} at pH 7.0, despite the high driving force for these reactions. The rate constants can be predicted assuming that the reactions take place via a concerted proton–electron transfer pathway and significantly low self-exchange rate constants for HNO/NO and RNO-H/RNO. NO does not react with piperidine and pyrrolidine nitroxides, but does add to HNO forming the highly oxidizing and moderately reducing hyponitrite radicals. In this work, the radicals are produced by pulse radiolysis and the rate constants of their reactions with 2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO), 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPOL) and 3-carbamoyl-PROXYL have been determined at pH 6.8 to be $(2.4\pm0.2)\times10^6$, $(9.8\pm0.2)\times10^5$, $(5.9\pm0.5)\times10^5$ M^{-1} s^{-1}, respectively. This low reactivity implies that NO competes efficiently with these nitroxides for the hyponitrite radical. The ability of TEMPOL and 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) to oxidize HNO and their different reactivity toward NO are used to quantify HNO formed via acetohydroxamic acid oxidation. The extent of TEMPOL or C-PTIO reduction was similar to the yield of HNO formed upon oxidation by $^{\bullet}OH$ under anoxia, but not by the metmyoglobin and H_2O_2 reaction system where both nitroxides catalytically facilitate H_2O_2 depletion and nitrite accumulation. In this system the conversion of C-PTIO into 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (C-PTI) is a minor reaction, which does not provide any mechanistic insight.

1. Introduction

Cyclic stable nitroxide radicals (RNO), also known as aminoxyl or nitroxyl radicals, have been used for many years as biophysical probes to monitor membrane dynamics, cellular pH and O_2 level [1–3]. RNO can shuttle through one-electron redox reactions among three oxidation states as shown in Scheme 1 for piperidine nitroxides. The rate constant of HNO reaction with the stable cyclic nitroxide 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPOL) has been determined to be 8×10^4 M^{-1} s^{-1} using the HNO donor Angeli's salt (AS) and competition kinetics against metmyoglobin ($MbFe^{III}$) assuming that TEMPOL oxidizes HNO [4]. Surprisingly, its structural analog 2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPO) has been reported to reduce HNO, and there is neither experimental evidence nor any explanation on how the authors arrived at this conclusion [5]. We have previously studied using AS and EPR spectrometry the reactions of HNO with TEMPOL, the nitronyl nitroxides 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) and 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO), and have shown unequivocally that HNO reduces these nitroxides to their respective hydroxylamines (reaction (1)) [6,7].

$$RNO + HNO \rightarrow RNO{-}H + NO \qquad (1)$$

Most RNO do not react with NO, which readily adds to HNO forming the hyponitrite radical $HN_2O_2^{\bullet}$ (reaction (2)) [8–10].

$$HNO + NO \rightarrow HN_2O_2^{\bullet} \qquad k_2 = 5.8\times10^6 M^{-1}s^{-1} \qquad (2)$$

$HN_2O_2^{\bullet}/N_2O_2^{\bullet-}$ ($pK_a = 5.5$) are strongly oxidizing (1.75 V/0.96 V) and moderately reducing (−0.06 V/−0.38 V) radicals [11], which at neutral solutions have sufficient driving force to oxidize as well as reduce RNO [12–17].

In contrast to piperidine and pyrrolidine nitroxides, which do not react with NO, nitronyl nitroxides react uniquely with NO forming the respective imino nitroxides as demonstrated below for C-PTIO

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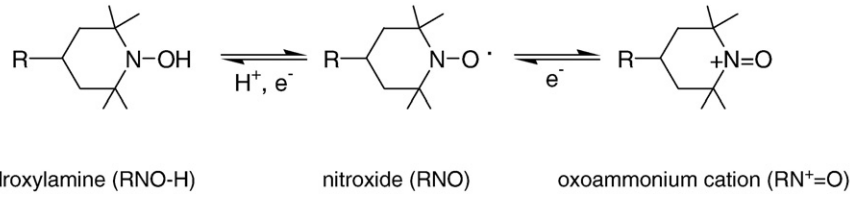
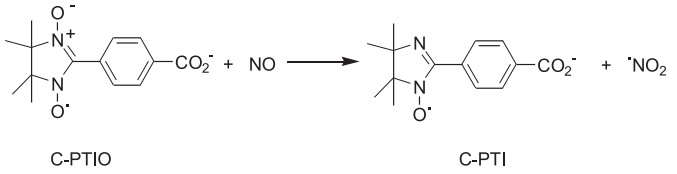


Scheme 1. Oxidation and reduction of piperidine nitroxides (R = H, TEMPO; R = OH, TEMPOL).

(reaction (3), $k_3 = 1\times10^4$ M^{-1} s^{-1} [18,19]). Both C-PTIO and C-PTI being nitroxides are reducible by HNO [6].



(3)

Since RNO has a characteristic EPR spectrum whereas RNO-H is diamagnetic and EPR-silent, the reduction of RNO by HNO can be monitored using EPR spectroscopy. The characteristic EPR spectra of nitronyl nitroxides and imino nitroxides are distinctly different, thus enabling the detection and in some cases quantification of NO by monitoring the conversion of nitronyl nitroxide to imino nitroxide [18–21].

