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A kinetic study on the reactivity of azanone (**HNO**) toward its selected scavengers: Insight into its chemistry and detection

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ABSTRACT

Recently, azanone (**HNO**), which is the protonated one-electron reduction product of **•NO**, has gained considerable attention due to its unique pharmacological effects. Although there has been much progress in understanding **HNO** biology and chemistry, it remains the most elusive reactive nitrogen species. Herein, we applied the competition kinetics method, based on two parallel **HNO** reactions with the different scavengers and molecular oxygen (k_{O2} = (1.8 ± 0.3) × 10^4 M^{-1} s^{-1}), to determine the rate constants for the reactions of **HNO** with its selected co-reactants. The rate constants for the reactions of **HNO** with nitrite (k = (5.0 ± 0.9) × 10^3 $M^{-1}s^{-1}$), hydroxylamine (k = (2.1 ± 0.4) × 10^4 $M^{-1}s^{-1}$), sulfite (k = (1.2 ± 0.2) × 10^6 M^{-1} s^{-1}), thiosulfate (k = (2.2 ± 0.7) × 10^4 M^{-1} s^{-1}), benzenesulfinate (k = (4.4 ± 0.9) × 10^4 M^{-1} s^{-1}), 2-bromobenzenesulfinate (k = (5.0 ± 1.2) × 10^4 M^{-1} s^{-1}), nitrosoglutathione (k = (2.4 ± 0.7) × 10^4 $M^{-1}s^{-1}$), nitrosobenzene (k > 1.5 × 10^5 M^{-1} s^{-1}), 2-nitroso-1-naphthol (k = (1.0 ± 0.2) × 10^6 M^{-1} s^{-1}), triphenylphosphine (k > 7.3 × 10^6 M^{-1} s^{-1}), triphenylphosphine-3,3′,3″-trisulfonate (k = (3.0 ± 0.5) × 10^6 M^{-1} s^{-1}), tris-carboxyethylphosphine (k = (1.2 ± 0.3) × 10^7 M^{-1} s^{-1}), a triphenylphosphine-based **P-CM** fluorogenic probe (k > 1.2 × 10^7 M^{-1} s^{-1}), the **TEMPO-9-AC** fluorogenic probe (k = (9 ± 2) × 10^4 M^{-1} s^{-1}) and **4-acetamido-TEMPO** (k = (8 ± 2) × 10^4 $M^{-1}s^{-1}$) are reported. The implications of these **HNO** reactions are also discussed. The data presented in this paper are a valuable contribution to the incompletely understood reactivity of **HNO.**

1. Introduction

Nitroxyl (**HNO, IUPAC** name azanone), which is formally the protonated product of one-electron reduction of nitric oxide (**•NO**), is an elusive reactive nitrogen species that demonstrates interesting biological chemistry and has high pharmacological importance [1–3]. To date, the chemistry and biological activities of azanone are still not comprehensively understood, although it has been established that **HNO** donors induce positive inotropic/lusitropic effects, elicit vasorelaxation and exert antiaggregating effects on platelets [4,5]. The positive effects of **HNO** that have been observed on the vascular system and the possible applicability of its donors as therapeutics in the treatment of heart failure [6] has significantly increased the interest in azanone in recent years.

There are several routes for the intracellular formation of **HNO**, although none of them has unambiguously been shown to occur. It has been proposed that **HNO** can be formed by the nitric oxide synthase-mediated oxidation of N^{ω}-hydroxy-L-arginine [7–9]. *In vitro* studies have shown that azanone can be produced upon **•NO** reduction by cytochrome *c*, xanthine oxidase, or ubiquinol [2,10–12]. Azanone can also be produced by the nitrosothiol reaction with other thiols or ascorbate, although kinetic considerations suggest that the biological relevance of this path is i negligible [13,14]. Recently, Doctorovich et al. showed that azanone can be formed during the reactions of **•NO** with ascorbate or phenols (e.g.

