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Nitroxyl accelerates the oxidation of oxyhemoglobin by nitrite

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Abstract

Angeli's salt ($\text{Na}_2\text{N}_2\text{O}_3$) decomposes into nitroxyl (HNO) and nitrite (NO_2^-), compounds of physiological and therapeutic interest for their impact on biological signaling both through nitric oxide and nitric oxide independent pathways. Both nitrite and HNO oxidize oxygenated hemoglobin to methemoglobin. Earlier work has shown that HNO catalyzes the reduction of nitrite by deoxygenated hemoglobin. In this work, we have shown that HNO accelerates the oxidation of oxygenated hemoglobin by NO_2^- . We have demonstrated this HNO mediated acceleration of the nitrite/oxygenated hemoglobin reaction with oxygenated hemoglobin being in excess to HNO and nitrite (as would be found under physiological conditions) by monitoring the formation of methemoglobin in the presence of Angeli's salt with and without added NO_2^- . In addition, this acceleration has been demonstrated using the HNO donor 4-nitrosotetrahydro-2H-pyran-4-yl pivalate, a water-soluble acyloxy nitroso compound that does not release NO_2^- but generates HNO in the presence of esterase. This HNO donor was used both with and without NO_2^- and acceleration of the NO_2^- induced formation of methemoglobin was observed. We found that the acceleration was not substantially affected by catalase, superoxide dismutase, c-PTIO, or IHP, suggesting that it is not due to formation of extramolecular peroxide, NO_2 or H_2O_2 , or to modulation of allosteric properties. In addition, we found that the acceleration is not likely to be related to HNO binding to free reduced hemoglobin, as we found HNO binding to reduced hemoglobin to be much weaker than has previously been proposed. We suggest that the mechanism of the acceleration involves local propagation of autocatalysis in the nitrite-oxygenated Hb reaction. This acceleration of the nitrite oxyhemoglobin reaction could affect studies aimed at understanding physiological roles of HNO and perhaps nitrite and use of these agents in therapeutics such as hemolytic anemias, heart failure, and ischemia reperfusion injury.

Keywords

Hemoglobin; kinetics; nitrite; nitroxyl; therapeutics

Introduction

Nitroxyl (HNO)¹ is one-electron reduced from nitric oxide (NO) and is associated with several biochemical processes that, though sometimes resembling processes involving NO,

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possess distinct mechanisms and pathways [1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13]. HNO has been considered as the basis of a therapeutic strategy in cardiac systems associated with heart failure [14; 15; 16; 17; 18; 19; 20; 21], in mitochondrial regulation [13], and in other pharmacological and biological signaling contexts both *in vitro* and *in vivo* [22; 23; 24; 25; 26; 27; 28]. Thus, Angeli's salt ($\text{Na}_2\text{N}_2\text{O}_3$, sodium α -oxyhyponitrite, AS) or other sources of nitroxyl (HNO) can potentially function as therapeutics for numerous conditions. The breakdown of Angeli's salt into HNO and nitrite has been studied for over a century [29; 30; 31; 32; 33; 34; 35].

Nitrite reacts with oxygenated hemoglobin (oxyHb) to form methemoglobin (metHb) [36; 37], and the reaction becomes very efficient at high nitrite concentrations, where the reaction becomes autocatalytic [38; 39; 40; 41; 42; 43; 44]. Interest in nitrite has been increasing lately due to its emerging role as a vasodilator and source of bioavailable nitric oxide [45; 46; 47; 48]. Nitrite has been shown to be a signaling molecule [49] with cytoprotective applications against ischemia-reperfusion injury [50; 51].

Angeli's salt decomposes under physiological conditions with a first order rate constant of $k = 6 \times 10^{-4} \text{ s}^{-1}$ to yield HNO and NO_2^- [32]. Early work [52] confirmed the stoichiometry of the reaction of HNO with oxyhemoglobin to form metHb and nitrate as

through a proposed two-step mechanism of oxyHb oxidation by HNO to form NO, followed by NO oxidation of a second oxyHb to form nitrate (Eq. 2–3)

Peroxide formed in Eq. 2 would be expected to rapidly form hydrogen peroxide at neutral pH. It should be noted that there has been debate regarding the pathway described in (Eq. 2), and alternative pathways have been proposed [53]. The rate constant for (Eq. 2) is likely to be on the order of $10^7 \text{ M}^{-1} \text{ s}^{-1}$, the rate reported for the same reaction with oxymyoglobin [12]. The rate of (Eq. 3) is somewhat faster ($k = 5\text{--}8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) [54; 55; 56].

The overall reaction of nitrite with oxyHb can be described by (Eq. 4).

This reaction was first studied in 1868 by Arthur Gamgee [36] and has since been studied by many others [40; 44; 55; 57; 58; 59; 60; 61; 62; 63]. The reaction progresses slowly at low nitrite concentrations ($k = 0.21\text{--}0.33 \text{ M}^{-1} \text{ s}^{-1}$), but becomes autocatalytic at nitrite concentrations that are high relative to the oxyHb concentration.