HNO ($pK_a = 11.4$ [22,23]) has been demonstrated as a unique species with potentially important pharmacological activities [24,25]. It is a relatively unstable species, which rapidly decomposes forming the transient hyponitrous acid that slowly produces nitrous oxide via dehydration (reaction (4) [22,26]).

$$HNO + HNO \rightarrow H_2N_2O_2 \rightarrow N_2O + H_2O \qquad k_4 = 8\times10^6 M^{-1}s^{-1} \qquad (4)$$

The question arises whether the different nitroxides might be used for HNO detection, quantification, and elucidation of the mechanism underlying its formation. In the present study the rate constants of the reactions of TEMPO, TEMPOL and 3-carbamoyl-PROXYL with HNO and with $N_2O_2^{\bullet-}$ have been determined. In addition, the ability of TEMPOL and C-PTIO to oxidize HNO and their different reactivity toward NO have been used to quantify HNO, which is known to be formed via the oxidation of acetohydroxamic acid [7,27].

2. Materials and methods

2.1. Materials

Water for solution preparation was purified using a Milli-Q system. All chemicals were of the highest available grade and were used as received: acetohydroxamic acid (aceto-HX), TEMPOL, TEMPO, 3-carbamoyl-PROXYL (3-CP), sodium *trans*-hyponitrite hydrate ($Na_2N_2O_2xH_2O$), myoglobin from horse heart and Griess reagent were purchased from Sigma-Aldrich. Catalase was purchased from Boehringer Biochemicals. AS and C-PTIO were purchased from Cayman Chemical Co. (San Diego, CA, USA). TEMPOL-H was prepared by bubbling HCl gas through ethanolic solution of TEMPOL followed by drying [28]. Fresh solutions of TEMPOL-H were prepared immediately before each experiment to minimize its oxidation to TEMPOL. Stock solution of AS was prepared in 10 mM NaOH and its concentration was determined by the absorbance at 248 nm ($\varepsilon = 8300$ M^{-1} cm^{-1} [29]). Stock solutions of $N_2O_2^{2-}$ ($pK_a(H_2N_2O_2) = 7.2$, $pK_a(HN_2O_2^-) = 11.5$ [30]) were prepared in 0.1 M NaOH, and the concentration was determined by the absorbance at 248 nm ($\varepsilon(N_2O_2^{2-}) = 6550$ M^{-1} cm^{-1}) [31]. The concentrations thus calculated correspond to 4 molecules of H_2O per $Na_2N_2O_2$ in the commercial salt. For pulse radiolysis experiments, solutions of sodium *trans*-hyponitrite (4 mM) were prepared by adding the salt to N_2O-saturated solutions containing 40 mM phosphate buffer (pH 6.8), and were used within 1 h.

Metmyoglobin ($MbFe^{III}$) was prepared by adding excess of 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 $MbFe^{III}$ was determined spectrophotometrically using $\varepsilon_{408} = 188{,}000$ M^{-1} cm^{-1} [32]. N_2O gas (Maxima, Israel) was purified from traces of O_2 by passing it through an oxygen trap (OXY-TRAP, Alltech Associate Inc.).

2.2. Analysis

Nitrite formation was assayed by mixing equal volumes of the sample and the Griess reagent. Analysis of nitrite produced under anoxia was done as follows. 1.5 mL of the reagent was placed in a 4 mL optical cell sealed with a rubber septum. The reagent was deoxygenated by passing N_2 through the solution for 10 min followed by injecting 1.5 mL of the anoxic sample through the rubber septum. The absorption at 540 nm was read 15 min after the addition of the sample. Calibration curves were prepared using known concentrations of nitrite. H_2O_2 concentration was determined by the molybdate-activated iodide assay ($\varepsilon_{352} = 25{,}800$ M^{-1} cm^{-1}) [33].

2.3. EPR

EPR spectra were recorded using a JEOL X band JES-RE3X spectrometer operating at 9.5 GHz with center field set at 3287 G, 100 kHz modulation frequency, 1 G modulation amplitude and 4–16 mW incident microwave power. Samples were injected into a flexible capillary, which was inserted into a quartz tube placed within the EPR spectrometer cavity.

2.4. Radiolysis

Pulse radiolysis experiments were carried out using a 5-MeV Varian 7715 linear accelerator (0.1–0.3 μs electron pulses, 200 mA current). A 200 W Xe lamp produced the analyzing light. Measurements were done using 2 or 4 cm spectrosil cells with three light passes. Dosimetry was performed with a N_2O-saturated solution containing 5 mM KSCN using $G\varepsilon((SCN)_2^{\bullet-}) = 5\times10^4$ radicals $(100\ eV)^{-1}$ M^{-1} cm^{-1} at 475 nm [34]. A ^{137}Cs source was used for steady-state radiolysis. Dosimetry was performed with Fricke dosimeter (10 mM M Fe^{II} and 1 mM NaCl in 0.8 N H_2SO_4) using $G(Fe^{III}) = 15.7$ and $\varepsilon(Fe^{III}) = 2197$ M^{-1} cm^{-1} at 302 nm. All experiments were carried out at room temperature.

