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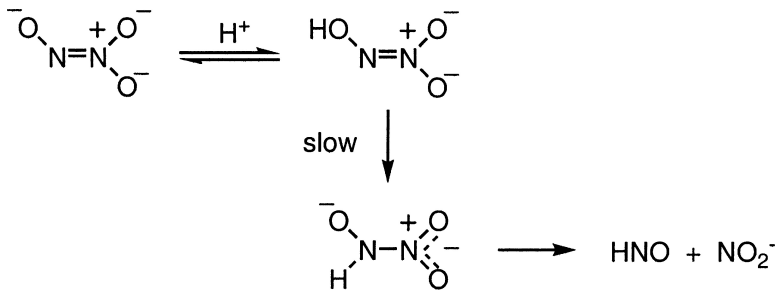

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Mechanism of Aerobic Decomposition of Angeli's Salt (Sodium Trioxodinitrate) at Physiological pH

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Abstract: The recent determination that Angeli's salt may have clinical application as a nitrogen oxide donor for treatment of cardiovascular diseases such as heart failure has led to renewed interest in the mechanism and products of thermal decomposition of Angeli's salt under physiological conditions. In this report, several mechanisms are evaluated experimentally and by quantum mechanical calculations to determine whether HNO is in fact released from Angeli's salt in neutral, aerobic solution. The mechanism of product autoxidation is also considered.

Introduction

Recent in vitro and in vivo comparisons of the effects of exposure to sodium trioxodinitrate (Angeli's salt) and nitric oxide (NO) donors have revealed that Angeli's salt elicits unique and generally orthogonal pharmacological responses to those of NO. For instance, Angeli's salt has been determined to enhance myocardial contractility (i.e., exert load-independent positive inotropy),[1] whereas the NO adduct of diethylamine (DEA/NO), which is a spontaneous NO donor with a similar decomposition rate to Angeli's salt,[2] has no such properties.[1] In fact, the cardiovascular effects induced by Angeli's salt may provide a novel therapy for heart failure.[3−5]

NO donors have been shown to be protective toward ischemia reperfusion injury of the heart, brain, liver, and gut mesentery.[6−9] Initial comparisons determined that infusion of Angeli's salt immediately prior to reperfusion exacerbated damage in the myocardial model.[10] However, alteration of the time of Angeli's salt exposure prior to the ischemic insult protectively preconditioned the heart against subsequent reperfusion injury to a greater extent than NO donors.[11] These results demonstrate a critical dependence on timing of donor delivery.

The contrast in the protective effects for NO donors and the deleterious consequences of Angeli's salt infusion observed in the initial reperfusion study[10] was similar to that of earlier cytotoxicity studies[12] in which necrosis induced by reactive oxygen species was alleviated by NO but augmented by Angeli's salt. Low millimolar concentrations of Angeli's salt itself were significantly toxic, with a magnitude similar to alkylhydroperoxides and several orders higher than NO donors. The cytotoxicity of Angeli's salt was dependent upon O_2, suggesting the involvement of an oxidized intermediate. Oxidation of the

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fluorophore dihydrorhodamine (DHR) by Angeli's salt has also been shown to be reliant upon O_2.[13]

The usefulness of Angeli's salt as a cardiovascular treatment requires understanding not only of the physiological effects but also of the chemical modifications and the chemistry of both the donor compound and the reactive intermediates. At physiological pH decomposition of Angeli's salt is generally accepted to be initiated by protonation of the dianion and to produce nitroxyl (HNO) and nitrite.[14−19]

$$N_2O_3^{2-} + H^+ \rightarrow HNO + NO_2^- \quad (1)$$

In 1970, Gratzel and colleagues[20] investigated the chemistry of the reduction products of NO in a pulse radiolytic study and reported that the pK_a of HNO is 4.7. This value indicates that HNO would deprotonate nearly quantitatively at physiological and higher pHs. Spectrophotometric observation of peroxynitrite ($ONOO^-$) during steady-state photolytic degradation of Angeli's salt in aerobic, highly alkaline solution[19] provided indirect evidence for the formation of the nitroxyl anion.

$$NO^- + O_2 \rightarrow ONOO^- \quad (2)$$

The autoxidation of NO^- is isoelectronic[21] to the nearly diffusion-controlled ($4-7 \times 10^9$ M^{-1} s^{-1})[22,23] reaction of NO and superoxide (O_2^-).

$$NO + O_2^- \rightarrow ONOO^- \quad (3)$$

The observation of significant $ONOO^-$ only at high pH was proposed[19] to be a result of the pH-dependent degradation rate[24] of $ONOO^-$.

Subsequent photochemical and thermal studies[25,26] confirmed that aerobic decomposition of Angeli's salt at high pH produces $ONOO^-$ nearly quantitatively. Formation of $ONOO^-$ was also observed[25] at neutral pH following UV flash photolysis, but only with a 25% yield. The mechanism is suggested to involve generation of excited states that are energetically favored to decay to NO^- rather than to HNO and, therefore, is unique to the high-energy system utilized and is not a function of thermal degradation. This finding is consistent with comparative aerobic studies[13,27] below pH 9 in which the overall chemical profiles of Angeli's salt and synthetic $ONOO^-$ were found to be distinct, although the oxidative properties were similar.

The condition-dependent dissimilarities[13,27] between the chemistry of Angeli's salt and $ONOO^-$ as well as the observations of discrete physiological effects for Angeli's salt and DEA/NO[1,3,10,11,28,29] led to reevaluation[25,30] of the acidity of HNO by quantum mechanical calculations and cyclic voltammetric and spectrophotometric analyses. The use of donor compounds in these two studies removed the complicating factor of excess NO required in the pulse radiolytic analysis,[20] and the pK_a for HNO was determined[25,30] to exceed 11. This high pK_a, which indicates that the predominant species at neutral pH is in fact HNO, is a function of the different ground states of the acid/base pair (^{1}HNO and $^3NO^-$). Due to the fast rate of consumption of HNO by dimerization (8×10^6 M^{-1} s^{-1})[25,31]

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

and to the extraordinarily slow, base-dependent rate of the spin-forbidden proton transfer (5×10^4 M^{-1} s^{-1}),[25]

$$^1HNO + OH^- \rightarrow {}^3NO^- + H_2O \quad (5)$$

formation of NO^- only becomes significant in alkaline solutions[5,25,32] or at low nanomolar concentrations of HNO.[33]

The oxidant resulting from aerobic decomposition of Angeli's salt at neutral pH has been speculated[13,25] to be a product of the reaction of HNO with O_2, through an undetermined mechanism. The dissimilar reactivity of Angeli's salt and synthetic $ONOO^-$ at neutral pH may then be explained by formation of different intermediates from the reaction of O_2 with HNO or NO^- (eq 2).[19,27] Alternatively, the possibility that O_2 reacts with Angeli's salt itself must be considered. Given the potential of Angeli's salt as a pharmacological agent in the treatment of cardiovascular diseases,[3−5] confirmation of the mechanism for Angeli's salt decomposition and for oxidant formation is of high interest. Here, we evaluate, both experimentally and with quantum mechanical calculations, the mechanisms that are under current consideration in the literature.