Recent kinetic models by Keszler and coworkers [60] support a mechanism by which autocatalysis is initiated in a multi-step process. This scheme postulates the addition of nitrite to heme-bound oxygen to form a ferrous-peroxynitrate intermediate (Eq. 5), which oxidizes nitrite to form nitrate and a ferrous-peroxynitrite intermediate (Eq. 6). This

Abbreviations: nitric oxide (NO), nitroxyl (HNO), Angeli's salt (AS), hemoglobin (Hb), oxygenated hemoglobin (oxyHb or $[\text{HbO}_2]^{2+}$), deoxygenated hemoglobin (deoxyHb), methemoglobin (metHb or $[\text{Hb}]^{3+}$), pig liver esterase (PLE).

proposed ferrous-peroxynitrite complex would then be reduced to nitric oxide and peroxide (Eq. 7), and the resulting NO would rapidly oxidize a second oxyHb (Eq. 3).

Allowing the reaction to progress slowly according to this scheme, peroxide would be expected to decompose to water and molecular oxygen, yielding a net reaction equivalent to (Eq. 4). However, at sufficiently high concentrations of metHb and peroxide, these components react to form a ferryl hemoglobin radical (Eq. 8), and it is this radical that is believed to initiate propagation of autocatalysis [44; 62]. Under the current model, the ferryl hemoglobin radical reacts with nitrite to form NO₂ radical and ferryl hemoglobin (Eq. 9), which reacts with an additional nitrite to form metHb and another NO₂ radical (Eq. 10). NO₂ radicals formed in this process can react with oxyHb to form a peroxynitrate adduct (Eq. 11), which decomposes to the ferryl hemoglobin radical and nitrate (Eq. 12). Thus, the autocatalytic mechanism is propagated by the NO₂ radical and the cycling of ferryl and ferryl radical hemoglobins.

At low concentrations of nitrite, such as those used in our experiments, autocatalysis is not expected to occur, and the oxidation of hemoglobin by nitrite is predicted to progress according to (Eqs. 5–7) and (Eq. 3).

In addition to oxidizing oxyHb, nitrite has also been shown to react with deoxyHb to form metHb and iron-nitrosyl hemoglobin [42; 64; 65]. Doyle and coworkers reported observing catalysis of the nitrite reaction with deoxyhemoglobin by HNO, but no such catalysis was reported for the reaction of nitrite with oxyHb [52]. In this paper, we show that HNO also accelerates the reaction of nitrite with oxyHb. We demonstrate this phenomenon using AS and using the newly developed HNO donor 4-nitrosotetrahydro-2H-pyran-4-yl pivalate [66] belonging to the recently described family of acyloxy nitroso compounds that yield HNO upon hydrolysis [67; 68]. Importantly, these experiments have been performed with oxyHb in excess to both nitrite and HNO, as would be the case under physiological conditions.

Materials and methods

Reagents

Angeli's salt, DEA NONOate, and carboxy-PTIO (c-PTIO) were purchased from Caymen Chemical. Superoxide dismutase, diethylene triamine pentaacetic acid (DTPA), and phytic acid were purchased from Sigma Aldrich. Other chemicals and supplies were purchased through Fisher Scientific. Packed red blood cells used in the preparation of hemoglobin solution were purchased from Interstate Blood Bank (Memphis, TN, USA).

Hb was purified as described previously [69; 70]. Red blood cells were washed in pH 7.4 PBS and lysed by dilution with distilled deionized water. The membranes were spun out by centrifugation at 17,000 g. and the Hb was dialyzed against distilled deionized water and PBS. The Hb was pelleted in liquid nitrogen and stored at -80°C for future use.

Angeli's salt and DEA NONOate stock solutions were prepared in 10 mM NaOH. The concentration of each stock solution was confirmed by absorbance at 250 nm, using an extinction coefficient (ϵ) of $8\text{ mM}^{-1}\text{ cm}^{-1}$ for Angeli's salt and $9\text{ mM}^{-1}\text{ cm}^{-1}$ for DEA NONOate [71]. Stock solutions of 10 mM NaNO_2 were also prepared in 0.01 M NaOH, with the concentration of NaNO_2 being determined by mass. Stock solutions of 10 mM c-PTIO were prepared in phosphate buffered saline, with the concentration of c-PTIO being determined by mass. Stock solutions of 10 mM inositol hexaphosphate (IHP) were prepared from phytic acid by titration with sodium hydroxide to pH 7.3.

4-nitrosotetrahydro-2H-pyran-4-yl pivalate was synthesized as described previously [66]. Esterase from porcine liver (pig liver esterase, PLE) was purchased from Sigma-Aldrich.