Irradiation of aqueous solutions produces several species with different yields as shown in Eq. (5). The numbers in parenthesis are G-values, which represent the radiation yields in neutral water (in number of molecules formed per 100 eV corresponding to 0.1036 μM Gy^{-1}) [35], and are about 7% higher in the presence of high solute concentrations.

$$H_2O \xrightarrow{\gamma} e_{aq}^-(2.6), {}^{\bullet}OH(2.7), H^{\bullet}(0.6), H_3O^+(2.6), H_2O_2(0.72) \qquad (5)$$

When solutions are saturated with N_2O ($[N_2O] = 25$ mM), the solvated electrons are converted into $^{\bullet}OH$ radicals (reaction (6)), and

under such conditions 90% of the primary radicals are $^{\bullet}OH$ and the rest $H^{\bullet}$ radicals [35].

$$e^-_{aq} + N_2O + H_2O \rightarrow {}^{\bullet}OH + N_2 + OH^- \qquad k_6 = 9.1 \times 10^9 M^{-1}s^{-1} \quad (6)$$

3. Results and discussion

3.1. HNO reaction with nitroxides

The reactions of TEMPOL, TEMPO and 3-CP with HNO have been studied using AS. The EPR-signal of 100 μM nitroxide progressively disappeared upon exposure to excess of AS in aerated solutions containing 40 mM phosphate buffer at pH 7.0. The initial signal was fully restored upon the addition of 1 mM ferricyanide indicating that RNO is reduced by HNO to its respective EPR-silent RNO-H.

The rate constants of HNO reactions with RNO have been determined using competition kinetics against $MbFe^{III}$. In the presence of AS, $MbFe^{III}$ undergoes reductive nitrosylation, producing a ferrous-nitrosyl complex, which is rather stable (reaction (7)) [36–38].

$$MbFe^{III} + HNO \rightarrow MbFe^{II}NO + H^+ \quad (7)$$

The addition of 40–50 μM AS to aerated solutions containing 10–30 μM $MbFe^{III}$ at pH 7 yielded, within the accuracy of the measurements, the same initial rate of $MbFe^{II}NO$ formation (V_o), which was monitored at 425 nm. This observation implies that under such experimental conditions the rates of reaction (4) and that of HNO with O_2 ($k \approx 3\times10^3$ M^{-1} s^{-1} [4]) are negligible as compared to the rate of reaction (7). Therefore, the initial rate of $MbFe^{II}NO$ formation (V) was followed upon mixing 44 μM AS with 10 or 22 μM $MbFe^{III}$ and varying [RNO]. Fig. 1 demonstrates typical results obtained in the case of TEMPOL. The effect [RNO] on the initial rate of $MbFe^{II}NO$ formation follows relationship 8.

$$1/V = 1/V_o + (k_1/k_7V_o)([RNO]/[MbFe^{III}]) \quad (8)$$

In Fig. 2 plots of $V_o/V-1$ vs. [RNO]/[$MbFe^{III}$] are given yielding $k_1/k_7 = 0.17 \pm 0.02$ for TEMPO and TEMPOL and 0.054 ± 0.004 for 3-CP. Using $k_7 = 8\times10^5$ M^{-1} s^{-1} [4], one calculates $k_1 = (1.4 \pm 0.2)\times 10^5$ M^{-1} s^{-1} for TEMPOL and TEMPO and $(4.3 \pm 0.4)\times10^4$ M^{-1} s^{-1} for 3-CP. The former value is ca. twice higher than the values reported in the literature and close to those previously determined for PTIO and C-PTIO (Table 1). The reactivity of HNO toward RNO is surprisingly

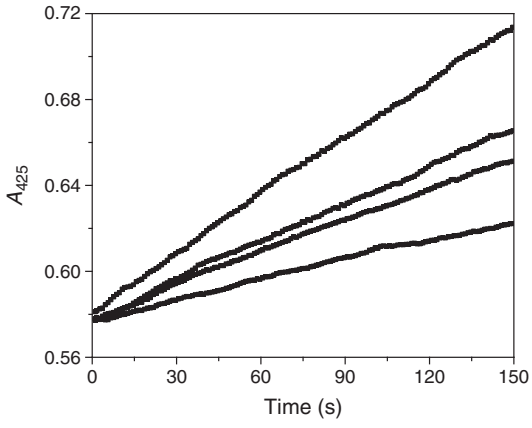


Fig. 1. TEMPOL effect on the initial rate of $MbFe^{II}NO$ formation. The formation of $MbFe^{II}NO$ was monitored at 425 nm upon mixing 44 μM AS with 10 μM $MbFe^{III}$ in the absence and presence of 21, 42 and 83 μM TEMPOL in aerated solutions containing 10 mM PB at pH 7.0 and 23 °C.