Material and Methods

Angeli's salt ($Na_2N_2O_3$), DEA/NO ($Na[Et_2NN(O)NO]$), and $ONOO^-$ were synthesized and used as previously described.[12,13,34] The concentrations of stock solutions (>10 mM, stored at pH 12 and −20 °C) were determined in 10 mM NaOH directly prior to use from the absorbance values at 250 nm for Angeli's salt and DEA/NO ($\epsilon = 8000$ M^{-1} cm^{-1})[2,35] and 302 nm for $ONOO^-$ ($\epsilon = 1670$ M^{-1} cm^{-1}).[36]

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich and used without further purification and stock solutions were prepared fresh daily at 100× in MilliQ filtered H_2O. The assay buffer consisted of the metal chelator diethylenetriaminepentaacetic acid (DTPA, 50 μM) in either calcium and magnesium-free Dulbecco's

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phosphate-buffered saline (PBS, pH 7.4) or 10 mM NaOH (pH 12). All reactions were performed at 37 °C.

Instrumentation. UV−visible spectroscopy was performed with a Hewlett-Packard 8453 diode-array spectrophotometer. Fluorescence measurements were acquired on a Perkin-Elmer LS50B fluorometer. Electrochemical detection was accomplished with a World Precision Instruments Apollo 4000 system equipped with NO, O_2, and H_2O_2 sensitive electrodes.

Rates of Angeli's Salt Decomposition. The complete decay profiles of Angeli's salt in either aerated or deaerated PBS or 10 mM NaOH were monitored at 37 °C while the solution was being stirred and protected from room light. The sample was not exposed to the instrument light source during time intervals. Deaerated solvent was prepared by purging with argon in bulk (>3 min/mL) and then again (5 min) following Hamilton syringe transfer (3 mL) to an argon-flushed, graded seal quartz cuvette (Spectrocell; Oreland, PA) stoppered with a Suba-Seal septum (Sigma-Aldrich). The spectrophotometer was blanked after warming the cuvette in the heat block for 5 min, Angeli's salt (<10 μL of stock) was added by syringe, and the solution was rapidly mixed by repeated inversion.

Kinetic analysis was performed by fitting the data to an exponential decay (absorbance $= Ae^{-kt} + c$). Fitting of truncated data sets resulted in significant deviations of the calculated rate constants, indicating that extrapolation to the end point is not a viable technique for this compound. The absorbance maximum for Angeli's salt is pH-dependent (250 nm at high pH for $N_2O_3^{2-}$, shifting to 237 nm for $HN_2O_3^-$ in PBS), and the tail of the absorbance peak for decomposition product nitrite (eq 1) extends past 240 nm. Therefore, all data were fit at 250 nm, since the absorbance values approached zero under all experimental decay conditions.

Fluorescence Assays. Two-electron oxidation was evaluated by formation of the fluorescent dye rhodamine 123 (RH) from DHR 123 (Molecular Probe, Eugene, OR). Formation of H_2O_2 was measured by catalysis of *p*-hydroxyphenylacetic acid to the fluorescent dimer by horseradish peroxidase. Nitrosation was monitored by conversion of 2,3-diaminonaphthalene (DAN) to the fluorescent 2,3-naphthotriazole. These assays have been previously described in detail.[13] Urate stock solutions were prepared at 10−100 mM in 50 mM NaOH and were prediluted in assay buffer, which was then brought to pH 7.4 with 6 M HCl.

Superoxide was produced by the oxidation of hypoxanthine by xanthine oxidase (XO), as previously described.[37] The initial rate of O_2^- formation was determined by observing ferricytochrome *c* reduction at 550 nm (ϵ = 21 000 M^{-1} cm^{-1})[38] in PBS containing 0.5 mM hypoxanthine.[37] The extent of DHR oxidation was assessed by varying the amount of XO relative to constant DEA/NO, and maximum oxidation conditions were subsequently utilized.

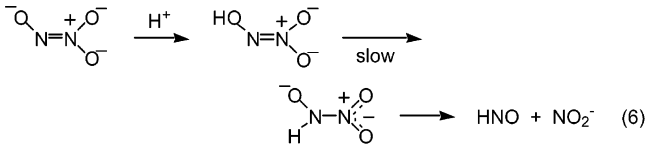
Electrochemical Measurement of H_2O_2. The functionality of the electrode was assessed with H_2O_2 and catalase, which catalytically consumes H_2O_2. Addition of Angeli's salt to PBS resulted in a small rise in electrode signal that was not sensitive to the HNO scavenger[39] glutathione (GSH). The signal persisted after the Angeli's salt was completely decomposed and was not affected by catalase. The electrode was found to be highly sensitive to hydroxylamine (NH_2OH; data not shown), which is a starting material in the synthesis of Angeli's salt.[34] Calibration determined that the Angeli's salt utilized contained less than 0.1% NH_2OH. A signal attributable to H_2O_2 was not observed with this preparation.

Quantum Mechanical Calculations. All structures and reaction transition states were optimized using the B3LYP method with a 6-311+G(d) basis set implemented with the Gaussian 98 program.[40] Aqueous solvation energies were calculated as single points on the B3LYP optimized gas-phase structures using the PCM model.[41] Structures were then recomputed using the CBS-QB3 method, which is known to give energies to within ±1 kcal/mol of experimental values for the G3 data set.[42] The free energy of a species in water was determined by combining the CBS-QB3 computed free energy with the aqueous solvation energy computed by the PCM model. Equations in the text include changes in gas-phase enthalpies (ΔH) and free energies (ΔG), with aqueous free energy changes (ΔG_{aq}) given in parentheses, in kcal/mol. Only ΔG_{aq} values are discussed in the text. For calculations involving Angeli's monoanion, $HN_2O_3^-$, the structure was assumed to be the most thermodynamically stable tautomer, protonated at the nitroso oxygen.[43]

Results and Discussion

Angeli's salt has been a compound of interest since the first report of its synthesis in the late 1800s[44] and the suggestion several years later[14] of the production of NOH upon decomposition. Detailed mechanistic studies quickly followed determination[45] of the crystal structure of Angeli's salt. The research groups of Bonner and Hughes utilized varied solution conditions, and isotopic labeling to determine that the mechanism of Angeli's salt decomposition in anaerobic aqueous solution involves protonation of the dianion followed by tautomerization and heterolytic cleavage of the N−N bond to produce HNO and nitrite.[15,16,18,19,46−48]

We recently explored this mechanism theoretically,[43] and the most thermodynamically stable monoanionic tautomer was indeed calculated to be protonated at the oxygen of the nitroso group. This species was computed to be stable toward heterolytic cleavage to HNO and nitrite by 16.4 kcal/mol, with a 53.8 kcal/mol barrier. The nitroso *N*-protonated tautomer was determined to lie 5.3 kcal/mol above the lowest energy monoanion, but heterolytic cleavage was predicted to be favorable by 3.8 kcal/mol, with an overall barrier for N−N cleavage of 13.1 kcal/mol. These quantum mechanical calculations support the mechanism of Angeli's salt decomposition in anaerobic solution through eq 6, with the slow rate due to the low equilibrium concentration of the nitroso *N*-protonated tautomer.