Spectroscopy

Angeli's salt, DEA NONOate, and initial oxyHb concentrations were verified on a Cary 50 bio-spectrometer (Varian, Inc.). Reactions involving Hb were monitored using time-resolved spectroscopy on a Cary 100 bio-spectrometer (Varian, Inc.) with a temperature controller set to maintain sample temperatures at 37°C and a six-cell sample changer that facilitated scanning of up to six samples simultaneously under the same conditions.

Hemoglobin reactions were analyzed by spectral deconvolution using a least-squares fit to known basis spectra (Figure 1A). A sample spectrum and the corresponding fit are shown in Figure 1B. Of the species included in the basis spectra, oxyHb and metHb always accounted for a sum total amount $> 97\%$ of the Hb species present. Data reported is for metHb levels, and remaining Hb is almost exclusively oxyHb, with occasional trace amounts ($< 2.5\%$) of deoxyHb found as the reactions progressed.

Nitrite Analysis

Nitrite levels were assessed using a Sievers Nitric Oxide Analyzer (NOA) (GE Instruments) according to standard procedures for a NaI assay provided by the manufacturer. Nitrite levels detected in the nitric oxide analyzer were quantified through comparison with stock NaNO_2 samples.

Experimental Reactions

Reactions were conducted using 1 mM oxyHb at 37°C . To one sample, 50 μM Angeli's salt was added. To another sample, 50 μM NaNO_2 was added. To a third sample, both 50 μM AS and 50 μM NaNO_2 were added. A fourth sample was used as a blank to account for any autoxidation and consisted of oxyHb with an amount of NaOH equivalent to that used for samples containing AS or NO_2^- . The procedure was repeated both with and without the

metal chelator DTPA, and DTPA had no observed effect on the reaction (data not shown). Additional time-resolved absorption spectroscopy was used to investigate reactions with higher nitrite concentrations: 100 μM NaNO_2 , 50 μM NaNO_2 , and a blank. Time-resolved spectra were collected, with scans taken every 10 min for two hours and then every hour thereafter for up to twelve hours total. Acceleration of the nitrite reaction with oxyHb was determined by comparing the methHb yield as a function of time in the above reactions.

In addition to spectroscopic analysis, reactions were conducted with aliquots being removed at 10 min intervals and analyzed in the NOA for nitrite content. A zero time point concentration could not be established for samples containing AS due to extreme signal broadening. The signal was sufficiently resolved to allow determination of NO_2^- concentrations after 10 min. However, samples at 20 min and beyond showed NO_2^- concentrations that were so low that they did not have a statistically significant difference given the margin of error in the experiment. Thus, only NO_2^- concentrations at 10 min are reported. The NOA would be expected to detect both free NO_2^- as well as NO_2^- and HNO from Angeli's salt that had not yet decomposed; at the reported time, 95% of AS is expected to have decomposed, and any remaining AS detected by the NOA can be accounted for using an AS control without added nitrite.

To examine the reaction of HNO and oxyHb in the absence of nitrite, and to observe the change in such a reaction upon addition of nitrite, Angeli's salt was replaced with 4-nitrosotetrahydro-2H-pyran-4-yl pivalate. This HNO donor is a water-soluble acyloxy nitroso compound that does not release NO_2^- but does generate HNO in the presence of esterase. Optimum HNO formation from this donor occurs in the presence of 9 units of pig liver esterase per μmol of donor. The donor (0.1 μmol) was added to 2 mL of 1 mM oxyHb with 0.9 units of PLE to create a system with 50 μM of HNO donor. Time-resolved spectra were collected as before. This HNO donor is roughly 15 fold slower than Angeli's salt, having a reported half-life of 39 min. However, it provides nitrite-free generation of HNO at our experimental pH; thus, experiments using this compound provide qualitative rather than quantitative comparison of the effects of HNO on the reaction of nitrite with oxyHb. Controls containing either PLE or 4-nitrosotetrahydro-2H-pyran-4-yl pivalate alone were run simultaneously with the experimental samples and an oxyHb blank; no significant differences were observed between the blank and the samples containing either PLE or 4-nitrosotetrahydro-2H-pyran-4-yl pivalate.

To isolate the effects of HNO from the effects of the NO created in the reaction of HNO with oxyHb, the reactions described above were also repeated with DEA NONOate in place of Angeli's salt. Time-resolved spectra were collected as above.

To explore the possibility of a peroxide initiated catalytic reaction, the procedure was repeated in the presence of 50 μM catalase. To investigate the possibility of a NO_2 radical propagated reaction, the procedure was repeated in the presence of 250 μM c-PTIO. The procedure was also repeated in the presence of 10 Ku/mL superoxide dismutase to investigate the possibility of a superoxide mediated reaction. To examine the potential role of allostery in the reaction, inositol hexaphosphate (IHP, in the form of phytic acid) was used to stabilize the T-state of hemoglobin and the procedure was repeated. Time-resolved spectra were collected as above.