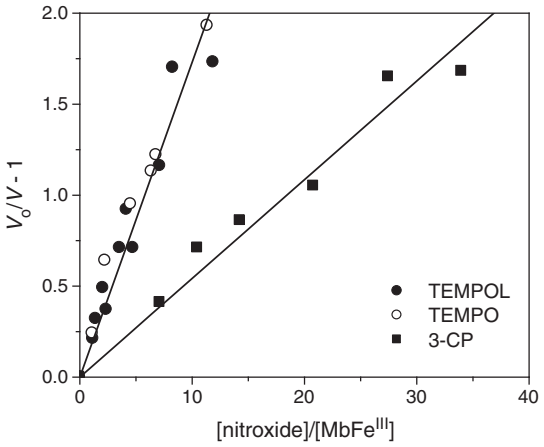


Fig. 2. Nitroxide effect on the initial rate of $MbFe^{II}NO$ formation analyzed according to Eq. (8). The formation of $MbFe^{II}NO$ was monitored at 425 nm upon mixing 42–44 μM AS with 10 or 22 μM $MbFe^{III}$ in aerated solutions containing 10 mM PB at pH 7.0 and 23 °C.

low in view of the relatively high driving force for this reaction calculated using $E_7(NO/HNO) = -0.55$ V [22,23] and $E_7(RNO/RNO\text{-}H) \approx 0.2$ V for TEMPO and TEMPOL [13,16], ≈0.1 V for 3-CP [13] and ≈0.27 V for C-PTIO [17] (Table 1).

Reaction (1) can proceed via a single concerted step, i.e., hydrogen atom transfer (HAT), or via stepwise processes involving initial electron transfer (ET) followed by proton transfer (PT), or PT followed by ET as shown in Scheme 2. It has been demonstrated that the TEMPO/TEMPOH and related redox couples are particularly valuable for proton-coupled electron transfer studies because of the their low O–H bond strengths, and their strong thermochemical bias toward HAT reactions [39,40]. Similar arguments also hold for HNO ($pK_a = 11.4$, $E^o(NO/NO^-) = -0.8$ V [22,23]) whose bond dissociation free energy (BDFE) in water is estimated according to Eq. (9) to be ≈55 kcal mol^{-1}, which is significantly lower than that of RNO-H, e.g., ≈71 kcal mol^{-1} for TEMPO-H [40].

$$BDFE(X-H)_{water} = 1.37pK_a + 23.06E^o + 57.6 \quad (9)$$

The rate constant of HAT reaction can be predicted using the Marcus cross relation, which was originally developed for electron transfer reaction, but has been demonstrated to provide a valuable new framework for understanding and predicting HAT reactivity [41,42]. Accordingly, k_1 is calculated using Eqs. (10) and (11) where $k_{HNO/NO}$ and $k_{RNO\text{-}H/RNO}$ are the HNO/NO and RNO-H/RNO self-exchange rate constants.

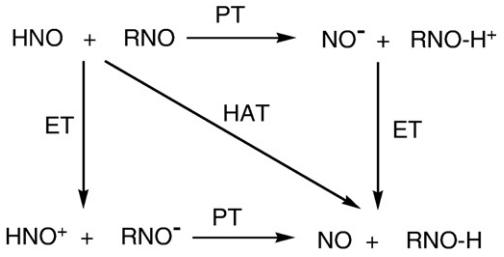
$$k_1 = \left(k_{HNO/NO}k_{RNO-H/RNO}K_1f\right)^{1/2} \quad (10)$$

$$\ln f = (\ln K_1)^2 / \left\{4\ln\left(k_{HNO/NO}k_{RNO-H/RNO}/10^{22}\right)\right\} \quad (11)$$

Table 1
Driving force and rate constants for the reaction of HNO with nitroxides at pH 7.

Nitroxide	ΔG_1° (kcal mol^{-1})[a]	k_1, $M^{-1}s^{-1}$	Ref.
TEMPO	−17.3	$(1.4 \pm 0.2)\times10^5$	This work
		$(6.3 \pm 0.8)\times10^4$	[5]
TEMPOL	−17.3	$(1.4 \pm 0.2)\times10^5$	This work
		8×10^4	[4]
3-CP	−15.0	$(4.3 \pm 0.4)\times10^4$	This work
C-PTIO	−18.9	$(1.4 \pm 0.2)\times10^5$	[6]

[a] Calculated using $E_7(NO/HNO) = -0.55$ V [22,23] and $E_7(RNO/RNO\text{-}H) = 0.2$ V for TEMPO and TEMPOL [13,16], 0.1 V for 3-CP [13] and 0.27 V for C-PTIO [17].



Scheme 2. Concerted vs. stepwise transfer of $H^+ + e^-$ demonstrated for reaction (1).

The TEMPO-H/TEMPO self-exchange rate constant has been estimated from pseudo-self-exchange reactions between similar nitroxides to be $4.7 \pm 1.0\ M^{-1}\ s^{-1}$ in MeCN [39]. We have estimated a value of $7.4 \pm 1.6\ M^{-1}\ s^{-1}$ in water using the Litwinienko and Ingold's kinetic solvent effect model, i.e., Eq. (12) [43], and the Abraham H-bonding parameters $\alpha_2^H(\text{TEMPO-H}) = 0.39$, $\beta_2^H(\text{MeCN}) = 0.44$ and $\beta_2^H(H_2O) = 0.38$ [44]. The origin of this very slow self-exchange rate constant has been discussed elsewhere [39].

$$\log(k_{solv1}) - \log(k_{solv2}) = -8.3\alpha_2^{H}(\text{TEMPO-H})\left[\beta_2^{H}(\text{solv1}) - \beta_2^{H}(\text{solv2})\right] \quad (12)$$