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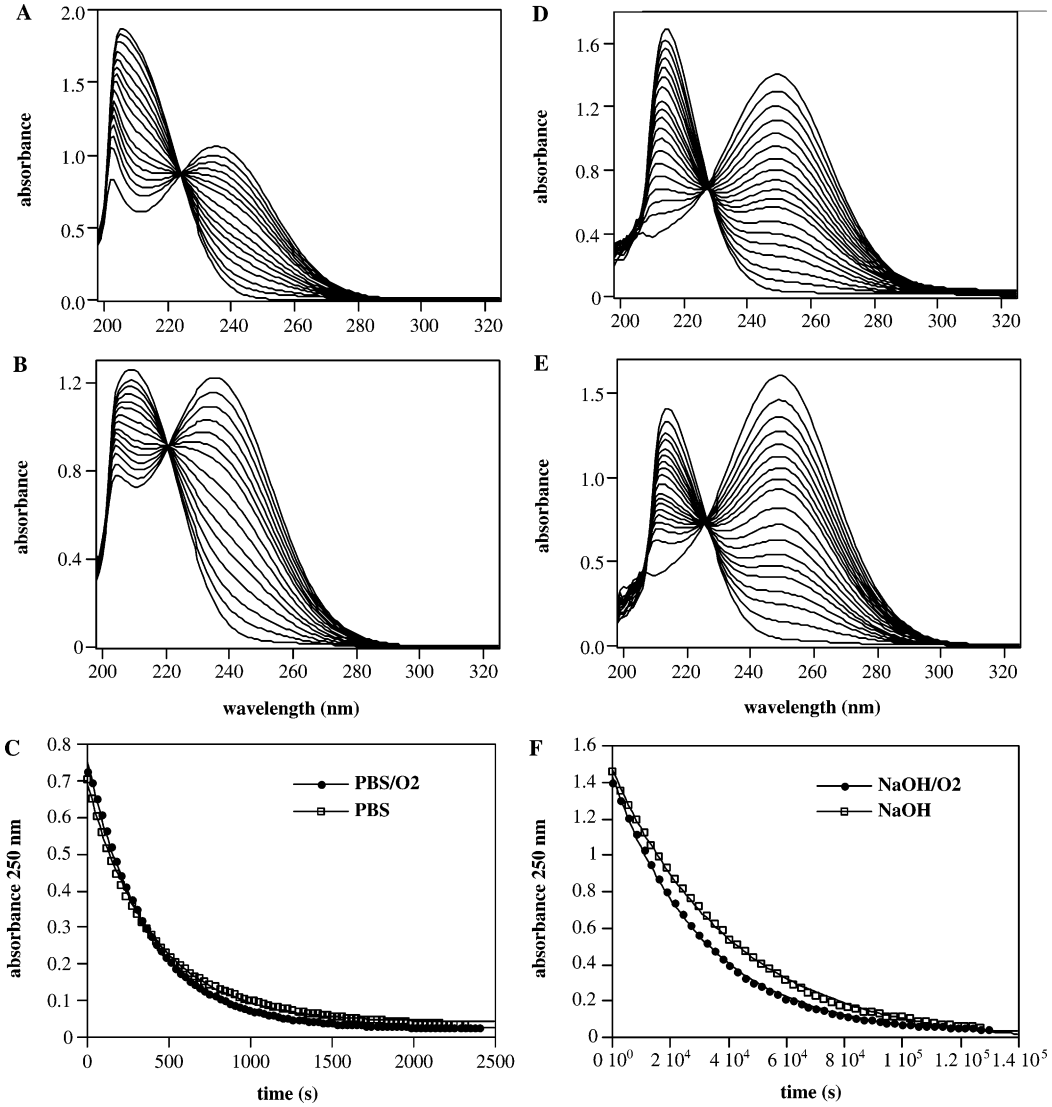

Figure 1. Spectrochemical monitoring of decomposition of Angeli's salt at 37 °C in aerobic (A) and anaerobic (B) PBS (initial intervals of 30 s; final spectra measured at 40 min) or aerobic (D) and anaerobic (E) 10 mM NaOH (initial intervals of 2700 s; final spectra measured at 36 h), containing 50 μM DTPA. Note that the initial concentrations vary slightly between each analysis. Representative decay rates are shown for pH 7.4 (C) and pH 12 (F). The anaerobic data sets, which were of slightly higher concentration than those of the corresponding aerobic sets, were plotted for this figure with the first three and one data points removed to provide a more accurate pictorial comparison. This did not affect the calculated rate constants, which for the full sets are (C) 2.7×10^{-3} s^{-1} in air and 2.5×10^{-3} s^{-1} anaerobically and (F) 3.2×10^{-5} s^{-1} in air and 2.4×10^{-5} s^{-1} anaerobically.

Currently, the mechanism and products of Angeli's salt decomposition under physiological conditions (pH 7.4, 37 °C, aerobic) are of interest due to the pharmacological potential of Angeli's salt in treatment of heart failure[3−5] and the observations[12,13,27,49,50] of formation of an oxidant with the capacity to cleave double stranded DNA.