Abbreviations: **4-acetamido-TEMPO**, 4-acetamido-2,2,6,6-tetramethylpiperidine 1-oxyl; **2-BrPA**, 2-bromo-*N*-hydroxybenzenesulfonamide (2-bromo substituted Piloty's acid); **dtpa**, diethylenetriaminepenta-acetic acid; **GSNO**, *S*-nitrosoglutathione; **PA**, *N*-hydroxybenzenesulfonamide (Piloty's acid); **PC1**, 3-oxo-3H-phenoxazin-7-yl pinacolatoboron (PeroxyCrimson 1); **P-CM**, 2-oxo-2H-chromen-7-yl 2-(diphenylphosphino)-benzoate; **PMT**, photomultiplier; **TCEP**, tris-carboxyethylphosphine; **TEMPO-9-AC**, 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-1-oxyl; **TEMPOL**, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; **TPPTS**, tris(3-sulfophenyl)phosphine trisodium salt; **TXPTS**, tris(4,6-dimethyl-3-sulfophenyl)phosphine trisodium salt.

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tyrosine, hydroquinone, salicylic acid, α-tocopherol or acetaminophen) [15,16]. Another possible path of azanone formation is the reaction of **•NO** and hydrogen sulfide (H_2S) [17].

Thiols, thiol proteins [18–20] and metalloproteins (e.g., superoxide dismutase, HRP, catalase, and cytochrome *c*) [21–26] are the most likely biological targets of **HNO** (reactions (1) and (2)).

$$HNO + RSH \rightarrow RSNHOH \quad (1)$$

$$HNO + M^n \rightarrow M^{n-1} + NO + H^+ \quad (2)$$

The most intriguing aspect of azanone reactivity is its reaction with molecular oxygen. Despite numerous studies on the reactivity of **HNO** with molecular oxygen, the product of this reaction has not been fully identified [27–29]. However, our recently published results unequivocally showed that **$ONOO^-$** is the major product of the reaction between **HNO** and molecular oxygen (reaction (3)) and that the second-order rate constant of that reaction was equal to $k_{O2} = (1.8 \pm 0.3) \times 10^4\ M^{-1}s^{-1}$ [20].

$$HNO + O_2 \rightarrow ONOO^- + H^+ \quad (3)$$

In contrast to nitric oxide, **HNO** is a strong electrophile that is highly reactive towards various nucleophiles. In aqueous solutions, **HNO** spontaneously dimerizes with a second-order rate constant of approximately $8 \times 10^6\ M^{-1}s^{-1}$ [30] to yield hyponitrous acid, which subsequently dehydrates to the final products, nitrous oxide and water (reaction (4)).

$$2HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O \quad (4)$$

The propensity of **HNO** to undergo this reaction requires the use of donor molecules that yield **HNO** as a product. Although numerous **HNO** donors have been reported [31–44], only two of them, Angeli's salt and Piloty's acid (*N*-hydroxybenzenesulfonamide, **PA**), are frequently used and have been studied extensively. Unfortunately, the latter cannot be utilized in biological assays due to the very slow release of **HNO** at physiological pH [31]. Recently, *ortho*-substituted Piloty's acid derivatives have been reported to be effective **HNO** donors under physiological conditions [32–34]. Among the various derivatives, one of the most promising is 2-bromo-Piloty's acid (**2-BrPA**, 2-bromo-*N*-hydroxybenzenesulfonamide), the decomposition of which under these conditions seems to be a facile and convenient process that leads to **HNO** generation [32].

Due to its high reactivity and short lifetime, **HNO** detection and kinetic studies are challenging. There are a number of strategies for the detection of **HNO,** including methods based on the detection of the final product of azanone dimerization, N_2O [2,32], electrochemical analysis [45], mass spectrometry [32] and methods based on the reaction of **HNO** with metal complexes or metalloporphyrins [46–55], thiols [14,56,57], phosphines [58–67], nitroso compounds [68] or the 2,2,6,6-tetramethyl-1-piperidinyloxy-based probe, **TEMPO-9-AC** [69]. The detection of **HNO** has recently been described in detail [1].

In this study, we applied a recently described competition kinetics method [20] to determine the reactivity of **HNO** towards its selected scavengers. The method is based on two parallel, competing **HNO** reactions: with an azanone scavenger or with molecular oxygen. The latter reaction results in the formation of peroxynitrite that can be easily detected fluorometrically using fluorogenic boronate probes [20,70]. In this study, we used the resorufin-based monoboronate probe, **PC1**. **PC1** reacts rapidly and directly with **$ONOO^-$** ($k = 1 \times 10^6\ M^{-1}s^{-1}$) [71] to form fluorescent resorufin as the major product. Herein, we present a kinetic study of the reaction of **HNO** with nitrite (**NO_2^-**), hydroxylamine (**NH_2OH**), sulfite (**SO_3^{2-}**), thiosulfate (**$S_2O_3^{2-}$**) benzenesulfinate (**$PhSO_2^-$**), 2-bromobenzenesulfinate (**2-BrPhSO$_2^-$**), selected nitroso compounds and selected phosphines (Scheme 1), fluorogenic probe **TEMPO-9-AC** and nitroxyl radical **4-acetamido-TEMPO**.