Using $k_{\text{TEMPO-H/TEMPO}} = 7.4\ M^{-1}\ s^{-1}$ and $K_1 = 5.4 \times 10^{12}$, the calculated k_1 approaches the experimental value ($1.4 \times 10^5\ M^{-1}\ s^{-1}$) when $k_{\text{HNO/NO}} \approx 0.03\ M^{-1}\ s^{-1}$. Within the limitations of this approach, the latter value can vary within 1–2 orders of magnitude [41]. The experimental k_1 and K_1 values for TEMPO and TEMPOL are the same implying that the $k_{\text{RNO-H/RNO}}$ values for these nitroxides are similar. Assuming that this is also the case for 3-CP, one calculates $k_1 = 2 \times 10^4\ M^{-1}\ s^{-1}$, which is ca. twice lower than the experimental value. It is concluded that the slowness of reaction (1) can be attributed to the low self-exchange rate constants of HNO/NO and RNO-H/RNO.

3.2. The reaction of hyponitrite radical with nitroxides and TEMPOL-H

Hyponitrite radicals were generated in situ upon pulse-irradiation of N_2O-saturated solutions containing 4 mM sodium *trans*-hyponitrite and 40 mM phosphate buffer (pH 6.8).

$$^{\bullet}OH + H_2N_2O_2/HN_2O_2^- \rightarrow HN_2O_2^{\bullet}/N_2O_2^{\bullet-} + H_2O \quad (13)$$

The apparent rate constant of the hyponitrite radicals formation at pH 6.8 is about $1 \times 10^9\ M^{-1}\ s^{-1}$ [11,45]. The $HN_2O_2^{\bullet}$ radical is weakly acidic ($pK_a = 5.5$ [11,45]) and at pH 6.8 the deprotonated radical predominates. The decay of $N_2O_2^{\bullet-}$ was monitored at 290 nm ($\varepsilon = 5900\ M^{-1}\ cm^{-1}$ [45]), and was found to obey second-order kinetics ($2k_{app} = 4.2 \times 10^8\ M^{-1}\ s^{-1}$) in agreement with the literature values [11,45]. The decay of $N_2O_2^{\bullet-}$ turned from second- to first-order kinetics in the presence of the nitroxides using a low pulse intensity (1.76 Gy, $[OH]_o \approx 1\ \mu M$) to minimize the contribution of the second-order decomposition. The observed first-order rate constants increased upon increasing the concentration of the nitroxides (Fig. 3), and the bimolecular rate constants were derived from the slopes of the lines in Fig. 3 to be $(2.4 \pm 0.2) \times 10^6\ M^{-1}\ s^{-1}$ for TEMPO, $(9.8 \pm 0.2) \times 10^5\ M^{-1}\ s^{-1}$ for TEMPOL and $(5.9 \pm 0.5) \times 10^5\ M^{-1}\ s^{-1}$ for 3-CP. The yield of $N_2O_2^{\bullet-}$ decreased upon increasing the concentration of the nitroxides due to the competition between $H_2N_2O_2/HN_2O_2^-$ and the nitroxides for $^{\bullet}OH$ ($k(^{\bullet}OH + \text{nitroxide}) = (3–5) \times 10^9\ M^{-1}\ s^{-1}$ [46,47]), but we assume that the resulting alkyl radicals and/or $RN^+{=}O$ have no effect on the decay of $N_2O_2^{\bullet-}$. The rate constant of TEMPOL-H reaction with $N_2O_2^{\bullet-}$ was estimated to be $k \approx 3 \times 10^4\ M^{-1}\ s^{-1}$ based on the decrease of the half-life of the $N_2O_2^{\bullet-}$ by about 50% in the presence of 4 mM TEMPOL-H.

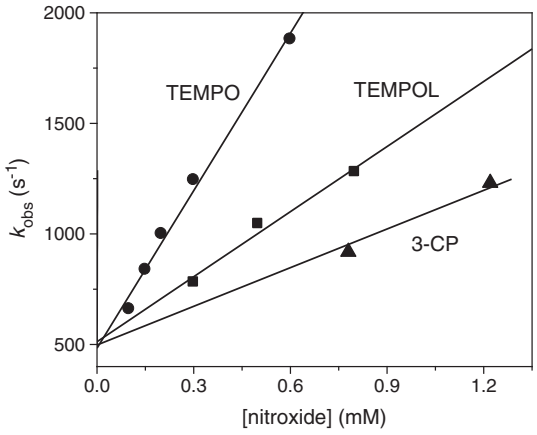


Fig. 3. Observed first order rate constant for the decay of the hyponitrite radical in the presence of various concentrations of TEMPO, TEMPOL and 3-CP. The decay of the hyponitrite radical was monitored at 290 nm upon pulse irradiation (1.76 Gy/pulse) of N_2O-saturated solutions containing 4 mM sodium *trans*-hyponitrite and 40 mM PB (pH 6.8).