Results

The three primary reactions examined were (1) oxyHb + HNO donor, (2) oxyHb + NO_2^- , and (3) oxyHb + HNO donor + NO_2^- . In each case, the methHb yield was determined as a function of time by spectral deconvolution and least squares fitting to the basis spectra

shown in Figure 1A, with a sample spectrum and fit shown in Figure 1B. We refer to the yield from each of these reactions as “[metHb] (x),” where x = 1, 2, or 3. Figure 2A shows time resolved spectra of the conversion of oxyHb to metHb by HNO and NO₂⁻; spectra such as these were fit to basis spectra to determine changes in the metHb concentration over time. Figure 2B shows the average metHb yield measured using time-resolved absorption from the three primary reaction mixtures; AS was used as the HNO donor, and a blank was used to account for autoxidation.

If the reaction of oxyHb with NO₂⁻ were independent of HNO, then one would expect

$$[\text{metHb}](1) + [\text{metHb}](2) = [\text{metHb}](3) \quad (\text{Eq. 13})$$

assuming a sufficient excess of oxyHb. Equivalently, examining the activity of NO₂⁻ in the presence or absence of HNO, independent reactions would yield

$$[\text{metHb}](3) - [\text{metHb}](1) = [\text{metHb}](2). \quad (\text{Eq. 14})$$

Figure 2C shows a significant difference between [metHb] (3) – [metHb] (1) compared to [metHb] (2), indicating that the two reactions are not independent. OxyHb + NO₂⁻ forms metHb at a higher rate in the presence of HNO, even after the metHb generated by HNO (and NO₂⁻ present in AS) has been accounted for.

NO₂⁻ itself is consumed more rapidly by the reaction with oxyHb in the presence of HNO. Figure 2D shows NO₂⁻ levels measured using the NOA in the three primary reactions after 10 min, as well as the difference between oxyHb + AS + NO₂⁻ and oxyHb + AS, hereafter referred to as the concentration of NO₂⁻ in the presence of HNO. The concentration of NO₂⁻ in the presence of HNO (27.3 ± 4.7 μM NO₂⁻) is only 54% of the concentration of NO₂⁻ in the absence of HNO (50.4 ± 6.2 μM). If the consumption of NO₂⁻ were independent of HNO, these two values would be expected to be the same. Moreover, the difference in the concentrations of NO₂⁻ in the presence and absence of HNO (23.1 ± 10.9 μM) is similar to the difference in metHb concentrations after 10 min between the corresponding samples (18.0 ± 8.0 μM). The difference in NO₂⁻ concentrations in the presence or absence of HNO can account for the difference in metHb concentrations under the same conditions if the NO₂⁻ reacted with oxyHb to make metHb on a one-to-one molar basis.

Angeli's salt decomposes into HNO + NO₂⁻, so a sample initially containing 50 μM AS and 50 μM NO₂⁻ could contain up to 100 μM NO₂⁻ following AS decomposition. Thus, it is reasonable to compare samples of oxyHb + 50 μM AS + 50 μM NO₂⁻ and oxyHb + 50 μM AS to samples containing 100 μM NO₂⁻ and 50 μM NO₂⁻. More precisely, it would be appropriate to compare the samples containing AS or AS + NO₂⁻ to samples titrated from 0 to 50 μM NO₂⁻ and from 50 to 100 μM NO₂⁻ at a rate comparable to AS decomposition. Figure 3A shows the oxidation of oxyHb to metHb by 100 μM NO₂⁻, 50 μM NO₂⁻, and autoxidation. Figure 3B demonstrates that the oxidation due to 50 μM NO₂⁻ is similar to the difference between the oxidation due to 100 μM NO₂⁻ and 50 μM NO₂⁻. If anything, using samples containing only 50 μM NO₂⁻ may slightly underemphasize the acceleration of the reaction by HNO. It would be expected that values found by titrating NO₂⁻ at a rate comparable to AS decomposition would lie between these two curves. Thus, oxidation due to 50 μM NO₂⁻ is considered to be an appropriate comparison for this study.

The initial reaction of oxyHb with HNO produces NO as well as metHb (Eq. 2). It is therefore reasonable to verify that the acceleration of the nitrite-induced oxidation of oxyHb is not due to a reaction with NO rather than HNO. To that end, the NO donor DEA NONOate was used in place of Angeli's salt. DEA NONOate has a half-life of 2 min at 37°C and pH 7.4 [71], comparable to the half-life of Angeli's salt (2.3 min) under the same

conditions. Figure 3C shows the conversion of oxyHb to metHb by 50 μM NO, 50 μM NO_2^- , and 50 μM NO + 50 μM NO_2^- . Figure 3D demonstrates that there is no significant difference in the concentration of metHb formed by NO_2^- in the presence or absence of NO.