Mechanism of Angeli's Salt Decomposition in Aerobic Solution. Here, we have evaluated, experimentally and by quantum mechanical calculations, two pathways by which a strong oxidant may be formed from aerobic decomposition of Angeli's salt at neutral pH: reaction of O_2 directly with Angeli's anion or dianion (pK_{a2} of 9.7),[51]

$$N_2O_3^{2-}/HN_2O_3^- + O_2 \rightarrow \text{oxidant} \qquad (7)$$

or with a nitrogen oxide intermediate following the rate-limiting step in the decomposition pathway.

$$HN_2O_3^- \rightarrow \text{intermediate} \xrightarrow{O_2} \text{oxidant} \qquad (8)$$

The first mechanism (eq 7) correlates to the reported[3,52−54] oxidation of Angeli's salt by metal complexes such as $Ru(NH_3)_6^{3+}$ and $Fe(CN)_6^{3-}$. The pH-dependence of the rates and product ratios in the presence of metal complexes is consistent with the dianion as the reactive species.

$$N_2O_3^{2-} + M^{3+} \rightarrow NO + NO_2^- + M^{2+} \qquad (9)$$

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Table 1. Experimental Rate Constants (±SEM; $n = 3$) for Decomposition of Angeli's Salt at 37 °C in Aerobic and Anaerobic PBS or 10 mM NaOH, Containing 50 μM DTPA

O_2 status	pH	k_{obs} (s^{-1})/10^5
normoxic	7.4	263 ± 8
	12	3.23 ± 0.06
anaerobic	7.4	240 ± 6
		291[a]
	12	2.48 ± 0.03

[a] pH 7.56 and 35 °C.[16]

If aerobic decomposition of Angeli's salt follows a similar redox mechanism to eq 9, the oxidant in eq 7 would conceivably be $ONOO^-$ produced via a two-step process:

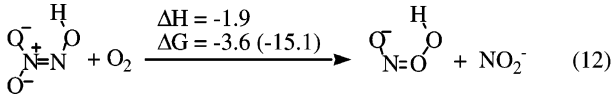
$$N_2O_3^{2-} + O_2 \rightarrow NO + NO_2^- + O_2^- \quad (10)$$

$$NO + O_2^- \rightarrow ONOO^- \quad (3)$$

Our quantum mechanical calculations predict that formation of $ONOO^-$ by this electron-transfer mechanism is favorable by 23.5 kcal/mol in water.

$$N_2O_3^{2-} + O_2 \xrightarrow[\Delta G = -133.2(-23.5)]{\Delta H = -131.9} ONOO^- + NO_2^- \quad (11)$$

In correlation to the higher reactivity of the dianion toward metal complexes[52,53] (eq 9), the reaction of O_2 with the most thermodynamically stable monoanionic tautomer[43] is less favorable.



(12)

To determine the effect of O_2, we spectrophotometrically monitored the decomposition rates of Angeli's salt at 37 °C and pH 7.4 or 12 in aerobic and anaerobic solution containing the metal chelator DTPA to eliminate a contribution from eq 9. Representative spectral changes and rate data are shown in Figure 1. Reproducibility in the calculated rate constants (Table 1) under these conditions required use of the full kinetic data sets and changes in absorbance above 240 nm. Our pH 7.4, anaerobic value (2.4×10^{-3} s^{-1}) compares favorably with the earliest reported value[16] of 2.9×10^{-3} s^{-1} at pH 7.56 and 35 °C. Aerobic conditions did not substantially affect the rate of Angeli's salt decomposition at neutral pH. This result correlates to previous analyses by Liochev and Fridovich,[55] in which the decomposition rates at 23 °C in saturated air or O_2 solutions were identical, and by Donald et al.[19] as well as Shafirovich and Lymar,[25] in which deaeration did not affect the rate of Angeli's salt decay during steady-state photolysis.

A larger perturbation of the rate by O_2 was observed at high alkalinity (Table 1), in accord with the calculated favorability of eq 11 and the higher redox reactivity of $N_2O_3^{2-}$ toward metal complexes[52,53] (eq 9). These data suggest that the reported[19,25,26] formation of $ONOO^-$ in aerobic, alkaline solution may occur via kinetically competitive pathways involving reaction of O_2 with both NO^- (eq 1, following deprotonation of HNO, and eq 2) and $N_2O_3^{2-}$ (eqs 10, 3). The contribution of two pathways is supported by the deviation from isosbestic behavior of aerobic Angeli's salt decomposition at pH 12 (compare Figure 1A,B with 1D,E). Significant accumulation of $ONOO^-$ ($\lambda_{max} = 302$ nm)[36] was not observed, although there was a slight elevation in absorbance above 300 nm in aerated, alkaline solution (Figure 1D).

The reaction of $N_2O_3^{2-}$ with metal complexes such as $Ru(NH_3)_6^{3+}$ and $Fe(CN)_6^{3-}$ (eq 9) has been suggested[52,53] to proceed through an outersphere mechanism. The inertness of the metal center to substitution would certainly be expected to preclude innersphere electron transfer to the $Ru(NH_3)_6^{3+}$ complex.[48] We thus used quantum mechanical calculations to investigate whether eq 11 also involves outersphere electron transfer. The $N_2O_3^-/N_2O_3^{2-}$ and $HN_2O_3/HN_2O_3^-$ couples were predicted to have potentials of 0.5 V ± 0.1 and 0.9 V ± 0.1 (vs NHE at pH 7; calculations not shown), respectively, which is consistent with assignment[52,53] of the dianion as the reactive species in eq 9. These calculated potentials suggest that the reaction of Angeli's salt with $Ru(NH_3)_6^{3+}$ is endergonic by ca. 10 kcal/mol, given the $Ru(NH_3)_6^{3+}/Ru(NH_3)_6^{2+}$ couple potential of 0.24 V (1 M vs NHE, H^+).[56] The more negative O_2/O_2^- couple potential (−0.16 V, 1 M vs NHE at pH 7)[57] is congruent with the substantially lower rates of the aerobic degradation of Angeli's salt (Table 1) compared to the reaction with $Ru(NH_3)_6^{3+}$ (maximal rate constant of 46 M^{-1} s^{-1} at pH 11.2, 2 °C).[52]

The calculated aqueous free energy changes predict that outersphere electron transfer during autoxidation of Angeli's salt is thermodynamically uphill by at minimum 25 kcal/mol.

$$N_2O_3^{2-} + O_2 \xrightarrow[\Delta G = -82.0(25.2)]{\Delta H = -80.8} N_2O_3^{-\bullet} + O_2^{-\bullet} \quad (13)$$

$$HN_2O_3^- + O_2 \xrightarrow[\Delta G = 60.4(37.7)]{\Delta H = 61.1} HN_2O_3^{\bullet} + O_2^{-\bullet} \quad (14)$$

$$HN_2O_3^- + O_2 \xrightarrow[\Delta G = 41.1(38.0)]{\Delta H = 44.0} N_2O_3^{-\bullet} + HO_2^{\bullet} \quad (15)$$

In contrast, the second step in formation of $ONOO^-$ (eq 11), cleavage of the $N_2O_3^-$ radical into NO and NO_2^-, is indicated to be highly favorable.

$$N_2O_3^- \xrightarrow[\Delta G = -28.1(-37.6)]{\Delta H = -17.8} NO + NO_2^- \quad (16)$$

Thus, decomposition of $N_2O_3^-$ may assist in driving outersphere electron transfer forward. Attempts to locate an associative reaction intermediate between O_2 and either $N_2O_3^{2-}$ or $HN_2O_3^-$ in the gas phase were unsuccessful, implying that these processes are substantially unfavorable.