$N_2O_2^{\bullet-}$ shows surprisingly low reactivity toward RNO given that the midpoint potential at pH 6.8 is ≈ 1.25 V [11] and $E^o(RN^+{=}O/RNO) = 0.74$, 0.81 and 0.87 V for TEMPO, TEMPOL and 3-CP, respectively [12,14–16], i.e., $\Delta G^o_{14} \approx -(9–12)$ kcal mol^{-1}.

$$N_2O_2^{\bullet-} + RNO \rightarrow N_2O_2^{2-} + RN^+{=}O \quad (14)$$

Low reactivity of $N_2O_2^{\bullet-}$ towards O_2 and $O_2^{\bullet-}$ at pH 9.2 has previously been reported for these strongly driven reactions with corresponding rate constants below 1×10^6 and $5 \times 10^7\ M^{-1}\ s^{-1}$ [11]. It has been assumed that there are large nuclear reorganization barriers for both reduction and oxidation of $N_2O_2^{\bullet-}$, and that these processes proceed via an inner-sphere electron transfer mechanism [11].

Oxidation of HNO by piperidine and pyrrolidine nitroxides yields NO, which does not react with these nitroxides but rather with HNO forming the hyponitrite radical (reaction (2)). This radical can either react with RNO/RNO-H or add to NO forming eventually N_2O and nitrite (reactions (15), (16)) [10].

$$N_2O_2^{\bullet-} + NO \rightarrow N_3O_3^- \qquad k_{15} = 5.4 \times 10^9\ M^{-1}s^{-1} \quad (15)$$

$$N_3O_3^- + NO \rightarrow N_2O + NO_2^- \qquad k_{16} = 300\ s^{-1} \quad (16)$$

Since $k_{14} = (5.9 - 24) \times 10^5\ M^{-1}\ s^{-1}$ is about 4-orders of magnitude lower than k_{15}, the contribution of reaction (14) is insignificant and net reaction (17) is obtained for RNO reduction by HNO.

$$2RNO + 3HNO \rightarrow 2RNO{-}H + N_2O + NO_2^- + H^+ \quad (17)$$

3.3. Oxidation of aceto-HX by $^{\bullet}OH$ in the presence of TEMPOL or C-PTIO

$^{\bullet}OH$ radical was generated upon irradiation of N_2O-saturated solutions containing 4 mM aceto-HX ($pK_a = 9.0$ [48]), 100 μM nitroxide, 55 U mL^{-1} catalase and 10 mM phosphate buffer at pH 8.3. Under such conditions $^{\bullet}OH$ reacts exclusively with the hydroxamate moiety forming the transient nitroxide radical (reaction (18)) [7].

$$^{\bullet}OH + CH_3C(O)NHOH/CH_3C(O)NHO^- \rightarrow CH_3C(O)NHO^{\bullet}/CH_3C(O)NO^{\bullet-} + H_2O/OH^- \quad (18)$$

Catalase was added to the reaction mixture to remove the radiolytically formed H_2O_2, which is capable of oxidizing RNO-H.

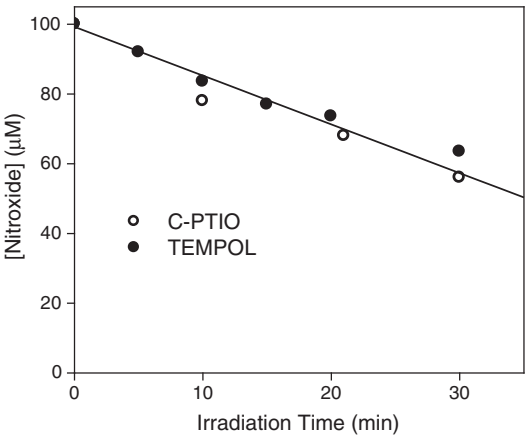


Fig. 4. TEMPOL and C-PTIO depletion induced upon aceto-HX oxidation by $^{\bullet}$OH. Solutions saturated with N_2O containing 100 μM nitroxide, 55 U mL^{-1} catalase, 4 mM aceto-HX and 10 mM phosphate buffer (pH 8.3) were irradiated at a dose rate of 7.8 Gy min^{-1}: (○) TEMPOL; (●) C-PTIO. The EPR signal intensity was monitored using modulation 1 G, power 8 mW, field center 3287 G and TC 0.1 s.

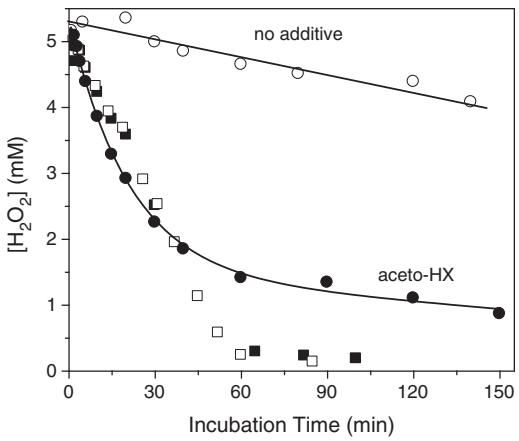


Fig. 5. Effects of TEMPOL and C-PTIO on H_2O_2 depletion. Depletion of 5 mM H_2O_2 in 10 mM phosphate buffer (pH 7.0) containing 25 μM $MbFe^{III}$: (○) no additive; (●) 4 mM aceto-HX; (■) 4 mM aceto-HX and 100 μM TEMPOL; (□) 4 mM aceto-HX and 100 μM C-PTIO.