The acceleration of the oxidation of oxyHb by NO_2^- in the presence of HNO can also be seen using HNO that does not originate from AS and does not inherently contain any NO_2^- . This was demonstrated using 4-nitrosotetrahydro-2H-pyran-4-yl pivalate in the presence of pig liver esterase (PLE), which releases HNO but does not release significant quantities of nitrite over the time period of interest. Time resolved spectra demonstrating the oxidation of oxyHb to metHb in the presence of this compound and NO_2^- are shown in Figure 4A. Figure 4B shows metHb yields as a function of time from the four different reaction mixtures using this compound instead of AS. HNO release from 4-nitrosotetrahydro-2H-pyran-4-yl pivalate and PLE ($k = 3 \times 10^{-4} \text{ s}^{-1}$) is slower than AS ($k = 5 \times 10^{-3} \text{ s}^{-1}$); thus, the quantitative results of experiments using the two HNO donors are different. However, both reactions demonstrate an increased rate of metHb formation due to NO_2^- in the presence of HNO. Figure 4C shows that metHb formation by NO_2^- in the presence of HNO 4-nitrosotetrahydro-2H-pyran-4-yl pivalate and PLE initially progresses at a faster rate than metHb formation by NO_2^- alone.

The addition of catalase to the reaction containing Angeli's salt had no substantial impact on the acceleration of the oxidation of oxyHb. Figure 5A shows metHb yields as a function of time from the four different reaction mixtures in the presence of 50 μM catalase. Figure 5B demonstrates that the acceleratory action remains in this system. Moreover, the addition of 250 μM c-PTIO had a similarly negligible effect. Although the overall metHb concentrations increased in the presence of c-PTIO (Figure 5C), these increases were uniform and did not prevent the acceleration of oxidation of oxyHb by nitrite (Figure 5D). Similarly, the addition of 10 Ku/mL superoxide dismutase had little impact on the system, either in the raw data (Figure 6A) or in the acceleration of oxidation of oxyHb (Figure 6B). Additionally, incubation of 1 mM oxyHb with 2 mM IHP for one hour showed no substantial effect on either the raw data (Figure 6C) or the acceleration of oxidation of oxyHb (Figure 6D).

In order to explore a role of metHb in the acceleration, the reactions were also conducted with an initial concentration of 250 μM metHb and 750 μM oxyHb (Figure 7A), and while the kinetics of the reaction were altered, the initial presence of metHb did not prevent acceleration of the oxidation of oxyHb by nitrite in the presence of HNO and the acceleration was similar to when no metHb was added initially (Figure 7B). Experiments were also performed at 200 μM oxyHb, the total amount of metHb expected from the reaction of 50 μM AS and 50 μM NO_2^- (Figure 7C), and while the kinetics were again affected by the change in initial conditions, the acceleration was still observed and the acceleration was similar to when no metHb was added initially (Figure 7D). In addition, we explored the possibility that HNO reacts with metHb bound nitrite or nitrite reacts with metHb bound HNO. We did experiments looking at any reactivity between nitrite, HNO, and metHb using high nitrite concentrations (>1 mM). Nitrite slightly impeded the formation of HbNO from metHb of HNO, and formed nitrite-bound metHb (data not shown).

Discussion

We have demonstrated that HNO accelerates the oxidation of oxyhemoglobin by nitrite. This acceleration affects the oxidation rate of oxyHb by Angeli's salt, as well as by sodium nitrite in the presence of HNO from a nitrite-free HNO donor. Nitrite from AS is often ignored in experiments involving heme proteins due to the assumption that the nitrite reaction is sufficiently slow compared to HNO so as to be negligible. However, our results

demonstrate that this is not the case, and that acceleration of the nitrite reaction must be considered in experiments involving AS.

The use of AS as an HNO donor has previously been shown by Sulc *et al.* to yield different end products from HNO donors such as the Piloty's acid analogue MSHA under aerobic conditions [72]. These reactions were performed at differing pH (pH 7 for AS and pH 10 for MSHA), so differences in product formation could be due to pH as well as to the presence of nitrite. However, the general findings were consistent with earlier work by Bazylnski *et al.* demonstrating complicated reactivity of AS with heme proteins [73; 74].

Allosteric processes might be considered as a possible cause of the acceleration of the nitrite reaction with oxyHb. If reaction of oxyHb with HNO alters the allosteric state of the Hb, and this altered allosteric state reacts faster with nitrite than the unreacted form, allostery could play a role in our observed kinetics. However, treating the hemoglobin with excess IHP, which stabilizes the T-state and inhibits the allostery of hemoglobin, had no substantial effect on the acceleration of the nitrite reaction. This suggests that the reaction is independent of allostery. It should be noted that this experiment was also attempted using myoglobin in place of hemoglobin incubated with IHP to examine allosteric effects; however, the autoxidation of the system dominated the reaction and impeded collection of statistically significant data.