These calculations suggest that although $ONOO^-$ formation from the autoxidation of Angeli's salt (eqs 11, 12) is thermodynamically favorable, there are no low-energy paths connecting reactants to products. The slow decomposition rate of Angeli's salt at high pH (Table 1) is thus due to the stability of $N_2O_3^{2-}$

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toward both decomposition[43] and autoxidation. While $HN_2O_3^-$ is predicted to have an even higher stability toward oxidation, heterolytic cleavage is accelerated by 100-fold (Table 1). Thus, decay of the dianion $N_2O_3^{2-}$, whether through decomposition or autoxidation, does not occur on a sufficient time scale to account for the observed neutral pH chemistry. The kinetic viability of eq 7 therefore requires the involvement of $HN_2O_3^-$. However, significant autoxidation of $HN_2O_3^-$ is not supported by the virtual insensitivity of the decomposition rate to O_2 at pH 7.4[25,55] (Table 1, Figure 1) and the calculated unfavorability of electron transfer (eqs 14,15). These data argue against eq 7 as a principal mechanism under physiological conditions and strongly indicate that the interaction with O_2 must occur after the rate-limiting step of decomposition (eq 8).

Since determination[45] of the crystal structure of Angeli's salt, the initial products of decomposition have been generally accepted to be HNO and nitrite (eq 6). However, the literature does contain the proposal[58] that Angeli's salt cleaves homolytically to produce different intermediates.

$$HN_2O_3^- \rightarrow HONO^{-\bullet} + NO^{\bullet} \qquad (17)$$

$HONO^-$ is reported[59] to decompose to NO and H_2O with a rate of 747 s^{-1}.

$$HONO^- + H^+ \rightarrow NO + H_2O \qquad (18)$$

However, if $HONO^-$ (or NO_2^{2-}) is a powerful reductant, which is likely considering that the reduction potential of nitrite is lower than -1 V (vs Ag/AgCl),[60] electron transfer from O_2 could be significant.

$$HONO^- + O_2 \rightarrow O_2^- + NO_2^- + H^+ \qquad (19)$$

These reactions would complete eq 8 as follows (excluding H^+ and NO_2^- from the equation),

$$HN_2O_3^- \rightarrow NO + HONO^- \xrightarrow{O_2} NO + O_2^- \rightarrow ONOO^- \qquad (20)$$

again providing a mechanism for $ONOO^-$ formation.

The homolytic mechanism in eq 17 was abandoned in favor of heterolytic cleavage (eq 6) due to reevaluation of the reactions of Angeli's salt with heme proteins[61,62] as well as by isotopic[47,48,63] and photochemical[19] analyses. Heterolytic degradation is supported by our recent calculations[43] predicting the favorability of eq 6 and here in which homolytic cleavage was calculated to be energetically unfavorable, due to an aqueous free energy change of 24.2 kcal/mol and an energy barrier of 31.0 kcal/mol.

$$N_2O_3^{2-} \xrightarrow[\Delta H^{\ddagger} = 26.5,\ \Delta G^{\ddagger} = 23.8(31.0)]{\Delta H = 75.6,\ \Delta G = 63.1(24.2)} NO_2^{2-} + NO \qquad (21)$$

Thus, there is every indication, both experimental[13−19,25−27,30,46−48,61−63] and theoretical,[43] that Angeli's salt decomposes by heterolytic decomposition to HNO (eq 6), which then interacts with O_2 to form a strong oxidant.[12,13,27,49,50]

Oxidant Characterization. Conversion of DHR to the fluorescent dye RH was previously used to demonstrate that the reaction of O_2 with HNO competes directly with HNO dimerization.[32] The rates of the two reactions were equivalent at approximately 15 μM Angeli's salt under conditions of excess DHR and O_2. RH production was linear with a slope of 1 at Angeli's salt concentrations below 2 μM, indicating that the ratio of HNO to O_2 to DHR is 1:1:1, as suggested previously.[13] Beyond this linear region, the ratio with respect to HNO will increase as dimerization (eq 4) begins to become kinetically significant. In fact, consumption of O_2 during Angeli's salt decomposition in the concentration range of 50−200 μM Angeli's salt has been shown[26] to be approximately 70%, with an apparent inverse dependence on Angeli's salt concentration. Thus, the conclusion can be made that oxidant formation is stoichiometric in HNO and O_2.

The mechanism by which the oxidant is formed has been suggested to proceed through direct association,[13] electron transfer,[26] or hydrogen atom transfer.[25] The composition and chemistry of the resulting oxidant vary for each mechanism. We have attempted experimentally and computationally to determine the correct mechanism.

Production of NO from decomposition of Angeli's salt in the presence of Cu,Zn superoxide dismutase[64−69] (SOD) has been proposed to result either directly[55,67] from metal-mediated oxidation of HNO

$$Cu(II) + HNO \rightarrow Cu(I) + NO + H^+ \qquad (22)$$

or indirectly[26] due to scavenging of O_2^-, and thus inhibition of $ONOO^-$ formation (eq 3), following electron transfer from HNO to O_2.[70]

$$HNO + O_2 \rightarrow NO + O_2^- + H^+ \qquad (23)$$

The concentration of NO, as well as the chemistry of NO, would be expected to be enhanced by SOD in either pathway, and these results are well documented.[65−67] In the latter scenario (eq 23), SOD would also abate the chemistry of both O_2^- and $ONOO^-$ and catalyze dismutation of O_2^- to H_2O_2. Each of these effects would be expected to be comparable for similar reactant fluxes from Angeli's salt and NO/O_2^- generating systems, such as SIN-1 or NO donor/XO.

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(70) These equations have been altered to reflect the current data on HNO/NO^- equilibria. Since the OH^--dependent deprotonation of HNO to $^3NO^-$ (eq 5) is the rate-limiting step, with a rate constant[25] of 5×10^4 M^{-1} s^{-1}, at pH 6−8 the half-life of this reaction would be 1400−14 s. However, the rate constant for dimerization of HNO (eq 4; 8×10^6 M^{-1} s^{-1})[25] reduces the lifetime of HNO above 1 μM to less than 1 s. This suggests that deprotonation and subsequent reaction with O_2 are not kinetically viable compared to dimerization due to the intersystem crossing barrier from ^{1}HNO to $^3NO^-$. Thus, O_2 is assumed to only react with HNO at neutral pH.