The EPR-signal of TEMPOL and C-PTIO decreased linearly with the radiation dose with a depletion rate of 1.4 ± 0.1 μM min^{-1} (Fig. 4). The conversion of C-PTIO to C-PTI was not observed, which can be attributed to the accumulation of C-PTIO-H and/or C-PTI-H. The addition of 1 mM ferricyanide restored most of the EPR signal of TEMPOL and C-PTIO, though not of C-PTI, indicating that C-PTI-H was not accumulated in this system. The rate of nitrite formation was significantly low, 0.06 ± 0.02 μM min^{-1}, and did not increase upon subsequent exposure of the anoxic irradiated samples to oxygen. The rate of RNO reduction by HNO is an upper limit value since the radiolytically produced $H^{\bullet}$ radical (0.47 μM min^{-1}) can also reduce RNO [46]. Thus, the lower reduction rate of RNO is 0.9 ± 0.1 μM min^{-1}, and since the rate of $^{\bullet}$OH production was 4.5 μM min^{-1}, the yields of HNO vary between 20 and 31% depending on the competition between 4 mM aceto-HX and 100 μM RNO for $H^{\bullet}$ radical.

These results demonstrate that the yield of HNO is in agreement with that previously determined by measuring the accumulation of N_2O at pH 7.0 (ca. 25% [27]), and that NO formed via the oxidation of HNO by TEMPOL or C-PTIO, is efficiently trapped by species other than HNO or C-PTIO. The spin density on the transient nitroxide radical derived upon one-electron oxidation of aceto-HX is evenly distributed over the O–C–N–O, and the potential tautomeric radicals are shown in Scheme 3. We have previously demonstrated that the transient radicals $CH_3C(O)NHO^{\bullet}/CH_3C(O)NO^{\bullet-}$ decompose bimolecularly [7,27]. The recombination of such tautomeric radicals can presumably produce various adducts where the O–O ones are expected to be unstable. We have assumed that these adducts decompose via heterolysis of the O–O bond yielding $CH_3C(O)NHOH$ and $CH_3C(O)N{=}O$, which forms HNO via hydrolysis. Since the yield of HNO derived upon TEMPOL/C-PTIO reduction is similar to that calculated from the yield of N_2O in the absence of these nitroxides, it is suggested that NO is efficiently trapped by the carbon-centered radical [49] and possibly by the nitrogen-centered one (see Scheme 2).

3.4. Oxidation of aceto-HX by $MbFe^{III}/H_2O_2$ in the presence of TEMPOL or C-PTIO

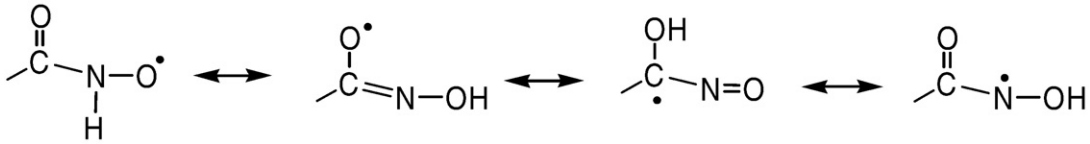
$MbFe^{III}$ in the presence of H_2O_2 demonstrates a modest catalase-like activity (reactions (19)–(21)), which is stimulated in the presence of aceto-HX [27] or nitroxides [50–52].

$$MbFe^{III} + H_2O_2 \rightarrow {}^{\bullet}MbFe^{IV}{=}O + H_2O \quad (19)$$

$${}^{\bullet}MbFe^{IV}{=}O + H_2O_2 \rightarrow MbFe^{IV}{=}O + O_2^{\bullet-} + 2H^+ \quad (20)$$

$$MbFe^{IV}{=}O + H_2O_2 \rightarrow MbFe^{III} + O_2^{\bullet-} + H_2O \quad (21)$$

The addition of 100 μM TEMPOL or C-PTIO to solutions containing 4 mM aceto-HX, 25 μM $MbFe^{III}$ and 5 mM H_2O_2 facilitates H_2O_2 depletion (Fig. 5) and nitrite accumulation (Fig. 6). Upon the addition of H_2O_2 to solutions containing $MbFe^{III}$, aceto-HX and nitroxide, the spectrum of $MbFe^{III}$ is converted within less than 30 s into that of $MbFe^{IV}{=}O$ implying that the steady-state concentration of $MbFe^{III}$ is extremely low as evident from the absence of its characteristic absorbance at 408 nm. About 30% of the EPR signal of TEMPOL was lost during the consumption of most H_2O_2. The EPR-signal could not be restored upon the addition of ferricyanide demonstrating the conversion of TEMPOL to diamagnetic species other than TEMPOL-H. In the case of C-PTIO, about 50% of its EPR signal decayed while only about 10% was converted into C-PTI during the consumption of most H_2O_2 (Fig. 7). The EPR-signals of C-PTIO and C-PTI were unaffected by the addition of ferricyanide indicating that the respective hydroxylamines were not accumulated. These results demonstrate that TEMPOL and C-PTIO act catalytically in facilitating H_2O_2 decomposition and nitrite formation. The 30–50% loss of the nitroxides during the complete consumption of 5 mM H_2O_2 can be attributed to their ability to react with carbon-centered radical, e.g., $^{\bullet}MbFe^{IV}{=}O$, $CH_3C(O)NHO^{\bullet}$ (Scheme 2), forming stable diamagnetic adducts [53,54].