Another possible mechanism for the HNO mediated increase in the rate of the nitrite/oxyHb reaction involves formation of reaction products/intermediates that are known to contribute to autocatalysis of the nitrite oxyHb reaction, such as peroxide and $\cdot\text{NO}_2$. In the autocatalytic system described in (Eqs. 8–12), peroxide initiates autocatalysis through formation of a ferryl hemoglobin radical and subsequent $\cdot\text{NO}_2$ propagation. However, even high concentrations (50 μM) of catalase did not prevent or delay the acceleration of the nitrite reaction; in contrast, Keszler and coworkers saw that catalase markedly delayed the initiation of the autocatalytic nitrite reaction. This suggests that peroxide is not a vital initiator in the acceleration we see of the nitrite/oxyHb reaction. Superoxide dismutase had a similarly negligible effect, arguing against a superoxide driven reaction as well. Catalase and superoxide dismutase react with HNO at rates of $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ respectively [12], and thus would not be expected to react significantly with HNO under these conditions. In addition to the observation that catalase did not impede the acceleration of the reaction, initiating the reaction in the presence of 250 μM metHb had little impact on the overall reaction; what impact was observed can be explained by a combination of the formation of nitrite-bound metHb and subsequent decrease in the amount of free nitrite, and by changes to the kinetics of the reaction due to decreasing available oxyHb. We also investigated the possibility of HNO reducing metHb to HbNO by attempting to detect HbNO in our samples using electron paramagnetic resonance (EPR) spectroscopy. No HbNO was detected (data not shown), indicating that any HbNO present was below the detection threshold of about 1 μM for this technique. Reducing the amount of free oxyHb without the addition of metHb also affected the kinetics, decreasing the total yield of metHb in the reaction.

Keszler *et al.* also found that addition of c-PTIO blocked propagation of autocatalysis through scavenging of the $\cdot\text{NO}_2$ radical to form nitrite and PTIO^+ as described by Goldstein *et al.* [75]. Addition of c-PTIO did not similarly block acceleration of the nitrite reaction in our experiments; however, this does not automatically exclude $\cdot\text{NO}_2$ radical as a possible mechanism. The reactions of PTIO and c-PTIO with NO, $\cdot\text{NO}_2$, and HNO, as well as reactions between the nitrogen oxides, constitutes a complicated chemical system. The previous experiments were conducted with 60 μM c-PTIO and only 30 μM oxyHb. In contrast, our experiments had oxyHb in excess to both nitrite and c-PTIO. Higher

concentrations of c-PTIO were not useable in our experiments as they rapidly oxidized the oxyHb independent of nitrite. In our reactions, it is likely that, if $\bullet\text{NO}_2$ radical was formed, some of it did react with c-PTIO to form PTIO^+ , as the rate constant for this reaction was reported to be $1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. However, as nitrite is produced by this reaction, and hemoglobin was not a limiting reagent, the nitrite produced by the oxidation of c-PTIO would have been free to react with another oxyHb. Thus, c-PTIO might not be expected to block an autocatalytic reaction at the concentrations we used, and any inhibition of the acceleration of oxidation by nitrite and HNO could have been masked by the oxidation of oxyHb by c-PTIO.

While we favor a mechanism that involves an autocatalytic pathway propagated by $\bullet\text{NO}_2$, we do not necessarily favor the initiation of this mechanism by peroxide. There are other possible sources of trace amounts of $\bullet\text{NO}_2$ that could be sufficient to start an autocatalytic reaction. HNO may lead to $\bullet\text{NO}_2$ formation through the creation and subsequent decay of peroxynitrite via direct reaction of HNO with molecular oxygen (Eqs. 15, 16) [76; 77; 78]

However, it has been reported by others that this reaction does not occur under physiologically relevant conditions [79; 80]. It is also conceivable that such a reaction occurs between HNO and heme-bound oxygen, forming a peroxynitrite adduct similar to that proposed by Keszler *et al.* in (Eq. 7) for the autocatalysis of nitrite. Indeed, the products of such a reaction are in agreement with (Eq. 2) for the reaction of HNO and oxyHb. If such an adduct were formed and dissociated from the heme as OONO^- , even in miniscule quantities, rather than oxidizing the heme as described in (Eq. 2), peroxynitrite could initiate an autocatalytic reaction rather than peroxide. Peroxynitrite has been shown to oxidize oxyHb by means of formation of an $\bullet\text{NO}_2$ radical [81; 82], and importantly, it was observed by Romero and coworkers that during the reaction of oxyHb and peroxynitrite, nitrite is consumed [81]. A catalytic mechanism involving $\bullet\text{NO}_2$ was also suggested to explain this phenomenon, invoking autocatalytic mechanisms [61] related to the autocatalysis of the reaction of nitrite and oxyHb. Thus, overall, we favor a mechanism whereby NO_2 is formed and leads to autocatalysis of the nitrite/oxyHb reaction in the presence of HNO. The c-PTIO used may have been insufficient to scavenge $\bullet\text{NO}_2$, or perhaps $\bullet\text{NO}_2$ formed could act locally within the same Hb tetramer.