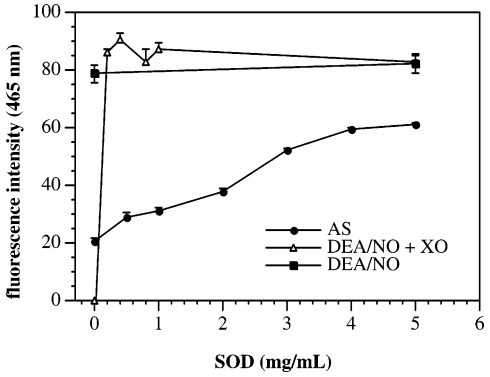

Figure 2. Nitrosation of DAN by DEA/NO, DEA/NO + XO, or Angeli's salt in the presence of Cu,Zn SOD. DEA/NO (5 μM) ± XO (45 μL of 20 U stock; 1.81 μM/min O_2^- production, conditions of maximum nitrosation) or Angeli's salt (10 μM) was added to 2 mL of PBS buffer containing DTPA (50 μM), DAN (30 μM), and hypoxanthine (500 μM). The solutions were immediately vortexed and incubated at 37 °C for 30 min. The fluorescence was measured at 465 nm with excitation at 360 nm with 2.5 mm slit widths. Data were collected in triplicate, and error bars represent SEM for the illustrative data set shown in the figure. Note that the data are end-point values, and the connecting lines were added to clarify the donor used.

Inhibition of oxidation by Angeli's salt to a similar extent as SIN-1 was shown in several studies[67,69] to require a minimum of 5-fold additional SOD, although generation of free NO was comparable.[67] The presence of NO was assessed here by nitrosation of DAN to the fluorescent triazole in aerobic assay buffer. The fluorescence signal induced by exposure of DAN to DEA/NO (5 μM) was reduced in a concentration-dependent manner by XO[37], due to scavenging of NO by O_2^-. Addition of 0.2 mg/mL SOD completely restored this signal (Figure 2). Triazole was also formed during decomposition of Angeli's salt or synthetic $ONOO^-$, although with a substantially lower yield than with NO donors[26,29] (Figure 2). The 4-fold lower signal from Angeli's salt (10 μM) was enhanced by addition of SOD toward that of DEA/NO (5 μM), which has a comparable decomposition rate to Angeli's salt[2]. Since DEA/NO decomposition produces 2 equiv of NO, stoichiometric signals would be expected at an Angeli's salt to DEA/NO ratio of 2:1. Augmentation to this level was not achieved even with addition of 5 mg/mL SOD, which is 25-fold higher than required to outcompete scavenging of NO by O_2^- (Figure 2). This suggests that triazole is formed as a result of enhanced NO, from oxidation of HNO by SOD (eq 22), rather than due to scavenging of O_2^- produced during decomposition of Angeli's salt (eq 23).

Decomposition in PBS of 20 or 50 μM AS, which is expected to exceed pharmacologically, and certainly physiologically, relevant amounts, did not produce detectable H_2O_2 by either fluorescent assay (Figure 3) or H_2O_2-sensitive electrode (data not shown). Addition of 1 mg/mL of SOD (62.5 μM SOD monomer), which was 5-fold in excess of the amount required to completely restore the signal in Figure 2, increased NO production, as measured electrochemically (data not shown), but did not significantly enhance H_2O_2 (Figure 3). These results contrast with those of Kirsh and de Groot,[26] who observed H_2O_2 during aerobic decomposition of Angeli's salt (500 μM) in the presence of SOD (100 or 500 U/mL), although at less than 10% yield. The absence of metal chelators and the 10−25-fold higher Angeli's salt concentrations in ref 26 may provide an explanation for the observed dissimilarities both here (Figure 3) and in ref 13.

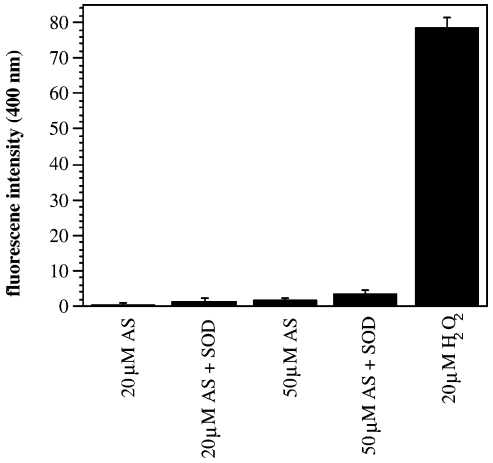

Figure 3. Cu,Zn SOD effect on H_2O_2 formation from AS. Angeli's salt (20 or 50 μM final concentration; 10 μL stock added) was added to 1 mL of PBS buffer containing DTPA (50 μM) ± Cu,Zn SOD (1 mg/mL). The solutions were immediately vortexed and incubated at 37 °C for 30 min. Subsequently, 100 μL of analyte solution (3 parts of 10 mg/mL *p*-hydroxyphenylacetic acid in H_2O to 1 part of 10 mg/mL HRP in H_2O) and 1 mL of buffer were added, and the fluorescence was measured at 326 nm with excitation at 400 nm with 2.5 mm slit widths. Fluorescence was compared to a H_2O_2 standard curve ranging from 0 to 20 μM. Data are plotted as mean + SEM ($n = 3$).

These data in conjunction with cited results[25,55] strongly support direct interaction of HNO and SOD (eq 22). Other direct association reactions between HNO and oxidized metal complexes are known, for instance with heme proteins[71] such as ferric myoglobin or ferricytochrome *c*. Further, SOD, which is suggested to react with HNO rather than Angeli's salt itself,[55] is reduced by Angeli's salt both aerobically and anaerobically,[69] indicating an independence from O_2.

Although NO^- is a strong reductant ($NO/^3NO^-$, <-0.7 V, 1 M vs NHE),[25,30] HNO is considerably less potent ($NO,H^+/^1HNO$, <-0.4 V 1 M vs NHE).[30,72] The reduction of ferricytochrome *c* by HNO from Angeli's salt has been reported[32] to be 100 times slower than by O_2^-. Assuming outersphere mechanisms, this indicates that HNO oxidation is of significantly more positive potential than O_2^- (O_2/O_2^-, −0.16 V, 1 M vs NHE).[57] The potential difference mandates that eq 23 is thermodynamically uphill, precluding formation of $ONOO^-$ by an outersphere electron-transfer mechanism.

From this analysis, electron transfer from HNO to O_2, and thus production of free NO and O_2^- (eq 23), is excluded as a major pathway of eq 8, leaving direct association or hydrogen atom transfer as potential mechanisms.

Direct association of HNO and O_2 is a spin-forbidden reaction that is predicted to be thermodynamically unfavorable by 13.7 kcal/mol.

$$^1\text{HNO} + {}^3\text{O}_2 \xrightarrow[\Delta G = 23.7\ (13.7)]{\Delta H = 12.7} \textit{cis}\text{-ON(H)OO} \qquad (24)$$

The rate constant for this reaction has been estimated[25,32,55] at $\ll 3 \times 10^5\ M^{-1}\ s^{-1}$. Hydrogen atom transfer has been proposed[25] to overcome the spin-forbidden aspects of direct association of

(71) Doyle, M. P.; Mahapatro, S. N.; Broene, R. D.; Guy, J. K. *J. Am. Chem. Soc.* **1988**, *110*, 593−599.
(72) The difference in potential is a function of different pathways of oxidation of ^{1}HNO or $^3NO^-$ to NO as a result of the requirement for a spin change during proton transfer.