Scheme 3. Tautomeric radicals derived from one-electron oxidation of $CH_3C(O)NHOH$.

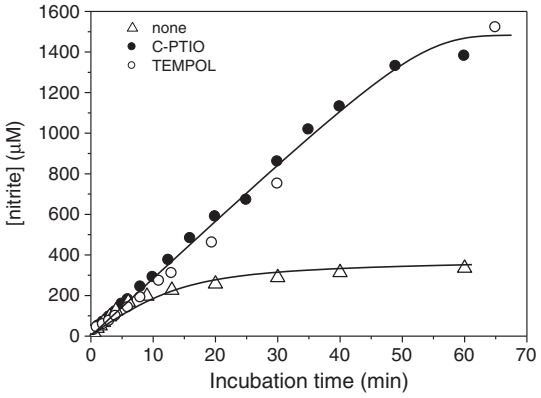


Fig. 6. Effects of TEMPOL and C-PTIO on nitrite accumulation. Solutions contained 25 μM $MbFe^{III}$, 5 mM H_2O_2 and 4 mM aceto-HX in 10 phosphate buffer at pH 7.0 (Δ) with 100 μM TEMPOL (O) or 100 μM C-PTIO (●).

Accumulation of nitrite and the conversion of C-PTIO to C-PTI have been previously demonstrated during the oxidation of hydroxyurea, SAHA and Trichostatin A by heme proteins and H_2O_2, which led to the conclusion that these hydroxamic acids release NO upon oxidation [55–57]. However, since C-PTIO catalytically facilitates H_2O_2 decomposition and nitrite accumulation, the minor conversion of C-PTIO to C-PTI demonstrates the formation of NO, but does not provide any mechanistic insight since NO can be produced via HNO oxidation by C-PTIO and/or $MbFe^{IV}=O$. The very low yield of C-PTI implies that $MbFe^{IV}=O$ competes efficiently with C-PTIO for NO, and that reaction (22) is the main source of nitrite in this system.

$$MbFe^{IV}=O + NO \rightarrow MbFe^{III} + NO_2^- \quad k_{22} = 1.79 \times 10^7 M^{-1}s^{-1} [58] (22)$$

4. Conclusions

The reactivity of HNO and $N_2O_2^{\bullet-}$ toward cyclic nitroxides is surprisingly low given the considerable driving force for these reactions. Cyclic nitroxides are reduced to their respective hydroxylamines by HNO. The rate constants for these HAT reactions can be predicted using the Marcus cross relation assuming that the self-exchange rate constants of HNO/NO and RNO-H/RNO are significantly low. Cyclic nitroxides cannot be used as quantitative HNO scavengers when hydroxamic acids are oxidized by the heme proteins and H_2O_2 reaction system since they catalytically facilitate the decomposition of H_2O_2 and accumulation of nitrite. In this system the conversion of C-PTIO into C-PTI is only a minor side reaction, which does not provide any mechanistic insight.

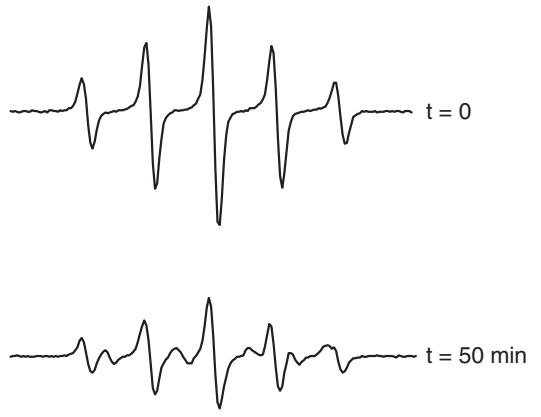


Fig. 7. Change of C-PTIO EPR spectrum upon exposure of aceto-HX to oxidative stress. C-PTIO at 100 μM C-PTIO, 4 mM aceto-HX, 25 μM $MbFe^{III}$ and 5 mM H_2O_2 in 10 mM phosphate buffer at pH 7.0 were incubated. Top: the five-line spectrum of C-PTIO before the addition of H_2O_2; Bottom: the decay of C-PTIO and the appearance of C-PTI after 50 min of incubation. The EPR spectra were monitored using modulation 1 G, Power 4 mW, field center 3287 G, gain 160 and TC 0.03 s.

Abbreviations

aceto-HX	acetohydroxamic acid
AS	Angeli's salt
BDFE	bond dissociation free energy
3-CP	3-carbamoyl-PROXYL
C-PTI	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl
C-PTIO	2-(4-carboxyphenyl)-4,4,5,5,-tetramethyl-imidazoline-1-oxyl-3-oxide
ET	electron transfer
HAT	hydrogen atom transfer
HX	hydroxamic acid
$MbFe^{IV}=O$	ferryl myoglobin
$MbFe^{III}$	metmyoglobin (MetMb)
PT	proton transfer
TEMPO	2,2,6,6-tetramethyl piperidine-1-oxyl
TEMPOL	4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl
TEMPOL-H	TEMPOL-hydroxylamine
RNO	nitroxide
RNO-H	hydroxylamine

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