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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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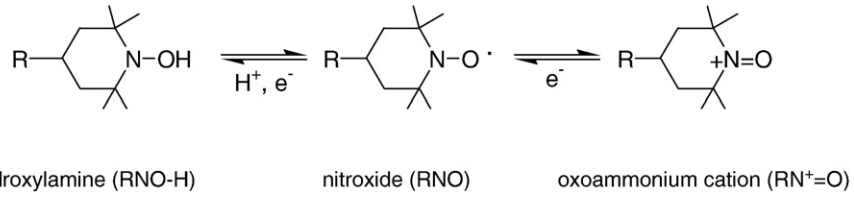
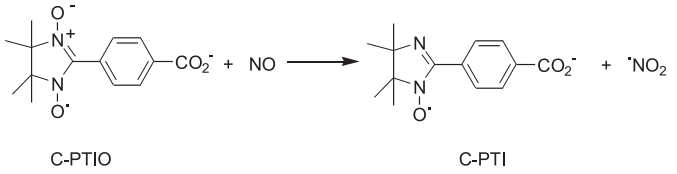


Scheme 1. Oxidation and reduction of piperidine nitroxides (R = H, TEMPO; R = OH, TEMPOL).

(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