The acceleration of the oxidation of oxyHb by nitrite in the presence of HNO could have significant implications for HNO biology and the potential use of HNO as a therapeutic, particularly for HNO sources that also produce nitrite such as Angeli's salt. The nitrite/oxyHb reaction is generally slow under physiological conditions where nitrite is generally much lower in concentration than hemoglobin or myoglobin so autocatalysis does not occur. However, we have shown here that HNO accelerates the nitrite/oxyHb reaction even when Hb is in excess. If AS were to be used as a therapeutic, it could result in elevated concentrations of both HNO and nitrite and, in addition to the effects of these two agents individually, the acceleration of the oxyHb/nitrite reaction could also have effects on biological systems. This is also likely to be the case where an HNO-based therapeutic would affect the rate of the reaction of oxyhemoglobin with intrinsic nitrite. It should be noted that since HNO also catalyzes the nitrite/deoxyHb reaction [52], nitrite/Hb reactions would be expected to be faster due to the presence of HNO at all oxygen saturations. The extent of effects of HNO on reactivity of nitrite and Hb as well as other heme proteins merits further exploration.

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- The oxidation of oxygenated hemoglobin by nitrite is accelerated by nitroxyl (HNO).
- Peroxide, superoxide, and metHb had little effect on the acceleration.
- Inhibiting allosteric modulation had no significant effect on the acceleration.
- Any Hb bound to HNO dissociates quickly, arguing against a role for an Hb-HNO adduct.
- The acceleration has implications for the use of Angeli's salt and HNO in therapeutics.

Figure 1.

Spectral deconvolution of hemoglobin species. (A) Basis spectra used to fit sample spectra for determination of hemoglobin species concentrations. Basis spectra include oxygenated Hb (oxyHb), deoxygenated Hb (deoxyHb), methemoglobin (metHb), nitrite bound metHb (metHb-NO₂⁻), ferrous nitrosyl Hb (HbNO), and ferric nitrosyl Hb (metHbNO). (B) A sample spectrum (red crosses) of 1 mM oxyHb + 50 μM AS + 50 μM NO₂⁻, incubated for 10 min at 37°C, fit by a least-squares regression to a linear combination of basis spectra (blue line).

Figure 2.

The reaction of oxyhemoglobin with Angeli's salt and/or nitrite. (A) Time-resolved spectra of 1 mM oxyHb upon addition of 50 μM AS and 50 μM NO_2^- under aerobic conditions. The decrease in absorbance at 542 nm and 577 nm, and the increase in absorbance at 500 nm and 630 nm, demonstrate the partial conversion of oxyHb to metHb. The arrows indicate the direction of spectral shift over time. (B) Methemoglobin levels due to the oxidation of oxyHb by AS and/or nitrite over time. Samples were made with 1 mM oxyHb + 50 μM AS + 50 μM NO_2^- , 1 mM oxyHb + 50 μM AS, 1 mM oxyHb + 50 μM NO_2^- , and 1 mM oxyHb + a blank (consisting of oxyHb alone plus an equivalent amount of NaOH that was added to other samples containing dissolved AS). Spectra were taken every 10 min for an hour, then every hour for four hours. Sample spectra were fit to basis spectra, and the evolution of metHb was tracked. The hemoglobin balance remained oxyHb, with occasional traces (<2.5%) of deoxyHb. No other species were present in quantities detectable by absorption spectroscopy. (C) MetHb formation in 1 mM oxyHb as a result of 50 μM nitrite in the presence (orange) or absence (teal) of 50 μM AS. The metHb concentration in 1 mM oxyHb + 50 μM AS was subtracted from the metHb concentration in 1 mM Hb + 50 μM AS + 50 μM NO_2^- to yield the metHb concentration due to nitrite in the presence of AS. The metHb concentration in 1 mM oxyHb + 50 μM NO_2^- was subtracted from the metHb concentration in 1 mM oxyHb + a blank to yield the metHb concentration due to nitrite in the absence of AS. (D) Nitrite levels after 10 min in samples of 1 mM oxyHb + 50 μM AS + 50 μM NO_2^- , 1 mM oxyHb + 50 μM AS, and 1 mM oxyHb + 50 μM NO_2^- , color coded to match the

corresponding lines in (B). The rightmost bar shows the nitrite concentration in 1 mM oxyHb + 50 μ M AS subtracted from the nitrite concentration in 1 mM oxyHb + 50 μ M AS + 50 μ M NO₂⁻, corresponding to the orange line in (C).

Figure 3.

Hemoglobin oxidation by nitrite at different nitrite concentrations. (A) The oxidation of oxyHb to metHb due to 100 μM NO₂⁻, 50 μM NO₂⁻, and autoxidation. (B) The difference between oxidation due to 100 μM NO₂⁻ and 50 μM NO₂⁻ compared to the difference between oxidation due to 50 μM NO₂⁻ and autoxidation. (C) Methemoglobin levels due to the oxidation of oxyHb by 50 μM DEA NONOate (NO donor) and/or nitrite over time. Conditions are otherwise as in Figure 2B. (D) MetHb formation in 1 mM oxyHb as a result of 50 μM nitrite in the presence (orange) or absence (teal) of 50 μM DEA NONOate. Conditions are otherwise as in Figure 2C.