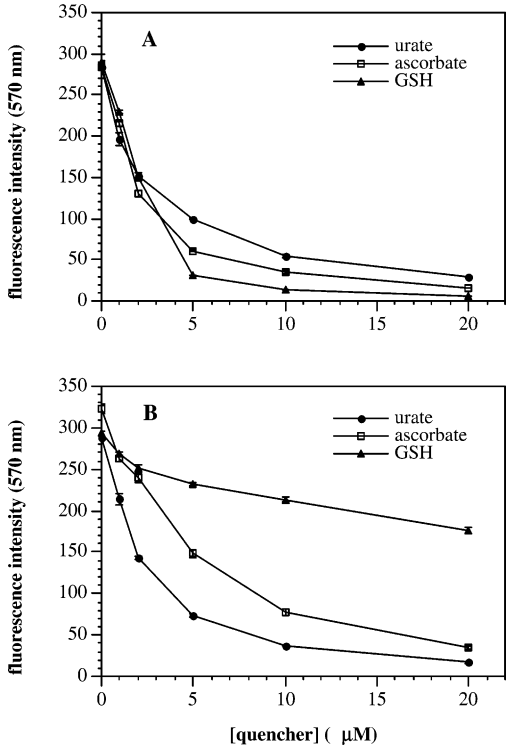

Figure 4. Comparison of quenching of Angeli's salt or $ONOO^-$-mediate oxidation of DHR by GSH, ascorbate and urate. (A) Angeli's salt or (B) $ONOO^-$ (5 μM final concentration; 10 μL of stock added) was added to 2 mL of PBS buffer containing DTPA (50 μM), DHR (50 μM), and scavenger (0−20 μM urate, GSH or ascorbate). The solutions were immediately vortexed and incubated at 37 °C for 30 min. Fluorescence was measured at 570 nm with excitation at 510 nm with 2.5 mm slit widths. Data were collected in triplicate.

HNO and O_2. This process was calculated to be thermodynamically favorable in water (ΔG_{aq} of −7.3 (eq 25) or −6[25] kcal/mol) but to have a relatively high activation barrier ($\Delta G^{\ddagger}_{aq}$ of 16.3 kcal/mol).

$$\mathrm{HNO} + \mathrm{O_2} \xrightarrow[\substack{\Delta G = -1.7(-7.3)\\ \Delta H^{\ddagger} = 7.4\\ \Delta G^{\ddagger} = 15.8(16.3)}]{\Delta H = -1.1} \mathrm{NO\bullet} + \mathrm{HO_2^{\bullet}} \qquad (25)$$

The rate constant has been estimated to be 5 orders of magnitude lower than dimerization (eq 4),[25] indicating severe kinetic constraints. Further, hydrogen atom transfer would result in formation of $ONOO^-$/ONOOH. Thus, the dissimilarity[13,27] between aerobic Angeli's salt and synthetic $ONOO^-$ (Figure 4) as well as the SOD results (Figures 2 and 3) argue against both electron and hydrogen atom transfer, leaving the possibility of an associative mechanism.

The direct associations of HNO with oxidized metal complexes, most notably with heme proteins[58,71,73] such as metMb, and with thiols[39] such as GSH are well established and are quite fast ($\sim 1 \times 10^6$; $M^{-1}\,s^{-1}$).[32,63] The high reactivity of HNO with thiols, resulting in RSNHOH,[39,71,74,75] led to examination[74] of the potential for nucleophilic addition of water to HNO. The resulting solvated species, dihydroxylamine, is analogous to the thiol product, RSNHOH.

$$\mathrm{HNO} + \mathrm{H_2O} \rightleftharpoons \mathrm{HN(OH)_2} \qquad (26)$$

This hydrated HNO may then react with O_2.

$$\mathrm{HN(OH)_2} + \mathrm{O_2} \rightarrow \mathrm{(OH)_2NH(O_2)^-} \qquad (27)$$

Bartberger et al.[74] have reported a computed equilibrium constant for hydration of HNO of 2×10^{-3}. Since k_{obs} for the association of HNO with O_2 is estimated[32] to be 4×10^3 $M^{-1}\,s^{-1}$, $k_{(eq\ 27)}$ via the hydrate would be 2×10^6 $M^{-1}\,s^{-1}$. If hydrated HNO exists as $HN(OH)_2$, then eq 27 may be analogous to the reaction of NH_2OH with O_2, which is slow, with a rate constant[76] on the order of 10^{-4} $M^{-1}\,s^{-1}$ under basic conditions. The difference of 10 orders of magnitude between the estimated rate constant for eq 27 and that for hydroxylamine oxidation seems unlikely to result from substitution of a hydrogen atom for a hydroxyl group. Further arguments against this mechanism are that $(OH)_2NH(O_2)^-$ would be expected to be short-lived and that isomerization to nitrate may be significant. However, a solvation mechanism cannot be disregarded at this juncture.

The above data leave few possibilities for the oxidant in eq 8, and the direct but spin-forbidden association of HNO with O_2 (eq 24) remains a viable reaction. The product would be expected to rapidly isomerize to the much more stable isomers of peroxynitrous acid and nitrous acid.

$$\mathrm{ON(H)OO} \xrightarrow[\Delta G = -39.5(-35.1)]{\Delta H = -38.7} \textit{trans}\text{-ONOOH} \qquad (28)$$

$$\mathrm{ON(H)OO} \xrightarrow[\Delta G = -42.1(-31.9)]{\Delta H = -41.9} \textit{cis}\text{-ONOOH} \qquad (29)$$

$$\mathrm{ON(H)OO} \xrightarrow[\Delta G = -71.4(-66.8)]{\Delta H = -71.7} \mathrm{HNO_3} \qquad (30)$$

Cis-peroxynitrous acid is proposed to be the initial reactive intermediate of $ONOO^-$. However, the chemical profiles between synthetic $ONOO^-$ and aerobic Angeli's salt have been shown[13,27] to vary substantially. Here, the relative reactivity of these nitrogen oxides was further assessed through competitive inhibition of DHR oxidation by the common biological scavengers GSH, urate, and ascorbate (Figure 4). The most noteworthy difference lies with GSH, which reacts rapidly[39] with HNO. The divergence in scavenging ability between Angeli's salt and $ONOO^-$ provides further evidence that Angeli's salt is not oxidized directly to NO and O_2^- (eq 23) but rather produces HNO upon decomposition (eq 1). The dissimilar scavenging of urate and ascorbate for Angeli's salt (Figure 4) is likely a function of unrelated reaction pathways, since ascorbate inhibits O_2 consumption by Angeli's salt,[13] implying a non-O_2-dependent association with HNO. The divergence in the reactivity of $ONOO^-$ toward GSH (50% quenching requiring >20 μM GSH) and urate (50% quenching with 2 ± 0.5 μM urate) is interesting, considering that the rate constants for reaction of NO_2 with these scavengers are nearly identical.[77] This suggests that NO_2, which,