2. Material and methods

2.1. Chemicals

Angeli's salt was synthesized according to previously described procedure [31]. The concentration of Angeli's salt in the stock solution in 1 mM **NaOH** was determined by measuring the absorbance at 248 nm ($\varepsilon = 8.3 \times 10^3\ M^{-1}cm^{-1}$) [31]. 2-Bromo-*N*-hydroxybenzenesulfonamide (2-bromo substituted Piloty's acid, **2-BrPA**) was synthesized from the corresponding sulfonyl chloride and hydroxylamine hydrochloride according to the published procedure [32]. Stock solutions of **HNO** donors (Angeli's salt in 1 mM **NaOH** and **2-BrPA** in 1 mM **HCl**) were kept on ice. 2-Bromobenzenesulfinic acid sodium salt, a coumarin-based **P-CM** probe for azanone detection, and the resorufin-based monoboronate probe **PC1** for peroxynitrite detection were synthesized according to the previously described procedures [32,60,72]. Nitrosoglutathione (**GSNO**) was synthesized from glutathione and sodium nitrite according to a published procedure [73]. **TEMPO-9-AC** was purchased from Synchem UG & Co. KG, Germany. All other chemicals (of the highest purity available) were from Sigma-Aldrich Corp., and all solutions were prepared using deionized water (Millipore Milli-Q system).

2.2. Competition kinetics method

First, we determined the kinetics of the decomposition of the **HNO** donors used in that study: Angeli's salt and **2-BrPA**. To determine the rate constant for the decomposition of **2-BrPA** at pH 7.4 and 25 °C, we used the **UPLC** method to monitor the decomposition of **2-BrPA** and the formation of its decomposition product, 2-bromobenzenesulfinic acid. Specifically, the **2-BrPA** solution in acetonitrile and the buffer solution (pH 7.4) containing **dtpa** were incubated at 25 °C before they were combined. Next, the progress of the decomposition reaction in the mixed sample (100 μM **2-BrPA**, 50 mM phosphate buffer, pH 7.4, 100 μM **dtpa**, 5% CH_3CN) was followed using **UPLC** with gradient elution. Before injecting the sample, the column was equilibrated with the water/acetonitrile mobile phase (90/10 v/v) containing 0.1% TFA. **2-BrPA** and 2-bromobenzenesulfinic acid were separated with a linear increase of the acetonitrile concentration to 100% over 1.5 min beginning 0.5 min after injection of the sample. The separation was performed on an Acquity UPLC BEH C_{18} (1.7 μm, 50 × 2.1 mm) column. The analytes were eluted at a flow rate of 0.3 ml/min. Under these conditions, the following retention times were observed: **2-BrPA**: 1.76 min; 2-bromobenzenesulfic acid: 1.56 min. The peak areas used for the kinetic analysis were detected by monitoring the absorption at 276 nm (**2-BrPA**) or 272 nm (2-bromobenzenesulfic acid). The decomposition of Angeli's salt was monitored spectrophotometrically by following the decrease in its characteristic absorbance at 235 nm. The **HNO** fluxes were determined from the determined rate of the decomposition of its donors: Angeli's salt ($k = (8.4 \pm 0.1) \times 10^{-4}\ s^{-1}$, $t_{1/2} = (13.8 \pm 0.2)$ min, 25 °C) and **2-BrPA** ($k = (7.4 \pm 0.2) \times 10^{-4}\ s^{-1}$, $t_{1/2} = (15.6 \pm 0.4)$ min, 25 °C).

In the studied system, the initial concentration of Angeli's salt or **2-BrPA** was equal to 3 μM. The initial flux of **HNO** was therefore below 0.15 μM/min (resulting in a very low **HNO** steady-state concentration), and the **HNO** dimerization process was negligible and was not taken into consideration. A reaction model illustrating the competition kinetic method is presented in Scheme 2. **HNO**

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