Figure 4.

The reaction of oxyhemoglobin with nitrite and/or HNO from 4-nitrosotetrahydro-2H-pyran-4-yl pivalate, a water-soluble acyloxy nitroso compound that releases HNO in the presence of pig liver esterase. (A) Time-resolved spectra of 1 mM oxyHb upon addition of 50 μM HNO donor and 50 μM NO_2^- under aerobic conditions. The decrease in absorbance at 542 nm and 577 nm, and the increase in absorbance at 500 nm and 630 nm, demonstrate the partial conversion of oxyHb to methHb. The arrows indicate the direction of spectral shift over time. (B) Methemoglobin levels due to the oxidation of oxyHb by HNO and/or nitrite over time. Samples were made with 1 mM oxyHb + 50 μM HNO donor + 50 μM NO_2^- , 1 mM oxyHb + 50 μM HNO donor, 1 mM oxyHb + 50 μM NO_2^- , and 1 mM oxyHb. Spectra

were taken every 10 min for two hours, then once an hour for two more hours. Sample spectra were fit to basis spectra, and the evolution of metHb was monitored. The hemoglobin balance remained oxyHb. No other species were present in quantities detectable by absorption spectroscopy. (C) MetHb formation in 1 mM oxyHb as a result of 50 μ M nitrite in the presence (orange) or absence (teal) of 50 μ M HNO donor. The metHb concentration in 1 mM oxyHb + 50 μ M HNO donor was subtracted from the metHb concentration in 1 mM Hb + 50 μ M HNO donor + 50 μ M NO_2^- to yield the metHb concentration due to nitrite in the presence of the HNO donor. The metHb concentration in 1 mM oxyHb + 50 μ M NO_2^- was subtracted from the metHb concentration in 1 mM oxyHb to yield the metHb concentration due to nitrite in the absence of the HNO donor.

Figure 5.

The reaction of oxyhemoglobin with Angeli's salt and/or nitrite in the presence of catalase or c-PTIO. (A) Methemoglobin levels due to the oxidation of oxyHb by AS and/or nitrite over time in the presence of 50 μM catalase. Conditions are otherwise as in Figure 2B. (B) MetHb formation in 1 mM oxyHb with 50 μM catalase as a result of 50 μM nitrite in the presence (orange) or absence (teal) of 50 μM AS. Conditions are otherwise as in Figure 2C. (C) Methemoglobin levels due to the oxidation of oxyHb by AS and/or nitrite over time in the presence of 250 μM c-PTIO. Conditions are otherwise as in Figure 2B. (D) MetHb formation in 1 mM oxyHb with 250 μM c-PTIO as a result of 50 μM nitrite in the presence (orange) or absence (teal) of 50 μM AS. Conditions are otherwise as in Figure 2C.

Figure 6.

The reaction of oxyhemoglobin with Angeli's salt and/or nitrite in the presence of superoxide dismutase (SOD) or inositol hexaphosphate (IHP). (A) Methemoglobin levels due to the oxidation of oxyHb by AS and/or nitrite over time in the presence of 10 Ku/mL SOD. Conditions are otherwise as in Figure 2B. (B) MetHb formation in 1 mM oxyHb with 10 Ku/mL SOD as a result of 50 μM nitrite in the presence (orange) or absence (teal) of 50 μM AS. Conditions are otherwise as in Figure 2C. (C) Methemoglobin levels due to the oxidation of oxyHb by AS and/or nitrite over time in the presence of 2 mM IHP. Conditions are otherwise as in Figure 2B. (D) MetHb formation in 1 mM oxyHb with 2 mM IHP as a result of 50 μM nitrite in the presence (orange) or absence (teal) of 50 μM AS. Conditions are otherwise as in Figure 2C.

Figure 7.

The reaction of oxyhemoglobin with Angeli's salt at differing initial concentrations of methHb and oxyHb. (A) Methemoglobin levels due to the oxidation of oxyHb by AS and/or nitrite over time with an initial concentration of 750 μM oxyHb and 250 μM methHb. Conditions are otherwise as in Figure 2B. (B) MetHb formation in 750 μM oxyHb and 250 μM methHb as a result of 50 μM nitrite in the presence (orange) or absence (teal) of 50 μM AS. Conditions are otherwise as in Figure 2C. (C) Methemoglobin levels due to the oxidation of oxyHb by AS and/or nitrite over time with an initial oxyHb concentration of 200 μM. Conditions are otherwise as in Figure 2B. (D) MetHb formation in 200 μM oxyHb as a result of 50 μM nitrite in the presence (orange) or absence (teal) of 50 μM AS. Conditions are otherwise as in Figure 2C.