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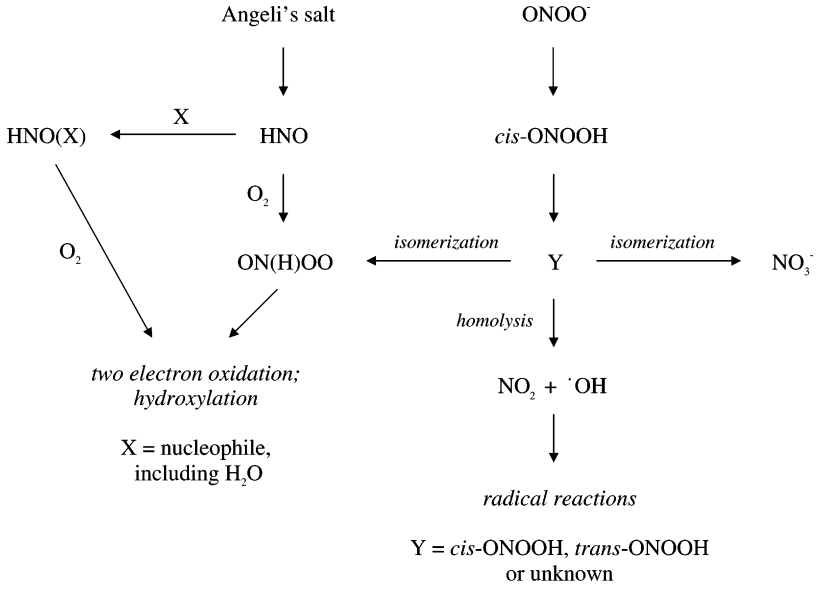
(77) Ford, E.; Hughes, M. N.; Wardman, P. *Free Radical Biol. Med.* **2002**, *32*, 1314−1323.

Scheme 1



along with •OH, is commonly assumed to be a significant product of dissociation of $ONOO^-$,[78] may not be responsible for DHR oxidation.

The observations reported and cited herein indicate that Angeli's salt initially releases HNO under both deaerated and aerated conditions (eq 6) and that HNO reacts with O_2 to form a species with similar oxidative properties but a distinct overall chemical profile compared to $ONOO^-$ formed from the NO/O_2^- reaction. The correlation[13] in the two-electron oxidation profiles of Angeli's salt and synthetic $ONOO^-$ could be envisioned to arise either from a common intermediate in the decomposition pathway or from similarity in the chemistry of constitutional isomers.

The decomposition pathway of $ONOO^-$ is complex and is not currently fully elucidated but is commonly suggested[79,80] to occur through protonation to *cis*-ONOOH followed by a combination of isomerization to nitrate or to the excited trans isomer and homolysis to a caged complex (NO_2/•OH) followed by release of the radicals. Quantum mechanical calculations suggest that, in analogy to the cis-trans relationship for ONOOH, the equilibrium between the HNO/O_2 adduct conformations favors the cis isomer by 5.6 kcal/mol, with a computed rotational barrier of approximately 22 kcal/mol (assumed from the highest energy dihedral angle connecting the isomers).

$$cis\text{-ON(H)OO} \xrightarrow[\Delta G = 2.6(5.6)]{\Delta H = 2.7} trans\text{-ON(H)OO} \qquad (31)$$

However, the formation of these species from the spin-forbidden association of HNO and O_2 is predicted to be endergonic by 13.7 kcal/mol (eq 24).

One possible explanation is that the caged complex of *cis*-ONOOH leads to radical reactions such as phenol oxidation and nitration, while isomerization of either *cis*-ONOOH or ON(H)OO to *trans*-ONOOH, or perhaps a different isomer of peroxynitrous acid, results in two-electron oxidation and oxygen insertion reactions with compounds such as benzoates (Scheme 1). This pathway is supported by the similarity in DHR quenching by urate (Figure 4), which, of the three scavengers utilized, does not inhibit O_2 consumption by Angeli's salt,[13] and would explain the lack of both nitration and a CO_2 effect on oxidation and hydroxylation by Angeli's salt.[27] This mechanism would have to preclude back rearrangement to the thermodynamically favored cis isomer. The rotational barrier has been computed to be 11−15 kcal/mol.[81,82]

Alternatively, HNO, which is a good electrophile, may react directly with the target molecule prior to reacting with O_2, in analogy to eqs 26 and 27 and to the reaction of HNO with GSH (Scheme 1). This intimates the existence of specific binding motifs in biological systems that favor covalent modifications mediated by HNO in an O_2-dependent manner. In this scenario, the absence of other species capable of binding to HNO could lead to the kinetic viability of the spin-forbidden reaction of HNO with O_2 followed by isomerization to nitrate. A combination of these mechanisms could potentially take place.

Although this study has determined that Angeli's salt is an HNO donor even in aerobic solution (eq 6), the mechanism of HNO autoxidation under physiological conditions remains elusive. This is similar to its highly studied nitrogen oxide cousins NO and $ONOO^-$, whose respective autoxidation and decomposition mechanisms have provoked substantial controversy. However, the slow rate of autoxidation of HNO may exclude this pathway from exogenous or endogenous mediation except under specific conditions[32] such as in cellular membranes or other hydrophobic regions where nitrogen oxide and O_2 concentrations are elevated[83] and where scavengers such as GSH are in reduced concentration. However, the potential for therapeutic use of HNO donors[3−5] coupled with the clonogenic toxicity of the HNO/O_2 product (albeit at suprepharmacological levels (50% cultured cell death at 2 mM))[12] requires further

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investigation of the HNO autoxidation mechanism. At present the speculation, currently without experimental or theoretical confirmation, is that the likely mechanism involves either direct association of O_2 and HNO or nucleophilic addition of HNO to a solvent or biological molecule followed by reaction with O_2 (Scheme 1).

Abbreviations

DAN, 2,3-diaminonaphthalene; DEA/NO, NO adduct of diethylamine ($Na[Et_2NN(O)NO]$); DHR, dihydrorhodamine; DTPA, diethylenetriaminepentaacetic acid; GSH, glutathione; HNO, nitroxyl; NO^-, nitroxyl anion; $ONOO^-$, peroxynitrite; O_2^-, superoxide; PBS, phosphate-buffered saline; RH, rhodamine; SOD, superoxide dismutase; XO, xanthine oxidase

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